Evidence that *p*-Fluorophenylalanine has a Direct Effect on Tubulin in *Aspergillus nidulans*

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Three temperature-sensitive alleles of *benA* (*benA11*, *17* and *21*) confer resistance to growth inhibition by *p*-fluorophenylalanine (FPA). FPA resistance cosegregates with the *benA* gene. Two back-mutations in *benA* which cause loss of temperature sensitivity cause loss of FPA resistance, and two indirect suppressors of *benA* temperature sensitivity also cause FPA resistance to be lost. These results indicate that FPA resistance is an intrinsic property of the *benA* mutations. The intracellular phenylalanine concentrations of these strains are normal as is their ability to take up phenylalanine from the medium. We conclude that FPA must inhibit growth and cause non-disjunction by a direct effect on the polymerization of tubulin.

INTRODUCTION

Benomyl and *p*-fluorophenylalanine (FPA) are commonly used in fungal genetics to induce loss of chromosomes from diploids during parasexual mapping of genes to chromosomes (Lhoas, 1961; Hastie, 1970). Benomyl is known to interact with the β -subunit of tubulin at a site closely related to the colchicine binding site and presumably causes chromosome loss by interfering with the polymerization or function of microtubules in the mitotic spindle (Davidse, 1973; Davidse & Flach, 1977; Sheir-Neiss *et al.*, 1978). The mechanism by which FPA causes chromosome loss has not yet been explained.

During the course of our studies of benomyl-resistant mutants of *Aspergillus nidulans* (Sheir-Neiss *et al.*, 1978), we noted that three strains with mutations (*benA11*, *17* and *21*) in the *benA* gene which were temperature-sensitive for growth (Morris *et al.*, 1979) were also cross-resistant to FPA. We have shown elsewhere that *benA* is a structural gene for β -tubulin (Sheir-Neiss *et al.*, 1978) and that the temperature sensitivity of the *benA11*, *benA17* and *benA21* alleles is an intrinsic property of these *benA* mutations (Morris *et al.*, 1979). In this paper, we demonstrate that resistance to FPA is another intrinsic property of the *benA11*, *17* and *21* mutations. From this we conclude that FPA probably affects mitosis by a direct effect on tubulin.

METHODS

Strains. B3 (*biA1 acrA1*), BEN1 to 25 (*benA* alleles *benA3* to 27 *biA1 acrA1*) and BEN28 (*benA28 suA1adE20 yA2 adE20 acrA1 sB3 riboB2*) were obtained from Dr J. M. Van Tuyl. FGSC 99 (*suA1adE20 adE20 yA2 pabaA1 proA1 acrA1*), FGSC 73 (*pabaA1 yA2 adE20 acrA1 phenA2 pyroA4 lysB5 sB3 riboB2*) and FGSC 273 (*adF17 pabaA1 yA2 fpaD11*) were obtained from the Fungal Genetics Stock Center at Arcata, California, U.S.A.

Genetic methods and media. These were standard for *A. nidulans* (Pontecorvo *et al.*, 1953; Clutterbuck, 1974). Back-mutations and strains carrying suppressor mutations were isolated as previously described (Morris *et al.*, 1979).

Chemicals. Benomyl was a gift from E. I. du Pont de Nemours and Co. FPA was purchased from Calbiochem.

Amino acid analysis. Cultures of B3, and strains carrying *benA11*, 17 and 21, were grown at 37 °C for 16 h from an inoculum of 3×10^6 conidia ml⁻¹, washed with cold saline and harvested by filtration. The mycelia were resuspended in 1 ml of cold 10% (w/v) sulphosalicylic acid and homogenized thoroughly with a Teflon-glass homogenizer. The debris was removed by centrifugation. The supernatant liquid was then adjusted to pH 2.2 with NaOH, centrifuged to remove the precipitate and 100 µl samples were applied to a Beckmann 121 MB amino acid analyser. Peaks were identified by their time of elution and the phenylalanine peak was verified by addition of excess phenylalanine to one sample. The peaks were cut out and weighed to determine their relative areas.

RESULTS

Strains carrying benA11, 17 and 21 are cross-resistant to FPA

Twenty-six *benA* mutants of *A. nidulans* originally isolated by Van Tuyl (1977) were tested for their ability to grow on FPA at 130 µg ml⁻¹, a concentration which is inhibitory to the wild-type parental strain B3. Many of the mutants were moderately resistant to FPA (Table 1), the most resistant being three temperature-sensitive *benA* mutants carrying *benA11*, 17 and 21 (Fig. 1). Whereas B3 showed 50% growth inhibition (LD₅₀) on 88 µg FPA ml⁻¹, the *benA11*, 17 and 21 strains were 50% inhibited at 310, 220 and 225 µg FPA ml⁻¹, respectively.

