Cytotoxic activity of *Bacillus thuringiensis* Cry proteins on mammalian cells transfected with cadherin-like Cry receptor gene of *Bombyx mori* (silkworm)

Yoko TSUDA, Fumiki NAKATANI, Keiko HASHIMOTO, Satoshi IKAWA, Chikako MATSUURA, Takashi FUKADA[1], Kenji SUGIMOTO and Michio HIMENO[2]

Laboratory of Applied Molecular Biology, Division of Applied Biochemistry, Graduate School of Agriculture and Biological Sciences, Osaka Prefecture University, Gakuen-cho 1-1, Sakai, Osaka, 599-8531, Japan

Cry1Aa, an insecticidal protein produced by *Bacillus thuringiensis*, has been shown to bind to cadherin-like protein, BtR175, in *Bombyx mori* (silkworm) midgut. We previously reported three variant alleles of BtR175 (BtR175a, b and c). When transiently expressed in COS7 cells, all the three BtR175 variants bound to Cry1Aa. We stably expressed BtR175b in HEK293 cells. These BtR175b-expressing cells swelled and died in the presence of activated Cry1Aa in a dose- and time-dependent manner, showing that BtR175b itself can impart Cry1Aa-susceptibility to mammalian cells. These cells were more susceptible to Cry1Aa than to Cry1Ab and Cry1Ac. Since dispersed *B. mori* midgut cells were reported to be highly susceptible to Cry1Ac, this result suggested that other Cry1Ac-specific receptor(s) were simultaneously working with BtR175 in the midgut cells. Advantages are also discussed of applying these transfected mammalian cells to toxicity assays of mutant Cry proteins.

Key words: Cry toxin, cytotoxicity assay, δ-endotoxin.

INTRODUCTION

Insecticidal Cry proteins produced by *Bacillus thuringiensis* are widely used as highly specific insecticides in agriculture and forestry. Their genes are also used to make insect-resistant transgenic crops, which are widely cultivated [1]. Accompanying the extensive use of Cry proteins as commercial insecticides, the emergence of Cry-resistant insects is becoming a potential problem.

Despite the practical detriment they cause, Cry-resistant insects have contributed to research on Cry-susceptibility determinants of insect cells. Since the midgut brush-border membrane of certain Cry-resistant insects were known to have reduced binding affinity to Cry proteins, loss or mutational changes of Cry receptors in midgut epithelial cells were thought to be involved in Cry-resistance development [2–5]. It is now well known that there are at least two classes of Cry-binding insect proteins, aminopeptidase N (APN) [6–8] and cadherin-like proteins {BT-R_1 in *Manduca sexta* (tobacco hornworm) [9,10] and BtR175 in *Bombyx mori* (silkworm) [11,12]}.

However, binding activity of brush-border membrane vesicles (BBMVs) to certain Cry proteins was occasionally detected in insects which were insensitive to those proteins [1]. One of the possible explanations of this discrepancy would be that functional Cry receptors induce specific conformational changes to the bound Cry proteins to activate their membrane-inserting activity, while there is another class of proteins which can bind to Cry proteins but cannot trigger their membrane insertion. Thus it is important to validate the functionality of Cry-binding proteins

as Cry receptors by examining whether they can impart Cry susceptibility to heterologous cell systems.

For this purpose, some attempts have been made to express the cloned Cry receptor genes in cultured insect and mammalian cells. BtR175, a *B. mori* homologue of BT-R_1, was expressed in Sf9 [*Spodoptera frugiperda* (fall armyworm)] cells by a baculovirus system, and Cry1Aa was reported to cause morphological change to these cells [13]. Although that report showed that BtR175 would be necessary for Cry susceptibility, the results obtained from an insect system could not rule out the involvement of other insect-specific factors in the determination of Cry susceptibility. It also seemed to be difficult to assess the toxicity of Cry1Aa to the cultured insect cells, since the recombinant protein expression by baculovirus is transient, and the virus propagation eventually kills the host cells. In mammalian cells, BT-R_1 was reported to show little expression on the cell surface, probably because of its inefficient transport to the plasma membrane [14]. Since BT-R_1 was prevented from interacting with Cry proteins on cell surface, the functionality of BT-R_1 as a Cry receptor was not determined. Therefore no distinct evidence has been reported about the ability of the Cry receptor to mediate susceptibility to Cry proteins in either insect or mammalian cells.

