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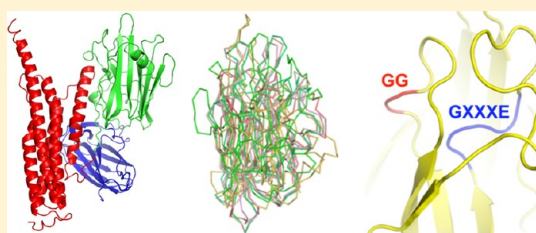
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Supporting Information

ABSTRACT: Crystal (Cry) proteins are globally used in agriculture as proteinaceous insecticides. They have also been recently recognized to have great potential as anthelmintic agents in targeting parasitic roundworms (e.g., hookworms). The most extensively characterized of the anthelmintic Cry proteins is Cry5B. We report here the 2.3 Å resolution structure of the proteolytically activated form of Cry5B. This structure, which is the first for a nematicidal Cry protein, shows the familiar three-domain arrangement seen in insecticidal Cry proteins. However, domain II is unusual in that it more closely resembles a banana lectin than it does other Cry proteins. This result is consistent with the fact that the receptor for Cry5B consists of a set of invertebrate-specific glycans (attached to lipids) and also suggests that domain II is important for receptor binding. We found that not only galactose but also *N*-acetylgalactosamine (GalNAc) is an efficient competitor for binding between Cry5B and glycolipids. GalNAc is one of the core arthroses tetrasaccharides of the Cry5B receptor and galactose an antennary sugar that emanates from this core. These and prior data suggest that the minimal binding determinant for Cry5B consists of a core GalNAc and two antennary galactoses. Lastly, the protoxin form of Cry5B was found to bind nematode glycolipids with a specificity equal to that of activated Cry5B, but with lower affinity. This suggests that the initial binding of Cry5B protoxin to glycolipids can be stabilized at the nematode cell surface by proteolysis. These results lay the groundwork for the design of effective Cry5B-based anthelmintics.



Crystal (Cry) proteins are widely used on a global scale as proteinaceous insecticides. These proteins target caterpillars, beetles, mosquitoes, and black flies but have no effects on higher animals; they also lack the harmful side effects that small molecule pesticides often have on higher animals.^{1–5} Cry proteins are produced by the Gram-positive soil bacterium *Bacillus thuringiensis* (Bt), and more than 200 varieties of such proteins are known (with sequence identities ranging from <20 to >90%).² These proteins are produced as crystalline inclusions in sporulating Bt and, following ingestion by invertebrates, are solubilized in the invertebrate gut as monomeric protoxins. Monomeric protoxins are then activated by host proteases, which remove N-terminal and, in almost all cases, C-terminal regions of the protoxins to yield ~60–70 kDa activated Cry proteins. Activated Cry proteins bind specific receptors² on midgut epithelial cells and insert into the plasma membrane of these cells to form cytotoxic pores. In some cases, more than one receptor has been identified, and receptor binding has been noted to occur sequentially.⁶ Receptor binding has also been noted to promote oligomerization of activated Cry proteins, which appears to enhance subsequent pore formation.⁷

The structures of seven insecticidal Cry proteins (Cry1Aa, Cry2Aa, Cry3Aa, Cry3Bb, Cry4Aa, Cry4Ba, and Cry8Ea1) have

been determined and are available in the Protein Data Bank.^{8–14} These proteins have a characteristic compact three-domain architecture. Domain I is an all- α -helical bundle responsible for pore formation.⁵ Evidence suggests that the pore is initiated by the insertion of an α -helical hairpin into the membrane, which is then followed by the insertion of the other helices.¹⁵ Domains II and III are rich in β -sheets and resemble lectins of the β -prism fold and jellyroll topology families, respectively.¹⁶ Domains II and III have been implicated in receptor binding,^{6,17–22} and domain III also modulates pore activity.²³

Cry proteins are active not only against insects but also against nematodes, including those that infest crops or are parasites of animals.^{24–29} The most extensively characterized nematicidal Cry protein is Cry5B, which has been shown to be a potent *in vivo* anthelmintic, providing therapeutic effects against the hookworm parasite *Ancylostoma ceylanicum* in hamsters and the intestinal roundworm parasite *Heligmosomoides bakeri* in mice.^{24,25} The potential use of Cry5B as an anthelmintic has immense implications as intestinal round-

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worms infect ~2.3 billion people worldwide, and there is an urgent need for new and better anthelmintics.^{30–32} The receptor for Cry5B consists of a set of invertebrate-specific glycans that are attached to lipids on the surface of intestinal epithelial cells.^{33,34} These glycans are composed of an arthroseries tetrasaccharide core (GalNAc β 1–4GlcNAc β 1–3Man β 1–4Glc) decorated with antennary glycans (Gal, Glc, Fuc, and 2-O-Me-Fuc), or single sugars (Figure S1 of the Supporting Information).^{33,35}

Because of the importance of Cry5B as an anthelmintic and as a roundworm-active Cry protein (as opposed to an insect-active Cry protein), we undertook the characterization of the structure of Cry5B and its glycan binding properties in further detail. We found that Cry5B is the most structurally divergent Cry protein described to date, with domain II being especially divergent. Domain II most closely resembles a banana lectin, suggesting that this domain is important for Cry5B glycolipid receptor binding. We found that GalNAc acts as an effective competitor for binding between Cry5B and nematode glycolipids, which in combination with prior results, suggests that the minimal binding determinant for Cry5B consists of a core GalNAc and two antennary galactoses. Lastly, we found that the protoxin form of Cry5B binds nematode glycolipids with the same specificity as activated Cry5B, but with weaker affinity. This suggests that the binding of Cry5B protoxin to the nematode cell surface may be strengthened by subsequent proteolysis. Our results provide a foundation for the design of effective anthelmintics based on Cry5B.

■ EXPERIMENTAL PROCEDURES

Expression, Purification, and Crystallization of Cry5B Protein. Cry5B protoxin was purified from crystal protein-deficient *B. thuringiensis* (Bt) strain HD1 that had been transformed with a plasmid encoding *cry5B*.³⁶ Cry5B protoxin was purified as previously described from spore crystal lysates²⁴ and stored as a precipitate in water at –80 °C. For experimental manipulations, aliquots of Cry5B protoxin were solubilized in 20 mM HEPES (pH 8.0) at a final concentration of 5 mg/mL. The protein concentration was determined using a calculated ϵ_{280} of 162510 M^{–1} cm^{–1}.

