Insect Biochemistry and Molecular Biology 94 (2018) 36–41

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

The intracellular region of silkworm cadherin-like protein is not necessary to mediate the toxicity of *Bacillus thuringiensis* Cry1Aa and Cry1Ab toxins

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ARTICLE INFO

Article history:
Received 30 November 2017
Received in revised form
11 January 2018
Accepted 31 January 2018
Available online 6 February 2018

Keywords:
Bacillus thuringiensis
Cry toxin
Cadherin-like protein

ABSTRACT

The cadherin-like protein in lepidopteran insects, known as a receptor for *Bacillus thuringiensis* Cry1A toxins, is a single-pass membrane protein that can be divided into extracellular and intracellular regions. The extracellular region is important for toxin binding and oligomerization, whereas the role of the intracellular region during Cry1A intoxication is unclear. In the present study, we generated a deletion mutant of *Bombyx mori* cadherin-like protein (BtR175) that lacked the intracellular region to investigate its role in mediating Cry1A toxicity. Like wild-type BtR175, the mutant protein conferred susceptibility to Cry1Aa and Cry1Ab toxins in Sf9 cells, suggesting that the intracellular region is not required to mediate intoxication. The deletion mutant maintained another role of cadherin-like proteins; that it, synergistic activity with *B. mori* ABC transporter C2 (ABCC2) when mediating Cry1Aa and Cry1Ab toxicity. In addition, we evaluated the effects of reagents that have been reported to inhibit Cry1A toxicity (e.g., protein kinase A inhibitors, EDTA, and sucrose) on Cry1A toxicity in BtR175-expressing cells. Our results suggest that Cry1Aa-induced cell death in BtR175-expressing cells was not caused by signal transduction but by osmotic lysis. Overall, our data indicate that BtR175 mediates the toxicity of Cry1Aa and Cry1Ab toxins entirely via its extracellular region. They also indicate that the synergism between cadherin-like protein and ABCC2 occurs outside of cells or in the cell membrane.

1. Introduction

Bacillus thuringiensis Cry toxins, insecticidal pore-forming toxins, have been utilized as biological control agents to manage pests in agriculture and as a gene source for genetically modified crops with pest resistance. Cry toxins form pores on the apical membrane of insect midgut cells after interacting with target molecule(s) or so-called receptor(s) (Bravo et al., 2011). The toxin pore disrupts the ionic balance in cells and generates high cytoplasmic osmotic pressure (Knowles and Ellar, 1987), driving an influx of water via aquaporins that leads to necrotic cell death (Endo et al., 2017). The evolution of resistance to Cry toxins is one of the major threats in agriculture (Tabashnik, 2015). In many cases, strong resistance to Cry toxins is generated by a receptor mutation. Two receptors have been identified as responsible agents for high-level resistance: cadherin-like protein and ATP-binding cassette transporter C2 (ABCC2) (Heckel, 2012).

Cadherin-like proteins have been studied since they were identified as Cry1A toxin-binding proteins (Vadlamudi et al., 1993). They are assigned to the cadherin superfamily, which is generally involved in cell adhesion; however, the native function of the molecules in the apical membrane of insect midgut cells remains unknown. Cadherin-like proteins are membrane proteins that consist of a conserved repeat motif called the cadherin repeat, a membrane proximal domain, a transmembrane domain, and a cytoplasmic domain. The several continuous cadherin repeats near the cell membrane are responsible for binding to Cry1A toxins (Nagamatsu et al., 1999). Cadherin-like protein binding promotes the cleavage of Cry1A toxin alpha-helix 1 (Gómez et al., 2001), resulting in oligomerization of the toxin to form a “pre-pore” that is believed to insert into the cell membrane (Jiménez-Juárez et al., 2007; Obata et al., 2009).

Cadherin-like proteins have been studied as Cry toxin receptors

Abbreviations: BtR175, *Bombyx mori* cadherin-like protein; ABC transporter C2, ATP-binding cassette transporter subfamily C2; PKA, protein kinase A; EDTA, ethylenediaminetetraacetic acid.

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https://doi.org/10.1016/j.ibmb.2018.01.005
0965-1748/

for more than two decades, but we still do not fully understand their role in the Cry toxin mode of action. Cry1A-resistant strains that lack cadherin-like protein have up to 1000-fold lower susceptibility compared to wild-type strains (Gahan et al., 2001; Morin et al., 2003; Xu et al., 2005; Yang et al., 2009). However, the susceptibilities of cultured cells to Cry1A toxins conferred by cadherin-like protein are quite low (~0.1–1 μM toxin level) or are undetectable (Tanaka et al., 2013; Bretschneider et al., 2016). The binding affinity and toxin oligomer-inducing activity of BtR175 to Cry1Aa toxins are apparently comparable to those of *Bombyx mori* (Bm) ABCC2 (Tanaka et al., 2016; Adegawa et al., 2017), but the toxin pore-inducing activity of BtR175 is at a trace level in agreement with its low susceptibility-conferring activity (Tanaka et al., 2013, 2016). Although the significant contribution of cadherin-like proteins to Cry1A intoxication is clear, their main role remains obscure.