To determine whether the FPA cross-resistance of these benomyl-resistant strains was related to mutation in the *benA* gene, the three strains were outcrossed to *A. nidulans* strain

Table 1. *FPA and temperature sensitivity of benA strains*

Strain	Genotype	LD ₅₀ FPA (µg ml ⁻¹)	Temperature sensitivity*
B3	<i>benA</i> ⁺ <i>biA1</i> <i>acrA1</i>	88	wt
BEN1	<i>benA3</i> <i>biA1</i> <i>acrA1</i>	135	wt
BEN2	<i>benA4</i> <i>biA1</i> <i>acrA1</i>	150	wt
BEN3	<i>benA5</i> <i>biA1</i> <i>acrA1</i>	72	wt
BEN4	<i>benA6</i> <i>biA1</i> <i>acrA1</i>	170	wt
BEN5	<i>benA7</i> <i>biA1</i> <i>acrA1</i>	78	wt
BEN6	<i>benA8</i> <i>biA1</i> <i>acrA1</i>	175	wt
BEN7	<i>benA9</i> <i>biA1</i> <i>acrA1</i>	160	CS
BEN8	<i>benA10</i> <i>biA1</i> <i>acrA1</i>	120	wt
BEN9	<i>benA11</i> <i>biA1</i> <i>acrA1</i>	310	TS
BEN10	<i>benA12</i> <i>biA1</i> <i>acrA1</i>	200	wt
BEN11	<i>benA13</i> <i>biA1</i> <i>acrA1</i>	105	wt
BEN12	<i>benA14</i> <i>biA1</i> <i>acrA1</i>	60	wt
BEN13	<i>benA15</i> <i>biA1</i> <i>acrA1</i>	170	wt
BEN14	<i>benA16</i> <i>biA1</i> <i>acrA1</i>	170	wt
BEN15	<i>benA17</i> <i>biA1</i> <i>acrA1</i>	220	CS, TS
BEN16	<i>benA18</i> <i>biA1</i> <i>acrA1</i>	108	wt
BEN17	<i>benA19</i> <i>biA1</i> <i>acrA1</i>	125	wt
BEN18	<i>benA20</i> <i>biA1</i> <i>acrA1</i>	68	wt
BEN19	<i>benA21</i> <i>biA1</i> <i>acrA1</i>	225	CS, TS
BEN20	<i>benA22</i> <i>biA1</i> <i>acrA1</i>	72	wt
BEN21	<i>benA23</i> <i>biA1</i> <i>acrA1</i>	128	CS
BEN22	<i>benA24</i> <i>biA1</i> <i>acrA1</i>	145	wt
BEN23	<i>benA25</i> <i>biA1</i> <i>acrA1</i>	62	wt
BEN24	<i>benA26</i> <i>biA1</i> <i>acrA1</i>	165	—
BEN25	<i>benA27</i> <i>biA1</i> <i>acrA1</i>	75	wt
BEN28	<i>benA28</i> <i>suA1adE20</i> <i>γA2</i> <i>adE20</i> <i>acrA1</i> <i>sB3</i> <i>riboB2</i>	30	wt
FGSC 273	<i>fpaD11</i> <i>adF17</i> <i>pabaA1</i> <i>γA2</i>	> 1000†	—

* CS, Poor growth relative to wild-type at 20 °C; TS, poor growth relative to wild-type at 44 °C; wt, normal growth response to high and low temperatures.

† From Sinha (1969).

Fig. 1. Effect of high temperature (44 °C) and *p*-fluorophenylalanine (FPA, 130 µg ml⁻¹) on the growth of *benA11*, 17 and 21 strains and the parental strain B3 (*benA*⁺). Plates containing YAG [0.5% (w/v) yeast extract (Difco), 2% (w/v) agar, 2% (w/v) glucose] were inoculated with conidia and incubated for 3 d (32 °C and 44 °C plates) or 5 d (FPA plate).

Table 2. Cosegregation of benomyl resistance and FPA resistance among the progeny of FGSC 99 × *benA11*, 17 or 21

The results indicate the number of progeny in each class: R, resistant; S, sensitive.