For expression in *Escherichia coli*, residues 1–772 of *cry5B* were cloned with an N-terminal His tag into vector pQE9 and expressed in *E. coli* M15. Bacteria were grown at 37 °C to midlog phase (OD₆₀₀ = 0.6–0.8), and expression of Cry5B(1–772) was induced with 0.15 mM isopropyl β -D-1-thiogalactopyranoside. After induction, bacteria were grown at 25 °C for 10 h and then harvested by centrifugation (6328g for 20 min at room temperature). Bacteria were lysed by sonication in phosphate-buffered saline (PBS), and the lysate was centrifuged (17418g for 20 min at 4 °C). The supernatant, which contained Cry5B(1–772), was applied to a Ni²⁺-nitrilotriacetic acid (NTA) agarose column; the column was washed with 3 column volumes of NiC buffer [0.5 M NaCl and 20 mM HEPES (pH 8.0)], and Cry5B(1–772) was eluted from the column with NiC buffer supplemented with 0.5 M imidazole. Cry5B(1–772) was dialyzed in 50 mM NaCl and 20 mM HEPES (pH 8.0) and concentrated by ultrafiltration to 5 mg/mL. The concentration of Cry5B(1–772) was determined using a calculated ϵ_{280} of 108525 M^{–1} cm^{–1}. For phase determination, selenomethionine (SeMet) was biosynthetically incorporated into Cry5B(1–772) as previously described,³⁷ and SeMet-labeled Cry5B(1–772) was expressed and purified as described above.

Cry5B protoxin produced in *B. thuringiensis* and Cry5B(1–772) produced in *E. coli* were activated by cleavage with elastase (overnight, room temperature, 200:1 Cry5B:elastase mass ratio) in 20 mM HEPES (pH 8.0). Activated Cry5B was purified by gel filtration chromatography (Superdex 200 26/60) in 200 mM NaCl and 20 mM HEPES (pH 8.0). Activated Cry5B was then dialyzed in 50 mM NaCl and 20 mM HEPES (pH 8.0) and concentrated to 5 mg/mL. The protein concentration of activated Cry5B was determined using a calculated ϵ_{280} of 72825 M^{–1} cm^{–1}. The site of elastase cleavage was determined by N-terminal sequencing. For this, activated Cry5B was run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted onto a polyvinylidene fluoride membrane. The molecular mass of elastase-cleaved Cry5B was determined by MALDI-TOF mass spectrometry to be 66145 Da (calculated value of 65815 Da for residues 112–170 and 173–698, the nicked protein that constitutes elastase-cleaved Cry5B).

Crystallization, Data Collection, Structure Determination, and Refinement. Crystals of elastase-activated Cry5B produced in *B. thuringiensis* were grown at room temperature by the vapor diffusion, hanging drop method. One microliter of 5 mg/mL activated Cry5B was mixed with 1 μ L of 20% PEG 3350 and 0.2 M NaCH₃COO as the precipitant. Crystals of SeMet-labeled, elastase-activated Cry5B, which had been prepared from Cry5B(1–772) produced in *E. coli*, were obtained under the same condition. Crystals of activated Cry5B were soaked in the precipitant solution supplemented with 15% glycerol as a cryoprotectant, before being mounted and cooled to 100 K in a N₂ stream.

Diffraction intensities from native and SeMet-labeled Cry5B crystals were collected at Advanced Light Source (ALS, Berkeley, CA) beamline 4.2.2 (Table S1 of the Supporting Information). Integration, scaling, and merging of intensities were conducted using Mosflm and Scala.³⁸ Phases were determined by the single-anomalous dispersion (SAD) method from crystals of SeMet-labeled Cry5B using Phenix³⁹ and were also refined using Phenix. Three selenomethionine positions were identified in the asymmetric unit, which contained a single molecule of elastase-activated Cry5B. The initial electron density map clearly showed an α -helical bundle corresponding to domain I and β -sheets corresponding to domains II and III. Residues 187–326 were built automatically with Phenix, and residues 112–161 (corresponding to helix α 4 in domain I) were manually built using Coot,⁴⁰ as were domains II and III. The partial model encompassing ~450 residues, which was missing the loops connecting the β -strands in domains II and III, was then refined against the higher-resolution native data set. The electron density map generated from the native data allowed tracing of the rest of the chain, except for residues 171 and 172, which were removed by elastase and are missing in the model. Simulated annealing with torsion angle dynamics was conducted from 3000 K using the slow-cool protocol of CNS.⁴¹ Ten cycles of maximum likelihood restrained refinement were subsequently conducted using REFMAC,³⁸ each cycle being followed by manual rebuilding into σ_A -weighted $2mF_o - DF_c$ and $mF_o - DF_c$ maps using Coot. Waters were added in the later stages of the refinement using Phenix with default parameters (3 σ peak height in $mF_o - DF_c$ maps), followed by inspection of maps.

Structure validation was performed using Procheck⁴² and Molprobity.⁴³ In the final model, 93.1 and 98.8% of residues were in allowed and generously allowed Ramachandran regions,

respectively. The final map had correlation coefficients of 0.997 and 0.995 for the main chain and side chains, respectively, as calculated with OVERLAPMAP.³⁸ The Molprobity clash score was 19.4 (61st percentile), and the overall score was 2.57 (55th percentile). The atomic coordinates and structure factors have been deposited as Protein Data Bank entry 4D8M.

Structural figures were produced with PyMOL.⁴⁴ Structure-based sequence alignments were generated using Expresso⁴⁵ and displayed using ESPrpt.⁴⁶ Calculations of structural superposition and sequence identity were conducted with Coot.

Purification of Upper Phase Glycolipids from Nematodes. *Caenorhabditis elegans* N2 and *Pristionchus pacificus* PS312 were grown on high-growth medium plates seeded with *E. coli* OP50. Once near starvation, nematodes were harvested from the plates, and pellets of mixed-life stage worms amounting to 0.5 mL were washed three times with water. The pellets were resuspended in 3 pellet volumes of water and sonicated five times for 2 min each at 11–14 W, with chilling on ice between sonication steps. Upper phase glycolipids were purified using the Svennerholm partitioning method.^{47–49}

Glycolipid Overlay Assay. The thin-layer chromatography (TLC) assay was conducted as previously described.⁴⁹ For biotinylation, elastase-activated Cry5B [6 μ L of a 2.5 mg/mL solution in 200 mM NaCl and 20 mM HEPES (pH 8.0)] was treated with a 3.16-fold molar excess of *N*-hydroxysuccinimido biotin in a 10 μ L total volume of 20 mM HEPES (pH 8.0). After 2 h at room temperature on a rocker, 90 μ L of 20 mM HEPES (pH 8.0) was added. Cry5B protoxin was biotinylated similarly, except that a 22-fold molar excess of *N*-hydroxysuccinimido biotin was used.

