Altered Binding of the Cry1Ac Toxin to Larval Membranes but not to the Toxin-Binding Protein in *Plodia interpunctella* Selected for Resistance to Different *Bacillus thuringiensis* Isolates

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Immunoblotting and cytochemical procedures were used to determine whether toxin binding was altered in strains of the Indianmeal moth, *Plodia interpunctella*, selected for resistance to various strains of *Bacillus thuringiensis*. Each of these *B. thuringiensis* subspecies produces a mixture of protoxins, primarily Cry1 types, and the greatest insect resistance is to the Cry1A protoxins. In several cases, however, there was also resistance to toxins not present in the *B. thuringiensis* strains used for selection. The Cry1Ab and Cry1Ac toxins bound equally well over a range of toxin concentrations and times of incubation to a single protein of ca. 80-kDa in immunoblots of larval membrane extracts from all of the colonies. This binding protein is essential for toxicity since a mutant Cry1Ac toxin known to be defective in binding and thus less toxic bound poorly to the 80-kDa protein. This binding protein differed in size from the major aminopeptidase N antigens implicated in toxin binding in other insects. Binding of fluorescently labeled Cry1Ac or Cry1Ab toxin to larval sections was found at the tips of the brush border membrane prepared from the susceptible but not from any of the resistant *P. interpunctella*. Accessibility of a major Cry1A-binding protein appears to be altered in resistant larvae and could account for their broad resistance to several *B. thuringiensis* toxins.

Bacillus thuringiensis produces protein inclusions during sporulation which are comprised of protoxins active on insects from at least three orders: Lepidoptera, Coleoptera, and Diptera (1). After ingestion, the protoxins are processed to toxins which bind with high affinity to receptors located on the insect's midgut membrane (10, 12, 24). This reversible binding is followed by what appears to be a more specific insertion of the toxin into the membrane (5, 11). An ion channel is then formed (or perhaps an existing one is altered) resulting in the disruption of the osmotic balance leading to feeding paralysis, cell lysis, and eventually death (1).

Insects resistant to *B. thuringiensis* toxins have been found in fields where commercial *B. thuringiensis* preparations have been used and have also been isolated in the laboratory (6). Both a narrow spectrum of resistance to a specific toxin, Cry1Ab or a related Cry1A toxin, as well as broad-spectrum resistance to many toxins which differ significantly in specificity (8, 19) have been reported. For example, selection for resistance of *Plodia interpunctella* (Indianmeal moth) to Dipel, a commercial formulation comprised of *B. thuringiensis* subsp. *kurstaki* HD1 (containing the *cryIAa*, *cryIAb*, *cryIAC*, *cry2A*, and *cry2B* genes), resulted in a >100-fold resistance to the Cry1Ab toxin, considerable resistance to the closely related Cry1Ac toxin but no resistance to the Cry1C toxin (28). In this case, resistance was primarily to toxins present in the subspecies used for the selection. Resistance was correlated with decreased binding of the Cry1Ab toxin to vesicles prepared from late instar larvae of the resistant insects whereas the binding of the Cry1C toxin was unaltered (29). In other cases, however, it was found that the spectrum of resistance in colonies of *P. interpunctella* selected with *B. thuringiensis* subspecies *aizawai* or *entomocidus* was much broader (18, 19). Colonies selected for resistance to *B. thuringiensis* subsp. *aizawai* HD133, *B. thuringiensis* subsp.

aizawai HD112, or *B. thuringiensis* subsp. *entomocidus* HD198 (all contain the *cryIAa*, *cryIAb*, *cryIC*, and *cryID* genes; *B. thuringiensis* subsp. *aizawai* HD112 also contains the *cryIG* and *cry2* genes) resulted in different extents of resistance to the Cry1Ab, Cry1C, Cry1Ac, Cry1B, and Cry2A toxins even though these strains do not produce the latter three toxins.

