Glycolipids as Receptors for 

Bacillus thuringiensis Crystal Toxin

Joel S. Griffitts,1 Stuart M. Haslam,2 Tinglu Yang,3 Stephan F. Garcynski,4 Barbara Mulloy,5 Howard Morris,6 Paul S. Cremer,5 Anne Dell,2 Michael J. Adang,4 Raffi V. Aroian1*

The development of pest resistance threatens the effectiveness of Bacillus thuringiensis (Bt) toxins used in transgenic and organic farming. Here, we demonstrate that (i) the major mechanism for Bt toxin resistance in Caenorhabditis elegans entails a loss of glycolipid carbohydrates; (ii) Bt toxin directly and specifically binds glycolipids; and (iii) this binding is carbohydrate-dependent and relevant for toxin action in vivo. These carbohydrates contain the arthroses core conserved in insects and nematodes but lacking in vertebrates. We present evidence that insect glycolipids are also receptors for Bt toxin.

The crystal (Cry) proteins produced by Bt are pore-forming toxins lethal to insects and nematodes but nontoxic to vertebrates (1, 2). In 2002, more than 14 million hectares of transgenic corn and cotton crops that express Cry proteins were planted worldwide, making these crops safe from specific insect pests and simultaneously resulting in substantial decreases in hazardous chemical pesticide use (3, 4). Cry proteins have now been shown to target nematodes as well, including the intestinal parasite Nippostrongylus brasiliensis, suggesting that Cry proteins may be used in the future to control parasitic nematodes of animals and plants (5). In the face of the enormous selective pressure generated by widespread use of Cry proteins in crops and organic farming, development of Cry toxin resistance among target populations is considered the major threat to their long-term use (6). The ability to detect resistance in the field, which is important for monitoring current resistance-management programs and making corrections before the resistance becomes a widespread problem, relies on molecular and genetic knowledge of the genes and pathways that give rise to resistance. Resistance can be mediated by multiple loci, the identities of which have remained largely elusive. To date, only insect cadherins, which serve as toxin receptors, have been definitively demonstrated to mutate to Cry toxin resistance (7, 8). Other candidates for resistance alleles include a second Bt toxin–binding protein, aminopeptidase N, and a host protease required to process the Bt toxin (9, 10). There are also a number of as yet unidentified loci that can mutate to Cry toxin resistance, including ones important for toxin binding (11, 12).

Using forward genetics, we identified four genes (called bre genes for Bt toxin resistant) that mutate to Bt toxin resistance in the nematode C. elegans (13–15). Loss-of-function mutants in this pathway resist at least two Cry proteins, Cry5B, which targets nematodes (Fig. 1A), and Cry14A, which targets nematodes and insects (13, 14). Cry5B and Cry14A are members of the main family of three-domain Bt toxins, which includes the commercially used Cry1, Cry2, and Cry3 toxins (16). The bre genes encode four glycosyltransferase proteins, act in a single pathway, and are required for the uptake of toxin into intestinal cells, suggesting that they might make a Bt toxin host cell receptor (13, 14). Based on their in vitro activities, the BRE-3 and BRE-5 counterparts in Drosophila—EGGHEAD and BRAINiac, respectively—have been suggested to synthesize the carbohydrate chains present on glycosphingolipids (14). We therefore hypothesized that the BRE enzymes might be involved in the biosynthesis of glycosphingolipids and that glycosphingolipids might be heretofore-unrecognized host cell receptors for Bt toxins.

