Eur. J. Biochem. **271**, 3127–3135 (2004) © FEBS 2004 doi:10.1111/j.1432-1033.2004.04238.x

Characterization of a Cry1Ac-receptor alkaline phosphatase in susceptible and resistant *Heliothis virescens* larvae

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We reported previously a direct correlation between reduced soybean agglutinin binding to 63- and 68-kDa midgut glycoproteins and resistance to Cry1Ac toxin from *Bacillus thuringiensis* in the tobacco budworm (*Heliothis virescens*). In the present work we describe the identification of the 68-kDa glycoprotein as a membrane-bound form of alkaline phosphatase we term HvALP. Lectin blot analysis of HvALP revealed the existence of N-linked oligosaccharides containing terminal *N*-acetylgalactosamine required for [^{125}I]Cry1Ac binding in ligand blots. Based on immunoblotting and alkaline phosphatase activity detection, reduced soybean agglutinin binding to HvALP from Cry1Ac resistant larvae of the *H. virescens* YHD2 strain was attributable to reduced amounts of HvALP in resistant larvae. Quantification of specific alkaline phosphatase activity in brush border membrane proteins from susceptible (YDK and F_1 generation from backcrosses) and YHD2 *H. virescens* larvae confirmed the observation of reduced HvALP levels. We propose HvALP as a Cry1Ac binding protein that is present at reduced levels in brush border membrane vesicles from YHD2 larvae.

Keywords: alkaline phosphatase; Cry1Ac; *Heliothis virescens*; resistance; *N*-acetylgalactosamine.

Specific binding to insect midgut receptors is a key step in the mode of action of insecticidal Cry toxins from the bacterium *Bacillus thuringiensis* (Bt). Despite exceptions [1], in most cases Cry toxin specificity and potency correlate with the extent of toxin binding to midgut brush border membrane receptors *in vitro* [2,3]. Effective toxin binding to receptors results in toxin insertion and oligomerization on the midgut cell membrane, leading to pore formation and cell death by osmotic shock [4].

In brush border membrane vesicles (BBMV) from *Heliothis virescens* (tobacco budworm) larvae, three groups of binding sites (A, B, and C) for Cry1A toxins were proposed based on their toxin binding specificities [5,6]. The A binding sites, which bind Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa and Cry1Ja toxins, include the cadherin-like protein HevCaLP (J. L. Jurat-Fuentes, L. Gahan, F. Gould, D. Heckel and M. Adang, unpublished observation) and a 170-kDa *N*-aminopeptidase (APN) [5,7–9]. Currently, there is evidence that both HevCaLP [10] (J. L. Jurat-Fuentes, L. Gahan, F. Gould, D. Heckel and M. Adang, unpublished observation); and the 170-kDa APN [8,10] function as Cry1A toxin receptors. In the B binding site group, a 130-kDa protein has been shown to recognize both Cry1Ab and Cry1Ac. The C binding site group includes Cry1Ac toxin-binding proteins smaller than 100-kDa in size [5]. We reported previously a correlation between altered glycosylation of 63- and 68-kDa glycoproteins that are part of the C binding site group and resistance to Cry1Ac in the *H. virescens* YHD2 strain [11].

Cry1 toxin-binding proteins of 60- to 80-kDa in size have been described in toxin overlays of BBMV proteins from *H. virescens* [5], *Manduca sexta* [1], and *Plodia interpunctella* [12]. In 2D proteomic analysis of *M. sexta* BBMV proteins, McNall and Adang [13] reported Cry1Ac binding to a 65-kDa form of alkaline phosphatase (ALP, EC 3.1.3.1). Membrane-bound ALP from *Bombyx mori* and *M. sexta* are attached to the brush border cell membrane by a glycosylphosphatidylinositol (GPI) anchor [13–15]. Specific interactions between Cry1Ac and ALPs under native conditions resulting in inhibition of phosphatase activity have been reported for *M. sexta* [16] and *H. virescens* [17]. However, the potential role for alkaline phosphatases in Cry1Ac intoxication has not been addressed directly.

The main goals of the present study were to identify the 68-kDa glycoprotein and characterize its oligosaccharide residues as a first step to investigate the specific alteration of this glycoprotein in Cry1Ac-resistant YHD2 larvae. Based on reported molecular sizes of insect alkaline phosphatases, and their interaction with Cry1 toxins, we hypothesized the 68-kDa glycoprotein to be a form of alkaline phosphatase. Immunoblotting and enzymatic activity experiments identified the 68-kDa protein as a GPI-anchored form of alkaline phosphatase we term HvALP (for *H. virescens* alkaline phosphatase). Ligand blots and glycosidase digestion

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Abbreviations: ALP, alkaline phosphatase; APN, *N*-aminopeptidase; BBMV, brush border membrane vesicles; Bt, *Bacillus thuringiensis*; CRD, cross-reacting determinant; dALP, digestive fluid alkaline phosphatase; GPI, glycosylphosphatidylinositol; GalNAc, *N*-acetylgalactosamine; HRP, horseradish peroxidase; HvALP, *Heliothis virescens* alkaline phosphatase; mALP, membrane-bound form of alkaline phosphatase; PBST, NaCl/P_i buffer containing 0.1% Tween-20; PIPLC, phosphatidylinositol-specific phospholipase C; PNG-F, peptide-*N*-glycosidase F; pNPP, *p*-nitrophenyl phosphate disodium; SBA, soybean agglutinin.