Insect resistance has been proposed to be due to altered protoxin activation by midgut proteases or altered binding of a specific toxin to midgut receptors (6, 28). In vitro binding to larval brush border membrane vesicles (BBMV) (10, 27), ligand blot analyses (7, 22), and tissue sectioning (4) have been used to identify toxin receptors. In many cases, there is a correlation between high-affinity binding and susceptibility to the particular *B. thuringiensis* toxin (4, 27) but there are exceptions (11, 23, 30). For example, two Cry1A toxins with very different activity for *Bombyx mori* bound with equal affinity to the same receptor, but only the more effective toxin displayed efficient irreversible binding (11). Loss of toxin binding was also correlated with resistance in some but not all cases (6, 15, 17). As noted previously, membrane insertion of these toxins involves a reversible binding step which is followed by irreversible insertion (5, 11), and the latter may be more specific (11). A defect in either step could result in resistance.

As mentioned above for *P. interpunctella* and as has also been found for other insects, the pattern of resistance varies from rather selective for the toxins (or *B. thuringiensis* subspecies) used (6, 28) to a rather broad cross-resistance including toxins not present in a particular isolate (6, 19, 25). Since a defect in the binding of the Cry1Ab toxin to midgut vesicles has been reported for the Dipel colony of *P. interpunctella* (28), the initial plan was to do similar binding studies with vesicles from the other resistant colonies. This was technically not feasible because of the very small size of these larvae and the difficulty of obtaining adequate quantities of midguts for the preparation of vesicles. A number of procedures were attempted but the presumptive vesicle fraction was very unstable, as indicated

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by the presence in vesicle extracts of degraded toxin-binding proteins in immunoblots (21). We were able, however, to examine the binding of toxins to midgut tissue sections (cytochemical analysis) and to proteins in immunoblots extracted from a vesicle fraction prepared from intact larvae. While there was loss of binding to midgut sections from resistant larvae, there were no detectable differences in the binding of the Cry1A toxins in immunoblots. An alteration in a membrane component with pleiotropic effects rather than changes in the toxin receptor per se would account for this difference as well as the broad pattern of cross-resistance in most of these colonies.

MATERIALS AND METHODS

Experimental insects. Colonies of the Indianmeal moth, *P. interpunctella* (Hubner), used in these studies were described by McGaughey and Johnson (18, 19). *P. interpunctella* RC688⁺ (wild type) was collected from a farm grain storage bin in Riley County, Kans., in 1988. Colony RC688⁺ had no known previous exposure to *B. thuringiensis*. *B. thuringiensis*-resistant colonies, designated Dipel^r, 133^r, 198^r, and 112^r, were derived from colony RC688⁺ by selection in the laboratory for resistance to the different *B. thuringiensis* spore-crystal preparations (18). Dipel^r (*B. thuringiensis* subsp. *kurstaki* HD1 [Dipel]) contains the *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry2A*, and *cry2B* genes; 133^r (*B. thuringiensis* subsp. *aizawai* HD133) contains the *cry1Aa*, *cry1Ab*, *cry1C*, and *cry1D* genes; 198^r (*B. thuringiensis* subsp. *entomocidus* HD198) contains the *cry1Aa*, *cry1Ab*, *cry1C*, and *cry1D* genes; and 112^r (*B. thuringiensis* subsp. *aizawai* HD112) contains the *cry1Aa*, *cry1Ab*, *cry1C*, *cry1D*, and *cry1G* genes plus a *cry2* gene.

Eggs from susceptible or resistant colonies were shipped overnight from the U.S. Grain Marketing Research Laboratory, USDA Agricultural Research Service, Manhattan, Kans. The eggs were shipped in mason jars on the standard laboratory larval diet of ground wheat, with or without the *B. thuringiensis* spore and crystal preparations mentioned above (20). The eggs were kept at 25°C and 60 to 70% relative humidity for hatching and subsequent larval growth.