To investigate these possibilities, lipids from wild-type and bre mutant animals were extracted, partitioned into two phases, resolved by thin-layer chromatography (TLC), and visualized with the orcinol reagent that stains carbohydrates (Fig. 1B). Wild-type animals contain multiple high-polarity glycolipid species (Fig. 1B, upper phase, components B to F). These glycolipids are ceramide-based (and hence glycosphingolipids) because the carbohydrates can be removed with leech ceramide glycanase (17). These upper phase glycolipids are completely absent in bre-3, bre-4, and bre-5 mutant animals. In bre-2 mutant animals, most (B, C, and F) but not all (D and E) upper phase components are missing. In contrast to what was seen in the upper phase, analysis of lower phase (presumably less complex) glycolipids from bre-4 and bre-5 mutant animals revealed the appearance of new glycolipid species (Fig. 1B), presumably each representing a different precursor that accumulates as a result of deficiencies in the biosynthetic pathway. Genetic epistasis allows us to infer that the BRE enzymes act in the following order in the synthesis of glycolipids: BRE-3, BRE-5, BRE-4, and lastly BRE-2 [supporting online material (SOM) text], in agreement with the known or proposed activities of these enzymes and the structures of their products. These data demonstrate that BRE enzymes are required to synthesize the carbohydrate chain of glycolipids. The lack of observable defects in protein-linked carbohydrates based on mass spectrometry analysis of N- and O-linked glycans from bre-3 animals suggests that BRE-3 is not involved in the synthesis of glycoproteins (fig. S5 and table S5). These data and the fact that linkages dependent on bre-3 and bre-5 have been found only in glycolipids indicate that glycolipids and not
glycoproteins are important for bre-mediated Bt toxin susceptibility.

We next tested whether Cry5B can directly bind glycolipids. An overlay technique was used in which crude C. elegans glycolipids were fixed in place on TLC plates and then incubated in an aqueous solution of activated, biotinylated Cry5B. After washing away unbound toxin, Cry5B bound to glycolipids was detected by enzyme-linked biotin detection. We found that Cry5B is able to bind to a number of glycosphingolipid species, namely components B, C, E, F, and other minor species (Fig. 2A). Specificity of binding is demonstrated by our observations that neither glycolipid species D (Fig. 2A, lanes 1 to 3) nor the simple glycolipids that accumulate in bre-4 and bre-5 mutants (17) nor mammalian glycolipid standards (Fig. 2A, lanes 7 and 8) bind Cry5B. As predicted for our resistant mutants, Cry5B-binding glycolipids are missing in bre-3, bre-4, and bre-5 mutant animals (Fig. 2A, lanes 4 to 6), and all but one is missing in bre-2 mutant animals (band E; Fig. 2A, lane 3). Because bre-2 mutant animals are as resistant as the other mutants (14, 15), expression of band E must not be sufficient for intoxication, perhaps because that glycolipid species is not expressed on the apical surface of intestinal cells.

The binding of Cry5B to purified C. elegans glycolipids was confirmed in supported lipid bilayers with microfluidic methods (18). Glycolipid component B was purified and incorporated into phosphocholine liposomes at 0.35 mol percent. These liposomes were allowed to form a continuous bilayer in hydrophilic microchannels, and the binding of fluorescently labeled Cry5B was evaluated with total internal reflection fluorescence microscopy. Cry5B binding to component B occurs in a saturable, dose-dependent manner and exhibits an apparent dissociation constant, $K_d$, of $0.73 \pm 0.06 \mu M$ at the particular ligand density tested (Fig. 2B). This $K_d$ falls near the low end of the range observed for many protein lectin–carbohydrate interactions (19). No specific binding in the absence of component B was detected. Thus, C. elegans glycolipid component B is sufficient to generate specific binding sites for Cry5B toxin in lipid bilayers.

We determined the chemical structures of components B, C, D, and E (Fig. 2C, Figs. S1 to S4, and Tables S1 to S4). All of these structures contain the core tetrascaccharide N-acetylgalactosamine (GalNAc) $\beta-1\rightarrow 4$ N-acetylglucosamine (GlcNAc) $\beta-1\rightarrow 3$ mannose (Man) $\beta-1\rightarrow 4$ glucose (Glc), which is an invertebrate-specific glycolipid signature conserved between nematodes and insects but lacking in vertebrates (20). Components D and E correspond to the previously described glycosphingolipid structures Nz2 and Nz3, respectively (21); the structures of components B and C were previously uncharacterized. The role of BRE-3, BRE-4, and BRE-5 in the synthesis of these structures can be assigned (Fig. 2C) on the basis of epistasis and the predicted or demonstrated biochemical activities of these enzymes (14, 22). We propose that BRE-2 initiates the synthesis of the branched moiety that distinguishes components B and C from D and E (SOM text).

