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An Inducible, Nondegradative Phytoalexin Resistance Mechanism in *Dictyostelium discoideum* Is Suppressed by Mutations That Alter Membrane Sterol Composition

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Pretreatment of *Dictyostelium discoideum* amoebae with a sublethal concentration of the pea phytoalexin pisatin was shown to induce nondegradative resistance to subsequent challenges with inhibitory concentrations. An alteration of membrane sterol composition either with the azasterol A25822B or by mutations in *nysC* that confer resistance to the polyene antibiotic nystatin suppressed the induction of pisatin resistance. Wild-type cells grown on pisatin medium acquired resistance to nystatin; however, after transfer to nystatin medium, they lost their pisatin resistance phenotype but remained nystatin resistant. To account for this asymmetry in the induction and maintenance of cross-resistance after growth on pisatin and nystatin media, we propose a model in which the two resistance phenotypes are governed by distinct mechanisms. This model presumes that growth on pisatin induces membrane alterations that predispose cells to acquire nystatin resistance but that the pisatin-induced membrane alterations are not maintained in the absence of pisatin.

Pisatin (6a-hydroxy-3-methoxy-8,9-methylenedioxypterocarpan) is the major isoflavonoid phytoalexin produced by pea (*Pisum sativum*) in response to microbial infection (7). Several fungal pathogens of pea circumvent its antifungal activity by inducing enzymes that demethylate pisatin to the less toxic metabolite 6a-hydroxymaackiain (reviewed in reference 12). In addition, these fungi possess inducible pisatin resistance mechanisms that are nondegradative. The induction of nondegradative pisatin resistance may serve as a rapid and possibly nonspecific response that enables the fungi to cope with a variety of phytoalexins until the more specific degradative enzymes begin to be synthesized. However, unlike the degradative mechanisms, the nondegradative pisatin resistance mechanism is poorly understood, because thus far no mutants have been available either to assess its significance in virulence or for biochemical studies.

We have, for the following reasons, undertaken to examine the cellular slime mold *Dictyostelium discoideum* for inducible, nondegradative resistance to pisatin. (i) One of us has studied the induction of resistance in *D. discoideum* to the polyene antibiotic nystatin (4) and found that pretreatment with the sterol biosynthesis inhibitor azasterol A25822B permitted growth of the cells on a medium containing an otherwise inhibitory concentration of nystatin (5). After induction by the azasterol, the nystatin resistance phenotype could be maintained by the nystatin in the medium. Contemporaneously, Denny and VanEtten (3) reported that mycelia of the pea pathogen *Nectria haematococca* in which nondegradative pisatin resistance had been induced developed cross-resistance to another polyene antibiotic, amphotericin B. Nystatin and amphotericin B have the same mode of action; both bind to membrane sterols and form aqueous pores through which cell contents leach out, ultimately causing cell death (6). Therefore, we wanted to determine whether *D. discoideum* also possessed inducible, nondegradative pisatin resistance and to examine its relationship, if any, to inducible nystatin resistance. If the two are related, one might expect the pisatin resistance phenotype to be affected by mutations that affect the nystatin resistance phenotype. Mutations in any one of three genes, *nysA*, *nysB*, or *nysC*, confer the ability to grow on medium supplemented with 80 μg of nystatin per ml (nys80) (9). The *nysB* and *nysC* mutations also alter membrane sterol composition, whereas *nysA* mutants have wild-type sterols (9). (ii) *D. discoideum* is a free-living soil amoeba that feeds on bacteria that grow on decaying vegetation and may encounter a variety of phytoalexins in its natural habitat. We anticipated that it would rely more on a nonspecific mechanism of phytoalexin resistance, such as nondegradative resistance, rather than on specific degradative enzymes. If this were true, *D. discoideum* would be a useful model for studying the mechanism(s) of nondegradative resistance. Moreover, *D. discoideum* represents a more tractable system for genetic and molecular studies than most phytopathogenic fungi.

We report here that inducible, nondegradative pisatin resistance does indeed occur in *D. discoideum* and that this resistance is suppressed by *nysC*- and azasterol-induced sterol alterations. Pisatin also appears to induce nystatin resistance in a manner analogous to that of the azasterol A25822B (5).

MATERIALS AND METHODS

Strains. All the *D. discoideum* strains used were derivatives of *D. discoideum* NC4 (8) and have been previously described (9). *N. haematococca* MP IV strains 272-26-1 (Pda^-) and 272-26-3 (Pda^+) were from the collection of H. D. VanEtten (University of Arizona).