Enzyme: alkaline phosphatase (EC 3.1.3.1).

(Received 21 April 2004, revised 20 May 2004, accepted 1 June 2004)

demonstrated that an N-linked oligosaccharide containing a terminal *N*-acetylgalactosamine (GalNAc) residue on HvALP was necessary for Cry1Ac binding. Immunoblotting and specific alkaline phosphatase activity of BBMV proteins from susceptible and resistant larvae provided evidence that decreased HvALP levels were produced in YHD2 larvae. Our results provide evidence that HvALP is involved in Cry1Ac toxicity to *H. virescens* larvae.

Materials and methods

Insect strains and brush border membrane vesicle (BBMV) preparation

H. virescens laboratory strains YDK and YHD2 have been described previously [18]. YDK is the unselected susceptible control colony for the Cry1Ac-selected YHD2 strain, which developed 10 000-fold resistance to Cry1Ac when compared to susceptible YDK larvae [19]. After continuous selection with Cry1Ac, levels of resistance increased to 73 000-fold [11]. Fifth instar larvae from each strain were dissected and midguts frozen and kept at −80 °C until used to prepare BBMV.

BBMV were isolated by the differential centrifugation method of Wolfersberger *et al.* [20]. BBMV proteins were quantified by the method of Bradford [21], using BSA as standard, and kept at −80 °C until used. APN activity using leucine-*p*-nitroanilide as the substrate was used as a marker for brush border enzyme enrichment in the BBMV preparations. APN activities were enriched six- to eight-fold in the BBMV preparations compared to initial midgut homogenates.

Cry1Ac toxin purification and labeling

B. thuringiensis strain HD-73 obtained from the Bacillus Genetic Stock Center (Colombus, OH, USA) was used to produce Cry1Ac. Mutated Cry1Ac QNR(509–511) → AAA(509–511) was expressed in *Escherichia coli* MV 1190 kindly provided by D. Dean (Ohio State University, OH, USA), and purified as described elsewhere [22]. This Cry1Ac mutant toxin lacks the GalNAc binding properties of the wild-type toxin [23]. Cry1Ac crystalline inclusions were solubilized, activated and purified as described previously [24]. Purified toxin samples (verified by 10% reducing SDS/PAGE) were pooled, the protein concentration determined as for BBMV proteins and stored at −80 °C until used.

Purified Cry1Ac (1 μg) was radiolabeled with 0.5 mCi of [^{125}I]Na by the chloramine T method [1]. Specific activities of labeled samples were 3–8 mCi·mg^{-1}, as determined using the bindability method of Schumacher *et al.* [25]. Labeled toxins were kept at 4 °C and used within 10 days.

Quantification of alkaline phosphatase and aminopeptidase activities

Specific alkaline phosphatase (ALP) and *N*-aminopeptidase (APN) enzymatic activities of BBMV proteins were measured using *p*-nitrophenyl phosphate disodium (*p*NPP) and leucine-*p*-nitroanilide (Sigma, St. Louis, MO, USA) as substrates, respectively. BBMV proteins (1 μg) were mixed with ALP buffer (100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) or NaCl/P_i buffer (10 mM Na_2HPO_4, pH 7.5, 135 mM NaCl, 2 mM KCl) containing 1.25 mM *p*NPP or 0.8 mM leucine-*p*-nitroanilide, respectively. Enzymatic activities were monitored as changes in the A_{405}-value for 5 min at room temperature (ALP) or at 37 °C (APN) in a microplate reader (Molecular Devices). One enzymatic unit was defined as the amount of enzyme that would hydrolyze 1.0 μmole of substrate to chromogenic product per min at the specific reaction pH and temperature. Data shown are the mean specific activities from at least four independent BBMV batches from each *H. virescens* strain measured in at least three independent experiments.

Ligand, lectin and immunoblots of BBMV proteins

BBMV proteins (15 or 2 μg) were separated by SDS/PAGE 8%, and gels were either stained or electrotransferred to poly(vinylidene difluoride) Q membrane filters (Millipore). After overnight transfer, filters were blocked for 1 h at room temperature with NaCl/P_i buffer containing 0.1% Tween-20 (PBST) and 3% BSA.

For immunoblots, blocked filters were probed with a 1 : 25 000 dilution of polyclonal serum against the membrane-bound form of alkaline phosphatase (mALP) from *B. mori* (kindly provided by M. Itoh, Kyoto Institute of Technology, Kyoto, Japan) for 1 h. After washing with PBST containing 0.1% BSA, blots were probed with anti-rabbit serum (Sigma) conjugated to horseradish peroxidase (HRP) or alkaline phosphatase. Filters were developed using enhanced chemiluminescence (ECL; Amersham Bio-Sciences) for peroxidase conjugates, or Nitro Blue tetrazolium and 5-bromo-4-chloroindol-2yl phosphate for alkaline phosphatase conjugates. No endogenous alkaline phosphatase activity was detected with Nitro Blue tetrazolium/5-bromo-4-chloroindol-2yl in blots of BBMV proteins when samples were boiled before electrophoresis. Periodate oxidation treatment of blots prior to immunoblotting did not alter antigenicity of BBMV proteins (data not shown); evidence that the serum used recognized protein and not sugar epitopes.