In the present study, we showed that expression of an allele of BtR175 [15] made mammalian cells susceptible to Cry1Aa without any additional insect-specific factors, and thus was sufficient to confer Cry1Aa-susceptibility to the heterologous cell system. It suggested that changes in Cry-binding proteins might play a pivotal role in the development of Cry resistance. This BtR175 allele was found to serve mainly as Cry1Aa receptor,
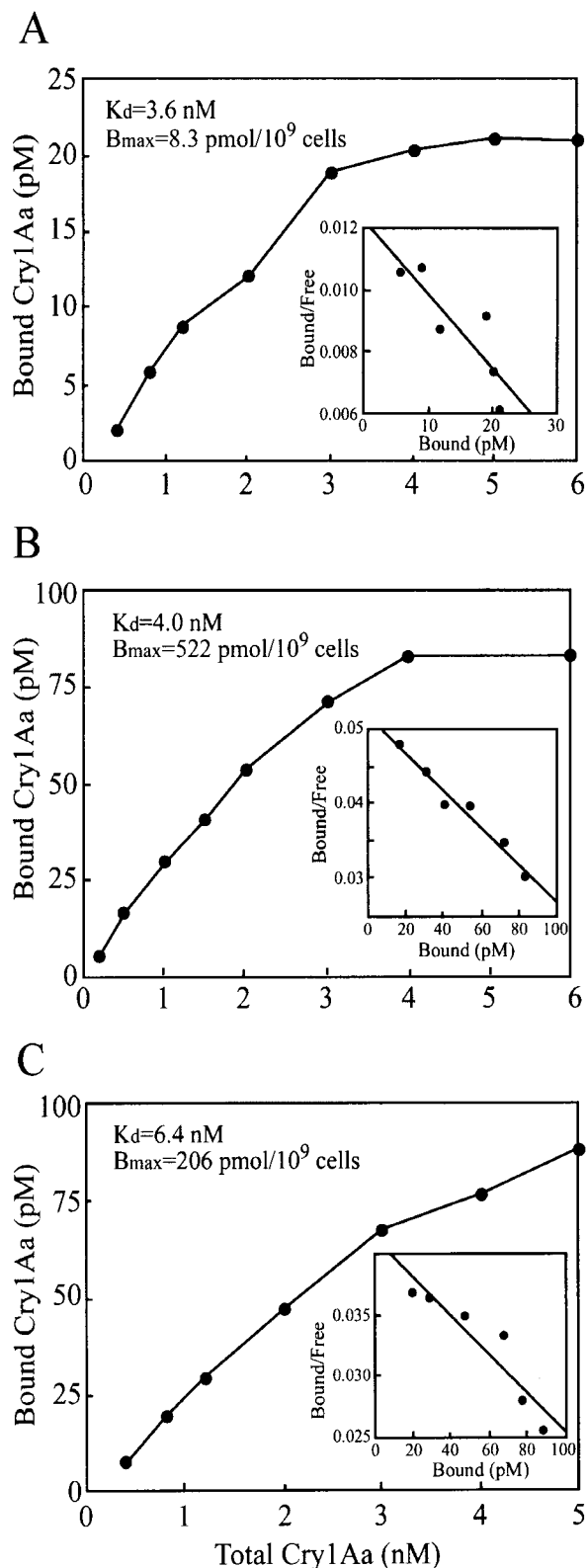

Figure 1   Quantitative analysis of Cry1Aa binding to BtR175 variants expressed in COS7 cells

The COS7 cells expressing BtR175a, BtR175b and BtR175c were fixed with paraformaldehyde and incubated with various concentrations of ^{125}I-labelled activated Cry1Aa. Unbound Cry1Aa was removed by filtration and the radioactivity of cell-bound Cry1Aa was measured. Non-specific binding to mock-transfected COS7 cells was subtracted from all the data. Dissociation constants (K_d) and numbers of Cry1Aa-binding sites (B_{max}) were calculated from Scatchard plots (insets). (**A**) BtR175a; (**B**) BtR175b; (**C**) BtR175c.
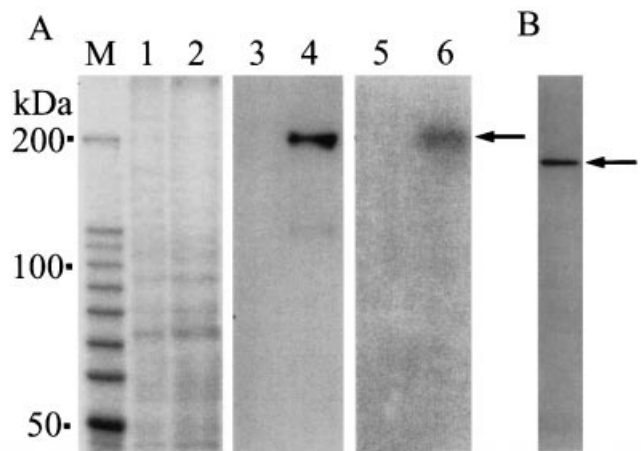
Figure 2   Analysis of BtR175b protein expressed in HEK293 cells

(**A**) Protein in the plasma-membrane fraction was separated by SDS/PAGE and stained with Coomassie Brilliant Blue (lanes 1 and 2), analysed by Western blotting with anti-Myc antibody (lanes 3 and 4), or by ligand-blot assay with ^{125}I-labelled activated Cry1Aa (lanes 5 and 6). Lane M, molecular-mass markers; lanes 1, 3 and 5, untransfected HEK293 cells; lanes 2, 4 and 6, HKb20 cells; arrow, 200 kDa bands of anti-Myc antibody- and Cry1Aa-binding protein. (**B**) BtR175b protein was synthesized *in vitro* using a rabbit-reticulocyte-lysate system and detected by anti-Myc antibody on a Western blot. A 180 kDa band was detected (arrow).

suggesting that *B. mori* larvae have other Cry1Ac receptor(s) in their midgut. These transfected mammalian cells could also provide an excellent alternative toxicity assay for Cry proteins.

MATERIALS AND METHODS

Plasmids and cell cultures

Cry1Aa, Cry1Ab and Cry1Ac proteins were expressed in *Escherichia coli* harbouring pKC6 [16], pOS4301 [17] and pOS4201 [17] respectively. They were solubilized, purified by anion-exchange chromatography, and activated by trypsin according to the solid-phase digestion method described in [18]. The Cry1Ab- and Cry1Ac-expressing *E. coli* strains were obtained from the *Bacillus* Genetic Stock Center (Department of Biochemistry, Ohio State University, Columbus, OH, U.S.A.).