Isolation and purification of protoxin inclusions and trypsin activation of the toxin. *B. thuringiensis* subsp. *kurstaki* HD73 was grown on G-Tris plates (3) at 30°C for 30 h. Spores and inclusions were scraped from the plates, washed with 5 ml of 1 M KCl–5 mM EDTA, and then washed twice with 20 ml of distilled water. Inclusions were separated from spores by centrifugation in Renografin gradients (3). Protoxin was solubilized and digested with trypsin, and the toxin was purified (31).

BBMV preparations. We used the method of Wolfersberger et al. (29) with some modifications to prepare brush border membrane fractions from *P. interpunctella*-susceptible and -resistant larvae. Twenty-five third-instar larvae were collected in Eppendorf tubes, immediately immersed in liquid nitrogen, and stored at –80°C until used. Frozen larvae were ground in a Broeck homogenizer in 1 ml of ice-cold buffer A {0.3 M mannitol, 5 mM EGTA [ethylene glycol-bis(beta-aminoethyl ether)-N,N',N'-tetraacetic acid], 20 mM Tris-HCl, pH 7.5}. The homogenate was adjusted to 12 mM MgCl₂ in a total volume of 2 ml. The suspension was allowed to stand on ice for 20 min and was then centrifuged at 3,000 × g for 10 min at 4°C. The supernatant was removed and centrifuged at 14,100 × g in a microcentrifuge for 10 min at 4°C. The pellet was resuspended in buffer A and kept on ice. The protein content was determined by the binding of Coomassie brilliant blue G-250 (Bio-Rad) with bovine serum albumin as standard. The resuspended pellet was distributed in sterile Eppendorf tubes and immediately immersed in liquid nitrogen. Samples were stored at –80°C. This procedure is similar to that used with *Plutella xylostella* fifth-instar larvae (16), and an 8- to 10-fold enrichment for leucine aminopeptidase activity was also obtained. In addition, a small quantity of BBMV was prepared directly from dissected midguts of fourth-instar larvae for comparative purposes. As noted previously, it was very difficult to obtain even a limited quantity of these BBMV, and they were not very stable.

Polyclonal antibody preparation. Antibodies against the Cry1Ac toxin were raised in New Zealand White rabbits. Preimmune serum was collected 2 weeks before immunization. About 200 µg of Cry1Ac toxin was suspended in an equal volume of Freund's complete adjuvant and injected subcutaneously. Booster injections of 200 µg of toxin in incomplete Freund's adjuvant were given at 6 weeks, 8 weeks, and every other week thereafter until the desired titers were reached. Immune serum was collected and incubated in a 37°C water bath for 30 min and then placed at 4°C overnight. The serum was separated from whole blood by centrifugation at 5,000 × g for 30 min., aliquoted, and stored at –20°C (9). The immunoglobulin G (IgG) fraction was purified on a protein A-Sepharose column according to the manufacturer's instructions (Pharmacia LKB Biotechnology). A midgut affinity column was used to reduce nonspecific binding of the antibody. Five milligrams of *P. interpunctella* midgut extract from a sensitive colony was coupled to cyanogen bromide-activated Sepharose 4B (Sigma). Column preparation and antibody purification were as described by Harlow and Lane (9).

Immunoblot analysis. For solubilization, 1 ml of the BBMV was suspended in

1 ml of 10 mM octylglucopyranoside–5 mM EGTA–10 mM Tris-HCl (pH 7.5), and centrifuged in an Eppendorf microfuge for 5 min. An equal volume of loading buffer (50 mM Tris-HCl, 2% sodium dodecyl sulfate [SDS], 100 mM dithioerythritol [pH 7.5]) was added to the supernatant, and the samples were heated at 95°C for 3 min. Proteins were fractionated on an SDS–10% polyacrylamide gel by using the buffer system of Laemmli (13) and transferred (26) to an Immobilon-P membrane by using a Hoefer TE 50 (Hoefer Scientific Instrument) electroblot apparatus for 40 min at 27°C. The membrane was washed twice for 5 min each time in Tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl [pH 7.5]) containing 0.2% Tween 20 (TBST). In order to block nonspecific binding, the membrane was incubated overnight at 4°C in Tris-buffered saline with 1% nonfat dry milk. Blots were then incubated for 1 h with a range of 0.1 to 1.0 µg of toxin in 10 ml of TBS plus 0.1% bovine serum albumin. Unbound toxin was removed by washing the blots three times in 50 ml of TBST. Bound Cry1Ac toxin was identified with purified polyclonal anti-Cry1Ac IgG diluted 1:10,000 in 1% nonfat dry milk in TBST. After a 1-h incubation, unbound antibody was removed by washing the blots three times (5 min each time) with TBST. The blots were then incubated with goat anti-rabbit IgG alkaline phosphatase conjugate, diluted 1:7,500, for 1 h at 27°C and in Western Blue-stabilized substrate for alkaline phosphatase (Promega) to visualize the binding protein-toxin-antibody complex.