For lectin blots, blocked filters containing separated BBMV proteins were incubated with lectins from *Canavalia ensiformis* (ConA, at 0.05 μg·mL^{-1}), *Artocarpus integrifolia* (Jac, at 0.5 μg·mL^{-1}), *Glycine max* [soybean agglutinin (SBA), at 1 μg·mL^{-1}], *Ricinus communis* (RCA-I, at 5 μg·mL^{-1}), *Dolichus biflorus* (DBA, at 5 μg·mL^{-1}), *Sophora japonica* (SJA, at 5 μg·mL^{-1}), *Wistaria floribunda* (WFL, at 1 μg·mL^{-1}), *Helix pomatia* (HPL, at 1 μg·mL^{-1}), or *Griffonia simplicifolia* (GSL-I, at 5 μg·mL^{-1}) for 1 h in blocking buffer (PBST plus 3% BSA). Con A, Jac, SBA, and HPL were purchased from Sigma; RCA-I, SJA, WFL, and GSL-I were from Vector laboratories (Burlingame, CA, USA).

Lectins conjugated to HRP were visualized by ECL. Blots of biotinylated lectins were probed with streptavidin–HRP conjugate (Vector) and then visualized as HRP-conjugated lectins. As controls for nonspecific lectin binding, lectins were incubated with specific hapten sugars (Table 1) for 30 min at room temperature before probing BBMV blots. This treatment eliminated or greatly decreased lectin binding to BBMV proteins on filters (see below, and data not shown).

For SBA binding competition, filters were blocked as above, and then 12 μg·mL^{-1} of Cry1Ac or the Cry1Ac

Table 1. Sugar specificities of lectins (based on [62]) used in blots and respective hapten sugars used for lectin specificity controls. Several lectins were selected according to their specificity of binding to Gal, *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), Man or Glc.

Lectin (abbreviation)	Sugar specificity	Hapten sugar
Canavalis ensiformis (ConA)	α-Man	0.2 M αmethylman/glc
	α-Glc	
Artocarpus integrifolia (Jac)	Galβ1 → 3GalNAc	0.8 M Gal
	Galβ1 → 3,4GlcNAc	
Glycine max (SBA)	α/βGalNAc	0.2 M GalNAc
	α/βGal	
Ricinus communis (RCA-I)	Galβ1 → 4GlcNAc	0.2 M Gal
	Galα1 → 3Gal	
Dolichus biflorus (DBA)	GalNAcα1 → 3GalNAc	0.2 M GalNAc
	GalNAcα1 → 3Gal	
Sophora japonica (SJA)	Galβ1 → 3GalNAc	0.2 M Gal
	Galβ1 → 3,4GlcNAc	
Wistaria floribunda (WFL)	α/βGalNAc	0.2 M GalNAc
Helix pomatia (HPL)	GalNAcα1 → 3GalNAc	0.2 M GalNAc
	GalNAcα1 → 3Gal	
Griffonia simplicifolia (GSL)	GalNAcα1 → 3Gal	0.2 M Gal
	Galα1 → 3,6Gal/Glc	

mutant protein QNR(509–511) → AAA(509–511) was added to the blocking buffer along with SBA lectin (1 $\mu g \cdot mL^{-1}$). After 1 h incubation and washing, filters were developed as described for lectin blots.

Ligand blots were performed as described previously [5]. [^{125}I]Cry1Ac (1 nM) was used to probe blotted BBMV proteins in blocking buffer for 1 h at room temperature. After washing, filters were exposed to photographic film at −80 °C for 24 h.

To detect HvALP in the filters used for lectin or ligand blotting, after development, filters were washed in PBST plus 0.1% BSA overnight. Blocking and HvALP immunodetection were performed as described above. To avoid interference with lectin or toxin detection, bound mALP antisera was detected by anti-rabbit sera conjugated to alkaline phosphatase.

Digestion of BBMV proteins with peptide-*N*-glycosidase F

Release of N-linked oligosaccharides from BBMV proteins was achieved by digestion of blotted BBMV proteins with peptide-*N*-glycosidase F (PNG-F). BBMV proteins (15 μg) were separated by 8% SDS/PAGE and transferred to poly(vinylidene difluoride) Q filters as above. Filters were incubated in 5 mL of NaCl/P_i buffer (pH 7.4) containing 0.1% SDS, 0.5% Triton-X-100 and 30 U of PNG-F (Boehringer-Mannheim) for 17 h at 37 °C. After treatment, filters were blocked and probed as for SBA lectin blots or [^{125}I]Cry1Ac ligand blots. Controls, which had no PNG-F in the incubation buffer, showed no differences in lectin or toxin binding when compared to SBA and [^{125}I]Cry1Ac blots (data not shown).

Detection of GPI anchors

The presence of glycosylphosphatidylinositol (GPI) anchors in BBMV proteins was detected following the method described by Luo *et al.* [8]. Briefly, after phosphatidylinositol-specific phospholipase C (PIPLC) digestion of BBMV blots, cleaved GPI anchors were detected by immunological detection of the exposed cross-reacting determinant (CRD) epitope contained in the residue of the GPI anchor by probing with anti-CRD sera (kindly provided by K. Mensa-Wilmot, University of Georgia, Athens, GA, USA). Blots were probed with anti-rabbit–HRP conjugate (Sigma) before developing with enhanced chemiluminescence as above. In controls, which had no PIPLC in the blocking buffer, no proteins were detected (data not shown).