BtR175 variant cDNAs were subcloned into pcDNA3.1(-) Myc-His vector (Invitrogen) to add Myc epitope tag to the C-termini of the proteins. These plasmids were used to transform COS7 cells. The Myc-tagged BtR175b cDNA was transfered into pIRESneo2 (Clontech) to stably transform HEK293 cells. COS7 and HEK293 cells were transfected by electroporation with Gene Pulser (Bio-Rad) [19]. The transfected HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) containing 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 0.1% NaHCO$_3$, 5 units/ml penicillin and 50 μg/ml streptomycin and selected in the presence of 800 μg/ml G418 (an antibiotic also known as geneticin). A BtR175b-expressing HEK293 clone (clone HKb20) was used in all subsequent experiments.

Preparation of plasma-membrane fractions

Cells were homogenized with a Dounce homogenizer in PBS containing 0.5 mM EDTA and 1% protease-inhibitor cocktail (Sigma, P8340). The homogenate was centrifuged at 1000 *g* for 10 min to remove cell debris and nuclei. The supernatant was
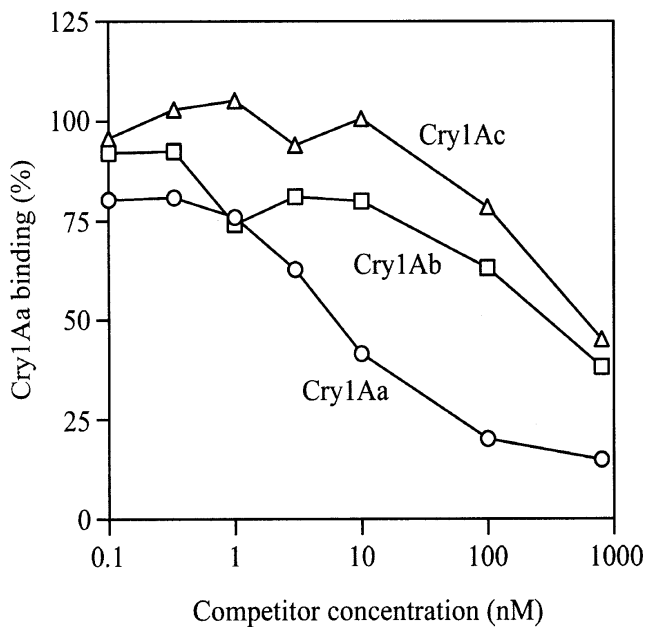
Figure 3   Competitive inhibition of Cry1Aa binding to BtR175b by Cry1Aa, Cry1Ab and Cry1Ac

The plasma-membrane fraction of HKb20 cells was suspended and incubated in buffer containing 0.2 nM ^{125}I-labelled Cry1Aa and various concentrations of either unlabelled Cry1Aa, Cry1Ab or Cry1Ac. Unbound ^{125}I-Cry1Aa was removed by filtration and remaining radioactivity was measured. Results were expressed as the proportion to the bound ^{125}I-Cry1Aa amount in the absence of the competitors. ○, Cry1Aa; □, Cry1Ab; △, Cry1Ac.

centrifuged at 10000 g for 30 min to obtain the pellet of plasma membranes.

Quantitative analysis of Cry1Aa binding to fixed cells and plasma-membrane fractions

Activated Cry1Aa was labelled with ^{125}I by the chloramine-T (sodium N-chloro-p-toluenesulphonamide) method [20]. To measure Cry1Aa binding to fixed cells, cells were detached from culture dishes with PBS containing 1 mM EDTA and pelleted by centrifugation. The pellet was suspended in PBS containing 4 % (w/v) paraformaldehyde, incubated at room temperature for 30 min and washed with PBS. The fixed cells were incubated with various concentrations of ^{125}I-labelled activated Cry1Aa in PBS containing 0.1 % BSA at 30 °C for 2 h. The cells were filtered on a nitrocellulose membrane (1 μm pore size) and washed twice with ice-cold PBS containing 0.1 % BSA. Radioactivity of the cell-bound Cry1Aa was measured in a COBRA 5003 γ-radiation counter (Packard). Non-specific binding to mock-transfected COS7 cells was subtracted from all the data. All experiments were done in duplicate. The paraformaldehyde fixation did not affect Cry1Aa-binding affinity of B. mori BBMV (results not shown).

