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Identification and partial purification of a *Bacillus thuringiensis* CryIC δ-endotoxin binding protein from *Spodoptera littoralis* gut membranes

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Immunoblotting experiments were performed using CryIC and CryIA(c) *Bacillus thuringiensis* δ-endotoxins to detect the presence of specific toxin binding proteins on *Spodoptera littoralis* brush border membrane vesicles. The CryIC toxin binds two proteins of 40 and 65 kDa and the CryIA(c) binds a protein of 40 kDa. The CryIA(c) toxin also binds faintly to a 120 kDa protein on *S littoralis* brush border membrane vesicles as does a polyclonal antiserum raised against a putative CryIA(c) 120 kDa binding protein from *Manduca sexta*. The 40 kDa CryIC binding protein was partially purified by affinity chromatography and is therefore a strong candidate for in vivo *S. littoralis* CryIC toxin receptor.

δ-Endotoxin; Toxin receptor; Membrane protein; *Spodoptera littoralis*; *Bacillus thuringiensis*

1. INTRODUCTION

During sporulation, the Gram-positive bacterium *Bacillus thuringiensis* produces insecticidal proteins designated δ-endotoxins that accumulate to high concentration and form parasporal crystalline inclusions within the cell [1]. Upon ingestion by susceptible insect larvae, the crystalline inclusions dissolve in the alkaline midgut lumen and the δ-endotoxins are activated by the action of the larval midgut proteases [2,3]. Subsequently, the toxins bind specific receptors located on the surface of the larval midgut epithelial cells [4–6] and disturb the osmotic balance of the cell membrane [7], probably through the formation of transmembrane pores [8], causing cell lysis and eventually the death of the insect larvae.

The insecticidal and activity spectrum of each strain is mainly determined by the composition of the crystal inclusion which contains one or more related δ-endotoxins varying in amino acid composition, size and toxic specificity [9]. Nevertheless, the molecular basis for the differences in insecticidal spectrum are still uncompletely understood. Differences in pH and proteases in the insect gut lumen have been shown to be factors determining the insecticidal specificity of the δ-endotoxins by altering solubilization or proteolytic activation of the crystal [10,11]. More recently, binding experiments using [^{125}I]-purified activated toxin and isolated brush border membrane vesicles (BBMV) prepared from midgut epithelial cells showed a positive correlation between larval susceptibility to a given toxin and the presence of high affinity binding sites for the toxin [4,5]. The results also indicated that both the affinity and the number of specific binding sites for the toxin are important factors in the determination of the insecticidal specificity [6]. However, a negative correlation between receptor affinity and toxicity was reported in one case [12] suggesting that binding of the toxin is necessary but not sufficient for toxicity. Recent work to identify putative insect BBMV binding molecules specific to *B. thuringiensis* CryIA δ-endotoxins showed that a variety of polypeptides of molecular masses 63, 81, 90, 103, 120, 140, 148, 155 or 170 kDa bind the toxins depending on the insect and CryIA toxin studied [13–15].

Lepidopteran insects belonging to the Noctuidae family, such as *Spodoptera littoralis*, are important agricultural pests which are poorly susceptible to most of the δ-endotoxins. In a previous study [16] we have reported the cloning and characterization of a new type of δ-endotoxin gene, designated *CryIC*, encoding an insecticidal crystal protein specifically toxic against *S. littoralis* and against several other species of the Noctuidae family. In the present study we describe the identification and partial purification of a strong candidate for in vivo *S. littoralis* CryIC toxin receptor.

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2. MATERIALS AND METHODS

2.1. S. littoralis *BBMV preparation*

S littoralis BBMV's were prepared according to the protocol described by Wolfersberger et al. [17] except that the following protease inhibitor cocktail was included: 0.1 mM 1.10 phenanthroline, 0.1 mM 3,4 DCI (3,4 Dichloroisocoumarin) and 0.05 mM E64 (*N*-[*N*-(L-3-*trans* carboxyrane-2-carbonyl)-L-leucyl]-agmatine). The protein con-

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tent of the BBMV's was determined using the BAC protein assay reagent. The midguts of 400 larvae (10 g of fresh tissue) were used and the yield was about 10 mg of BBMV per 10 g of guts. 1 mg aliquots of BBMV were immediately frozen in liquid nitrogen and stored at −80°C.