Tissue preparation and sectioning. Tissues were prepared and sectioned as described by Bravo et al. (4) with some modification. Late third-instar larvae from susceptible and resistant insects were chilled on ice for 30 min, and the midguts were extruded by dissection under a binocular microscope at 4°C. Peritrophic membranes and Malpighian tubules were removed. The midguts were immediately immersed in Bouin Holland's 10% sublimite fixative, left in the fixative for 30 min, and then placed in a fresh solution for 5 days. The tissues were washed overnight in distilled water; dehydrated twice for 1 h in each of the following: 70% ethanol, 95% ethanol, 100% ethanol, xylol-ethanol 50/50 (vol/vol), and 100% xylol; and then dehydrated for 12 h at 56°C in xylol-Paraplast (Sigma) 50/50 (vol/vol). The tissues were then incubated at 56°C in 100% Paraplast twice for 24 h each time and then embedded in Paraplast. Transverse sections (5-µm thick) were cut with a Spencer microtome and attached to mounting glass slides which had been coated with a 0.1% polylysine solution.

Fluorescent labeling of the Cry1Ac toxin. Purified Cry1Ac toxin was labeled with fluorescein isothiocyanate (FITC) as per the manufacturer's directions (Molecular Probes, Inc.). Five micrograms of toxin was dissolved in 1 ml of 0.1 M NaHCO₃. FITC was dissolved in dimethyl sulfoxide at a concentration of 10 mg/ml immediately before starting the reaction, and 50 µg was added slowly to the protein while stirring. The mixture was incubated for 1 h at 27°C with stirring, and the coupling was terminated by adding 0.1 ml of freshly prepared 1.5 M hydroxylamine, pH 8.0. The mixture was incubated an additional 30 min, and the unreacted labeling reagent was separated from the conjugate by dialysis against three changes of 2 liters each of Tris-buffered saline (0.02 M Tris-HCl, 0.2 M NaCl [pH 8.7]). The labeled toxin was then aliquoted and stored at –80°C until used. The FITC-labeled toxin was as toxic to *Manduca sexta* as unlabeled toxin (8).

Binding of toxin to tissue sections. Glass mounts with tissue sections from susceptible and resistant strains of *P. interpunctella* were deparaffinated and hydrated as follows (4): twice for 5 min each in 100% xylol, three times in 100% ethanol for 3 min each, and then once for 1 min in distilled water. The slides were then immersed twice for 2 min each in 0.5% iodine–1% KI and once for 2 min in 5% sodium thiosulfate to remove the sublimate. The sections were washed in distilled water and equilibrated in TSTB (10 mM Tris-HCl, 150 mM NaCl, 1 mM thimerosal [pH 7.6], 0.1% Triton X-100) for 5 min. The glass mounts with tissue sections were covered with 0.5 ml of 5-µg/ml FITC-Cry1Ac toxin solution and incubated for 1 h at 27°C in a moist chamber in the dark. The sections were washed in TSTB for 1 min to remove all unbound toxin and then mounted with Vectashield mounting medium (Vector Laboratories) which contains an anti-fade. The sections were viewed under an Axiophot photomicroscope (Carl Zeiss, Inc., Thornwood, N.Y.). At least three different batches of larvae were used to prepare sections and in each case, a minimum of 20 sections were scanned.