Developed HPTLC plates were fixed for 60 s in 40 mL of hexanes (mixture of isomers, 99% pure, Acros Organics) and 40 mL of hexanes containing 0.02% polyisobutylmethacrylate. After being dried for at least 5 min at 45 °C, the plates were blocked with 9.9 mL of blocking buffer (PBS containing 0.5% bovine serum albumin and 0.02% Tween). When monosaccharides were added to compete for binding, the HPTLC plates were blocked in 8.9 mL of blocking buffer and 1 mL of 1 M monosaccharide. After 30 min on a rocker, 100 μ L of labeled Cry5B was added. After a further 2 h on the rocker, the plates were washed with 10 mL of blocking buffer for 1 min and then for a second time for 5 min. To visualize Cry5B, the plates were incubated for 1 h with 40 μ L of avidin DH and biotinylated alkaline phosphatase H (Reagent A and B, Vectastain ABC-AP kit) dissolved in 6 mL of blocking buffer. The plates were washed with 10 mL of blocking buffer three times for 1 min each and then incubated in the alkaline phosphatase substrate NBT/BCIP (30 μ L of each NBT and BCIP per 5 mL of solution, Vector Laboratories) for 2–4 h. There were slight variations in the sharpness of bands from experiment to experiment, but the result that Gal and GalNAc blocked binding by Cry5B to glycolipids was consistent throughout.

RESULTS

Overall Structure of Cry5B. The structure of elastase-activated Cry5B (residues 112–698) was determined by single anomalous diffraction (SAD) and refined to 2.3 Å resolution (Table S1 of the Supporting Information). The electron density map was unambiguous, and the entirety of the protein chain was clearly defined, except for residues 171 and 172, which are missing in the final model. Elastase-activated Cry5B is nicked at these residues and, as such, is composed of two polypeptide chains (residues 112–170 and 173–698) connected by

disulfide bonds. This conclusion was verified by N-terminal sequencing and MALDI-TOF mass spectrometry (data not shown). Although the sequence of the nematocidal Cry5B protein is only ~16–19% identical with those of insecticidal crystal proteins, Cry5B has a three-domain structure that resembles that of insecticidal crystal proteins (Figure 1). The

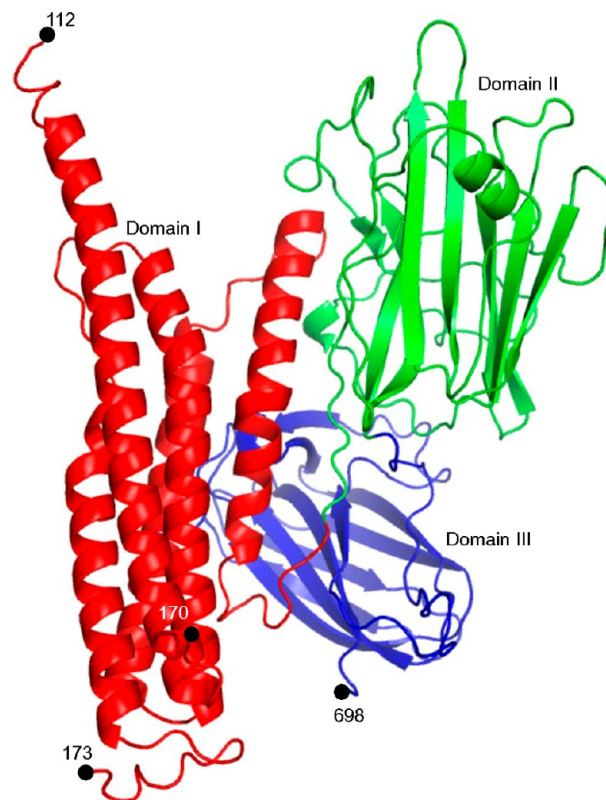


Figure 1. Structure of Cry5B. Cry5B is shown in a ribbon diagram. Domain I (red) is a five- α -helix bundle, domain II (green) a β -prism domain, and domain III (blue) a β -sandwich domain.

insecticidal crystal proteins with known structures (i.e., Cry1Aa, Cry2Aa, Cry3Aa, Cry3Bb1, Cry4Aa, Cry4Ba, and Cry8Ea1) are highly similar to one another. Their α positions vary by 1.0–2.1 Å root-mean-square deviations (rmsds) in pairwise comparisons, except for the most dissimilar member of this group, which is Cry2Aa (pairwise rmsd of 2.5–2.8 Å).¹⁰ By comparison, Cry5B has an average rmsd of ~3 Å in pairwise comparisons with the insecticidal crystal proteins. This indicates that Cry5B is the most structurally divergent crystal protein described to date, in accordance with its differing host tropism.

Domain I. Domain I is implicated in pore formation,⁸ and in accord with its central mechanistic role, domain I is the most structurally conserved of the three domains of Cry5B. Cry5B domain I (residues 112–328), which is composed of a five- α -helix bundle, can be superimposed on domain I of the insecticidal Cry proteins with an average rmsd of 1.97 Å (153 α positions on average) (Figure 2a), despite this domain being only 22% identical in sequence on average (Figure 3). The five helices of activated Cry5B match the five helices of activated Cry4B, in agreement with the fact that these two proteins are cleaved at equivalent locations. In comparison, protoxin Cry2Aa is uncleaved and therefore extends much farther in the N-terminal direction than does Cry5B; thus, the