2.2. *Immunovisualisation of the affinity binding of the CryIC and CryIA(c) toxins to* S. littoralis

BBMV preparations (50 μg total protein) were separated by SDS-PAGE and electrotransferred to nitrocellulose membranes. The blots were then soaked overnight in Tris-buffered saline (TBS) to renature the proteins. Non-specific binding was blocked using 3% haemoglobin in TBS for 60 min. After that, blots were incubated for 90 min with 300 μg of trypsin-activated CryIC or CryIA(c) toxin in 50 ml of blocking buffer. Unbound toxin was removed by washing in three changes ot TBS and the bound toxin was identified by incubating the blots in 50 ml of blocking solution containing 10 μl of antitoxin polyclonal antibodies for 90 min. The unbound antibodies were removed by washing in three changes of TBS and the membrane-bound complex was visualized using 50 ml of blocking solution containing 25 μl of peroxidase-labelled secondary antiserum. The colour reaction was developed in 50 ml TBS containing 30 mg 4-chloro-1-napthol dissolved in 10 ml methanol and 25 μl hydrogen peroxide.

2.3. *CHAPS extraction of* S. littoralis *BBMV proteins*

10 mg of *S. littoralis* BBMV's prepared as described above (~10 ml) were centrifuged 10 min at 4°C and 10,000 rpm in a minifuge and washed twice with distilled water. The pellet was then resuspended in 10 ml of 1% CHAPS in 50 mM sodium carbonate buffer pH 9.5 and kept on ice for 30 min. The mixture was then spun at 100,000 × *g* (36,000 rpm) in a Ti 50 rotor for 1 h at 4°C. The supernatant (~10 ml) was concentrated to 2 ml using Centricon 10 concentrators and the protein content was determined using the BCA protein assay reagent. 4 to 5 mg of soluble proteins were generally obtained.

2.4. *Affinity purification of* S. littoralis CryIC binding proteins

An Epoxy-spacer Activated Agarose 4XL matrix purchased from ACL (Affinity Chromatography Ltd) was used for the immunobilisation of CryIC trypsin-activated toxin. The pre-activated support supplied as a freeze-dried preparation was rehydrated and washed with water as described by the manufacturer prior to a final wash with 50 mM sodium carbonate buffer pH 10 (coupling buffer). 2 ml of wet gel and 10 mg of CryIC toxin were used for the coupling reaction that was performed at room temperature by mixing gently for 6 h. 2 mg of CryIC-activated toxin were coupled, using these conditions. The residual reactive groups were blocked by incubating the gel for 16 h at room temperature with 2 M ethanolamine pH 9.5. The gel was then loaded on a column, washed with 10 volumes of coupling buffer and stored at 4°C in 50 mM sodium carbonate buffer pH 7.5 containing 0.02% sodium azide until required for purification. The column was equilibrated with 10 ml of sample buffer (50 mM sodium carbonate pH 9.5 containing 1% CHAPS). 2 ml of CHAPS solubilised *S. littoralis* BBMV proteins (4 to 5 mg of proteins) were applied to the affinity column. The column was washed with 20 ml of sample buffer and eluted with NaCl 1.5 M. 2 ml fractions were collected and concentrated to 100 μl using Centricon 10 concentrators, 25 μl of 5× gel sample buffer were added to the concentrated samples and 50 μl were loaded on SDS-PAGE. The gel was run, blotted and developed as described for the immunovisualisation of the CryIC binding proteins.

3. RESULTS

The binding of CryIC to *S. littoralis* BBMV's has been investigated by immunoblotting. Fig. 1A shows an SDS-PAGE of the *S. littoralis* BBMV preparation (50 μg total protein per lane) and the CryIC activated toxin used in the experiments. The *S. littoralis* BBMV's were electrophoresed, transferred to nitrocellulose membranes and incubated with CryIC toxin as described in section 2. The results indicated that the CryIC toxin bound to a 65 kDa protein and a 40 kDa protein (Fig. 1C). The 65 kDa protein was also observed in controls where the toxin incubation was not performed (Fig. 1D) indicating that it could correspond to a non-specific signal.