Growth conditions. *D. discoideum* amoebae were grown on lawns of *Enterobacter aerogenes* at 22°C on SM agar plates (10). Stock solutions (10 mg/ml) of the azasterol A25822B (Eli Lilly & Co.) and nystatin (Sigma) in dimethyl formamide and of pisatin in dimethyl sulfoxide were used to supplement SM agar plates just before the latter were poured. Nystatin agar plates were shielded from light by

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TABLE 1. EOPs of wild-type and mutant *D. discoideum* amoebae on pisatin agar

Strain	*nys* allele[a]	EOP[b] on:		EOP[b] of pis50 medium-derived cells on pis150
		pis50	pis150	
DdB	+	0.32	1.66×10^{-5}	0.75
M28	+	0.125	$<5 \times 10^{-6}$	0.25
TS12M	+	0.05	$<1.25 \times 10^{-5}$	0.5
HK5	*nysB203*	0.5	$<1.66 \times 10^{-6}$	0.225
HK10	*nysC208*	0.04	$<4 \times 10^{-6}$	$<5.7 \times 10^{-6}$
HK12	*nysC210*	0.006	$<3.57 \times 10^{-6}$	$<1.75 \times 10^{-5}$
HK14	*nysA212*	0.014	$<1.66 \times 10^{-6}$	0.29

[a] +, wild-type allele from NC4.
[b] The EOP was calculated as the ratio of the number of plaques on pisatin SM agar to that on pisatin-free SM agar.

being wrapped with aluminum foil. At the concentrations used, dimethyl formamide and dimethyl sulfoxide themselves had no detectable effects on the growth, development, or drug resistance phenotypes of the slime molds. In experiments to determine efficiency of plating (EOP), Bonner's salt solution (1) was used to suspend the amoebae and to make serial dilutions. The *N. haematococca* strains were maintained on slants of V-8 agar.

Pisatin extraction. Pisatin was extracted from germinated pea seeds (*P. sativum*) by the procedure of Sweigard and VanEtten (11).

Assay for pisatin degradation. To assay for pisatin demethylation by pisatin-resistant amoebae, we used pisatin that was specifically labelled with ^{14}C at the 3-*O*-methyl position (13). In all other systems examined, the first step in pisatin detoxification is demethylation of the 3-*O*-methyl group (12). Labelled or unlabelled pisatin (150 μg/ml) was incorporated in SM agar in 10-ml liquid scintillation vials. Pisatin-resistant amoebae were streaked on bacterial lawns grown on the agar. After 5 days, when the amoebae had cleared the bacteria, the vials were filled with scintillation fluid (5.5 g of 2,5-diphenyloxazole per liter of toluene), and the amount of ^{14}C that was partitioned into the organic phase (toluene) was determined in a liquid scintillation counter. Demethylation of the 3-*O*-methyl group results in the loss of the ^{14}C label from the organic phase (13). Control vials were inoculated with *N. haematococca* strains that either possess or lack pisatin demethylase activity (272-26-3 and 272-26-1, respectively).

RESULTS AND DISCUSSION

Induction of pisatin resistance in wild-type *D. discoideum* and in *nys* mutants. The EOPs of all three wild-type *D. discoideum* strains tested (DdB, TS12M, and M28) were relatively unaffected on SM agar supplemented with pisatin at 50 μg/ml (pis50) but dropped about 5 orders of magnitude on SM agar containing pisatin at 150 μg/ml (pis150) (Table 1). However, when wild-type amoebae were grown on pis50, the EOP of these cells when transferred to pis150 was about 4 to 5 orders of magnitude higher than that of cells transferred from pisatin-free medium (Table 1). Since the EOP was unaffected on pis50, we can conclude that there was virtually no selection for pisatin-resistant mutants during growth on pis50. Consequently, growth on the sublethal concentration must induce resistance to the higher pisatin concentration. Maintenance of the resistance phenotype required pisatin in the growth medium. When resistant cells were transferred to pisatin-free medium, they rapidly reverted to pisatin sensitivity. In one experiment, pis150 medium-derived TS12M cells were passaged on pisatin-free medium; upon retesting on pis150 medium, the EOP of these cells had dropped to $<5.5 \times 10^{-6}$, a value comparable to that of cells that had never before encountered pisatin. The EOP of control cells passaged on pis150 was 0.5.

