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# *The role of helices of domain I for the insecticidal activity of Bacillus thuringiensis Cry4A toxin*

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An active form of Cry4A is a heterodimer of the 20- and 45-kDa fragments that are derived from the 130-kDa Cry4A protoxin. To investigate the function of these two fragments, several deletion mutants were constructed and expressed in *E.coli* as the GST (glutathione-*S*-transferase) fusion proteins. The results of the bioassay against *Culex pipiens* larvae showed that the interaction of two fragments of Cry4A was necessary for the toxicity, and that the C-terminal 67 amino acids of the 20-kDa fragment corresponding to the helices α4 and α5 were involved in determining the insecticidal activity. Surprisingly the lack of helix α5 did not affect the toxicity to *C. pipiens*, suggesting that the role of helix α5 of Cry4A was different from that postulated in the case of Cry1A toxins.

## 1. INTRODUCTION

During sporulation, *Bacillus thuringiensis* produces crystalline protein inclusions consisting of highly specific insecticidal proteins called δ-endotoxins which are toxic, upon ingestion, to lepidopteran, dipteran, and coleopteran insect larvae [1]. There are more recent reports of *B. thuringiensis* isolates active against other insect orders such as hymenoptera, homoptera, orthoptera, and mallophaga, and against nematodes, mites, and protozoa [2]. Moreover, *in vitro* cell-killing activity of *B. thuringiensis* isolates on human cancer cells was reported [3]. These isolates may pave the way for the use of *B. thuringiensis* inclusion proteins for medical purposes.

δ-Endotoxins or Cry proteins show homology in the primary sequence and probably have similar three-dimensional structures comprising three domains. Nevertheless, Cry proteins show a great variety of insecticidal specificity. The mostly lepidopteran-specific δ-endotoxins biosynthesized as protoxins are solubilized and proteolytically activated by gut proteases in the susceptible insect larvae [4]. The activated toxin binds to a receptor in the apical microvilli of epithelial cells of the midgut [5-7]. It is believed that specificity is determined largely by the interaction of the toxin with the receptor. The conformational change in the toxin molecule bound to the receptor triggers the insertion of its channel-forming domain into the membrane [8, 9]. Colloid-osmotic swelling and lysis of the cell results in the death of the larvae [10].

The three-dimensional structures of Cry1Aa [11], Cry2Aa [12], and Cry3A [13] have been determined by X-ray crystallographic analyses, and reveal the presence of three domains. Domain I consists of seven α helices and is involved in membrane partitioning and ion-channel regulation. Domain II consists of three β sheets in a so-called Greek key conformation, and is proposed to be involved in the determination of insect specificity and in recognition of receptor molecules on midgut epithelial cells of the target insects. Domain III, which consists of two β sheets in a jelly-roll conformation, has also been assumed to be involved in ion-channel function and receptor binding [14].

The mode of action of dipteran-specific δ-endotoxins is rather poorly elucidated. Previously

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we reported that the 130-kDa Cry4A, a dipteran-specific δ-endotoxin produced by *B. thuringiensis* subsp. *israelensis*, was processed into the 20- and 45-kDa fragments, and that the active form of Cry4A was a complex of the 20- and 45-kDa fragments [15]. The 20-kDa fragment of activated Cry4A includes α1 to α5 helices of domain I, and the 45-kDa fragment contains α6 and α7 helices of domain I, in addition to domain II and III. What is the role of these two fragments produced by the interhelical cleavage in domain I? Taking notice of roles of the α helices of domain I, we constructed several deletion mutants to answer the question. Our results of bioassays suggested that the association of the two fragments was necessary for the toxicity. We also found that the α4 and α5 helices were crucial for the insecticidal activity, and that the α5 helix was unlikely to perform a direct role for the toxicity.

## 2. MATERIALS AND METHODS

### 2.1 Construction of plasmids

The plasmid pGST4A20del30 (Fig. 1) was obtained by deleting the 0.37-Kb *Spe*I-*Xho*I fragment from pGST4AC20X [15]. This plasmid encoded GST-20 del30, the fusion protein of GST linked to the segment spanning from $Pro^{30}$ to $Leu^{205}$ of Cry4A. The plasmid pGST4A20del67 (Fig. 1) was constructed by inserting the 0.35-Kb *Eco*RI fragment of pCR347, which was obtained by TA cloning (Invitrogen) of 347-bp PCR fragment encoding the region spanning from $Gly^{58}$ to $Gln^{168}$ of Cry4A, into *Eco*RI site of pGEX-4T-2 (Amersham Pharmacia Biotech). The primers used for PCR were 5'-CAGAATCAGCAGTATGGTGG-3' as the forward primer and 5'-CTGAGTATTTTGTGGGTTTGG-3' as the reverse primer. This plasmid encodes GST-20 del67, the fusion protein of GST linked to the segment spanning from $Gly^{58}$ to $Gln^{168}$ of Cry4A. The plasmid pGST4A45del32 (Fig. 1) was constructed by inserting the 1.26-Kb *Bam*HI (blunt-ended) –*Sal*I fragment of pBS4A45del32 into the *Eco*RI (blunt-ended) –*Sal*I site of pGEX-4T-2 (Amersham Pharmacia Biotech). The plasmid pBS4A45del32 was obtained by inserting the 0.61-Kb *Nde*I-*Sal*I fragment of pBS622, which was constructed by subcloning the 0.62-Kb *Bam*HI-*Eco*RI fragment of pCR622 into the *Bam*HI-*Eco*RI site of pBSIISKplus, into the *Nde*I-*Sal*I site of pLH4-B2-sal [15]. The plasmid pCR622 was obtained by TA-cloning (Novagen) of the 622-bp PCR fragment encoding the region spanning from $Pro^{279}$ to $Val^{527}$ of Cry4A.