Reaction of aminopeptidase N antibody with larval proteins. Anti-aminopeptidase N antibody to the *B. mori* larval enzyme was sent courtesy of Donald Dean, Ohio State University. The polyclonal antibody had been raised in rabbits after injection with purified aminopeptidase N from *B. mori*. Solubilized BBMV from *M. sexta* and *P. interpunctella* were fractionated in duplicate by SDS-polyacrylamide gel electrophoresis (PAGE), and the gel contents were transferred to an Immobilon-P membrane as described above. One portion of the membrane was incubated with the polyclonal antibody against aminopeptidase N (serum diluted 1:10,000 in 1% nonfat dry milk in TBST). The second portion was incubated with Cry1Ac toxin and then treated with the anti-Cry1Ac antibody. Both portions of the membrane were visualized as described above.

RESULTS

Identification of a Cry1Ac-binding protein. Because of the difficulty of preparing adequate membrane vesicles from *P. interpunctella* larvae, binding of the Cry1Ab and Cry1Ac toxins to *P. interpunctella* RC688⁺ and resistant strains Dipel^r, 133^r, 198^r,

FIG. 1. Detection of the Cry1Ac-binding protein from various colonies of *P. interpunctella*. Larval extracts in lanes 2 to 11 were fractionated on an SDS-10% polyacrylamide gel and transferred to an Immobilon-P membrane for binding as described in Materials and Methods. Lanes 2 to 6 were incubated only with Cry1Ac antibody, whereas lanes 7 to 11 were incubated with 0.6 μ g of Cry1Ac toxin and then the antibody. Lanes 1 and 12, Cry1Ac toxin alone; lane 13, molecular mass standards [myosin (200 kDa), β -galactosidase (116 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa)]; lanes 2 and 7, extracts from HD112^r; lanes 3 and 8, extracts from HD198^r; lanes 4 and 9, extracts from HD133^r; lanes 5 and 10, extracts from Dipel^r; lanes 6 and 11, extracts from RC688^r.

and 112^r was investigated initially by immunoblotting (7, 22). Solubilized BBMVs (40 μ g of total protein per lane) were electrophoresed, transferred to Immobilon-P membranes, and incubated with the Cry1Ac (Fig. 1) or Cry1Ab toxin (21). In both cases, these toxins bound only to an 80-kDa protein. Neither the small variations in binding intensities in Fig. 1 nor the apparent doublet band in lane 8 were reproducible. The amount of toxin added to the blots was varied from a concentration in which there was less than maximal binding to the wild-type blot (0.1 μ g) to a saturating concentration (1.0 μ g/ml). No differences in binding intensities were detected over this range of toxin concentrations, indicating that the affinity of this major binding protein for toxin (at least in immunoblots) did not differ between the wild type and the various mutants. When the toxin or the antibody was omitted there was a weak band of the same molecular weight as the toxin (Fig. 1), probably due to a small amount of the toxins present in the selective diets binding to the larvae and copurifying with the BBMVs from isolated midguts for immunoblotting, and similar results were obtained.

Since only a single Cry1Ac-binding protein was found, it is likely to be involved in the initial reversible binding of this toxin. Further evidence was provided by examination of a mutant Cry1Ac toxin (S504I) which was demonstrated to be defective in such binding and thus less toxic for *M. sexta* and *Heliothis virescens* (2). There was little binding of the S504I toxin to the 80-kDa protein (Fig. 2) as well as lower toxicity for *P. interpunctella*. There was only very weak binding of the Cry1C toxin to several bands both larger and smaller than the 80-kDa binding protein and no differences among the strains (21).