Detection of alkaline phosphatase activity in SDS/PAGE gels and blots

To detect alkaline phosphatase activity in BBMV, proteins (15 or 2 μg) solubilized in sample buffer [26] were not heat-denatured before gel loading. After 8% SDS/PAGE and transfer to poly(vinylidene difluoride) Q, filters were washed with ALP buffer for 15 min at room temperature. After addition of 330 $\mu g \cdot mL^{-1}$ of Nitro Blue tetrazolium and 165 $\mu g \cdot mL^{-1}$ of 5-bromo-4-chloroindol-2yl to the ALP buffer, alkaline phosphatase activity was visualized by the formation of a purple-red precipitate. Reactions were stopped by incubation of filters in 50 mL of NaCl/P_i, pH 7.5 containing 200 μL of 500 mM EDTA pH 8.0.

Results

Identification of the 68-kDa BBMV glycoprotein as alkaline phosphatase

To test the hypothesis that the 68-kDa protein with altered glycosylation in the Cry1Ac-resistant YHD2 larvae was a form of ALP, we used sera developed against the mALP from *B. mori* [27] to detect homologs of this protein in BBMV from *H. virescens*. Although no protein amount differences were detected in Coomassie blue stained gels (Fig. 1A), the 68-kDa protein had reduced SBA binding in BBMV from YHD2 larvae (Fig. 1B). This protein was recognized by sera against mALP (Fig. 1C) and

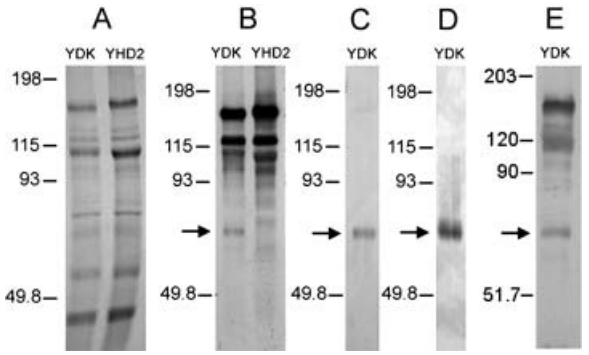

Fig. 1. Identification of the 68-kDa BBMV glycoprotein as HvALP, a form of alkaline phosphatase. BBMV proteins from *H. virescens* strains specified in the figure were separated by electrophoresis and Coomassie blue stained to control for equal protein loads (A) or transferred to poly(vinylidene difluoride) Q filters. After blocking, filters were probed with SBA lectin (B) or sera against the mALP from *B. mori* (C). Blots were developed using enhanced chemiluminescence. Alkaline phosphatase activity in separated BBMV proteins (D) was detected by incubating filters in Nitro Blue tetrazolium/5-bromo-4-chloroindol-2yl until purple precipitate was visualized in the region of enzymatic activity. For detection of GPI-anchored proteins in BBMV protein blots (E), protein blots were treated with PIPLC and cleaved GPI anchors detected by probing with sera against the CRD determinant. BBMV proteins containing cleaved GPI anchors were visualized by enhanced chemiluminescence. Arrows indicate the electrophoretic position of HvALP on the filters.

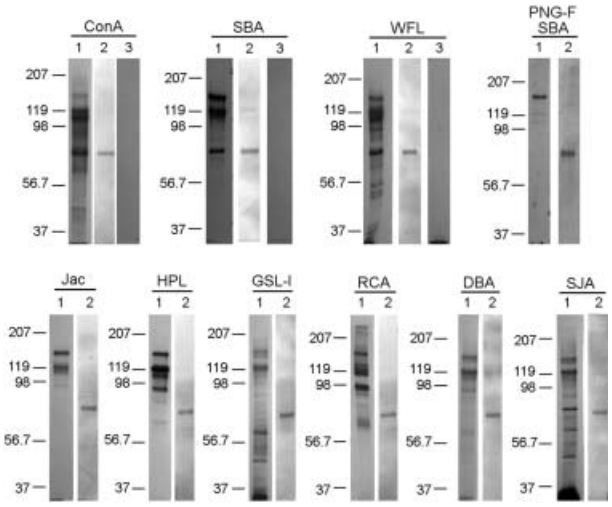

Fig. 2. Analysis of oligosaccharides on HvALP by lectin blotting. BBMV proteins from YDK larvae were separated by electrophoresis and transferred to poly(vinylidene difluoride) Q filters. After blocking, filters were probed with specific lectins as indicated in the figure. Lane 1: bound lectins were visualized by enhanced chemiluminescence. Lane 2: immunodetection of HvALP using sera against the mALP from *B. mori*. HvALP was visualized by anti-rabbit–alkaline phosphatase conjugate and Nitro Blue tetrazolium/5-bromo-4-chloroindol-2yl, so that both lectin blots and HvALP immunodetection could be performed using the same filter. Lane 3: competition of lectin binding with the respective hapten sugar (Table 1). For release of N-linked oligosaccharides from BBMV proteins (PNG-F/SBA), filters were treated with PNG-F. After washing, filters were probed with SBA and developed as for SBA lectin blots. All treatments were replicated at least three times to confirm reproducibility.

displayed ALP activity in blots of BBMV proteins (Fig. 1D), demonstrating that this protein is a form of alkaline phosphatase. PIPLC digestion was used to determine whether the 68-kDa protein was GPI anchored to BBMV in *H. virescens*. As shown in Fig. 1E, after PIPLC digestion, anti-CRD sera recognized the 68-kDa protein in *H. virescens* BBMV, suggesting that this protein is GPI-anchored to the brush border membrane. Based on these results, we named the 68-kDa GPI-anchored glycoprotein as HvALP for *H. virescens* alkaline phosphatase.