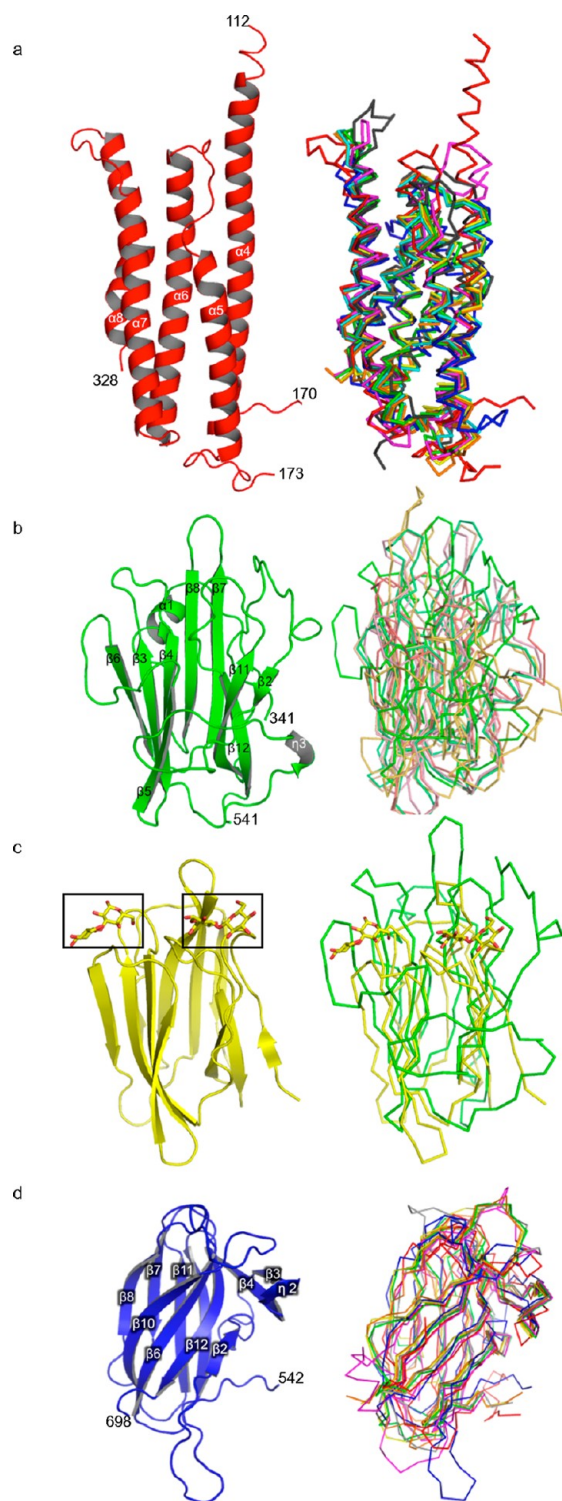


Figure 2. Domains of Cry5B. (a) Domain I in ribbon representation (left) and superposition (right) of Cry5B domain I (red) with domain I from Cry1Aa (green), Cry2Aa (blue), Cry3Aa (yellow), Cry3Bb (orange), Cry4Aa (gray), Cry4Ba (magenta), and Cry8Ea1 (cyan), all in chain representation. (b) Domain II in ribbon representation (left) and superposition (right) with domain II from other Cry proteins, as in panel a. (c) Banana lectin in ribbon representation (left) with bound glycans (yellow) boxed and superposition (right) of banana lectin (yellow), including glycans (laminaribiose), with Cry5B domain 2 (green), in chain representation. (d) Domain III in ribbon representation (left) and superposition (right) with domain III from other Cry proteins, as in panel a.

five helices of Cry5B match the last five helices ($\alpha 4$ – $\alpha 8$) of Cry2Aa. Similarly, some of the activated Cry proteins (i.e., Cry1Aa, Cry3Aa, Cry3Bb, and Cry4Aa) are cleaved at positions N-terminal to activated Cry5B, and here again, the five Cry5B helices match the last five helices ($\alpha 3$ – $\alpha 7$) of these activated proteins.

Helix $\alpha 6$ of Cry5B is the central helix in the five-helix bundle and is surrounded by the four other helices. This central α -helix is the most conserved of the α -helices in domain I, containing three residues (Leu233, Ala240, and Leu244) that are absolutely conserved among the structurally characterized Cry proteins (Figure 3). The central helix along with the preceding helix has been suggested to act as a “helical hairpin” that inserts into the plasma membrane of host midgut epithelial cells and initiates the formation of a cytotoxic pore.^{15,50} Mutagenesis studies have demonstrated the crucial role of this central α -helix role in toxicity.^{2,51–53}

There are some small differences between Cry5B and the other Cry proteins in domain I. First, helix $\alpha 4$ of Cry5B at ~45 residues is unusually long for Cry proteins. Second, Cry5B has two disulfide bridges (Cys163–Cys180 and Cys177–Cys186) connecting the long loop between helices $\alpha 4$ and $\alpha 5$, whereas other crystal proteins lack these disulfides or, as in the case of Cry4Aa, have only one disulfide at this location. The long $\alpha 4$ – $\alpha 5$ loop contains the nick generated by elastase, which results in the loss of residues 171 and 172. Residues 170 and 173 apparently shift position after the nicking, as deduced from the fact that these residues are too far apart for the missing residues to span the intervening space.

Domain II. Domain II (residues 341–541) consists of a β -prism, as found in other Cry proteins, as well as in the jacalin-related superfamily of lectins⁵⁴ (Figure 2b). β -Prism domains generally consist of three four-stranded β -sheets, each with a Greek key topology. These sheets are all parallel to an approximate 3-fold axis of symmetry and form the sides of a prism. In Cry5B, one of the β -sheets has four long strands ($\beta 5\beta 4\beta 3\beta 6$), whereas the other two have a mixture of two long and two short strands. These latter two are composed of $\beta 1\beta 12\beta 11\beta 2$, with $\beta 1$ and $\beta 2$ being short, and $\beta 9\beta 8\beta 7\beta 10$, with $\beta 9$ and $\beta 10$ being short; this latter sheet has an α -helix intervening between $\beta 9$ and $\beta 10$.

