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21. The 5′ SS RNA represents a 172-nt T7 RNA polymerase transcribed RNA molecule including 37 nt of the polylinker region of Bluescript plasmid (Stratagene) and 135 nt from the rabbit β-globin gene between positions 476 and 611. This RNA molecule encompasses the 5′ splice site of the second intron from rabbit β-globin. The 3′ SS RNA comprises the 94-nt region from rabbit β-globin gene between positions 1022 and 1116. This RNA encompasses the branch point and the 3′ splice site of the second intron. In addition the 3′ SS RNA contains at its 5′ end 49 nt from the polylinker region of the Bluescript plasmid. The 5′ SS containing T7 transcription plasmid was constructed by insertion of the second intron of rabbit β-globin gene flanked by some exon sequences as Bam HI–Bgl II fragment (nt 476 to 1196) into the Bam HI linearized Bluescript plasmid. Before transcription the construct has been linearized with Nco I at nucleotide position 611. The 3′ SS containing T7 transcription plasmid was prepared by insertion of a fragment of rabbit β-globin gene between the restriction sites for Apa I (position 1022) and Bgl II (position 1196) into the Bluescript plasmid which was opened at the Pst I and Hinc II sites after creation of appropriate ends. Before transcription the construct was linearized with Eco RI at position 1116. All restriction site positions refer to the sequencing data of van Ooyen *et al.* [A. van Ooyen, J. van den Berg, N. Mantei, C. Weissmann, *Science* **206**, 337 (1979)].
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Mechanism of Insect Resistance to the Microbial Insecticide *Bacillus thuringiensis*

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Receptor binding studies show that resistance of a laboratory-selected *Plodia interpunctella* strain to a *Bacillus thuringiensis* insecticidal crystal protein (ICP) is correlated with a 50-fold reduction in affinity of the membrane receptor for this protein. The strain is sensitive to a second type of ICP that apparently recognizes a different receptor. Understanding the mechanism of resistance will provide strategies to prevent or delay resistance and hence prolong the usefulness of *B. thuringiensis* ICPs as environmentally safe insecticides.

PROSPECTS FOR THE FUTURE OF MICROBIAL insecticides are bright. In addition to conventional applications, recent advances in plant transformation with ICP genes from the entomopathogenic bacterium *Bacillus thuringiensis* (*1*) provide an exciting new approach to insect control in which transgenic plants produce their own protective proteins. Moreover, increasing limitations on the use of chemical pesticides stimulate interest in alternative strategies in pest control. Obviously, the extent to which insects are able to develop resistance to *B. thuringiensis* ICPs will be an essential determinant for the continued success of these insecticides. Significant resistance to *B. thuringiensis* has not been observed during its use over more than three decades. However, in laboratory selection experiments, high levels of resistance were obtained in the Indian meal moth (*Plodia interpunctella*) (*2*). In strains selected by rearing on a *B. thuringiensis*–treated diet, the median lethal dose (LD_{50}) increased to as much as 250 times those of the sensitive parent strains (*3*). Subsequently, lower levels of resistance have been selected in the almond moth (*Cadra cautella*) (*3*) and the tobacco budworm (*Heliothis virescens*) (*4*). Conventional applications of *B. thuringiensis* are currently increasing and commercialization of transgenic crops expressing ICPs is envisaged within a few years. Thus, the challenge is to develop deployment strategies that will minimize the potential for development of field resistance (*5*). Therefore, it is critical to understand the mechanism involved in pest resistance to these toxins.

We investigated the mechanism of resistance to *B. thuringiensis* ICPs in a *P. interpunctella* strain (strain 343) selected for a high level of resistance against Dipel, a commercial formulation of a crystal-spore mixture of *B. thuringiensis* var. *kurstaki* (*2*). It has been shown that this *P. interpunctella* strain did not exhibit similar levels of resistance to certain other *B. thuringiensis* strains (*6*). In this context, it should be noted that crystals of most *B. thuringiensis* strains contain more than one type of ICP. Different classes of ICPs exhibit striking differences in their insecticidal spectrum (*7, 8*) and may bind to distinct receptor sites (*9*), presumably of glycoprotein nature (*10, 11*). We have taken into account this substantial heterogeneity in *B. thuringiensis* ICPs by studying two distinct ICPs. Two essential factors in the pathway of toxic action of *B. thuringiensis* ICPs are proteolytic activation of the protoxin (around 130,000 daltons) to the active toxin (around 66,000 daltons) (*12*) and binding of the toxin to receptors on the brush border membrane of the midgut epithelium (*9–11*). The contribution of receptor binding and protease activity in the mechanism of resistance has been studied.