Characterization of the glycan moiety of HvALP by lectin blotting

To investigate the oligosaccharides present on HvALP from Cry1Ac susceptible larvae, we performed lectin blotting using selected lectins (Table 1) and BBMV proteins from YDK larvae. After lectin blotting, HvALP on blots was detected by sera against *B. mori* mALP to confirm lectin binding to HvALP. As shown in Fig. 2, HvALP was recognized by lectins from *Canavalia ensiformis* (ConA), *Glycine max* (SBA), and *Wistaria floribunda* (WFL). The different pattern of BBMV proteins being recognized by both SBA and WFL (both bind terminal GalNAc) was probably due to the existence of terminal GalNAc in linkages poorly recognized by one of the lectins. Conversely, no binding to HvALP was detected using lectins from *Artocarpus integrifolia* (Jac), *Ricinus communis* (RCA), *Dolichus biflorus* (DBA), or *Helix pomatia* (HPL). Although proteins of similar size to HvALP were bound by *Griffonia simplicifolia* (GSL) and *Sophora japonica* (SJA) lectins, immunodetection of HvALP in these filters demonstrated that the detected lectin binding proteins were not HvALP. To further test the existence of terminal GalNAc on N-linked oligosaccharides on HvALP, we performed digestion of blotted BBMV proteins with PNG-F, which releases N-linked oligosaccharides as N-glycosides from polypeptide chains. Digestion of BBMV proteins with PNG-F eliminated binding of SBA to HvALP (Fig. 2), supporting the hypothesis that this protein has N-linked oligosaccharides with terminal GalNAc residues. Binding of SBA to other BBMV proteins was also decreased after PNG-F digestion, suggesting the presence of GalNAc or galactose on N-linked oligosaccharides in these proteins.

Importance of ALP glycosylation for Cry1Ac binding

To test the hypothesis that Cry1Ac toxin bound to the terminal GalNAc residue on HvALP, we competed SBA binding to HvALP with Cry1Ac. We did not perform the reciprocal competition assay due to the 10^6-fold lower affinity of SBA for GalNAc (K_d = 0.3 mM [28]); when compared to Cry1Ac affinity for its binding sites (K_d = 1.1 nM [5]). When comparing SBA binding to BBMV with Cry1Ac competition blots (Fig. 3A), Cry1Ac prevented SBA binding to HvALP as well as to other BBMV proteins, indicative of toxin binding to terminal GalNAc residues on these proteins. Binding of SBA to the 170-kDa APN was almost unaffected by the presence of Cry1Ac. As a control for toxin binding not due to GalNAc recognition, we competed SBA

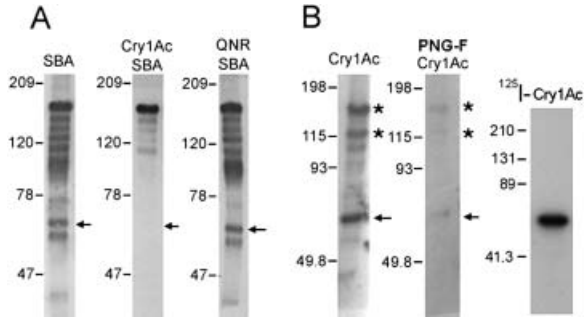

Fig. 3. Investigation of Cry1Ac binding to N-linked oligosaccharides on HvALP. For competition of SBA binding (A), blocked poly(vinylidene difluoride) Q filters containing separated BBMV proteins from YDK larvae were probed with SBA lectin (SBA) or SBA lectin plus either Cry1Ac (Cry1Ac/SBA) or the Cry1Ac mutant QNR(509–511) → AAA(509–511) (QNR/SBA), which lacks GalNAc binding. Bound SBA lectin was detected by enhanced chemiluminescence. For ligand blots (B), BBMV proteins binding Cry1Ac were detected by probing blocked filters with 1 nM [^{125}I]Cry1Ac for 1 h (Cry1Ac). The importance of N-linked oligosaccharides for [^{125}I]Cry1Ac binding (PNG/Cry1Ac) was tested by digestion of BBMV proteins with PNG-F glycosidase. After digestion, filters were washed, blocked and treated as described for ligand blots. Bound toxin was detected by autoradiography. Asterisks indicate the electrophoretic position of the 170- and 130-kDa proteins, arrows indicate the position of HvALP in the filters. Radiography of the radiolabeled Cry1Ac toxin used for these experiments ([^{125}ICry1Ac) is included.