Domain II is the most structurally divergent of the three domains of Cry5B, with an average rmsd of 3.1 Å (122 average C α atoms, 8% average level of sequence identity) as compared to domain II of the insecticidal Cry proteins. A superposition of these domains makes this point clearly (Figure 2b). In fact, a structural homology search revealed that Cry5B domain II is most similar not to a Cry protein domain II but to a banana lectin (2.7 Å rmsd, 114 C α atoms, Z score of 9.6, 14% identical sequence)⁵⁵ (Figure 2c). Notably, the structure of the banana lectin (BanLec) has been determined with two different glycans bound, laminaribiose (Glc β 1–3Glc) and Xyl- β 1,3-Man- α -O-methyl.⁵⁵ Two separate glycan-binding sites were observed, both formed by loops at the tips of the β -sheets. The equivalent of the first site in Cry5B consists of the $\beta 1\beta 2$ and $\beta 11\beta 12$ loops (emanating from the $\beta 1\beta 12\beta 11\beta 2$ sheet) and the $\beta 3\beta 4$ and $\beta 5\beta 6$ loops (emanating from the $\beta 5\beta 4\beta 3\beta 6$ sheet) for the second site (Figure 3). The closer relationship of Cry5B domain II with BanLec as compared to a crystal protein suggests that this domain has a primary role in glycan binding.¹⁷

Domain III. Domain III (residues 542–698) is a β -sandwich with a jellyroll topology, composed of two four-stranded