To measure competitive inhibition of Cry1Aa binding, a suspended membrane fraction of HKb20 cells (0.6 mg/ml) was incubated with 0.2 nM ^{125}I-labelled activated Cry1Aa, and various concentrations of unlabelled competitor in PBS containing 0.1 % BSA at room temperature for 2 h. Unbound Cry1Aa was removed by filtration through a nitrocellulose membrane (1 μm pore size) and two rounds of washing with ice-cold PBS containing 0.1 % BSA. The radioactivity of membrane-bound Cry1Aa was measured as described above.
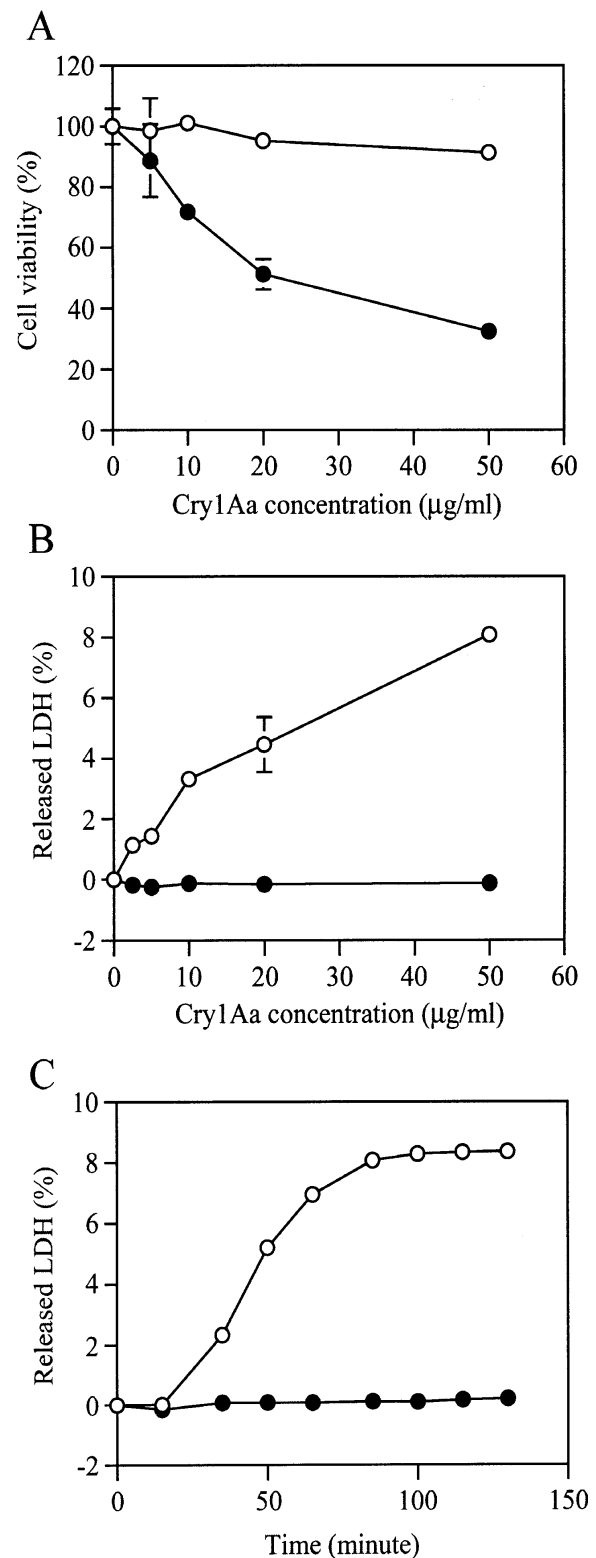

Figure 4   Cry1Aa susceptibility of BtR175b-expressing HEK293 cells

(**A**) Cell viability was measured by tetrazolium salt reduction after a 1 h incubation in the presence of various concentrations of activated Cry1Aa. Error bars indicate the S.D. (n = 3). (**B**) Cry1Aa-induced cell death was measured by extracellular release of LDH activity after a 1 h incubation with various concentrations of activated Cry1Aa. Error bars indicate the S.D. (n = 3). (**C**) Kinetics of LDH release was measured by taking aliquots of extracellular medium at the indicated time points during the incubation of the cells with 50 μg/ml activated Cry1Aa. ○, HKb20 cells; ●, untransfected HEK293 cells.
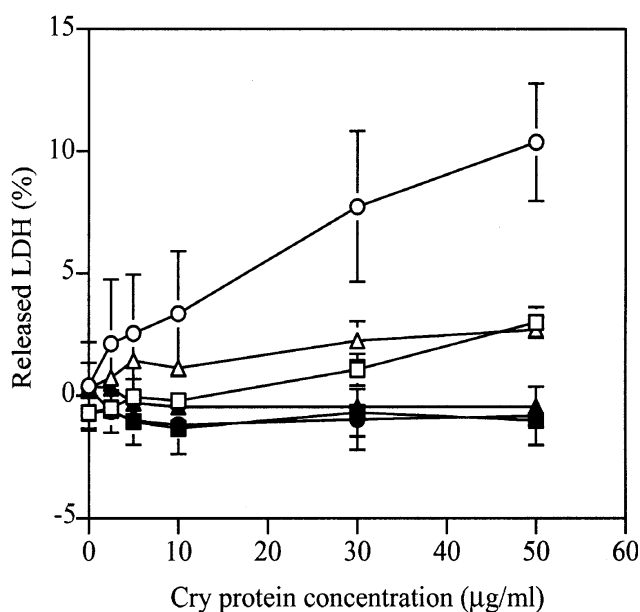
Figure 5   Specificity of Cry1A-susceptibility of BtR175b-expressing HEK293 cells

Susceptibility of HKb20 cells to activated Cry1Aa, Cry1Ab and Cry1Ac was measured by LDH release. Error bars indicate S.D. ($n = 3$). ○, ●, Cry1Aa; □, ■, Cry1Ab; △, ▲, Cry1Ac; ○, □, △, HKb20 cells; ●, ■, ▲, untransfected HEK293 cells.

In vitro transcription and translation of BtR175b cDNA

BtR175b protein was synthesized *in vitro* from the cDNA cloned in pcDNA 3.1(-) Myc-His by using the TnT Quick Coupled Transcription/Translation System (a rabbit reticulocyte lysate translation system from Promega).

Western-blot and ligand-blot analyses

HEK293 plasma-membrane fractions were separated by SDS/PAGE and transferred to an Immobilon membrane (Millipore). Detection of BtR175 variant proteins with anti-Myc antibody and ^{125}I-labelled activated Cry1Aa was described previously [12,15].

Cell-viability and lactate dehydrogenase (LDH)-release assays

Cells were seeded in 96-well microplates at the density of 1×10^5 cells/well and cultured overnight. Cell viability was assayed by tetrazolium salt reduction as follows. The culture medium was replaced with 100 μl of Hepes-buffered DMEM containing various concentrations of activated Cry1Aa. The cells were incubated at 37 °C for 1 h. Then 10 μl of Premix WST-1 reagent (Takara Biomedicals, Shiga, Japan) was added and incubated at 37 °C for 2 h. Formazan production was measured by light absorbance at 490 nm using 655 nm as a reference with a Model 550 microplate reader (Bio-Rad). The amount of formazan produced in the absence of Cry1Aa was taken as 100 %. All experiments were performed in triplicate.