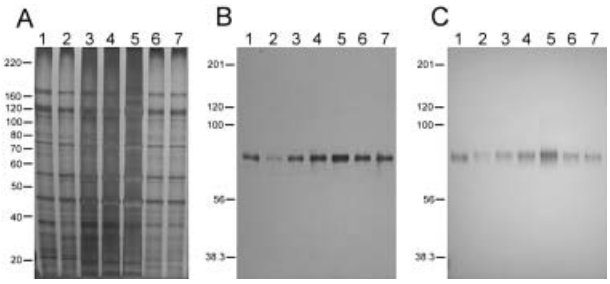

Fig. 4. Comparison of HvALP levels and alkaline phosphatase activity between BBMV from susceptible and resistant *H. virescens* larvae. BBMV proteins from YDK (lane 1), YHD2 (lane 2), F_1 generation of YDK males crossed with YHD2 females (lane 6), or F_1 generation of YDK females crossed with YHD2 males (lane 7), were separated by electrophoresis. For comparison, lanes 3, 4 and 5 contained YHD2 BBMV proteins at three-, five- and ten-fold, respectively, the protein concentration used for YDK and F_1 lanes. Gels were Coomassie blue stained (A), or transferred to poly(vinylidene difluoride) Q filters (B and C). After blocking, blot in (B) was probed with sera against the mALP from *B. mori* to detect HvALP. For visualization of alkaline phosphatase activity (C), the filter was washed in ALP buffer, and then Nitro Blue tetrazolium/5-bromo-4-chloroindol-2yl included in the buffer as described in Materials and methods. Alkaline phosphatase activity was visualized as a purple precipitate.

binding with a Cry1Ac mutant, QNR(509–511) → AAA(509–511), which lacks GalNAc binding [23]. SBA binding to HvALP was unchanged by QNR(509–511) → AAA(509–511), demonstrating that Cry1Ac bound to terminal GalNAc on HvALP.

To provide further support for the hypothesis of Cry1Ac binding to GalNAc on HvALP, we performed ligand blots with [^{125}I]Cry1Ac. Cry1Ac bound to several BBMV proteins, including HvALP (Fig. 3B). When N-linked oligosaccharides were released from HvALP by PNG-F digestion, Cry1Ac did not bind to this protein, demonstrating that toxin binding was dependent on the presence of N-linked oligosaccharides on HvALP. Binding to other Cry1Ac binding proteins was also decreased greatly by PNG-F digestion, indicating the importance of N-linked protein glycosylation for Cry1Ac binding on blots.

Reduced HvALP correlates with resistance to Cry1Ac

To investigate the possibility that reduced SBA binding to HvALP from YHD2 larvae (Fig. 1B) was a result of decreased HvALP protein levels, we compared HvALP from YHD2, YDK, and larvae from the F1 generation of backcrosses between YDK and YHD2 adults, using immunodetection and alkaline phosphatase activity blots. Two different types of F_1 larvae, according to the sex of the susceptible parent, were used to determine the potential existence of sex linkage. As shown in Fig. 4B, sera against the membrane-bound form of alkaline phosphatase from *B. mori* recognized HvALP in BBMV from YDK, YHD2 and F_1 larvae. No differences in intensity of recognition were observed between HvALP from YDK and F_1 vesicles, while recognition of HvALP in YHD2 was clearly reduced. To confirm reduction in HvALP antigen in BBMV from YHD2, we increased the protein load by three-, five- and tenfold to compare to YDK and F_1 vesicles. Increased BBMV protein concentrations as observed in the stained gel (Fig. 4A), resulted in augmented HvALP recognition (lanes 3, 4 and 5 in Fig. 4B), clearly suggesting a reduction in HvALP protein levels in BBMV from YHD2 larvae. Visual comparison of the lanes with increasing YHD2 protein loads and the YDK and F1 lanes in the blots (Fig. 4B) suggested a three- to fivefold reduction in HvALP antigen levels in BBMV from YHD2 larvae when compared to YDK or F1 vesicle proteins.

We predicted that reduced HvALP amounts in BBMV from YHD2 larvae would result in reduced alkaline phosphatase activity. Alkaline phosphatase activity in blots of BBMV proteins from YDK and F1 larvae was similar, and higher than activity in YHD2 vesicles (Fig. 4C). In agreement with reduced protein levels observed in Fig. 4B, specific alkaline phosphatase activity in suspensions of BBMV from YHD2 insects was reduced three- to fourfold when compared to YDK or F_1 vesicles (Table 2). *N*-aminopeptidasespecific activity was used as control, with no significant differences found between BBMV from YDK, YHD2 or F_1 larvae. These results were evidence for reduced amounts of HvALP in BBMV from YHD2 larvae resulting in reduced alkaline phosphatase activity and correlating with resistance to Cry1Ac and reduced Cry1Ac toxin binding.

Discussion

In the Cry1Ac-resistant *H. virescens* strain YHD2, knockout of the cadherin-like protein HevCaLP [10] resulted in reduction of Cry1Aa but not Cry1Ab or Cry1Ac binding

Table 2. Specific alkaline phosphatase (ALP) and *N*-aminopeptidase (APN) activities of BBMV suspensions from YDK, YHD2 and F_1 larvae. Specific activity of BBMV suspensions is expressed in units per milligram of BBMV protein ($U \cdot mg^{-1}$). One enzymatic unit was defined as the amount of enzyme that would hydrolyze 1.0 μmole of substrate to chromogenic product per min at the specific reaction pH and temperature. SD; standard deviation of the mean based on at least six independent measurements.