Immunoblotting experiments performed using the CryIA(c) toxin and *S. littoralis* BBMV's to detect the presence of putative CryIA(c) binding proteins on *S. littoralis* BBMV's showed (Fig. 2A) that the CryIA(c) toxin bound to a 40 kDa protein which is not detected in the control lacking the toxin (Fig. 2B). A very faint binding to 120 kDa protein was also detected (Fig. 2A, lane 2). The 116 kDa β-galactosidase present in the molecular weight standards (Fig. 2A, lane 3) also reacted positively when preincubated with toxin but did not react in the control in which the toxin was omitted (Fig. 2B, lane 3) indicating this is not only the result of binding of primary or secondary antibody but that this protein seems to bind the toxin with some affinity.

A polyclonal antiserum raised against a putative CryIA(c) 120 kDa binding protein purified from a *M. sexta* gut preparation (P.J. Knight, personal communication) was also used for an immunoblot analysis of *S. littoralis* BBMV's. These antibodies cross-reacted with the CryIC toxin and recognized a 120 kDa polypeptide (Fig. 2C) that did not react when the preimmune serum was used as a control (data not shown).

In order to further characterize the putative *S. littoralis* CryIC binding proteins we decided to purify these proteins using affinity purification techniques. The starting material for the purification of the putative receptors was 4 mg of CHAPS-solubilised *S. littoralis* BBMV proteins and the purification was performed as described in section 2. The Epoxy spacer activated support (12 atoms hydrophilic spacer arm) was chosen to allow immobilisation of the toxin at a reasonable distance from the support to ensure that the toxin can interact with the receptor after immobilisation. A further point in choosing this type of support was that the secure epoxyde linkage requires one covalent bond for attachment. Therefore, the immobilised support was saturated with toxin when coupling to ensure that a maximum of toxin molecules was immobilised via the minimum of bonds and therefore that the 3D structure of the toxin was not disrupted. Moreover, as the reactivity of this matrix is not very good, the coupling time was also shortened and the coupling temperature lowered to reduce as much as possible the number of covalent bonds for attachment. Finally, to enhance the stability of the CHAPS protein extract the following protease inhibitor cocktail: 0.1 mM 1.10 phenanthroline, 0.1 mM 3,4 DC1 and 0.05 mM E64 were added to the CHAPS extraction buffer.

The presence of CryIC-binding proteins in the 2 ml concentrated fractions eluted from the CryIC Epoxy-