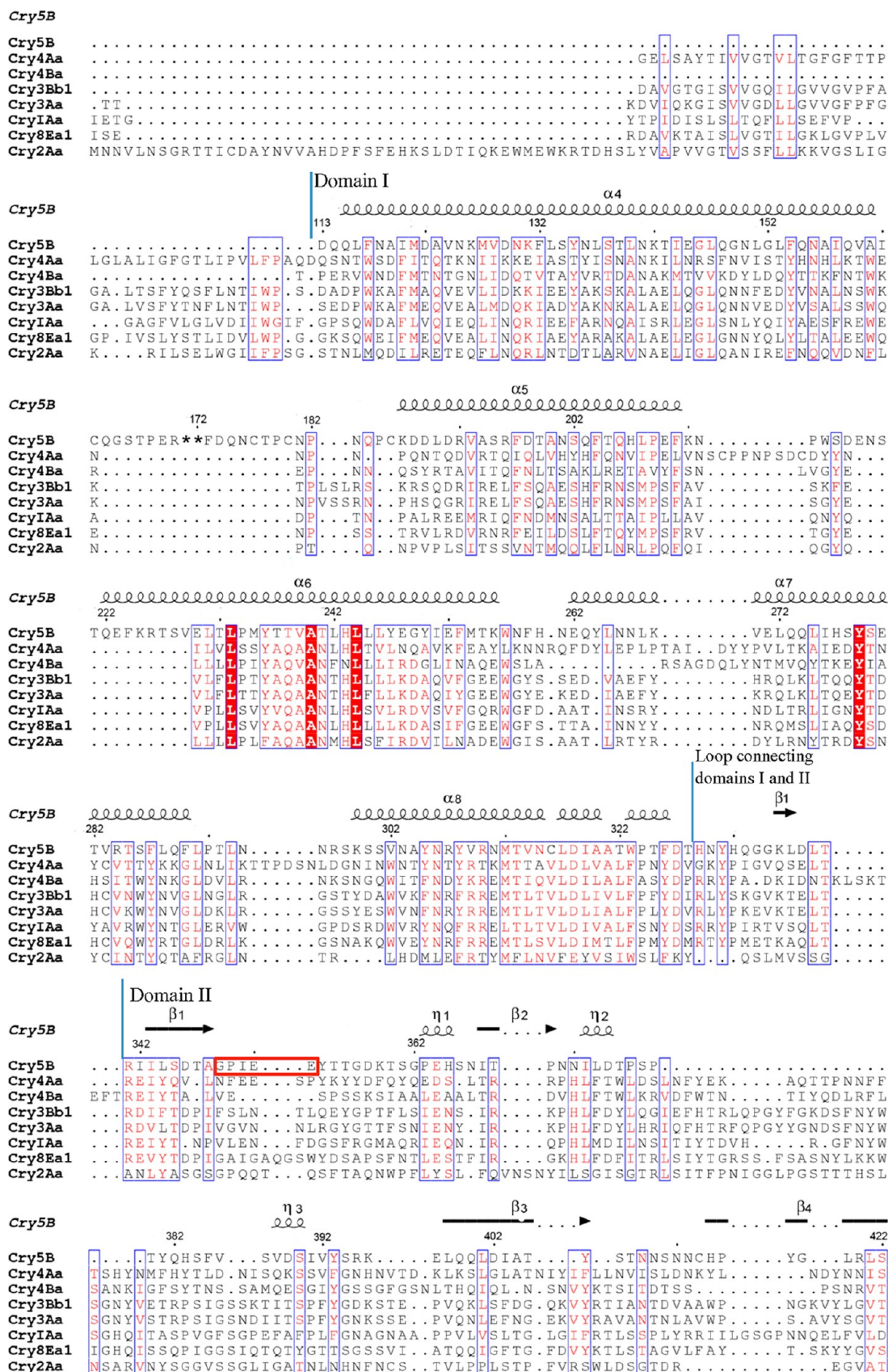


Figure 3. continued

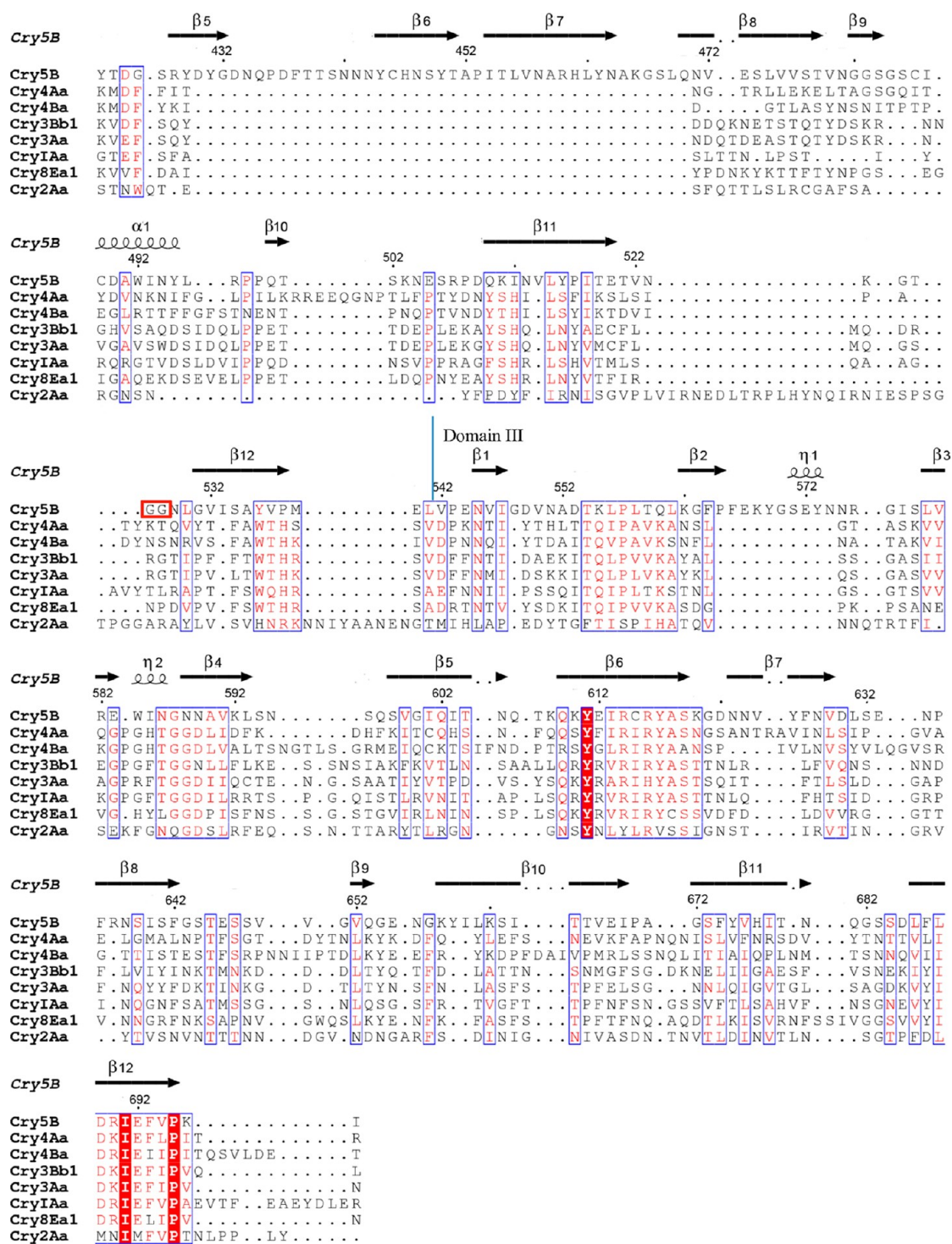


Figure 3. Sequence alignment. Structure-based sequence alignment of Cry5B and other structurally characterized Cry proteins was conducted with Expresso⁴⁵ and annotated with the secondary structure of Cry5B, indicated at the top, with ESPript.⁴⁶ Horizontal red boxes indicate the GXXXE and GG sequences in domain II.

antiparallel β -sheets (Figure 2d). The $\beta 10\beta 6\beta 12\beta 2$ sheet packs against domain II, whereas the $\beta 5\beta 11\beta 7\beta 8$ sheet faces solvent. Strands $\beta 1$ and $\beta 9$ are short and part of the $\beta 5\beta 11\beta 7\beta 8$ and $\beta 10\beta 6\beta 12\beta 2$ sheets, respectively. Two additional β -strands ($\beta 3$ and $\beta 4$) and two short 3_{10} -helices lie roughly perpendicular to the β -sandwich and connect $\beta 2$ to $\beta 5$. In contrast to domain II, domain III is structurally most similar to domain III of other Cry proteins (2.2 Å average rmsd, 127 average C α atoms, 20% average level of sequence identity). As with other Cry proteins,

domain III also is structurally similar to carbohydrate binding modules, such as the one in β -agarase (2.4 Å rmsd, 121 C α atoms, 15% identical sequence, Z score of 13.0). The structure of β -agarase bound to a hexasaccharide is known,⁵⁶ and the glycan-binding site in β -agarase is equivalent in Cry5B to the solvent-exposed face of the $\beta 5\beta 11\beta 7\beta 8$ sheet (Figure 2d).

Interactions of Cry5B with Glycolipids. Cry5B has been shown to bind several *C. elegans* glycolipid species,³⁵ all containing a core arthroses tetrasaccharide (GalNAc β 1–

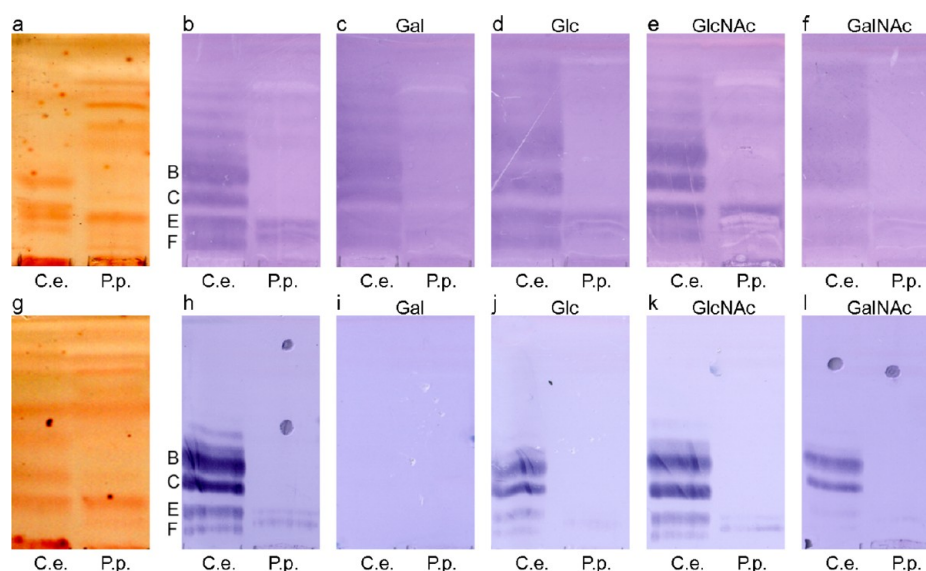


Figure 4. Binding of activated Cry5B and Cry5B protoxin to upper phase glycolipids. Upper phase glycolipids of *C. elegans* (C.e.) and *P. pacificus* (P.p.) were resolved by thin-layer chromatography and stained with orcinol (a and g) and overlaid with elastase-activated Cry5B (b–f) or Cry5B protoxin (h–l) in the presence of 100 mM galactose (Gal, c and i), 100 mM glucose (Glc, d and j), 100 mM *N*-acetylglucosamine (GlcNAc, e and k), or 100 mM *N*-acetylgalactosamine (GalNAc, f and l). The glycolipid band species are indicated to the left of panels b and h. This is a representative set from several experiments that were conducted.