BBMV sample	ALP activity ($U \cdot mg^{-1} \pm SD$)	APN activity ($U \cdot mg^{-1} \pm SD$)
YDK	223 ± 91	2192 ± 427
YHD2	77 ± 37	2364 ± 290
YDK♀ × YHD2♂	375 ± 12	3156 ± 62
YHD2♀ × YDK♂	292 ± 12	2921 ± 275

[19] (J. L. Jurat-Fuentes, L. Gahan, F. Gould, D. Heckel and M. Adang, unpublished results). The patterns of Cry1Ac binding molecules in BBMV from YDK and YHD2 larvae, including the 170-kDa APN, were identical [11]. To explain decreased Cry1Ac toxin binding after continuous selection of YHD2 larvae with Cry1Ac, we hypothesized a key role for two BBMV glycoproteins of 63- and 68-kDa in Cry1Ac binding and toxicity [11].

In this study we identified the 68-kDa glycoprotein as a membrane-bound form of alkaline phosphatase we term HvALP (*H. virescens* alkaline phosphatase). As observed in other insect alkaline phosphatases, HvALP was GPI-anchored to the cell membrane. In insect larvae, alkaline phosphatases have been localized along the midgut, in Malpighian tubules, and in embryos [29]. Serum used to detect HvALP was developed originally against the mALP from *B. mori*, which was localized to the brush border of columnar cells along the middle and posterior midgut [27]. As GPI anchored proteins, alkaline phosphatases are located preferentially in lipid rafts [30]. Zhuang *et al.* [31] reported isolation of lipid rafts from *H. virescens* midgut epithelium containing a GPI-anchored protein of 66-kDa. Based on molecular size, the GPI anchor, and localization in rafts, we believe HvALP and the 66-kDa protein reported by Zhuang *et al.* [31] are equivalent. Alkaline phosphatases have been reported previously to interact with Cry1Ac toxin in ligand blots of BBMV from *M. sexta* [13,16]. Moreover, direct inhibition of alkaline phosphatase activity by Cry1Ac has been reported in *H. virescens* [17] and *M. sexta* [16]. Together with our current results, these observations are evidence of a direct interaction between Cry1Ac and membrane-bound forms of alkaline phosphatase.

As reported for other insect alkaline phosphatases [32], HvALP was glycosylated [11]. Binding of ConA to HvALP was evidence for the presence of N-linked oligosaccharide structures, as this lectin recognizes the trimannosidic core characteristic of N-linked glycans [33]. Binding of both SBA and WFL suggested the presence of either GalNAc or galactose at the nonreducing end of the oligosaccharide. Absence of RCA-I binding to HvALP suggested lack of terminal galactose, confirming that SBA and WFL were binding to a terminal GalNAc residue. Terminal GalNAc in glycoproteins is usually part of an O-linked glycan [34]. Interestingly, none of the lectins with high specificity for O-linked oligosaccharide structures (Jac, DBA, HPL, SJA) bound HvALP, indicating that terminal GalNAc bound by SBA and WFL was part of a complex or hybrid type N-linked oligosaccharide.

Even though N-linked oligosaccharides with complex type cores are rare in insects [35], mALP from *B. mori* was found to possess oligosaccharides of the biantennary complex type [32]. Terminal GalNAc has been proposed as binding site for Shiga-like and heat-labile toxins from *E. coli* [36,37]. Additionally, the role of GalNAc as binding epitope for Cry1Ac toxin has been studied extensively [38–41]. Lack of DBA and HPL binding is evidence that the terminal GalNAc on HvALP is not in a GalNAcα1 → 3 linkage. Considering that terminal GalNAc in other α-linkages has not been reported to occur on N-linked oligosaccharides, and both SBA and WFL bind α- as well as β-linked GalNAc, terminal GalNAc on HvALP is probably β-linked. Terminal βGalNAc has been reported in N-linked oligosaccharides of proteins synthesized by the parasite *Dirofilaria immitis* [42] and in microvillar glycoproteins of 68-kDa in size from *Anopheles stephensi* midguts [42,43]. Even though both terminal GalNAcβ1 → 3 and GalNAcβ1 → 4 can be found in biological samples, only terminal GalNAcβ1 → 4 has been described to occur on glycoproteins. Lepidopteran insect cell lines express a β1 → 4-GalNAc transferase that functions in the synthesis of complex-type carbohydrate chains [44]. N-linked oligosaccharides containing terminal GalNAcβ1 → 4 have been reported in hemocyanin from the pond snail *Lymnaea stagnalis* [45], bovine milk [46], antigenic glycoproteins from *Schistosoma mansoni* [47], and bee venom [48]. Terminal GalNAcβ1 → 4Gal has been proposed as adherence receptor for *Streptococcus pneumoniae* and *E. coli* infection in humans [49,50].