### 2.2 Bioassay of the mosquitocidal activities of GST-Cry4A fusion proteins

GST-Cry4A fusion proteins were expressed in *E. coli* BL21 cells in 200 ml of TB medium. The cells were harvested, resuspended in 40 ml of 100 mM $Na_2CO_3$ (pH10.5)/ 20 mM β-mercaptoethanol containing 100 μg/ml p-APMSF, and disrupted by sonication followed by centrifugation to obtain the supernatant. To the supernatant was added 200 μl of 50% slurry of Glutathione-Sepharose 4B (Amersham Pharmacia Biotech). After rotating for 2 h at 4°C, the beads were washed three times with the above buffer. GST-Cry4A fusion proteins were eluted with 100 μl of 0.2 M Tris-HCl (pH8.8) containing 20 mM reduced glutathione. The concentration of the purified protein was determined with the Bio-Rad Protein Assay using bovine serum albumin (Sigma) as a standard. The bioassay of GST-Cry4A fusion proteins was performed essentially by the methods of Schnell *et al.* [16]. The proteins were added to 1 ml of 0.1 M Tris (pH7.5), 0.1% latex beads (Sigma) of 0.8 μm in diameter to give 10 μg/ml of a final protein concentration. After a brief vortex, the samples were rotated for 1 h at room temperature. The mosquitocidal activities were assayed on 4th instar larvae of *C. pipiens*. The mosquito larvae were grown in a container (35×25×3 cm) at 25°C. Before the assays, each larva was transferred to 200 μl of distilled water in each well of 96-well plates. After 8 h, the GST-Cry4A fusion proteins adsorbed to latex beads were added. In each experiment, 96 larvae were tested at a protein concentration of 0.5 μg/ml, and the assays were performed more than 3 times. The mortality was scored after 12-h incubation at 25°C. The efficiency of adsorption of protein to the latex beads was almost 100% in a preliminary experiment.

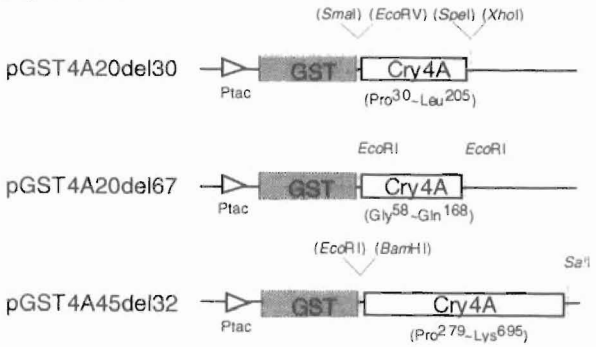


**Fig. 1 Physical maps of the constructed plasmids**
Three deletion mutants of GST fusion proteins and two deletion mutants of the 6∞His-tagged proteins were constructed. The pGST4A20del30, pGST4A20del67, and pGST4A45del32 were constructed by inserting the corresponding DNA fragment into pGEX-4T vector (Amersham Pharmacia biotech). The genes encoding all GST-Cry4A fusion proteins were transcribed from *tac* promoter. See Materials and Methods for details about the strategy construction of these plasmids.