To evaluate the carbohydrate dependence of Cry5B binding to glycolipids, we examined the ability of simple sugars to inhibit the
binding of toxin to glycolipids. Glycolipid component B was purified and immobilized in polystyrene wells and then probed with biotinylated Cry5B in the absence and presence of various monosaccharides (Fig. 3A). Galactose is the most potent of the monosaccharide inhibitors, exerting 92 ± 2% binding inhibition at 15 mM. GalNAc also had a significant effect. The galactose analog β-methylgalactoside (conferring 92 ± 4% inhibition at 3 mM) was more inhibitory than the related compound α-methylagalactoside; β-methylglucoside was noninhibitory (Fig. 3B). β-galactose–mediated inhibition also occurred in our microfluidic lipid bilayer system (17). Galactose inhibits Cry5B binding to the entire bre-dependent glycolipid series in overlay assays (Fig. 3C), suggesting a common glycolipid-dependent binding mechanism. These data confirm that carbohydrates are key mediators of Cry5B binding to glycolipids and point to the β-galactose–rich terminus of these receptors as an important binding epitope.

An in vivo prediction from these results is that β-methylgalactoside fed to C. elegans should provide an antidote to Cry5B toxin by competing with intestinal glycolipids for toxin binding. C. elegans hermaphrodites were fed doses of Cry5B that moderately inhibit nematode growth along with β-methylagalactoside, β-methylglucoside, or no exogenous carbohydrate. Neither of the carbohydrate treatments resulted in major growth differences in the absence of Cry5B (17). In the presence of Cry5B, β-methylgalactoside specifically protected animals at the toxin doses tested (Fig. 3D). Control glucoside-treated animals exhibited no protection from the toxin. Thus, the same treatment that directly interferes with the Cry5B-glycolipid interaction also specifically diminishes Cry5B toxicity, confirming the functional importance of these carbohydrate receptors to Cry toxin function in vivo.

Considering the substantial conservation of bre-dependent glycolipids in nematodes and insects and the conservation of Cry toxin structures (including lectin-like domains), it seems likely that insecticidal Cry toxin activity is also modulated by glycolipid host cell receptors. Consistent with this, we found that Cry1Ac toxin binds to glycolipids extracted from the midguts of the tobacco hornworm, Manduca sexta (Fig. 4A) (23). Competition of binding with unlabeled Cry1Ac indicates that binding is specific (Fig. 4A). Furthermore, Cry1AA and Cry1AB toxins bind the same M. sexta glycolipids as do Cry1AC, consistent with glycolipids serving as general host cell receptors for these toxins (Fig. 4B).

Previously, glycolipid levels have been found to be substantially reduced in a Cry1Ac-resistant Plutella xylostella strain (24). It was proposed in the same report that alterations in glycolipids are involved in the evolution of P. xylostella resistance to Cry1Ac, although possible glycolipid receptor functions were not discussed. In a second study, a capacity for nonpurified Bt kurstaki toxins to bind to insect glycolipids was shown, but it was postulated that the in vivo toxin receptors were other glycoconjugates, such as glycoproteins (25). In addition, we have shown that Cry14A, a toxin
active against both nematodes and insects, requires the bre pathway for full activity against C. elegans (13, 14). Taken together, our data and these studies suggest that both nematicidal and insecticidal three-domain Bt toxins use invertebrate glycolipids as host cell receptors and that the loss of glycolipid receptors represents an important mechanism for Bt toxin resistance. The ease with which glycolipids can be isolated and analyzed suggests that it will be feasible to monitor glycolipid-mediated resistance in field and laboratory populations of insects and nematodes.

Glycolipids, however, are not the only class of Bt toxin host cell receptors. For insects, high-affinity protein receptors, such as insect cadherins and aminopeptidases, have been shown to play functional roles as Cry1 receptors, although correlating protein receptor defects with binding defects has not always been simple. For example, in at least two cases, Cry1Ac has been found to bind specifically to membranes of cadherin receptor mutants that are Cry1Ac resistant (26, 27), leading to the hypothesis that a multistep binding process involving multiple receptors is required for proper pore formation. For nematodes, our data suggest that although bre-dependent glycolipids are important for Cry14A function, there is likely another binding factor involved in Cry14A toxicity (14). We hypothesize that glycolipid and protein receptors may both play a role, sequentially or simultaneously, in positioning Bt toxins appropriately at the bilayer or in inserting toxins into the bilayer.