For the LDH-release assay, the culture medium was replaced with 200 μl of Hepes-buffered DMEM containing 1 % BSA and various concentrations of activated Cry protein. The cells were

incubated at 37 °C for 1 h and centrifuged at 250 g for 10 min. A 100 μl portion of the supernatant was transferred to another microplate. The LDH activity in the supernatant was measured using the LDH Cytotoxicity Detection Kit (Takara Biomedicals, Shiga, Japan) following the manufacturer's instructions. The amount of released LDH activity was indicated as proportion of the total LDH activity, which was measured by disrupting Cry-untreated cells with 1 % Triton X-100. Background leakage of LDH activity, which occurred without Cry protein during the incubation, was separately measured and subtracted from all the data, including the total LDH activity. All experiments were performed in triplicate.

To measure the kinetics of Cry1Aa-induced LDH release, cells were cultured overnight in 35-mm-diameter dishes and the culture medium was replaced with 3 ml of Hepes-buffered DMEM containing 50 μg/ml activated Cry1Aa and 1 % BSA. The cells were incubated at 37 °C and a 120 μl aliquot of the medium was sampled at each time point. The LDH activity in the aliquots was measured as described above and adjusted according to the remaining volume of medium in the culture dish at the time of sampling.

RESULTS

Cry1Aa-binding properties of BtR175a, BtR175b and BtR175c expressed in mammalian cells

We previously reported three BtR175 variant alleles, BtR175a, BtR175b and BtR175c, which have one, six and five amino acid substitutions respectively. Four and three of the substitutions in BtR175b and BtR175c respectively resided in the reported Cry1Aa-binding region of BtR175 [15].

cDNAs of the BtR175 variants were transfected and transiently expressed in COS7 cells. Quantitative analysis of ^{125}I-Cry1Aa binding to the transfected COS7 cells showed that the dissociation constants of Cry1Aa binding were 3.6, 4.0 and 6.4 nM for BtR175a, BtR175b and BtR175c respectively (Figure 1), suggesting that their binding affinities to Cry1Aa were not significantly different under these experimental conditions. By contrast, the numbers of Cry1Aa-binding sites were 8.3, 522 and 206 pmol/10^9 cells for the BtR175a, BtR175b and BtR175c-expressing cells respectively. It suggested that BtR175a was less efficiently expressed or transported to the cell surface than the other variants.

Analysis of BtR175b protein expressed in a mammalian cell line

We further analysed BtR175b protein, since it was most efficiently expressed on the cell surface of COS7 cells among the three variants and thus suitable for examining its functionality as a Cry1Aa receptor. BtR175b cDNA was transfected into HEK293 cells and a stable cell line expressing BtR175b protein (clone HKb20) was cloned. BtR175b in HKb20 cells was detected as a 200 kDa protein on Western blot (Figure 2A, lane 4). A protein of the same molecular mass showed ^{125}I-Cry1Aa binding ability on a ligand blot (Figure 2A, lane 6). We previously showed that the molecular mass of BtR175b expressed in COS7 cells was also 200 kDa [15]. This was larger than that of the Cry1Aa-binding protein purified from *B. mori* midgut (180 kDa) [11,12], suggesting that the post-translational processing of BtR175b in mammalian and insect cells differed. We observed that only a 180 kDa protein was produced by *in vitro* transcription and translation of BtR175b cDNA in a rabbit-reticulocyte-lysate system without microsome membranes (Figure 2B). Thus it was
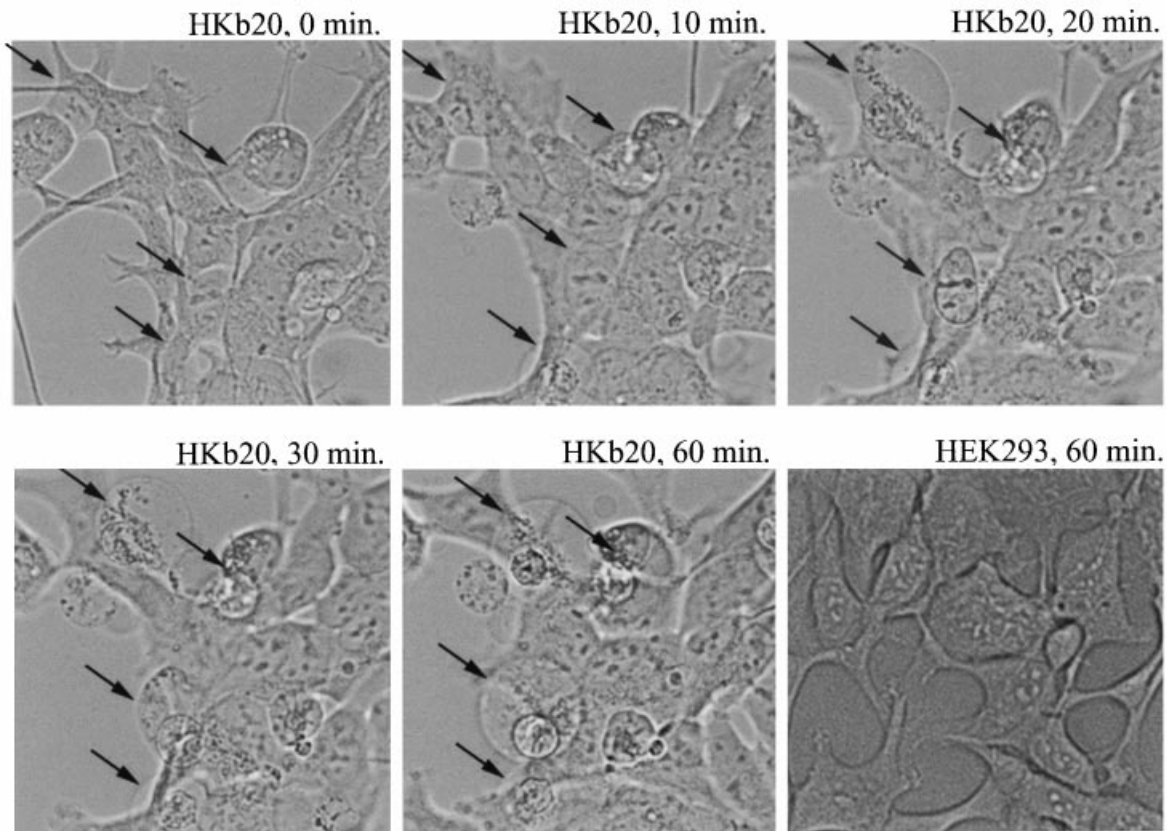
Figure 6   Cry1Aa-induced morphological change of BtR175b-expressing HEK293 cells

