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Identification of scFv Molecules that Recognize Loop 3 of Domain II and Domain III of Cry1Ab Toxin from *Bacillus thuringiensis*

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A phage repertoire was constructed using antibody genes from the bone marrow and the spleen of a rabbit immunized with Cry1Ab toxin. Biopanning against either the Cry1Ab toxin or a domain II loop 3 synthetic peptide resulted in the identification of monoclonal antibodies in scFv format. They inhibited binding and toxicity of Cry1Ab toxin against *Manduca sexta*. Toxin overlay assays, using the scFv antibodies as competitors, revealed that the anti-loop 3 molecule competed with Cry1Ab toxin binding to the cadherin receptor (Bt-R_1) of *M. sexta*, while anti-domain III antibodies interfered with the binding of Cry1Ab toxin to the aminopeptidase N (APN) receptor of this insect.

Introduction

Insecticidal Cry1 proteins from *Bacillus thuringiensis* (Bt) are used in biopesticides and transgenic crops (2). In susceptible insects, proteinases in the alkaline midgut activate the Cry protein to a toxin that binds with high affinity to receptors of the brush border epithelium membrane. In the case of the cadherin receptor Bt-R_1, toxin binding initiates a conformational change that results in the assembly of a pre-pore toxin oligomer (5). Aminopeptidases also bind Cry1 toxins and facilitate toxin-induced pore formation (8). An emerging model suggests that after binding cadherins, toxins bind aminopeptidases and insert into membrane microdomains called lipid rafts (3). Domain II determines specificity, because it represents the most divergent part of the toxin sequence, and exchanging domain II, or domains II and III, between closely related toxins resulted in active hybrids showing altered specificity (4,6).

Materials and methods

Phage display libraries construction. Total RNA from spleen tissue and bone marrow of an immunized rabbit was used for first strand cDNA synthesis. Heavy- and light-chain genes were amplified separately and recombined by three subsequent PCR, essentially as described (7). In order to construct the scFv libraries, scFv DNA and phagemid vector were digested with *Sfi*I and *Not*I (New England BioLabs, Beverly, MA, USA), and ligated. The purified DNA was electroporated into TG1 electrocompetent cells. Each library was grown on large TYE AMP GLU agar plates. For panning, phage preparations were purified and concentrated by polyethylene glycol precipitation.

Selection and characterization of phage-displayed antibodies. Panning was carried out essentially as described previously (7) using 50 μg of Cry1Ab. 10^{11} phage were used in each round of selection. Binders were eluted with 1 ml of triethylamine (100 mM) and the eluant was neutralized and mixed with 8.5 ml of exponentially growing TG-1 cells. An aliquot was removed for titration, measured as colony forming units on agar plates. Infected bacteria were plated on TYE AMP GLU agar plates and bacteria were harvested after ON growth at 37°C. Phages were rescued for the next selection cycle.

*Insect bioassay***.** Bioassays were performed on *M. sexta* neonate larvae by the surface contamination method. The toxin solution was preincubated with selected phages for 1 h then poured on the diet surface and allowed to dry. Neonate *M. sexta* larvae were placed on the dried surface and mortality was monitored after 7 days.

Results

Phage antibody library construction and characterization. After cDNA synthesis by reverse transcription from spleen and bone marrow RNA samples, the VH and VL region gene repertoires were amplified separately by PCR. In the second PCR reaction, a DNA linker coding a $(Gly_4Ser)_3$ peptide linker sequence was added using

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modified 3´-heavy and 5´-light chains primers. Finally, a third PCR reaction was performed to fuse heavy and light chain genes by overlapping extension. PCR products from the third PCR reaction were digested with *Sfi*I and *Not*I, and cloned into phagemid vectors pSyn2 or pCANTAB that allow the display of the cloned fragment on M13 phage. After transformation, libraries sizes of 2.0 x 10^6 members were obtained.