Strains HK14, HK5, and HK12 (which are, respectively, *nysA*, *nysB*, and *nysC* mutants derived from TS12M) were examined on pis50 and pis150 media. There were no major differences in EOPs among these isogenic strains and their parent, TS12M, when grown on either medium (Table 1). However, when pis50 medium-grown cells of these strains were transferred to pis150 medium, only the *nysA* and *nysB* mutants exhibited inducible pisatin resistance, whereas the *nysC* mutant (HK12) remained pisatin sensitive (Table 1). The association between a mutation in *nysC* and the inability to acquire pisatin resistance was also confirmed with another *nysC* mutant, HK10, derived from parental strain M28 (Table 1). These results suggested that the sterol changes caused by the *nysC* mutation but not those caused by the *nysB* mutation were associated with the suppression of the pisatin resistance phenotype.

The azasterol A25822B suppresses the expression of pisatin resistance in the wild type. Wild-type amoebae grown in the presence of 15 μg of the azasterol A25822B per ml accumulate a novel sterol in place of the wild-type sterol stigmastenol, and this change is reversible upon removal of the azasterol from the growth medium (5). Azasterol-grown amoebae mimic the *nysC* mutant phenotype (4, 5); both azasterol-grown cells and *nysC* mutant cells are capable of growth on nystatin medium when plated either as amoebae or as spores (unlike *nysA* and *nysB* mutants, which can grow on nys80 plates only when plated as amoebae). They also make smaller fruiting bodies and display temperature-sensitive morphogenesis. In addition, azasterol-grown cells and *nysC* mutant cells contain similar sterols, as judged by a comparison of their mass spectra (4). With these similarities in mind, experiments were done to determine whether, in the presence of the azasterol, wild-type cells mimic the unresponsiveness toward pisatin characteristic of *nysC* mutants.

The addition of the azasterol at 15 μg/ml to pis50 medium (pis50 + aza15 medium) did not inhibit wild-type growth. Cells derived from pis50 + aza15 medium, however, did not grow on pis150 + aza15 medium. These results are consistent with the behavior of *nysC* mutants on pis50 and pis150 media and indicate that the presence of the azasterol induces the *nysC* mutant phenotype in the wild type.

The ability to mimic the *nysC* mutant phenotype by treatment of the wild type with A25822B made it possible to dissect this phenomenon further. Cells grown on both pis50 and pis50 + aza15, when transferred to pis150 medium (without the azasterol), expressed pis150 resistance, indicat-

TABLE 2. Degradation of ^{14}C-labelled pisatin

Strain	Mean cpm of undegraded pisatin (no. of replicates) in expt:	
	1	2
N. haematococca Pda$^+$	474.9 (2)	919.46 (3)
N. haematococca Pda$^-$	1,062.0 (1)	2,671.18 (3)
DdB Pis^r	1,203.30 (2)	2,425.13 (3)

ing that the azasterol-induced alterations of sterol composition did not interfere with the pis50-induced alterations required for growth on pis150. In contrast, cells grown on both pis50 and pis50 + aza15 were severely inhibited on pis150 + aza15 medium, indicating that the azasterol-induced sterol alterations, when maintained by the continued presence of the azasterol, interfered with the expression of pisatin resistance despite the occurrence of pis50-induced alterations.

These results suggest that pis150 resistance involves at least two components, one of which (produced during growth on pis50) is not suppressed by the azasterol- and possibly *nysC*-induced sterol changes and the other of which (required for growth on pis150) is suppressed by the sterol alterations.

Resistance is nondegradative. An assay for pisatin degradation was done to determine whether the inducible pisatin resistance of *D. discoideum* involved the production of pisatin-detoxifying enzymes. Pisatin-resistant DdB was grown on medium containing radiolabelled pisatin, and the amount of intact pisatin remaining after 5 days was measured and compared with that remaining in control vials inoculated with *N. haematococca* strains that either possess pisatin demethylase activity (Pda$^+$) or lack it (Pda$^-$). The results (Table 2) indicated that the resistant amoebae and the *N. haematococca* Pda$^-$ strain did not degrade the pisatin, whereas the *N. haematococca* Pda$^+$ strain did.

Growth on pisatin induces wild-type cells to acquire nystatin resistance. *D. discoideum* amoebae that had been induced for pisatin resistance were tested for their ability to develop cross-resistance to nystatin (Table 3). On nys80, the EOP of pis150 medium-derived cells was 10^3- to 10^4-fold higher than that of cells derived from pisatin-free medium. Cells derived from pis50 medium also developed cross-resistance to nystatin but with less reproducibility than pis150 medium-derived cells. Cells transferred from pis150 to pis50 remained as nystatin resistant as cells maintained continuously on pis150 (data not shown). The nystatin resistance was indistinguishable from the transient nystatin resistance (induced, for example, by growth on the sterol biosynthesis inhibitor azasterol A25822B and maintained by continued growth on nys80) previously reported for *D. discoideum* (5). pis150 medium-grown cells that had subsequently been passaged once on pisatin-free medium became as nystatin sensitive as cells that were maintained throughout the experiment on SM medium.