HKb20 cells and untransfected HEK293 cells were incubated with 50 μg/ml activated Cry1Aa and observed by time-lapse video microscopy. Rounding and swelling of HKb20 cells was observed (arrows). These results can also be viewed as time-lapse movies at: http://www.BiochemJ.org/bj/369/bj3690697add.htm

likely that post-translational modification(s), glycosylation for example, increased its molecular mass in mammalian cells.

Binding specificity of BtR175b was examined by competitive inhibition assay on ^{125}I-labelled Cry1Aa binding (Figure 3). BtR175b showed the highest binding affinity to Cry1Aa in the three Cry1 proteins examined. Cry1Ab and Cry1Ac could also compete for the binding of labelled Cry1Aa to BtR175b, although less efficiently than Cry1Aa.

Cry-susceptibility of BtR175b-expressing mammalian cells

Cry1Aa-susceptibility of HKb20 cells was measured by a cell-viability assay based on tetrazolium salt reduction. Incubation of the cells with 50 μg/ml of activated Cry1Aa for 1 h reduced formazan production by $\approx 70\%$ (Figure 4A). The Cry1Aa susceptibility was further confirmed by a cytotoxicity assay based on extracellular release of LDH activity. Cry1Aa at 50 μg/ml caused a 8 % release of the total intracellular LDH activity of HKb20 cells into the medium in 1 h (Figure 4B). This release began 30 min after the addition of Cry1Aa and reached a maximum in 85 min (Figure 4C). Untransfected host cells were not affected by Cry1Aa in either assay.

Cry1Ab and Cry1Ac also showed detectable cytotoxicity to HKb20 cells, but the amount of LDH released was $\approx$ 4-fold smaller than that caused by the same concentration of Cry1Aa (Figure 5). Together with the results of the competitive binding assay in Figure 3, it indicated that BtR175b primarily serves as

Cry1Aa receptor, though it can mediate binding and suscep-tibility to Cry1Ab and Cry1Ac with lower efficiency.

Cry1Aa-induced morphological changes in BtR175b-expressing mammalian cells

We observed the morphology of HKb20 cells in the presence of activated Cry1Aa at 1 min intervals. Some of the cell images are shown in Figure 6. Some HKb20 cells began to change their shape within 10 min after the addition of 50 μg/ml activated Cry1Aa. They showed remarkable swelling and detached from the culture dish in 30 min. Lysis or burst of the swollen cells, however, was not observed. Untransfected HEK293 cells showed no morphological change in the presence of 50 μg/ml activated Cry1Aa (time-lapse movies of HKb20 and HEK293 cell mor-phology in the presence of Cry1Aa are available at: http://www.BiochemJ.org/bj/369/bj3690697add.htm; the movies and the still images in Figure 6 were compiled from the same data, but represent different parts of the field; at least three swelling cells are shown near the centre of the field in the HKb20 movie; in the movies, 1 s corresponds to 10 min of real time).

DISCUSSION

Our results showed that BtR175b, an allele of BtR175, im-parted Cry1Aa-susceptibility to mammalian cells, indicating that this Cry1Aa receptor essentially required no additional insect-

specific factors to mediate the toxicity of Cry1Aa. It suggested that loss or changes of this receptor might play a pivotal role in naturally developed Cry resistance.

Cry1Aa susceptibility of the BtR175b-expressing HKb20 cells ($LC_{50} \cong 20$ μg/ml in the cell-viability assay) was difficult to compare with the reported Cry susceptibility of cultured insect cells, since most of the reported data were described as threshold doses on lawn assays [21–24]. *B. mori* larvae seem to be more sensitive to Cry1Aa ($LC_{50} = 0.1$–0.2 μg/g of diet) [22] than HKb20 cells. However, the initial rate of Cry1C-induced volume increase of Sf9 cells required ≈ 5 μg/ml of Cry1C to approach the maximum value [25] and the LC_{50} value of dispersed midgut epithelial cells of *B. mori* was ≈ 100 μg of activated Cry1Aa/ml in the cell-viability assay (T. Nakata, M. Nishigaki, T. Fukada, K. Sugimoto and M. Himeno, unpublished work). From these results, the Cry1Aa-susceptibility of HKb20 cells seemed to be comparable with that of *B. mori* midgut epithelial cells. Probably the lower LC_{50} value for *B. mori* larvae compared with their midgut cells likely just indicates that disruption of only a small number of midgut epithelial cells would be sufficient to kill a larva. The BtR175b-expressing mammalian cells could be more sensitive to Cry1Aa if BtR175b were more efficiently transported to the cell surface, since most of the BtR175b protein was found to remain in intracellular structures in HKb20 cells (results not shown). Their Cry1Aa-susceptibility could be also affected by mammalian-specific modification of BtR175b protein, which might have lowered its Cry1Aa-binding affinity, since the recombinant BtR175b protein in HKb20 cells had a higher molecular mass than the purified *B. mori* protein (Figure 2).