4GlcNAc β 1–3Man β 1–4Glc) and antennary glycans (Gal, Glc, Fuc, and 2-O-Me-Fuc) (Figure S1 of the Supporting Information). Significantly, interaction of Cry5B with these glycolipids is effectively blocked by one of the antennary glycans, galactose,³⁵ suggesting that galactose serves at least in part as a Cry5B-binding determinant. To further characterize the interaction of Cry5B with glycolipids, we purified polar glycolipids from *P. pacificus*, a model nematode that is sensitive to Cry5B,^{29,57} along with those from *C. elegans*. The polar glycolipids from these nematodes were separated by thin-layer chromatography (TLC) and visualized by orcinol staining (Figure 4a,g). This staining showed that *P. pacificus* has a different set of glycolipids versus those of *C. elegans*. Next, Cry5B protoxin and elastase-activated Cry5B, which had both been biotinylated, were overlaid on the TLC plates and visualized. As shown previously, activated Cry5B bound to a number of the *C. elegans* glycolipids, including the B, C, E, and F species (Figure 4b and Figure S1 of the Supporting Information), and these interactions were inhibited specifically by galactose (Figure 4c).³⁵ For *P. pacificus*, a double band near the bottom of the TLC plate bound activated Cry5B, and this binding was also inhibited by galactose, demonstrating its specificity (Figure 4b,c). The same pattern of interaction was seen with Cry5B protoxin (Figure 4h,i), but these interactions were much weaker than for activated Cry5B. This was deduced from the fact that galactose completely eliminated glycolipid interactions in the case of Cry5B protoxin but only diminished them in the case of activated Cry5B (cf. Figure 4c,i). These results indicate that proteolytic activation is not required for the interaction of Cry5B with its glycolipid receptors and does not change the specificity of such interactions. Activation does, however, increase the affinity of these interactions.

We next asked which other monosaccharides competed with binding of *C. elegans* and *P. pacificus* glycolipid by Cry5B to gain further insight into the binding of Cry5B with its glycolipid receptors. We found that neither glucose nor *N*-acetylglucosamine (GlcNAc) affected interaction to the same extent as

galactose, although there was perhaps a slight diminution with glucose (Figure 4d,e,j,k). Glucose is at the base of the arthroses tetrasaccharide core (i.e., attached to ceramide) and is also one of the antennary sugars, and GlcNAc is part of the arthroses tetrasaccharide core (Figure S1 of the Supporting Information). Notably, we found that the addition of *N*-acetylgalactosamine (GalNAc) substantially weakened the binding of both Cry5B protoxin and activated Cry5B to the glycolipids (Figure 4f,l). GalNAc is part of the arthroses tetrasaccharide core and the sugar from which the antennary glycans emanate. These results provide evidence that Cry5B interacts with not only galactose but also GalNAc.

DISCUSSION

We report here the first structure of a nematocidal Cry protein and find it to be the most structurally divergent of the Cry proteins characterized to date. The previously characterized Cry proteins, which are all insecticidal, are highly similar in structure to one another.^{8,9,11–14} Of this insecticidal group, Cry2Aa is the most dissimilar,¹⁰ which may be related to the fact that it is toxic against both Lepidoptera and Diptera, while the other insecticidal Cry proteins act against only a single order, either Lepidoptera (Cry1Aa),⁹ Diptera (Cry4Aa and Cry4Ba),^{12,13} or Coleoptera (Cry3Aa, Cry3Bb, and Cry8Ea1).^{8,11,14} Cry5B is structurally even more divergent than Cry2Aa, with the largest divergence between Cry5B and the insecticidal Cry proteins occurring in domain II. This domain has been implicated in the receptor specificity in other Cry proteins,^{17,19–22} and in Cry5B, this activity would fit its divergent organismal specificity. In contrast, domain I is highly conserved between Cry5B and the insecticidal Cry proteins, which likely reflects its conserved role in pore formation.⁸ Domain III of Cry5B also bears a close relationship to insecticidal Cry proteins, although not as great as domain I but greater than domain II. This may reflect a role of domain III in modulating both receptor specificity and pore activity, as has been seen in other Cry proteins.^{6,18,21,23}

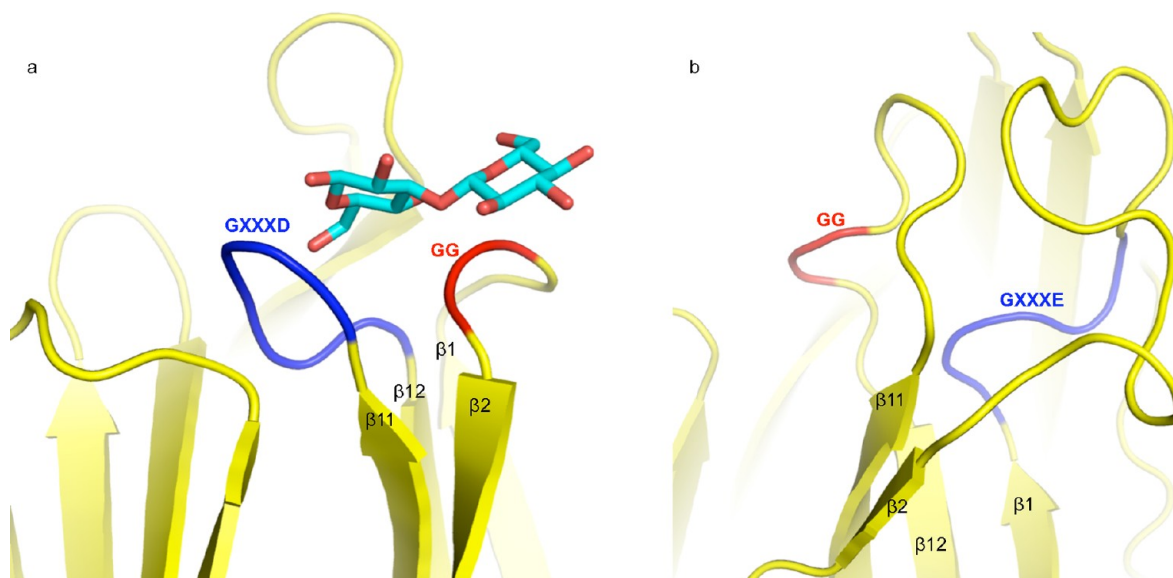


Figure 5. Glycan-binding motifs. (a) The $\beta 1/\beta 2$ loop of BanLec containing the GG motif (red) and the $\beta 11/\beta 12$ loop containing the GXXXD motif (blue) in ribbon representation. The structure of bound laminaribiose is shown in bond representation with carbons colored cyan and oxygens red. (b) $\beta 1/\beta 2$ loop of Cry5B domain II containing the GXXE motif (blue) and $\beta 11/\beta 12$ loop containing the GG motif (red) in ribbon representation.