Crystals of Dipel preparations consist of a mixture of ICPs belonging to the CryIA and to the CryII family (*7*). We compared resistance levels to Dipel and cloned ICPs of the CryIA(b) type [Bt2, from *B. thuringiensis* var. *berliner* 1715 (*13*)] and the CryIC type [Bt15, from *B. thuringiensis* var. *entomocidus* HD110 (*14*)]. Resistance levels to both the protoxin and the activated toxin were determined. Resistance to Dipel was confirmed by the toxicity data for the sensitive strain (S strain) and for the strain selected for Dipel resistance (R strain) (Table 1). High levels of resistance are also observed for CryIA(b) protoxin and CryIA(b) toxin. Since similar resistance levels were obtained with CryIA(b) protoxin and in vitro–activated CryIA(b) toxin, resistance is apparently not due to lack of proteolytic activation of protoxin in the midgut of the R strain. In contrast to the ICP of the CryIA(b) type, there is no resistance to CryIC protoxin and CryIC toxin. This ICP is not present in Dipel crystals. Moreover, we observed a marked increase in sensitivity to CryIC pro-

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toxin and CryIC toxin in the R strain.

Autoradiography of labeled toxin incubated with brush border membrane vesicles revealed unaltered protein profiles and showed that resistance is not correlated with toxin degradation by membrane-associated proteases (*15*). Also, evidence is available that soluble midgut proteases are not involved (*16*). Consequently, we concluded that proteolytic activity does not contribute to resistance.

To investigate whether a change in toxin-membrane binding was responsible for resistance, we performed receptor binding studies with ^{125}I-labeled CryIA(b) toxin and CryIC toxin with brush border membrane vesicles derived from larval midguts of the R and S strains (Table 2). High-affinity, saturable binding of CryIA(b) toxin to membranes of the S strain was observed. A dissociation constant (K_d) of 0.72 ± 0.26 n*M* and a binding site concentration (R_t) of 1.44 ± 0.35 pmol per milligram of membrane protein was calculated (n = 6). LIGAND analysis (*17*) indicated that the best fit to the data was obtained with a single-site model. When similar experiments were done with the R strain, increased concentrations of labeled toxin and of vesicles were required in the binding assay in order to obtain measurable levels of toxin binding. Competition experiments revealed a 50-fold reduction in binding affinity (K_d = 36.3 ± 22.7 n*M*) whereas R_t remained virtually unchanged (1.77 ± 0.58 pmol per milligram of membrane protein) (n = 6).

Binding studies were also performed with CryIC toxin. In the S strain, two populations of binding sites were demonstrated with the following characteristics: K_{d1} = 0.31 ± 0.12 n*M*, R_{t1} = 0.38 ± 0.07 pmol per milligram of membrane protein, K_{d2} = 154 ± 108 n*M*, R_{t2} = 6.17 ± 3.8 pmol per milligram of membrane protein (n = 7) (where the subscripts 1 and 2 refer to the first and second binding sites, respectively). For all seven experiments, a two-site model gave a significantly better fit than a one-site model ($P < 0.05$ for two experiments, $P < 0.005$ for five experiments). High-affinity binding of CryIC toxin was also shown in the R strain. Calculated values are K_d = 0.18 ± 0.07 n*M* and R_t = 1.15 ± 0.20 pmol per milligram of membrane protein (n = 7). Best fits were obtained with a single-site model. Thus, in parallel with the higher sensitivity of the R strain to CryIC toxin as compared to the S strain, the binding of this toxin is increased in the R strain. The increase in R_t is statistically significant at $P < 0.001$ (t test). The difference in K_d for the high-affinity site in the two strains is only marginally significant (P = 0.029).

Mutual competition experiments (Fig. 1) demonstrated that CryIC toxin could displace all saturably bound ^{125}I-labeled CryIA(b) toxin in the S strain with an affinity of 237 ± 145 n*M* (n = 2). This value is not significantly different from the mean K_{d2} of CryIC toxin, which suggests that the low-affinity site for CryIC toxin in the S strain represents the CryIA(b) toxin binding site. We assume that this site, which in the R strain exhibits a 50-fold reduction in affinity for CryIA(b) toxin, would also display a reduced affinity for the CryIC toxin. This would result in a very low affinity, explaining why a second site for CryIC toxin is not detected in the R strain. We did not observe any significant displacement of bound ^{125}I-labeled CryIC toxin by unlabeled CryIA(b) toxin. Indeed, only very low displacement would be expected as ^{125}I-labeled CryIC toxin binding to the low-affinity site represents only a minor fraction of total binding (less than 10%).

Table 2. Binding characteristics of *B. thuringiensis* ICPs to brush border membrane vesicles from sensitive and resistant *P. interpunctella* (*19, 20*).