Binding of FITC-Cry1Ac toxin to *P. interpunctella* tissue sections. The presence in BBMVs extracts from the Dipel^r colony of an 80-kDa protein which bound the Cry1Ab and Cry1Ac toxins with about the same affinity as in the wild type was surprising given the much poorer binding of the former

toxin to BBMVs from these insects (28). In order to study the binding of toxin to intact larval membranes, FITC-Cry1Ac toxin was incubated with sections of larvae prepared from *P. interpunctella*-sensitive and -resistant colonies as well as to sections from *M. sexta* larvae. Toxin binding was detected at the tips of the brush border of susceptible *P. interpunctella* RC688^r (Fig. 3a) but not that of the resistant larvae (Fig. 3b and c). In both sensitive and resistant colonies, there was some binding of the toxin to the peritrophic membrane but not to any other tissues. A magnification of the apical microvilli from Fig. 3a shows fairly extensive coverage by FITC-Cry1Ac toxin (Fig. 4). Sections of *M. sexta* midguts served as a positive control (Fig. 3d). The much more extensive labeling of *M. sexta* was due to the larger larvae and thus greater surface area of the epithelium in these sections with perhaps a higher concentration of the Cry1Ac-binding protein. The indirect method for detecting toxin binding employing antibodies (4) was found to be less sensitive.

Binding of an aminopeptidase N antibody. In ligand blots, the Cry1Ac toxin binds to a 120-kDa protein in *M. sexta* BBMVs (7) which is the enzyme aminopeptidase N (12, 24). In confirmation of these results, an antibody to *B. mori* aminopeptidase N bound to the 120-kDa Cry1Ac toxin-binding protein from brush border membranes of *M. sexta* (Fig. 5, lanes 1 and 6). This antibody also bound to proteins in extracts of the *P. interpunctella*-sensitive and -resistant colonies. There were major reactive bands of ca. 120 kDa and a relatively minor band of ca. 90 kDa (Fig. 5).

DISCUSSION

There were no detectable differences in immunoblots in the binding of the Cry1Ab or Cry1Ac toxins to *P. interpunctella* proteins extracted from wild type or various resistant larvae over a range of toxin concentrations and times of incubation. The binding was to a ca. 80-kDa protein which differed in size from the major aminopeptidase N antigen (ca. 120 kDa) but is very close in size to a minor antigen (Fig. 5). The aminopeptidase N antigens were detected with a *B. mori* antibody which reacted with the Cry1Ac-binding protein from *M. sexta* so the cross specificity is adequate. This 80-kDa protein is very likely to be the toxin receptor since (i) it was the only major binding protein detected and (ii) a mutant toxin with lower toxicity for other Lepidoptera due to defective binding (2) had the same properties with *P. interpunctella*.

In all of these colonies, there was a high level of resistance to the Cry1Ac toxin and substantial resistance to Cry1Ab (19).

FIG. 2. Binding of the wild-type Cry1Ac toxin and mutant toxin S504I to immunoblots as described in the legend for Fig. 1. Lane 1, 0.3 μ g of Cry1Ac toxin; lanes 2 and 4, binding of 1.0 and 0.3 μ g of wild-type toxin; lanes 3 and 5, binding of 1.0 and 0.3 μ g of S504I toxin. The major 80-kDa binding protein is indicated by the arrow. Other minor binding bands of higher molecular mass were not reproducibly found. The short marks on the right are molecular mass standards β -galactosidase, bovine serum albumin, and ovalbumin (from top to bottom).

FIG. 3. Binding of FITC-CryIAc toxin to tissue sections from *P. interpunctella*-susceptible RC688⁺ (a), Dipe1⁺ (b), HD133⁺ (c), and *M. sexta* (d) larvae. The toxin bound to apical microvilli throughout the midgut of the susceptible insect as indicated by the arrow in panel a but not to midguts of any of the resistant insects (panels b and c). BM, basement membrane and connective tissue; L, lumen; AMV, apical microvilli. Micrographs were taken with a fluorescent microscope. Bar = 24 μ m (panels a, c, and d); bar = 15.36 μ m (b).

FIG. 4. An approximately fourfold blowup of the apical microvilli from Fig. 3a including the one indicated by the arrow and two to the immediate left.