Interestingly, the number of Cry1Aa-binding sites in the BtR175a-expressing COS7 cells was significantly less than that of the BtR175b- or BtR175c-expressing cells. It is likely that some of the amino acid substitutions in BtR175a affect its transport efficiency to the cell surface. If a similar transport defect is observed in insect cells, the inefficiency of BtR175a transport may lead to reduced Cry1Aa susceptibility of BtR175a-expressing larvae.

Among Cry1Aa, Cry1Ab and Cry1Ac, Cry1Aa shows the highest toxicity to *B. mori* larvae in feeding assays, and Cry1Ac is practically non-toxic in such assays. However, Cry1Ac was reported to show the highest toxicity of these three toxins in an *in vitro* assay on dispersed *B. mori* midgut cells [22]. The report stated that this discrepancy was not caused by degradation of Cry1Ac by digestive enzymes in midgut, since the results were not affected by treating Cry1A proteins with *B. mori* gut juice. Although the reason for the resistance of *B. mori* larvae to Cry1Ac in feeding assays is unclear, their midgut epithelial cells are, when dispersed, likely to be highly susceptible to Cry1Ac. On the other hand, the mammalian cells expressing BtR175b were more susceptible to Cry1Aa than to Cry1Ac. Together with the reported result mentioned above, this result suggested that BtR175b essentially served as a Cry1Aa receptor and *B. mori* midgut cells may express additional Cry1Ac receptor(s). Although APN is a good candidate for this putative Cry1Ac receptor, Jenkins and Dean recently reported that purified *B. mori* APN did not bind to Cry1Ac [26]. It suggested an interesting possibility that a third, as-yet-uncharacterized, class of Cry receptor might exist. Response of dispersed *B. mori* midgut cells to Cry proteins would repay more intensive investigation.

Since artificial Cry mutant proteins are often labile to digestive proteases in larval midgut, the potential cytotoxicity of such mutants cannot be detected by *in vivo* assays on living larvae. This has hampered the analysis of structure–activity relationship of Cry proteins, which is necessary for rational improvement of Cry insecticides. To exclude interference by digestive enzymes, *in*

vitro toxicity assays of Cry proteins have been made with dispersed midgut cells of target insects or cultured insect cell lines. The mammalian cells expressing a Cry receptor would provide an excellent alternative for this purpose. They are easier to handle and maintain than dispersed midgut cells, and have the advantage that a single, defined receptor species is involved in the assay, while the receptors expressed in cultured insect cell lines may be different from those working in midgut epithelial cells of their parental insects. The methods for stable gene expression and monitoring cell death are also more established for mammalian cells than insect cells.

In summary, we successfully made a cadherin-like Cry receptor expressed on the mammalian cell surface, where it mediated Cry1Aa-susceptibility without additional insect-specific factors. Among Cry1Aa, Cry1Ab and Cry1Ac, Cry1Aa was most toxic to the transfected cells, suggesting that *B. mori* midgut cells may have other Cry1Ac receptor(s). The quick methods for quantifying cell death of the transfected mammalian cells used in the present study would make an *in vitro* cytotoxicity assay of Cry proteins easy to conduct and applicable to high-throughput primary screening of novel Cry insecticides.

REFERENCES

1   Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D. R. and Dean, D. H. (1998) *Bacillus thuringiensis* and its pesticide crystal proteins. Microbiol. Mol. Biol. Rev. **62**, 775–806

2   Wright, D. J., Iqbal, M., Granero, F. and Ferré, J. (1997) A change in a single midgut receptor in the diamondback moth (*Plutella xylostella*) is only in part responsible for field resistance to *Bacillus thuringiensis* subsp *kurstaki* and *B. thuringiensis* subsp *aizawai*. Appl. Environ. Microbiol. **63**, 1814–1819

3   Iqbal, M., Verkerk, R. H. J., Furlong, M. J., Ong, P. C., Rehman, S. A. and Wright, D. J. (1996) Evidence for resistance to *Bacillus thuringiensis* (Bt) subsp *kurstaki* HD-1, *Bt* subsp *aizawai* and abamectin in field populations of *Plutella xylostella* from Malaysia. Pestic. Sci. **48**, 89–97

4   Tabashnik, B. E., Liu, Y.-B., Malvar, T., Heckel, D. G., Masson, L., Ballester, V., Granero, F., Ménsua, J. L. and Ferré, J. (1997) Global variation in the genetic and biochemical basis of diamondback moth resistance to *Bacillus thuringiensis*. Proc. Natl. Acad. Sci. U.S.A. **94**, 12780–12785

5   Gahan, L. J., Gould, F. and Heckel, D. G. (2001) Identification of a gene associated with bt resistance in *Heliothis virescens*. Science **293**, 857–860

6   Knight, P. J. K., Crickmore, N. and Ellar, D. J. (1994) The receptor for *Bacillus thuringiensis* CryIA(c) δ-endotoxin in the brush-border membrane of the lepidopteran *Manduca sexta* is aminopeptidase N. Mol. Microbiol. **11**, 429–436

7   Luo, K., Sangadala, S., Masson, L., Mazza, A., Brousseau, R. and Adang, M. J. (1997) The *Heliothis virescens* 170 kDa aminopeptidase functions as "receptor A" by mediating specific *Bacillus thuringiensis* Cry1A δ-endotoxin binding and pore formation. Insect Biochem. Mol. Biol. **27**, 735–743