ICP	Strain	K_{d1} (n*M*)	R_{t1} (pmol/mg protein)	K_{d2} (n*M*)	R_{t2} (pmol/mg protein)
CryIA(b) toxin	Sensitive	0.72 (±0.26)	1.44 (±0.35)		
	Resistant	36.3 (±22.7)	1.77 (±0.58)		
CryIC toxin	Sensitive	0.31 (±0.12)	0.38 (±0.07)	154 (±108)	6.17 (±3.79)
	Resistant	0.18 (±0.077)	1.15 (±0.20)		

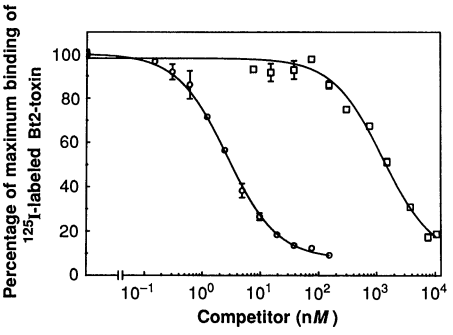

Fig. 1. Binding of ^{125}I-labeled CryIA(b) toxin to membranes of the sensitive *P. interpunctella* strain as a function of increasing concentrations of competitor: CryIA(b) toxin (circles), CryIC toxin (squares). Procedures for the binding assay were as in Table 2 (*19*). Data points represent the mean of duplicate measurements. Error bars representing the range are given when larger than data points.

Our data provide evidence that resistance in this *P. interpunctella* strain is due to an alteration in toxin-membrane binding. Resistance to CryIA(b) toxin is correlated with a reduction in affinity of CryIA(b) toxin binding, whereas increased sensitivity to CryIC toxin is reflected in an apparent increase of the CryIC binding site concentration. It remains to be seen whether similar mechanisms will occur in other insects exhibiting resistance to *B. thuringiensis* ICPs.

It is surprising that apparently two distinct molecular changes have occurred in *P. interpunctella* in parallel with resistance to *B. thuringiensis*. The increase in CryIC toxin sensitivity was not observed in a strain that was less resistant to Dipel. Assuming that the toxin receptor is a physiologically important molecule in the midgut, a possible explanation could be that the CryIA(b) toxin receptor not only has reduced affinity for

Table 1. Toxicity of *B. thuringiensis* ICPs to sensitive and resistant *P. interpunctella* (*18*). Values given are the LD_{50} (the dose required to kill 50% of the insects tested) and the 95% confidence intervals (CI_{95}) (in parentheses).

ICP	LD_{50} (microgram per larva) of *P. interpunctella* strain*	
	Sensitive	Resistant
Dipel	1.21 (0.88–1.84)	>30†
CryIA(b) protoxin	0.12 (0.08–0.18)	>12.8‡
CryIA(b) toxin	0.03 (0.02–0.05)	26.3§ (11.8)
CryIC protoxin	0.20 (0.14–0.30)	0.02 (0.00–0.04)
CryIC toxin	0.11 (0.08–0.15)	0.03 (0.00–0.07)

*Resistance was induced by rearing colony 343 on a diet treated with Dipel at 500 mg per kilogram of body weight after nine generations of selection at 62.5 mg per kilogram of diet (*2, 3*). †LD_{50} could not be estimated: less than 10% response at maximum dose (30 μg per larva) tested. ‡LD_{50} could not be estimated: less than 16.7% response at maximum dose (12.8 μg per larva) tested. §Estimated value: 45% response at maximum dose (12.3 μg per larva) tested.

the toxin, but is also functionally affected. This loss is compensated for by an increase in the concentration of the CryIC receptor after several generations of selection.

Strategies for resistance management are needed to extend the lifetime of chemical insecticides. It is equally important to implement such strategies with *B. thuringiensis* to maintain its usefulness as a safe and environmentally sound insect control agent. Our results provide a basis on which to build such strategies. When different ICPs are available with activity against the same insect species, resistance to one ICP does not always imply resistance against other ICPs. ICPs with different binding properties could be used in combination or sequentially for greater effectiveness or to delay resistance.