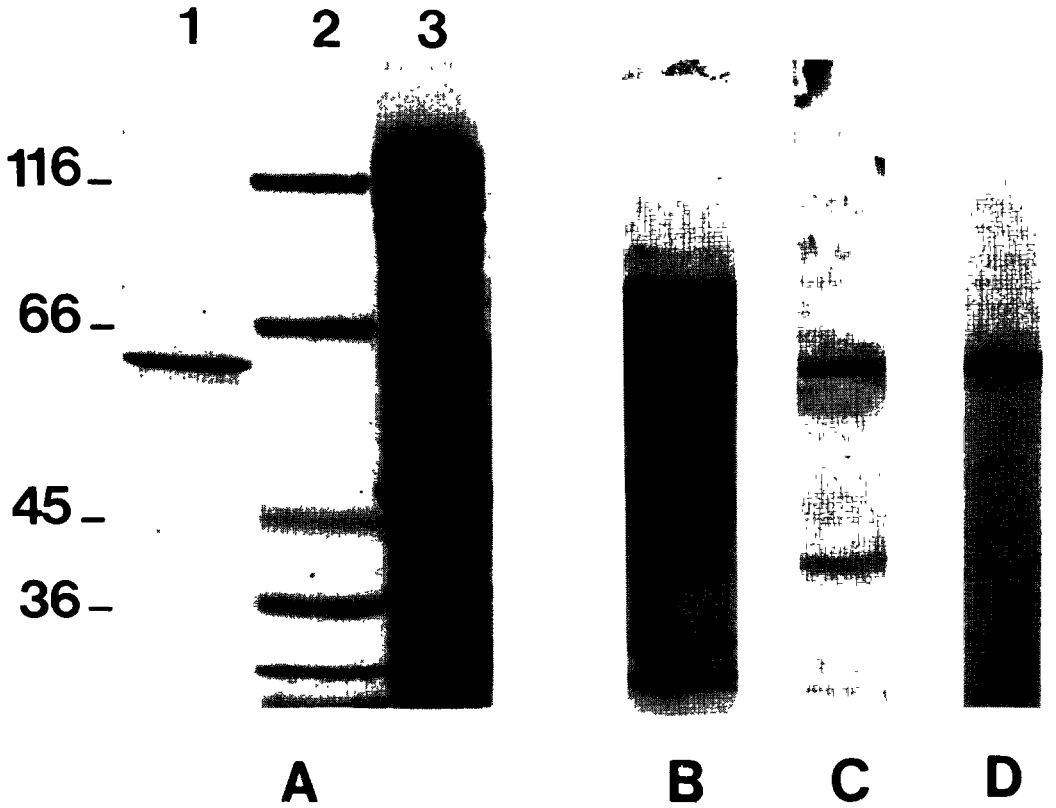

Fig. 1. Identification of *S. littoralis* CryIC binding proteins. (A) SDS-10% PAGE of CryIC activated toxin and *S. littoralis* BBMV's. Lane 1, CryIC activated toxin; lane 2, molecular weight markers; lane 3, *S. littoralis* BBMV's. The toxin in (B) and the BBMV's in (C) were electrotransferred to nitrocellulose and the membranes were incubated with 300 μg of CrilC toxin. Bound toxin was detected by immunovisualisation as described in section 2. Track (D) is a control in which the preincubation with CryIC toxin was omitted after electrotransfer of *S. littoralis* BBMV's to nitrocellulose.

spacer affinity column was analysed by immunoblotting. Two fractions containing CryIC binding proteins were identified: in one fraction (Fig. 3, lane 2), the CryIC bound to a 65 kDa band and to a 40 kDa band, whereas in an other fraction (Fig. 3, lane 3), the toxin bound to a 110 kDa polypeptide never detected before. This result shows that the affinity column used was functional and that it is possible to purify CryIC binding proteins using this strategy.

4. DISCUSSION

In this report, we have investigated the binding of CryIC and CryIA(c) toxins to *S. littoralis* BBMV's using a ligand blotting technique to visualize the interaction of CryIC and CryIA(c) toxins with proteins extracted from brush border membrane vesicles and transferred to nitrocellulose. Using this procedure, we have shown that the CryIC toxin binds to a 65 kDa protein and a 40 kDa protein on *S. littoralis* BBMV's. The 65 kDa protein was also seen in controls in which the toxin was omitted but not in controls where the preimmune serum was used instead of the primary anti-CryIC antibody or in controls where the primary anti-CryIC antibody was omitted, indicating that this was the result of binding of the toxin specific primary antibody. Therefore, this 65 kDa CryIC-binding protein must share some antigenic determinants with the CryIC toxin. The 40 kDa binding protein for which all the controls were negative, is therefore a possible candidate for in vivo *S. littoralis* CryIC toxin receptor. The fact that the CryIA(c) toxin also bound to a 40 kDa protein on *S. littoralis* BBMV's (Fig. 2A, lane 2) also supports this conclusion and indicates that in *S. littoralis* these two toxins bind putative receptors that have the same size. Whether these two binding proteins are identical or related must await their isolation and structural comparison.

The immunoblotting of the CryIC toxin with polyclonal antibodies directed against a purified 120 kDa CryIA(c) binding protein from *M. sexta* showed that the CryIC toxin was specifically recognized by this antiserum (Fig. 2C, lane 1). This result could indicate that the *M. sexta* CryIA(c) 120 kDa putative receptor shares some homology with the CryIC toxin. However, as the 120 kDa putative receptor used to raise the antibodies was purified using a CryIA(c) 130 kDa protoxin affinity column, a leakage of protoxin from the column during the purification and contamination of the putative 120 kDa receptor with protoxin cannot be completely ex-