Mammalian cells do not bind three-domain Bt Cry toxins (2), and the results presented here provide a plausible molecular basis for the lack of toxicity of Cry toxins toward vertebrates. Vertebrates lack arthroseries glycolipids, which contain the conserved invertebrate-specific core tetrasaccharide GalNAcβ1-4GlcNAcβ1-3Manβ1-4Glc that is synthesized by the BRE pathway. Although the β-linked galactose important for Cry5B binding is not present in this core sequence, our unpublished data indicate that the intact receptor is, by three orders of magnitude, a better competitive inhibitor than β-methylgalactoside. Thus, higher-order structure is likely important for binding. We hypothesize that three-domain Bt Cry toxins evolved to at least partly recognize the core arthroseries tetrasaccharide and thus target the invertebrates, nematodes, and insects that synthesize these molecules.

The high degree of conservation between glycolipids present in C. elegans and in the human parasitic nematodes Ascaris suum and Onchocerca volvulus, which are phylogenetically divergent from C. elegans, suggests that most, if not all, nematodes will be susceptible to Cry5B toxin. All the nematodes we have tested to date are susceptible to Cry5B, and the one animal parasite we have tested has been shown to succumb to both Cry5B and Cry14A.

References and Notes

17. J. S. Griffitts et al., data not shown.
28. Materials and methods are available as supporting material on Science Online.
29. We thank R. Schnar for technical advice on glycolipids. B. Hayes and N. Preece of the UCSD Glycotechnology Core for help with glycan analysis, T. Huxford for assistance with toxin purification, D. Huffman for comments, and members of the R.V.A. laboratory for stimulating discussions. This work was supported by NSF grant MCB-9983013 and grants from the Burroughs–Wellcome Foundation and the Beckman Foundation (to R.V.A.).
30. Supporting Online Material
www.sciencemag.org/cgi/content/full/307/5711/922/DC1
Materials and Methods
SOM Text
Figs. S1 to S5
Tables S1 to S5
References
24 August 2004; accepted 19 November 2004
10.1126/science.1104444

Lymphotoxin-Mediated Regulation of γδ Cell Differentiation by αβ T Cell Progenitors
Bruno Silva-Santos,* Daniel J. Pennington,* Adrian C. Hayday†
The thymus gives rise to two T cell lineages, αβ and γδ, that are thought to develop independently of one another. Hence, double positive (DP) thymocytes expressing CD4 and CD8 coreceptors are usually viewed simply as progenitors of CD4+ and CD8+ αβ T cells. Instead we report that DP cells regulate the differentiation of early thymocyte progenitors and γδ cells, by a mechanism dependent on the transcription factor RORγt, and the lymphotoxin (LT) β receptor (LTβR). This finding provokes a revised view of the thymus, in which lymphoid tissue induction-processes coordinate the developmental and functional integration of the two T cell lineages.

Cell-mediated immunity involves T cell receptor (TCR) αβ+ cells, which recognize antigenic peptides presented by major histocompatibility complex (MHC) proteins, and unconventional, non-MHC-restricted T cell, of which TCRγδ+ cells are the prototype. There is increasing evidence that αβ and γδ T cell progenitors do not develop independently. This has been suggested by studies performed on murine systems: γδ T cells are functionally integrated, but their relatedness and how they may “cross-talk” are incompletely understood (1). To clarify this situation, we identified a “γδ-biased” gene profile (2). Unexpectedly, full expression of this profile by TCRγδ+ thymocytes depended in trans on CD4+ CD8− (DP) cells, which are late-stage αβ T cell progenitors that form the most abundant thymocyte subset (Fig. 1A). Reflecting this situation, γδ cell function is altered in TCRβ−/− mice that lack normal DP s (2).

We hypothesized that DP cells might exert their effects directly on maturing γδ T