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18. Cubes (2 mm) were cut from dehydrated apple slices and placed singly into each well of a 24-well tissue culture plate. Toxin solution (2 μl) (dilutions ranging from 0.001 mg/ml to 10 mg/ml) was applied to each apple cube, which was then allowed to dry. A single second or third instar larva was placed into each well and the plates were covered and incubated at 25°C with 60% relative humidity. Larvae that consumed the toxin-coated apple cubes were subsequently fed normal diet (*2*) and were maintained until death or pupation. Larval mortality was scored every 4 to 5 days over a period of 20 to 25 days. Twelve or more larvae were used per dose. LD_{50} and CI_{95} for each toxin were calculated by a probit analysis program (written by G. A. Milliken, Kansas State University, Manhattan, KS). Protoxin purification and activation was performed as described in (*13*). Purification of the toxic fragment was done according to the method described by Hofmann *et al.* (*9*).
19. Duplicate samples of ^{125}I-labeled toxin (*20*), either alone or in combination with varying concentrations of unlabeled toxin, were incubated at room temperature with brush border membrane vesicles in a total volume of 100 μl tris buffer (20 m*M* tris and 150 m*M* NaCl, *p*H 7.4) with 0.1% bovine serum albumin (BSA). Vesicles were prepared according to the method of M. Wolfersberger *et al.* [*Comp. Biochem. Physiol.* **86A**, 301 (1987)]. In experiments with CryIA(b) toxin on the S strain, mixtures were incubated for 60 min; all other experiments were done with a 90-min incubation period. In experiments with ^{125}I-labeled CryIA(b) toxin (0.37 n*M*, S strain; 32 n*M*, R strain), vesicle concentrations of 100 and 200 μg of membrane protein per milliliter of S and R strain, respectively, were used. Experiments were set up with 12 concentrations of unlabeled ligand (from 0 to 10 μg/ml, S strain; 0 to 300 μg/ml, R strain). In experiments with ^{125}I-labeled CryIC toxin (0.15 n*M*), vesicle concentrations of 150 and 70 μg of membrane protein per milliliter of the S and R strain, respectively, were used. Twenty-one concentrations of unlabeled ligand were used (from 0 to 100 μg/ml). Bound toxin was quantitated by ultrafiltration through Whatman GF/F glass-fiber filters. Each filter was rapidly washed with 5 ml of ice-cold tris buffer with 0.1% BSA. Binding data were analyzed with the LIGAND computer program (*17*). Molar R_t values were transformed to picomoles per milligram of protein, on the basis of the amount of vesicle protein [M. M. Bradford, *Anal. Biochem.* **72**, 248 (1976)]. Two or three binding experiments were performed on each of three independently prepared batches of membrane vesicles from both S and R strains. No significant differences in K_d or R_t were found between batches (one-way analysis of variance). Therefore, only the mean values and the standard deviations for the total set of repeat experiments are presented.
20. CryIA(b) toxin was labeled with chloramin T, essentially as described by W. M. Hunter and F. C. Greenwood [*Nature* **194**, 495 (1962)] to a specific radioactivity of 155,100 Ci/mol. CryIC-toxin was labeled with the Iodogen method (*9*); a specific radioactivity of 462,990 Ci/mol was obtained. Specific activity of iodinated toxins was determined with a "sandwich" enzyme-linked immunosorbent assay (ELISA) technique [A. Voller, D. E. Bidwell, A. Barlett in *Manual of Clinical Immunology*, N. R. Rose and H. Friedman, Eds. (American Society of Microbiology, Washington, DC, 1976), pp. 506–512].

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Derepression of Ferritin Messenger RNA Translation by Hemin in Vitro

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Incubation of a 90-kilodalton ferritin repressor protein (FRP), either free or complexed with an L-ferritin transcript, with hemin or Co^{3+}-protoporphyrin IX prevented subsequent repression of ferritin synthesis in a wheat germ extract. Neither $FeCl_3$ in combinations with H_2O_2, nor Fe^{3+} or Fe^{2+} chelated with EDTA, nor Zn^{2+}-protoporphyrin IX, nor protoporphyrin IX caused significant inactivation of FRP. FRP that had been inactivated by hemin remained chemically intact, as revealed by SDS–polyacrylamide gel electrophoresis. Inclusion of chelators of iron or free radical scavengers did not alter the inactivation produced by hemin. These and other results indicate that hemin derepresses ferritin synthesis in vitro.

FERRITINS ARE IRON STORAGE PROteins that are found in virtually all cells (*1*). Their synthesis in vertebrates is regulated in response to the extracellular iron supply at the translational level (*2*). Kinetic evidence suggests that a significant number of other vertebrate mRNAs are similarly regulated (*3*). The ferritin translational regulatory machinery consists of a conserved sequence of 28 nucleotides [the iron-responsive element (IRE)] in the mRNA 5′ untranslated region (5′ UTR), which makes translation responsive to iron (*4*), and a 90-kD ferritin repressor protein (FRP) that binds to the IRE in the paucity of iron (*5–9*).

We now describe properties of a third regulatory component, the "ferritin inducer." The ferritin inducer should be a metabolite whose concentration rises and falls in response to the rate of iron uptake, and which interacts with the FRP, either directly or indirectly, in such a way as to relieve the repression of ferritin mRNA translation. Evidence is presented in this report that hemin fulfills these criteria, and therefore may be a ferritin inducer.

To study the effect of iron compounds on ferritin synthesis, FRP was ordinarily incubated with hemin, or analogs thereof, in a redox buffer made up of glutathione plus oxidized glutathione (termed GSB) for various times and temperatures. Wheat germ lysate, L-ferritin and apolipoprotein A-1 transcripts, [^{35}S]methionine, and other

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