To examine the integrity of the libraries, 20 clones of each library were picked at random and 95% of the clones were found by PCR to contain scFv genes having the expected size. To determine the diversity of the gene content of the libraries, cloned scFv genes were amplified from the same colonies and digested with the *Alu*I restriction enzyme. PCR fingerprinting analysis of bone marrow and spleen libraries showed that the libraries were diverse since all restriction patterns analyzed were different.

Identification of anti-loop 3 and anti-domain III phage antibodies. For the identification of anti-loop 3 scFv antibodies, the libraries were panned against a synthetic biotinylated peptide with a sequence corresponding to the Cry1Ab loop 3. The panning procedure consisted in two selection rounds against the whole Cry1Ab toxin and a third panning round against the biotinylated loop 3 synthetic peptide. Fingerprinting analysis revealed five different restriction patterns. In ELISA, all clones bound to Cry1Ab and Cry1Ac, but did not bind to Cry1Aa which has a different loop 3 amino acid sequence, suggesting that these scFv bind loop 3 of Cry1Ab. After two rounds of panning against Cry1Ab, a final panning round was conducted against Cry1Ab in the presence of soluble Cry1Ac to ensure binding to Cry1Ab toxin of phages that do not recognize Cry1Ac. Fifty colonies from the fourth round were amplified by PCR and characterized by fingerprinting analysis. Five different restriction patterns were identified. Analysis of binding to the three Cry1A proteins revealed that three scFv phages bound to Cry1Ab, but not to Cry1Ac which has a different domain III sequence, suggesting that these scFv antibodies bind Cry1Ab toxin through domain III.

Effect of phage antibodies on toxicity of Cry1Ab toxin to *M. sexta* larvae. First instar larvae were fed Cry1Ab toxin either alone or Cry1Ab pre-incubated with 10^8 phage preparation of the different monoclonal scFv antibodies selected after four rounds of selection. None of the phages were toxic to *M. sexta* larvae. All of the anti-loop 3 phages reduced the toxicity of Cry1Ab to 30-60% while the two anti-domain III phages reduced it to 10-20%.

Discussion

The striking dissimilarity between domain II and domain III amino acid sequences of different Cry toxins motivated us to investigate the role of these regions in insect specificity, toxicity and binding using phage display antibodies against these regions. We generated immune libraries in the scFv format that could be used for efficient selection of high-affinity and specific scFv antibodies against Cry1Ab toxin. We identified scFv phages that recognized domain II loop 3 or domain III, since these regions are likely to be involved in receptor interaction (1, 6).

Rabbit immune repertoires from spleen or bone marrow yielded a great diversity. Using phage display technology, after three or four rounds of panning using Cry1Ab toxin, we isolated specific scFv antibodies from the pool of the two libraries, that inhibited toxicity. Among these, antibody M22 was particularly interesting. M22, in contrast to the other anti-Cry1Ab scFv molecules analyzed, recognized Cry1Ab but did not bind to Cry1Ac. Therefore, scFv M22 most likely recognized a certain domain III region involved in toxicity, since this antibody reduced the toxicity of Cry1Ab toxin. In the case of Cry1Ac toxin, domain III is important on APN binding (4). We were able also to obtain five specific scFv antibodies against loop 3 of domain II. All anti-loop 3 scFv molecules analyzed recognized Cry1Ab but did not bind to Cry1Aa, consistent with the fact that these toxins do not share sequence similarity in the loop 3 region. Our results show the efficacy of our libraries for the generation of highly specific reagents against specific regions of Cry toxins to study toxin-receptor interaction. Our set of recombinant scFv antibodies against Cry1Ab toxin represents the first demonstration of the recombinant-antibody approach to the study of toxin-receptor and structure-function relations. With these tools it should be possible now to characterize more thoroughly the toxin-receptor interactions of Cry proteins and to ascertain the role of these interactions in the mode of action of these important toxins.

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