8   Yaoi, K., Kadotani, T., Kuwana, H., Shinkawa, A., Takahashi, T., Iwahana, H. and Sato, R. (1997) Aminopeptidase N from *Bombyx mori* as a candidate for the receptor of *Bacillus thuringiensis* Cry1Aa toxin. Eur. J. Biochem. **246**, 652–657

9   Vadlamudi, R. K., Ji, T. H. and Bulla, L. A., Jr (1993) A specific binding protein from *Manduca sexta* for the insecticidal toxin of *Bacillus thuringiensis* subsp, *berliner*. J. Biol. Chem. **268**, 12334–12340

10   Vadlamudi, R. K., Weber, E., Ji, I., Ji, T. H. and Bulla, Jr, L. A. (1995) Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*. J. Biol. Chem. **270**, 5490–5494

11   Nagamatsu, Y., Toda, S., Koike, T., Miyoshi, Y., Shigematsu, S. and Kogure, M. (1998) Cloning, sequencing and expression of the *Bombyx mori* receptor for *Bacillus thuringiensis* insecticidal CryIA(a) toxin. Biosci. Biotechnol. Biochem. **62**, 727–734

12   Ihara, H., Uemura, T., Masuhara, M., Ikawa, S., Sugimoto, K., Wadano, A. and Himeno, M. (1998) Purification and partial amino acid sequencing of the binding protein from *Bombyx mori* for CryIAa δ-endotoxin of *Bacillus thuringiensis*. Comp. Biochem. Physiol. B, **120**, 197–204

13   Nagamatsu, Y., Koike, T., Sasaki, K., Yoshimoto, A. and Furukawa, Y. (1999) The cadherin-like protein is essential to specificity determination and cytotoxic action of the *Bacillus thuringiensis* insecticidal CryIAa toxin. FEBS Lett. **460**, 385–390

14   Keeton, T. P. and Bulla, Jr, L. A. (1997) Ligand specificity and affinity of BT-R1, the
     Bacillus thuringiensis Cry1A toxin receptor from *Manduca sexta*, expressed in
     mammalian and insect cell cultures. Appl. Environ. Microbiol. **63**, 3419–3425

15   Ikawa, S., Tsuda, Y., Fukada, T., Sugimoto, K. and Himeno, M. (2000) cDNA cloning
     of the Cry1Aa receptor variants from *Bombyx mori* and their expression in
     mammalian cells. Biosci. Biotechnol. Biochem. **64**, 2682–2685

16   Shimizu, M., Oshie, K., Nakmura, K., Takada, Y., Oeda, K. and Ohkawa, H. (1988)
     Cloning and expression in *Escherichia coli* of the 135-kDa insecticidal protein gene
     from *Bacillus thuringiensis* subsp. *aizawai* IPL7. Agric. Biol. Chem. **52**, 1565–1573

17   Ge, A. Z., Shivarova, N. I. and Dean, D. H. (1989) Localization of *the Bombyx mori*
     specificity domain of a *Bacillus thuringiensis* δ-endotoxin protein. Proc. Natl. Acad.
     Sci. U.S.A. **86**, 4037–4041

18   Nagamatsu, Y., Itai, Y., Hatanaka, C., Funatsu, G. and Hayashi, K. (1984) A toxic
     fragment from the entomocidal crystal protein of *Bacillus thuringiensis*.
     Agric. Biol. Chem. **48**, 611–619

19   Sugimoto, K., Tasaka, H. and Dotsu, M. (2001) Molecular behavior in living mitotic
     cells of human centromere heterochromatin protein HP1 alpha ectopically expressed
     as a fusion to red fluorescent protein. Cell Struct. Funct. **26**, 705–718

20   Hunter, W. M. and Greenwood, F. C. (1962) Preparation of iodine-131-labelled human
     growth hormone of high specific activity. Nature (London) **194**, 495–496

21   Gringorten, J. L., Witt, D. P., Milne, R. E., Fast, P. G., Sohi, S. S. and Van
     Frankenhuyzen, K. (1990) An *in vitro* system for testing *Bacillus thuringiensis* toxins:
     the lawn assay. J. Invert. Pathol. **56**, 237–242

22   Masson, L., Mazza, A., Gringorten, L., Baines, D., Aneliunas, V. and Brousseau, R.
     (1994) Specificity domain localization of *Bacillus thuringiensis* insecticidal toxin
     is highly dependent on the bioassay system. Mol. Microbiol. **14**,
     851–860

23   Smith, G. P. and Ellar, D. J. (1994) Mutagenesis of two surface-exposed loops of the
     Bacillus thuringiensis CryIC δ-endotoxin affects insecticidal specificity. Biochem. J.
     302, 611–616

24   Potvin, L., Laprade, R. and Schwartz, J. L. (1998) Cry1Ac, a *Bacillus thuringiensis*
     toxin, triggers extracellular Ca^{2+} influx and Ca^{2+} release from intracellular stores in
     Cf1 cells (*Choristoneura fumiferana*, Lepidoptera). J. Exp. Biol. **201**, 1851–1858

25   Villalon, M., Vachon, V., Brousseau, R., Schwartz, J. L. and Laprade, R. (1998) Video
     imaging analysis of the plasma membrane permeabilizing effects of *Bacillus
     thuringiensis* insecticidal toxins in Sf9 cells. Biochim. Biophys. Acta **1368**, 27–34

26   Jenkins, J. L. and Dean, D. H. (2001) Binding specificity of *Bacillus thuringiensis*
     Cry1Aa for purified, native *Bombyx mori* aminopeptidase N and cadherin-like
     receptors. BMC Biochem. **2**, 12–19