TABLE 3. EOPs on nys80 of wild-type *D. discoideum* amoebae derived from pis150 SM medium or pisatin-free SM medium

Strain	EOP[a] of cells derived from the following medium:	
	pis150 SM	Pisatin-free SM
DdB	0.057	$<5 \times 10^{-5}$
M28	0.068	$<2 \times 10^{-5}$
TS12M	0.021	$<2 \times 10^{-5}$

[a] Amoebae were harvested either from pis150 SM agar plates or from pisatin-free SM agar plates and suspended in Bonner's salt solution. The EOP was calculated as described in Table 1, footnote *b*.

Surprisingly, cells transferred from pis150 to nys80 lost their pisatin resistance phenotype during growth on nystatin medium and became as pisatin sensitive as cells from SM medium. In one representative experiment, the EOP on pis150 of nys80 medium-grown DdB cells was 2.5×10^{-5}, comparable to the EOP (1.66×10^{-5}) of SM medium-grown cells. This was true even though the nystatin resistance had been induced by pretreatment with pis150. To verify that this result was not an artifact resulting from the degradation of nystatin in the plates accompanied by the concomitant loss of the resistance phenotype, we simultaneously tested and confirmed that the cells from nys80 medium retained their nystatin resistance phenotype (data not shown).

A model to explain the asymmetry in the induction of pisatin and nystatin resistance. The fact that *D. discoideum* amoebae are pisatin and nystatin resistant when derived from pis150 but are pisatin sensitive and nystatin resistant when derived from nys80 may argue against models in which the two resistances are mediated by the same mechanism. If they were, one would expect cross-induction to work more efficiently in both directions: from pisatin to nystatin and from nystatin to pisatin. Also, it should be noted that mutations that confer resistance to nystatin (Table 1) do not confer resistance to pisatin, suggesting that these phenotypes are due to separate mechanisms.

We prefer an alternative model to account for the pisatin induction of nystatin resistance. This model is analogous to the one proposed earlier for the induction of nystatin resistance following growth on the azasterol A25822B (5). In the present model, growth on pisatin induces membrane alterations that allow the cells to survive longer in the presence of otherwise cytotoxic concentrations of nystatin and thereby predisposes them to acquire nystatin resistance. Presumably, after transfer to nystatin medium, the pisatin-induced changes are not maintained and the cells become pisatin sensitive, but the induced nystatin resistance phenotype persists. This model readily accommodates the fact that *nysC*- and azasterol-induced sterol changes promote nystatin resistance but suppress pisatin resistance because the two resistances are presumed to be due to quite distinct mechanisms. Although the induction of resistance to nystatin suggests that growth on pisatin induces membrane alterations, it is premature to conclude that the same alterations are also responsible for the pisatin resistance phenotype.

Concluding remarks. We have shown here that by pretreating *D. discoideum* amoebae with sublethal concentrations of the pea phytoalexin pisatin, nondegradative resistance to otherwise inhibitory pisatin concentrations can be induced. The molecular changes that accompany growth on pisatin and how such changes predispose cells to acquire nystatin resistance have not yet been determined.

Given that growth on pisatin can induce nystatin resistance, it may appear paradoxical that mutations that confer nystatin resistance do not confer pisatin resistance and that *nysC* mutations instead suppress the induction of pisatin resistance. We postulate that the *nysC*-dependent sterol alterations and pisatin induce nystatin resistance by different mechanisms. That is, pisatin may induce a change in membrane structure which is different from and perhaps incompatible with the *nysC*-induced sterol alteration. Pisatin-

induced membrane modifications have previously been invoked to explain the decrease in pisatin accumulation by *N. haematococca* mycelia expressing nondegradative resistance (2).

Our results differ from those obtained with amphotericin B and *N. haematococca* (3), in that pisatin resistance in *D. discoideum* cannot be cross-induced by nystatin. This difference may reflect differences in the actions of the two polyene antibiotics. Assuming that nondegradative resistance occurs by a similar mechanism in various phytopathogenic fungi, it may be possible to overcome it in such fungi by using sterol biosynthesis inhibitors, such as A25822B, as synergists to potentiate the effects of phytoalexins. Finally, like *nysC* mutants, sterol mutants may block nondegradative pisatin resistance in *N. haematococca*. This idea suggests that an approach similar to that of selecting for nystatin resistance mutations may be applied to obtaining such mutations in other systems, in particular, the phytopathogenic fungi. Such mutations will allow us to evaluate the role of the nondegradative resistance mechanism in phytopathogenesis.

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