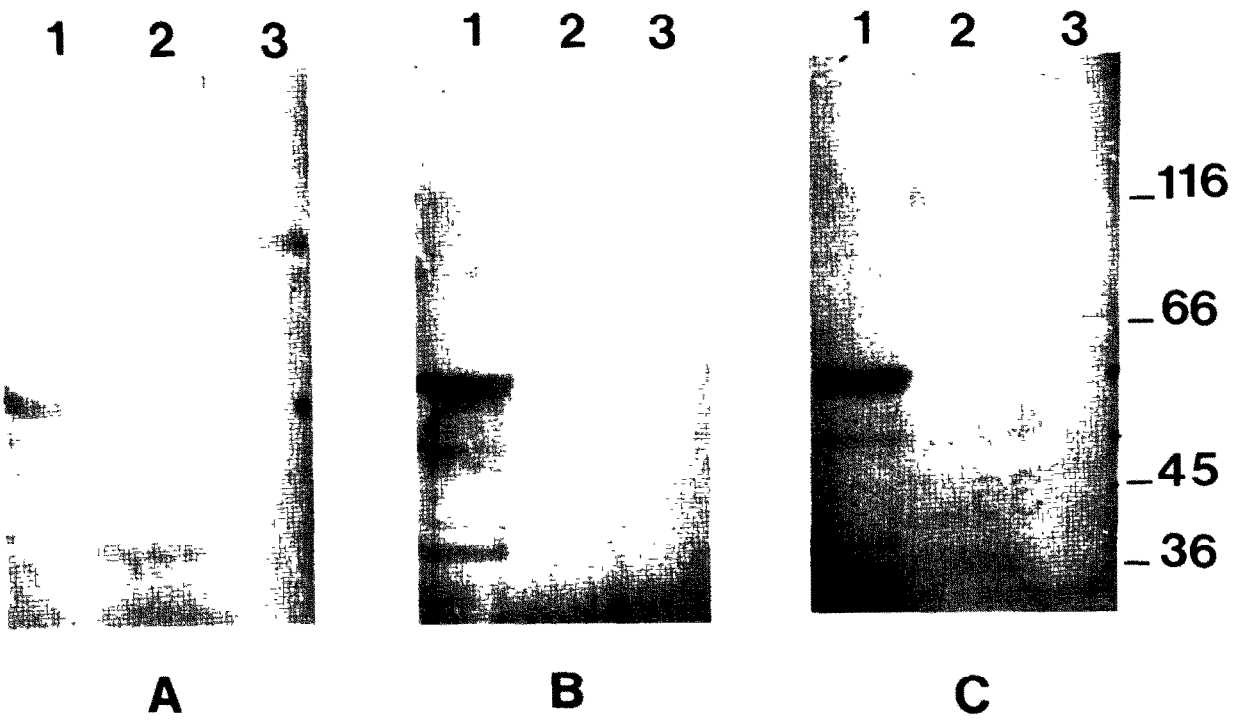

Fig. 2. Binding of the CryIA(c) toxin to *S. littoralis* BBMV's. The BBMV's were subjected to SDS-10% PAGE and electrotransferred to nitrocellulose. In (A) the membrane was incubated with 300 μg of CryIA(c) toxin and bound toxin was detected by immunovisualisation using anti-CryIA(c) polyclonal antibodies as described in section 2. (B) control in which the preincubation with the CryIA(c) toxin was omitted. In (C) immunoblot of *S littoralis* BBMV's with polyclonal antibodies directed against the 120 kDa CryIA(c) binding protein from *M. sexta*. Lane 1, CryIC activated toxin; lane 2, *S littoralis* BBMV's; lane 3, molecular weight markers.

cluded. Therefore, the antiserum obtained could also contain anti-CryIA(c) antibodies which could explain the cross-reaction with CryIC.

Another point is that an antiserum directed against a CryIA(c) 120 kDa binding protein of *M. sexta* does bind to a protein of the same size on *S. littoralis* BBMV's (Fig. 2C, lane 2) and that the CryIA(c) toxin does also seem to bind to a 120 kDa polypeptide (although very weakly) on *S. littoralis* BBMV's (Fig. 2A, lane 2). Taken together these results could indicate that the CryIA(c) binding protein of *M. sexta* is also present in *S. littoralis* and that the CryIA(c) binds at least two proteins in *S. littoralis*: a 40 kDa polypeptide that could also be the CryIC binding protein and a 120 kDa polypeptide that could be CryIA(c) specific and immunologically related to the CryIA(c) binding protein present in *M. sexta*. Moreover, as *S. littoralis* is much less susceptible to CryIA(c) than to CryIC, it is possible that the region corresponding to the toxin binding site is different between the two 120 kDa proteins in the two insect explaining both the weak binding of CryIA(c) to *S. littoralis* BBMV's and its low toxicity to *S. littoralis*.