Binding of Cry1Ac to proteins of 68-kDa in size in ligand blots of *H. virescens* BBMV has been reported previously [1,5,11]. Our ligand blotting and competition results are evidence for Cry1Ac binding to the terminal GalNAc residue on HvALP. An interesting possibility is that terminal GalNAcβ1 → 4 may serve as a general recognition epitope for Cry1Ac toxin on alternative toxin receptors. Zhuang *et al.* [31] proposed a potential role for GPI anchored proteins such as HvALP in toxin action after observing a correlation between partition of Cry toxin to lipid rafts, toxin aggregation, and pore formation. Although speculative, Cry1Ac may bind to GalNAcβ1 → 4 on HvALP to initiate toxin oligomerization and pore formation, due to putative HvALP localization in lipid rafts. Similarly, the aerolysin enterotoxin from the bacterium *Aeromonas hydrophila* binds to βGlcNAc on the GPI anchor of alkaline phosphatase before insertion on target cell membranes [51,52]. In support of the terminal GalNAcβ1 → 4 as a Cry toxin binding epitope, mutations in a predicted UDP-GalNAc:GlcNAc β1,4-*N*-acetylgalactosaminyltransferase resulted in resistance to Cry5B and Cry14A Bt toxins in *Caenorhabditis elegans* [53]. Further analysis of purified oligosaccharides from HvALP as well as other putative toxin receptors would be necessary to obtain more conclusive and detailed linkage information on oligosaccharides with terminal GalNAc.

As we did not previously observe Cry1Aa or Cry1Ab binding to HvALP on ligand blots [5], we propose that HvALP is part of the C group of binding sites. According to

the current toxin binding model, alteration of C binding sites would explain reduced Cry1Ac binding, as observed in BBMV from YHD2 insects [11]. Our initial hypothesis, to explain reduced Cry1Ac and SBA binding to HvALP in YHD2 larvae, was based on possible alteration of protein glycosylation in resistant insects. Results from immunoblotting and alkaline phosphatase activity detection revealed instead that HvALP protein levels were decreased in BBMV from YHD2 larvae. Therefore, decreased SBA binding to HvALP from YHD2 vesicles was due to reduced protein levels and not to altered glycosylation. Due to limiting YHD2 materials, oligosaccharide analysis was only performed in BBMV from YDK larvae, hence potential alterations of HvALP glycosylation in YHD2 larvae cannot be excluded. BBMV from the F_1 generation of reciprocal crosses recovered HvALP levels observed for the susceptible parents independently of the sex of the susceptible progenitor, demonstrating autosomal recessive transmission of this trait. Considering that F_1 generation larvae bound Cry1Ac toxin and were only twofold resistant to Cry1Ac [11], our results are evidence for a direct correlation between decreased HvALP levels and increased resistance to Cry1Ac.

Electrophoretic variations of alkaline phosphatase between different strains or developmental stages have been reported for *Drosophila melanogaster* [54], *Aedes aegypti* [55], and *B. mori* [56,57], although the physiological consequences of these variations are not clearly understood. In the Tsunomata *B. mori* strain, reduced mALP activity correlated with undetectable levels of mALP antigen, while there were no alterations in gene copy or transcript size [57]. These results suggested that electrophoretic mALP polymorphisms were due to post-transcriptional processes. The fact that Tsunomata larvae were viable and fertile under normal conditions suggests lack of dramatic fitness costs associated with reduced mALP levels. Interestingly, YHD2 larvae do not survive through pupation when grown in cotton or Bt cotton [58], suggesting dramatic fitness costs associated with resistance in this species. We believe these costs are the result of the existence of multiple resistance mechanisms in YHD2 larvae. The existence of such effects is crucial when designing approaches to delay evolution of resistance against Bt crops.

Insect alkaline phosphatases have been proposed to function in active absorption of metabolites and transport processes [29], although there is also evidence for participation in cell adhesion and differentiation [59]. Interestingly, knockout of HevCaLP, another protein predicted to function in cell adhesion processes, results in Cry1 resistance in YHD2 larvae [10]. According to these important functions, significant fitness costs associated with reduced ALP activity would be expected, although information from the Tsunomata *B. mori* strain may suggest the contrary.

The specific mechanism by which YHD2 larvae reduce HvALP expression needs further investigation. As stated above, information from *B. mori* mALP suggests that decreased HvALP activity may not be related to changes in gene copy number or transcription. An alternative hypothetical mechanism to reduce HvALP in midgut brush border membranes was proposed previously by Lu and Adang [60]. According to this hypothesis, GPI-anchored proteins would be selectively solubilized by endogenous PIPLC digestion in Bt-resistant insects. Such treatment would result in elimination of potential Cry toxin binding sites such as aminopeptidases and alkaline phosphatases from the midgut epithelium. In support of this hypothesis, *B. mori* mALP is solubilized by midgut epithelium enzymes to form digestive fluid alkaline phosphatase (dALP), which is highly resistant to degradation by midgut proteases [61].

Our results demonstrate a direct correlation between decreased HvALP levels and resistance in *H. virescens*. HvALP may be a critical component in toxicity, or alternatively, the reduced HvALP levels observed in resistant larvae may indicate broader alterations in the brush border membrane. One possibility is that resistant larvae have altered membrane components such as lipid rafts that affect the amounts of HvALP localized to the brush border membrane. The specific role of HvALP in Cry1Ac intoxication needs further investigation. We believe HvALP has potential as a resistance marker, so that biochemical and DNA-based tests may be developed to detect emergence of resistance to Bt crops in field populations. These questions are currently being addressed in our laboratory.

Acknowledgements

The authors express their gratitude to Dr. Fred Gould (North Carolina State University, Raleigh, NC, USA) for providing the *Heliothis* materials used for this research.

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