Differences in binding properties may have been lost because of alterations in the properties of the binding protein during extraction from the BBMV and/or electrophoresis under denaturing conditions (although they could have been renatured upon transfer to the Immobilon-P membrane). No differences in binding of the Cry1Ab or Cry1Ac toxins were detected over a range of toxin concentrations from subsaturating to saturating amounts. While this is a qualitative analysis, such blotting experiments have been useful for detecting toxin binding differences (as in Fig. 2) which have been confirmed by direct kinetic measurements (2). In addition, the HD133^r, HD198^r, and HD112^r colonies are resistant to several toxins and at least some (Cry1C, for example) use different receptors than that for Cry1Ac (28). Given the rather high frequency of occurrence of these multiply resistant colonies, it is unlikely that in each case more than one mutation in the genes encoding separate receptors had occurred.

While altered binding of toxin to BBMV from *P. interpunctella* larvae has been reported for the Dipel^r colony (28), such experiments have not been performed for the other resistant colonies because of the small size of the larvae and the prohibitive effort required to obtain adequate stable BBMV from midguts for extensive binding studies. Binding was measured in tissue sections, therefore, and there was no detectable binding of the Cry1Ac toxin to sections of any of the resistant colonies.

A correlation between poor binding to BBMV (6) and to tissue sections (4) has been reported for *P. xylostella* selected for resistance to Dipel. In another resistant colony, however,

no differences in the association or dissociation rates of the Cry1Ac toxin to sonicated vesicles were found (17). As noted by Masson et al. (17), the use of sonicated vesicles as well as the different techniques for measuring binding may account for the discrepancy, but there are other cases in which direct correlations between altered binding to BBMV and resistance were not found (6). In *H. virescens* selected for resistance to

FIG. 5. Binding of the *B. mori* aminopeptidase N antibody to BBMV extracts from *M. sexta* and *P. interpunctella* RC688^r, Dipel^r, HD133^r, and HD198^r. BBMV proteins were resolved by SDS-10% PAGE and transferred to an Immobilon-P membrane. Duplicate lanes containing 10 μ g of protein for *M. sexta* and 40 μ g of protein for each of the *P. interpunctella* colonies were treated either with a 1:10,000 dilution of the aminopeptidase N antibody (lanes 1 to 5) or with the Cry1Ac toxin plus Cry1Ac antibody (as described in the legend for Fig. 1) (lanes 6 to 10). The arrows on the left indicate the *M. sexta* and one of the *P. interpunctella* bands reacting with the aminopeptidase N antibody. The arrow on the right indicates the *P. interpunctella* Cry1Ac-binding protein. Lanes 1 and 6, *M. sexta*; lanes 2 and 7, HD198^r; lanes 3 and 8, HD133^r; lanes 4 and 9, Dipel^r; lanes 5 and 10, RC688^r; lane 11, molecular mass standards [β -galactosidase (116 kDa) and bovine serum albumin (67 kDa)].

Cry1Ac, there was no detectable change in binding to BBMV except for the Cry1Aa toxin, which is not very active on these larvae (14).

There are also cases of natural resistance to certain toxins but no detectable differences in binding to BBMV (11, 23, 30). In *B. mori*, the K_d values for the Cry1Aa and Cry1Ab toxins were about the same (and they competed for sites on BBMV) and yet the former was 17 times more effective (11). These toxins differed, however, in the extent of irreversible binding, indicating that the insertion step is very specific and critical for activity (5, 11). Irreversible binding should be measured in cases in which there are no detectable differences in binding to vesicles or to extracted proteins.

The results reported here with *P. interpunctella* can be explained by a mutation in the binding protein which alters its accessibility (and thus its orientation in the membrane) but not its toxin-binding properties when extracted. Such a mutation could be pleiotropic if the accessibility of other toxin-binding proteins was also affected. Alternatively, a similar phenotype could result from a mutation in some other membrane protein which disrupts the accessibility of the binding proteins. Either of these changes could account for insects with broad cross-resistance (6, 19, 25). As discussed above, there were no detectable differences in binding to the 80-kDa protein in immunoblots so the possibility of other membrane alterations should be considered.

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