Finally it should be noted that the toxins bound repeatedly to the 116 kDa β-galactosidase present in the molecular weight standards. This result is very difficult to explain and may be caused by non specific binding of the toxins to the β-galactosidase due to the high amount of β-galactosidase present per experiment but it may also reflect some affinity of the β-galactosidase for the CryIC and CryIA(c) toxins.

Affinity chromatography using CryIC-activated toxin immobilised on an Epoxy-spacer activated agarose support was used for the separation and screening of fractions containing CryIC binding proteins. Immunodetectable amounts of three CryIC binding proteins with molecular weights of about 40, 65 and 110 kDa were purified by affinity chromatography. This result is in agreement with the reported heterogeneity of putative *B. thuringiensis* toxin receptors [13,14] and the demonstration by Van Rie et al. [6] that at least two high-affinity binding sites are recognized by the CryIC toxin on *S. littoralis* BBMV's. Therefore one or several of the affinity purified polypeptides are likely candidates for in vivo *S. littoralis* toxin binding molecules. Nevertheless, although the affinity purification technique described in this report appears to be a valuable method for the purification of CryIC binding proteins, the amounts of purified putative receptor(s) obtained were not sufficient to permit further analysis. Indeed, it was necessary to use the totality of the concentrated fractions to visualise these proteins by immunoblotting. As it is difficult to increase the amount of starting material for purification when using *S. littoralis* as a source of experimental animals, this insect cannot be easily used as a source of material from which to purify CryIC receptors.

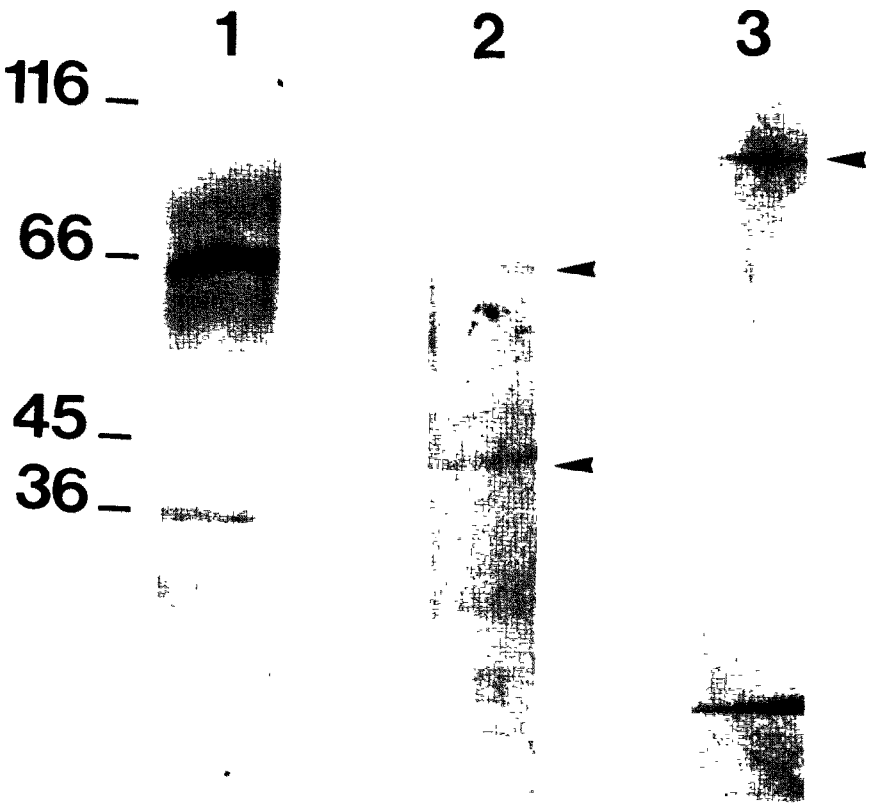

Fig. 3. Immunodetection of CryIC binding proteins on fractions from affinity chromatography. The concentrated fractions purified by affinity chromatography were analysed by immunoblotting as described in section 2. Lane 1, CryIC-activated toxin; lanes 2 and 3, affinity chromatography fractions containing CryIC binding proteins

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