	<i>benA11</i> × FGSC 99		<i>benA17</i> × FGSC 99		<i>benA21</i> × FGSC 99	
	BenR	BenS	BenR	BenS	BenR	BenS
FPAS	0	58	0	42	0	36
FPAR	38	0	34	0	58	0

FGSC 99 which is sensitive to both FPA and benomyl. Progeny from these crosses were then tested for the presence of the *benA* mutation by their ability to grow with an inhibitory concentration of benomyl (2.4 µg ml⁻¹) and for the presence of the mutation conferring FPA resistance by their ability to grow on FPA. FPA resistance cosegregated perfectly with the *benA* gene among the segregants of the outcrosses of *benA11*, 17 and 21 to FGSC 99 (Table 2). Thus FPA resistance appears to be caused either by the mutations *benA11*, 17 and 21 or by mutations very closely linked to the *benA* gene. Compelling evidence that FPA resistance is an intrinsic property of *benA17* and *benA21* has been provided by studies of revertants of strains carrying these mutations.

Revertants of *benA11*, 17 and 21 strains

The *benA11*, 17 and 21 strains are not only benomyl-resistant and FPA-resistant but they are also temperature-sensitive (*ts*⁻) for growth. The *benA17* and 21 strains are also cold-sensitive for growth. Because it is not possible to select revertants from drug resistance to drug sensitivity, we used the temperature sensitivity of these strains to select revertants from *ts*⁻ to *ts*⁺. The methods used for selecting these revertants have been described elsewhere (Morris *et al.*, 1979). Three *ts*⁺ revertants were isolated from *benA11*, nine from *benA17* and six from *benA21*. Each of the *ts*⁺ revertants was then classified as being due to a back-mutation in *benA* or to a suppressor mutation external to *benA* by being outcrossed to FGSC 99, a *ts*⁺ wild-type strain. At least 50 segregants from each cross were tested for temperature sensitivity. The appearance of *ts*⁻ segregants among the progeny of such a cross indicated that the reversion was caused by an indirect suppressor mutation and the absence of *ts*⁻ segregants was taken to indicate that the reversion was probably caused by a back-mutation in *benA*. Four revertants, *benA17R10*, *benA17R11*, *benA17R13* and *benA21R7*, gave no *ts*⁻ segregants and, therefore, probably represent back-mutations in *benA*. The remaining 14 revertants all produced *ts*⁻ progeny and must carry indirect suppressor mutations external to the *benA* gene (Table 3).

Table 3. *Revertants of temperature-sensitive benA mutants*

Revertants were examined (i) for resistance (R) or sensitivity (S) to benomyl; (ii) for resistance or sensitivity to FPA; (iii) for temperature sensitivity at 44 °C (ts⁻), cold sensitivity at 20 °C (CS) or normal wild-type growth responses (ts⁺) [at least 50 segregants from each cross were tested]; (iv) for the presence of a suppressor (Su) or back-mutation (bm).

Revertant strain	Benomyl	FPA	ts	Su/bm
BEN9 (<i>benA11</i>)	R	R	—	
R1	R	R	+	Su
R7	R	R	+	Su
R8	R	R	+	Su
BEN15 (<i>benA17</i>)	R	R	—	
R3	R	R	+	Su
R6	S	S	CS	Su
R7	R	S	+	Su
R8	R	R	+	Su
R9	R	S	+	Su
R10	S	S	+	bm
R11	R	R	+	bm
R12	R	S	+	Su
R13	S	S	+	bm
BEN19 (<i>benA21</i>)	R	R	—	
R7	R	R	+	bm
R8	R	R	+	Su
R9	R	R	+	Su
R10	R	R	+	Su
R11	S	S	CS	Su
R12	R	R	+	Su

The ts⁺ *benA* revertants were analysed for resistance to benomyl and to FPA and for temperature sensitivity (Table 3). In most cases reversion to ts⁺ did not cause reversion to benomyl or FPA sensitivity. However, two extragenic suppressor strains, *benA17R7* and *benA17R9*, were sensitive to FPA although still resistant to benomyl; and two other suppressor strains, *benA17R6* and *benA21R11*, were sensitive to both benomyl and FPA. Two of the strains with probable back-mutations in *benA*, *benA17R10* and *benA17R13*, were also revertant with respect to both drug resistance markers, whereas two other back-mutation strains, *benA17R11* and *benA21R7*, remained resistant to both drugs. The fact that two of the back-mutations and two of the suppressor mutations caused the reversion not only of temperature sensitivity but also of benomyl and FPA resistance is convincing evidence that benomyl resistance, FPA resistance and temperature sensitivity are properties of the *benA17* and *benA21* mutations. By extrapolation it is likely that these are also properties of the *benA11* mutation.

The biochemical basis for FPA resistance has been described for three previously isolated FPA-resistant mutants of *A. nidulans*. Strains carrying the mutations *fpaA* (*tyrA*) and *fpaE* (*trypA*) are resistant to FPA because they have defects in the pathways for aromatic amino acid biosynthesis which by a feedback mechanism elevate the intracellular concentration of phenylalanine, thereby reversing FPA toxicity. A strain carrying *fpaD* is resistant to FPA because it has a permease defect which prevents the uptake of FPA (and phenylalanine) from the medium. Because mutations in tubulin might by some unknown mechanism affect intracellular amino acid levels or amino acid permease activity, we have compared phenylalanine and tyrosine levels in B3 and the *benA11*, 17 and 21 strains and have also tested the ability of these strains to utilize extracellular phenylalanine.

