**Note**

Binding of an Engineered 130-kDa Insecticidal Protein of *Bacillus thuringiensis* var. *israelensis* to Insect Cell Lines

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*B. thuringiensis* var. *israelensis* (BTI) produces crystalline protein bodies (CPBs) consisting of several kinds of insecticidal proteins, the molecular masses of which are 130 kDa, 70 kDa, 28 kDa, and so on, and which are toxic to diptera such as mosquitoes and black flies.1) The insecticidal proteins are solubilized and proteolytically activated, and then break the cells in the midgut to kill the insect.2) Several investigators have cloned and sequenced the two genes for 130-kDa insecticidal proteins which we named ISRH3 and ISRH4.3–5) which are identical to CrylVB and CryIVA, respectively.6) We have delineated the insecticidal N-terminal fragment of ISRH4 by deletion analysis.7) In this report, we show that an N-terminal insecticidal fragment of ISRH4 protein binds to two insect cell lines.

To construct an assay system, interaction between insect cell lines and truncated gene products of ISRH4 fused with β-galactosidase (β-gal) was examined. The recombinant plasmids, pM4B2 and pM4B7 (Fig. 1), were constructed from pL4H4-B2 and -B7, respectively.7) The plasmid pM4B2 encoded a dipherous active fragment of ISRH4 fused with β-gal derived from pMC1403,8) and pM4B7 encoded an inactive one.

The protein extracts prepared from *Escherichia coli* cells carrying the recombinant plasmids were electrophoresed on an SDS-polyacrylamide gel and analyzed by immunoblotting with antibody against β-gal (Fig. 2). The intact fused proteins were produced, but the majority of each fused protein was decomposed. Therefore the amount of intact fused proteins in the protein extracts was too small to measure (approximately 0.1–0.2% of the total protein in our estimation), and the amounts of intact products of pM4B2 and pM4B7 in the extracts were estimated to be nearly equal, judging from their signals on the immunoblot. An assay of insecticidal activity was done as previously described,9) and the product of pM4B2 was mosquitoicidal but that of pM4B7 was not (data not shown).

The TN-368 cells were typically susceptible to CPBs of BTI and active fragments of ISRH4.10) As shown in Fig. 3, TN-368 cells treated with the insecticidal fused protein were disrupted and stained after the color reaction catalyzed by β-gal (panel B), which

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**Fig. 1. Schematic Organization of the Genes Encoding Truncated ISRH4 Fused with β-Galactosidase.**

The names of recombinant plasmids are indicated at the left. The directions of transcription of the genes are indicated by thick arrows. The solid boxes indicate the stretch of the β-gal encoded by ISRH4 derived from pMC1403.8) The shadowed boxes indicate the stretch of a collagen linker.11) The numbers in the thick arrows are the ordinal numbers of amino acid residues of ISRH4. B, BamHI; C, Cid; E, EcoRI; H, HindIII; S, SalI; Sm, Smal; Ss, SstI; V, EcoRV.

**Fig. 2. Expression of the Genes for Truncated ISRH4 Fused with β-Galactosidase.**

An SDS-PAGE (panel A) and a corresponding immunoblot (panel B) are shown. **E. coli** MC4100β cells carrying the plasmids pM4B2, pM4B7, and placZ, which encodes lacZ, were cultured in 10 ml of 2×YT medium (1.6% Bactotryptone, 1% yeast extract, and 0.5% NaCl) containing 50 μg/ml ampicillin and 1 mM isopropyl-β-D-thiogalactopyranoside (1 mM IPTG) and harvested by centrifugation (10000 rpm, 4°C). Cells were washed with 1 M NaCl and suspended in 200 μl of PBS (136 mM NaCl, 2.7 mM KCl, 8.1 mM NaHPO₄, and 1.5 mM KH₂PO₄) containing 0.1 M β-mercaptoethanol and disrupted by shaking with zirconium beads (0.5 mm in diameter) for 3 min using a mini-beadbeater (BioSpecProducts). After centrifugation, the supernatant solutions were used as protein extracts. Lane 1, β-gal (2 µg). Each of the lanes 2 to 5 contained 50 µg of protein extract from **E. coli** MC4100 carrying pMC1403 (lane 2), placZ (lane 3), pM4B2 (lane 4), and pM4B7 (lane 5). The small arrows indicate the positions of the intact fused proteins. Lane M contains the standard marker proteins.

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**Abbreviations:** A, *Aspia picta*; Aedes aegypti; β-gal, β-galactosidase; BTI, *Bacillus thuringiensis* var. *israelensis*; CPBs, crystalline protein bodies; SDS, sodium dodecyl sulfate.
Fig. 3. Two Insect Cell Lines after the Color Reaction.

The cell strain TN-368 was derived from ovary cells of Trichoplusia ni, and the culture methods were previously described.\(^{12}\) The cell strain NIAS-AeAl-2 was derived from the first instar larvae of A. albopictus.\(^ {10}\) The cells were cultivated in MTCM-1103 medium\(^ {10}\) with 3% fetal bovine serum. The cells in 5 ml of the medium were cultivated in a plastic flask (25 cm\(^2\)) (Falcon) at 28°C for 4-6 days. The medium was removed gently and the cells were suspended in the new medium. The cell suspension containing approximately 1.0 × 10\(^5\) cells/ml was subcultured into a 96-well microtiter plate (200 µl/well), and the plate was incubated at 28°C for 3 days. The medium in the wells was gently removed, the cells were washed with PBS (pH 6.0), and 200 µl of the protein extract diluted (3 mg/ml) with PBS was put on the cells. The cells were further incubated at 28°C for 2 h and washed twice with PBS. Two hundred µl of the reaction solution (125 mM sodium phosphate buffer (pH 6.0), 0.5 mM MgSO\(_4\), 0.1 mM MnSO\(_4\), 0.05 mM X-gal) was poured into the well and incubated at 37°C until the blue color developed (several hours). The color development was stopped by washing the cells with PBS several times. Panels A to D are the photographs of the TN-368 cells after the coloring reactions, and panels E to H are those of NIAS-AeAl-2 cells. The cells were treated with the protein extracts from E. coli MC4100 cells carrying placZ (A and E) as negative controls. pM482 (B and F), and pM487 (C and G). The cells were treated with solubilized CPBs of BTT (15 µg/ml) and β-gal (15 µg/ml) (D and H). Typically disrupted cells are indicated with arrows.

gave a blue color produced by hydrolysis of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and those treated with the inactive fused protein or the placZ product were not stained (panels C and A, respectively). Moreover, the cells treated with a mixture of solubilized CPBs of BTT and purified β-gal which was about ten-fold more concentrated than the placZ product (panel A) were disrupted but not stained (panel D). Because this reaction occurred in the region where the β-gal located, the β-gal activity found in the stained cells would indicate that the β-gal was anchored to the cell through the fused insecticidal fragment of ISRH4 protein. NIAS-AeAl-2 cells,\(^ {10}\) which were derived from a larva of mosquito, Aedes albopictus, were treated with the fused proteins (Fig. 3). While the cells treated with insecticidal proteins were disrupted (panels F and H), the others were not disrupted, though the shape of them was not changed so obviously as that of TN-368 cells (panels E and G). And those treated with the insecticidal fused protein were stained after the color reaction (panel F), and the others were not stained (panels E, G, and H). Therefore, the active fragments of ISRH4 were toxic and bound to the NIAS-AeAl-2 cells. These results suggested that a diptera active truncated 130-kDa protein bound to these two insect cell lines unspecifically, but the difference in the affinity was not measured in our experiment, because the production of fused protein and the activity of the color reaction were too weak.

References