The roles of the extracellular region of cadherin-like proteins in toxin binding and oligomerization are clear, whereas those of the intracellular region are unclear. Zhang et al. (2005, 2006) proposed a signal transduction model in which binding of the Cry1Ab toxin to *Manduca sexta* cadherin-like protein induces Mg^{2+}-dependent signal transduction via adenyl cyclase and protein kinase A (PKA). In that model, signaling, not the toxin pore, causes oncosis-like cell death. Notably, the authors did not provide any direct evidence to support the involvement of the intracellular domain in the Cry1A mode of action. Researchers have attempted to verify this hypothesis for more than a decade, with almost no success. Portugal et al. (2017) showed that the adenylate cyclase/PKA signaling pathway is not involved in toxicity of the Cry1A toxin to CF cells from *Choristoneura fumiferana*, which express a cadherin-like protein. However, the role of the intracellular region in the Cry1A mode of action cannot be ruled out because non-recessive Cry1Ac-resistant *Helicoverpa armigera* strains have been reported (Zhang et al., 2012). The Xj-r15 and AY-r15 strains, which show 140- and 82-fold resistance to Cry1Ac toxin, respectively, lack 55 amino acids in the intracellular region of the cadherin-like protein (Zhang et al., 2012). Yet, the cadherin-like protein from these resistant strains confers Cry1Ac susceptibility to Sf9 cells, and the difference in receptor activity is about two-fold (Zhang et al., 2012). Therefore, resistance is not likely to be simply conferred by the lack of toxicity mediated by cadherin-like protein alone.

Some studies have reported synergism between the cadherin-like receptor and ABCC2 in inducing pore formation. We showed previously that Sf9 cells co-expressing BtR175 and BmABCC2 were highly sensitive to Cry1A toxins compared to Sf9 cells expressing either one alone (Tanaka et al., 2013). A voltage clamp experiment using *Xenopus* oocytes revealed that the synergism results in an increase in the number of toxin pores permeable to cations (Tanaka et al., 2016). However, cooperation in pore formation is not likely to fully explain the synergism because the synergistic effect is often greater in Sf9 cells (up to 1000-fold) than that in *Xenopus* oocytes (up to 8-fold). Accordingly, other factor(s), including intracellular signaling triggered by toxin binding to cadherin-like protein, may also generate the synergistic effect between cadherin-like protein and ABCC2. Bretschneider et al. (2016) showed the synergistic role of the cadherin-like protein and ABCC2 from *Heliothis virescens*, and hypothesized that the cadherin-like protein helps to remove inserted pore structures from ABCC2 through a putative interaction between the intracellular region of the protein and the cytoskeleton.

In the present study, we generated a BtR175 mutant that completely lacked the intracellular region and expressed it in Sf9 cells to elucidate the role of the intracellular region of cadherin-like protein in the Cry1A toxin mode of action. Our data suggest that the intracellular region is not responsible for Cry1Aa and Cry1Ab intoxication mediated by BtR175 itself in cooperation with BtR175 and BmABCC2. Accordingly, the roles of cadherin-like proteins, including synergistic receptor activity with ABCC2 during Cry1Aa and Cry1Ab intoxication, are likely to rely entirely on the extracellular region.

2. Material and methods

2.1. Cry toxin preparation

Cry1Aa and Cry1Ab toxins were produced as recombinant proteins from *Escherichia coli* as described before (Tanaka et al., 2013). Solubilized proteins obtained from inclusion bodies were activated and purified using HPLC system as described elsewhere (Obata et al., 2009).

2.2. Heterologous expression of cadherin-like protein mutants lacking intracellular region

We used Sf9/baculovirus expression system for heterologous expression of BtR175, its mutant BtR175-DEL, and BmABCC2 as we conducted previously (Tanaka et al., 2013). The intracellular region of BtR175 (1593-1715aa) was predicted using SMART (http://smart.embl-heidelberg.de/). Partial cDNA coding the intracellular region of BtR175 was deleted by means of site-directed mutagenesis using the pBAC4x-BtR175 construct (Tanaka et al., 2013) as a template using primers 5′-TCATCATCCACCACCACCACCACCAC-3′ and 5′-TCATCATCCACCACCACCACCACCAC-3’. Recombinant baculovirus harboring BtR175-DEL was generated using BacMagic™ kit (Merck Millipore, Tokyo, Japan) according to manufacturer's instructions. Sf9 cells were infected with recombinant virus and used for cell swelling assay after 72 h culture.

2.3. Western blotting

Approximately 5×10^7 cells expressing BtR175, BtR175-DEL, or EGFP (enhanced green fluorescence protein) were collected and washed twice with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). After fixed with 10% trichloroacetic acid/PBS for 1 h, cells were solubilized using solubilization buffer (7 M urea, 2 M thiourea, 3% 3-((3-Cholamidopropyl) dimethylammonium)-1-propanesulfonate (CHAPS), 1% TritonX-100) for 30 min at 4 °C and centrifuged at 14 800×*g* for 15 min. The same volume of supernatants applied to SDS-PAGE followed by Western blotting using anti-BtR175 antiserum (Tanaka et al., 2013). The expression levels of BtR175 and BtR175-DEL were evaluated by densitometry using image J ver. 1.6.0 software (NIH, Bethesda, MD, USA).

2.4. Immunostaining

Cells were washed with Tris-NaCl-Tween buffer (TNT, Tris-NaCl-Tween buffer, 0.15 M Tris-HCl, 0.01 M NaCl, 0.05% Tween20) and fixed with 4% paraformaldehyde/PBS for 10 min. After wash with PBS, fixed cells were blocked with 2% BSA/TNT for 30 min at room temperature. Subsequently cells were incubated with anti-BtR175 serum (Tanaka et al., 2013) that can detect the toxin-binding extracellular region (1108–1464 aa) for overnight at 4 °C. The Alexa 555-conjugated anti-rabbit IgG (Thermo Fisher Scientific, Tokyo, Japan) was used as a secondary antibody.

2.5. Cell swelling assay and viability assay

Cell swelling assay was conducted as described elsewhere (Tanaka et al., 2013). Briefly, Sf9 cells infected by single virus or double viruses were seeded on the cover glass and then placed on a