To determine the relative phenylalanine and tyrosine levels of the wild-type and the three mutant strains, mycelia from overnight cultures were extracted with cold sulphosalicylic acid and subjected to amino acid analysis. The relative amounts of phenylalanine and tyrosine in the extracts were determined by comparing the areas of the absorption peaks for these

Table 4. Ratios of phenylalanine and tyrosine to leucine in acid-soluble extracts of wild-type *benA*⁺ (B3) and temperature-sensitive *benA* mutants (*benA11*, 17 and 21) of *A. nidulans*

The results show the ratios of the areas of the phenylalanine, leucine and tyrosine peaks from the amino acid analyser.

	B3	<i>benA11</i>	<i>benA17</i>	<i>benA21</i>
Phe/Leu	0.23	0.22	0.28	0.22
Tyr/Leu	0.38	0.32	0.33	0.38

amino acids to the area of the absorption peak for leucine in the extracts (Table 4). In all three mutants the ratios of phenylalanine and tyrosine to leucine were essentially the same as in the wild-type parental strain B3. Thus, unless the concentration of leucine in these strains was abnormally elevated, which seems unlikely since the area of the leucine peak was normal with respect to all the other amino acids in the extracts, we can conclude that the FPA resistance associated with *benA11*, 17 and 21 is not caused by a high intracellular concentration of phenylalanine. To rule out the possibility that the temperature-sensitive *benA* mutations significantly decrease the uptake of FPA (or phenylalanine) from the medium, *benA21* was crossed with a phenylalanine requiring (*phenA*) strain, FGSC 73. Approximately 50% of the benomyl-resistant segregants of the *benA21* × FGSC 73 cross were *phenA*. Moreover, the *benA phenA* segregants were indistinguishable from the *benA phenA*⁺ segregants with respect to colony size at phenylalanine concentrations between 0.05 and 100 µg ml⁻¹, indicating that the double mutants were not limited in their ability to take up phenylalanine (and presumably FPA) from the medium. In contrast, the corresponding cross between *fpaD* and *phenA* has been shown to yield no *fpaD phenA* segregants (Sinha, 1969), presumably because the double mutant can neither synthesize nor take up extracellular phenylalanine. Because of the difference between the results of these crosses, we consider it unlikely that the temperature-sensitive *benA* mutations cause FPA resistance by affecting amino acid uptake.

DISCUSSION

FPA is known to induce non-disjunction in fungi and to block mitosis in mammalian cells (Hastie, 1970; Siskin, 1973). Since FPA is structurally much simpler than most other drugs known to block mitosis, its mechanism of action is of some interest. In this paper, we have demonstrated that moderate resistance to FPA is caused by certain temperature-sensitive mutations in the gene for β -tubulin (*benA*) in *A. nidulans*. We have shown that FPA resistance cosegregates with *benA*, and that back-mutations in *benA* and suppressors of *benA* cause reversion of FPA resistance.

Resistance to FPA has been studied extensively in *A. nidulans* (Sinha, 1967, 1969; Srivastava & Sinha, 1975). Ten gene loci which cause FPA resistance have been identified previously in this organism and it has been estimated that mutation at as many as 28 different loci may cause FPA resistance (Srivastava & Sinha, 1975). The mechanisms of resistance to FPA of the genes *fpaA*, *D* and *E* are known. *FpaA* and *E* are blocked in tyrosine and tryptophan synthesis, respectively (Sinha, 1967). Normal metabolic control mechanisms cause organisms carrying these mutations to over-produce phenylalanine which then competes with FPA to prevent toxicity. The *fpaD* mutation is known to interfere with amino acid uptake in *A. nidulans* (Sinha, 1969). We have determined that the intracellular pool of phenylalanine and the ability to utilize external phenylalanine are essentially normal in the temperature-sensitive *benA* mutants. Resistance to FPA is probably therefore an intrinsic property of these *benA* mutations in β -tubulin and not a consequence of an effect on metabolism or amino acid permease. This result suggests that FPA causes non-disjunction and blocks mitosis by directly affecting either the polymerization of tubulin or the function of microtubules in the mitotic spindle. It also implies that the site at which FPA acts may

be related to the benomyl binding site on β -tubulin. Whether FPA acts as a ligand by binding to tubulin at this site or by being incorporated into tubulin in place of phenylalanine should be amenable to biochemical investigation.

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