The structure of Cry5B was modeled several years ago on the basis of the structure of Cry1Aa.⁵⁸ The modeled structure, however, is dissimilar in detail from the experimental one reported here, with an overall rmsd of 2.8 Å (401 C α) between the two; the greatest differences occur in domain II (rmsd of 3.4 Å, 131 C α atoms). This is not surprising given our finding that Cry5B is the structurally most divergent Cry protein characterized to date, and that domain II is the most dissimilar of all the domains.

We found that Cry5B domain II is most similar in structure not to another Cry protein, but instead to the lectin BanLec.⁵⁵ BanLec is a member of the mannose-specific jacalin-related superfamily of lectins and binds both glucose and mannose. The structure of BanLec with two different bound glycans is known, with each of these glycans being observed to bind at two separate sites.⁵⁵ Site 1 is conserved in all lectins and in BanLec is formed by the $\beta 1/\beta 2$ loop, which contains a GG sequence, and the $\beta 11/\beta 12$ loop, which contains a GXXXD sequence (Figure 5). Site 2 is specific to BanLec and some related lectins and is formed by the $\beta 3/\beta 4$ loop, which contains a GXXXD sequence, and the $\beta 5/\beta 6$ loop, which contains a GG sequence. In BanLec, most of the hydrogen bonds to the glycans are through main chain amides, while the Asp of the GXXXD sequence makes the only side chain hydrogen bonds. The equivalent of site 1 potentially exists in Cry5B. The $\beta 11/\beta 12$ loop in Cry5B contains a GG sequence, and the $\beta 1/\beta 2$ loop has a GXXXE sequence, closely approximating the GXXXD sequence (Figures 3 and 5). These loops are considerably longer than in BanLec and may accommodate longer saccharide chain lengths. The equivalent of site 2 does not appear to exist in Cry5B, as the $\beta 3/\beta 4$ and $\beta 5/\beta 6$ loops lack GG and GXXXD sequences. The conservation of the BanLec glycan-binding motifs in Cry5B domain II provides further evidence that domain II is likely to be responsible for recognizing nematode glycolipids.

In contrast, domain III of Cry1Ac has been implicated in binding GalNAc moieties that decorate the surface of its putative receptor aminopeptidase N.^{59,60} The GalNAc-binding site in Cry1Ac was suggested by mutational evidence to reside

on the solvent-exposed face of the β -sandwich fold in domain III (equivalent to the solvent-exposed surface of the $\beta 5/\beta 11/\beta 7/\beta 8$ sheet in Cry5B). This supposition was based on modeling, as no structure of Cry1Ac is available, despite the fact that Cry1Ac was reported to be crystallized a number of years ago.⁶¹ The putative GalNAc-binding site of Cry1Ac may be unique, in that the Cry1Ac residues identified to be involved in binding GalNAc are dissimilar from those of other Cry toxins, including the highly related Cry1Aa and the more distant Cry5B.⁵⁹ Additionally, the functional significance of the interaction between Cry1Ac and GalNAc is questionable, as disruption of this interaction fails to reduce toxicity.⁵⁹

It is worth noting that other Cry proteins besides Cry5B are able to bind glycolipids. This includes some insecticidal Cry proteins. Members of the Lepidoptera-specific Cry1A family bind the glycolipids of *Manduca sexta* and *Plutella xylostella*.^{34,62} This raises the question of why the BanLec-like domain II of Cry5B does not occur in Cry1Aa as well. One possibility is that the interaction of Cry proteins with roundworm and insect glycolipids is quantitatively different, e.g., that Cry5B has developed a more dominant reliance on glycans as receptors. This possibility is in accord with the observation that *C. elegans* lacking glycolipid receptors is resistant to as much as 1 mg/mL Cry5B.⁶³ Alternatively, there may be a different, as yet unknown, carbohydrate receptor for Cry5B that is present in worms but not insects.

We also found that GalNAc, a part of the arthroseries tetrasaccharide core, is a competitor for interaction between Cry5B and its glycolipid receptors. The competition experiment required a high monosaccharide concentration (i.e., 100 mM), indicative of the much higher affinity of Cry5B for its intact glycolipid receptor than for simple sugars. This is also consistent with the observation that Cry5B is effective in vivo as an anthelmintic,^{24,25} demonstrating that dietary sugars and glycans do not inhibit it from interacting with its receptor. Along with our identification of GalNAc as a binding determinant, prior results provide further definition. These results have shown that Cry5B binds *C. elegans* glycolipid band E but not band D (Figure S1 of the Supporting Information).³⁴

Significantly, band E has two antennary galactoses attached to the tetrasaccharide core, while band D has only one antennary galactose attached to the tetrasaccharide core. Thus, this prior observation combined with our current results suggests that the minimal binding determinant for Cry5B consists of GalNAc from the arthroses core decorated with at least two antennary galactoses.

Lastly, we found that the protoxin and activated forms of Cry5B bind nematode glycolipids with similar specificities but markedly differing affinities. The protoxin form has weaker receptor affinity, suggesting that the receptor-binding site is partially occluded by the N- or C-terminal regions, or both, that are present in the protoxin but absent in the activated protein. Furthermore, these results suggest that an initially weak binding event between the protoxin and glycolipid receptors could be stabilized by subsequent proteolysis of the protoxin on the nematode cell surface.

In summary, this first structure of a nematocidal Cry protein coupled with biochemical characterization of its interactions with nematode glycolipids has laid the groundwork for detailed dissection of Cry5B function, with the ultimate aim of devising an effective anthelmintic to combat parasitic worms.

■ ASSOCIATED CONTENT

● Supporting Information

Supplemental Figure 1 and Supplemental Table 1. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

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■ ABBREVIATIONS

BanLec, banana lectin; Bt, *B. thuringiensis*; Cry, crystal; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcNAc, *N*-acetylglucosamine; NTA, Ni²⁺-nitrilotriacetic acid; rmsd, root-mean-square deviation; SAD, single anomalous diffraction; SeMet, selenomethionine; TLC, thin-layer chromatography.

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