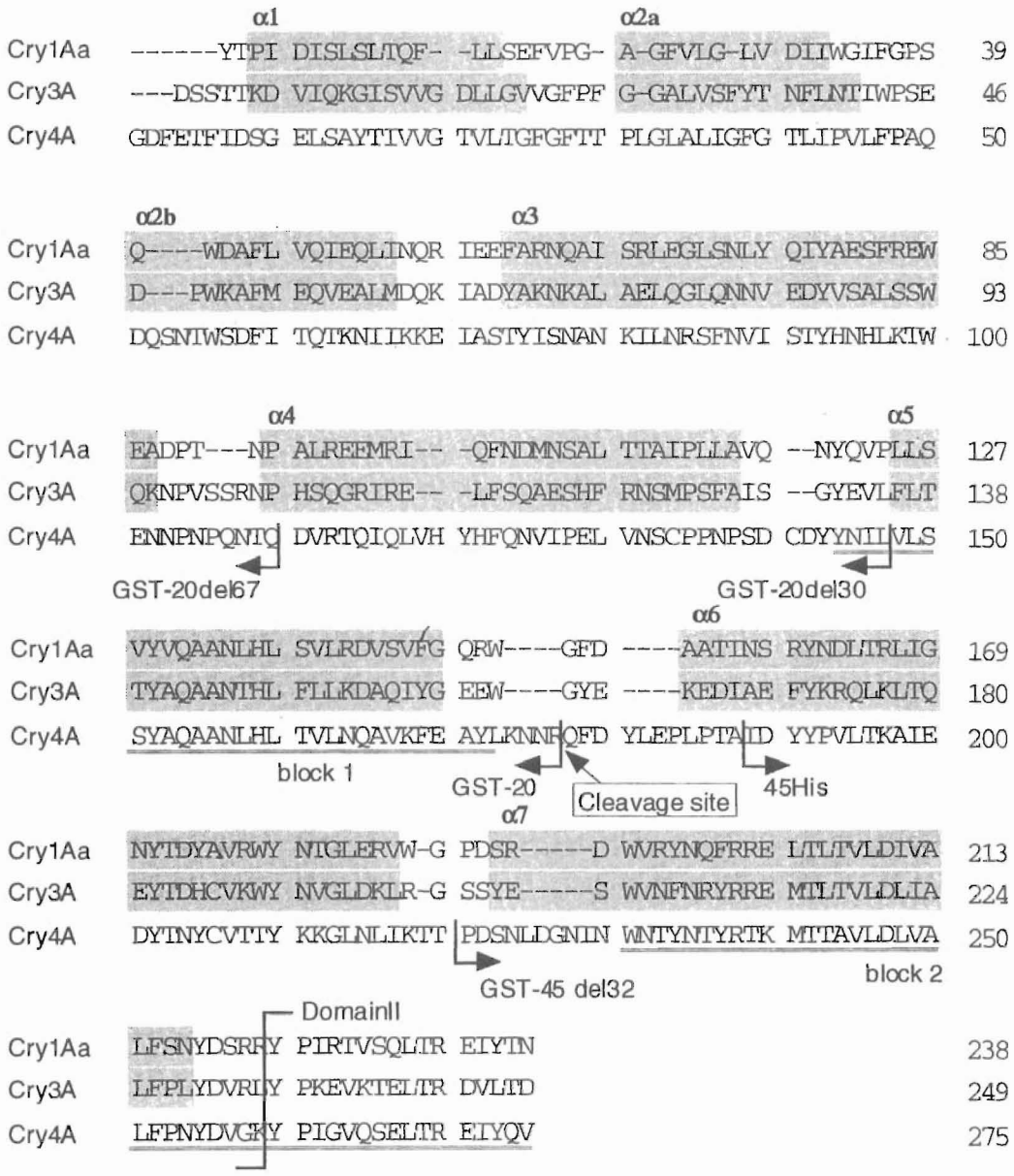


**Fig. 2 Domain I alignment of activated Cry1Aa, Cry3A, and Cry4A**
Alignment was done by GeneWorks (IntelliGenetics, Inc.). Block determination was based on the results of Schnepf *et al* [3] and the helices were located by tertiary structure of Cry1Aa and Cry3A. Rectangular arrows indicate the N- or C-terminus of deletion mutants.

## 3. RESULTS

### 3.1 Structures of the Cry4A deletion mutants

The 20-kDa fragment of activated Cry4A consists of five helices according to the alignment of domain I (Fig. 2). To investigate the function of 20-kDa fragment, GST-20 del30 and GST-20 del67 were constructed by deleting the 30 or 67 amino acids of C-terminus of GST-20. GST-20 del30 is devoid of the predicted α5 helix (Fig. 2) and consists of GST and the segment from $Pro^{30}$ to $Leu^{205}$ of Cry4A (Fig. 3). GST-20 del67 is devoid of the predicted α4 and α5 helices and comprises GST (Fig. 2) and the segment from $Gly^{58}$ to $Gln^{168}$ of Cry4A (Fig. 3).

### 3.2 Mosquitocidal activity of the deletion mutants

The purified GST fusion proteins of the deletion mutants were subjected to the bioassay for the insecticidal activity against *C. pipiens* larvae. The proteins were adsorbed to latex beads and given to the larvae at a working concentration of 0.5 μg/ml. The mortality after 12 h was calculated through a correction for the mortality when latex beads alone were given. All of the individual GST fusion proteins were not toxic to *C. pipiens* larvae. When GST-20 and GST-45 coexisted, a significant insecticidal activity was observed [15]. The combination of GST-20 del30 and GST-45 was also as toxic as that of GST-20 and GST-45. However, when GST-20 del67 and GST-45 coexisted, no such toxicity was observed (Fig. 4). These results suggested that the C-terminal 67 amino acids of the 20-kDa fragment which corresponded to the α4 and α5 helices was essential for the expression of insecticidal activity against *C. pipiens* larvae. The combination of GST-20 and GST-45 del32, which did not associate (data not shown), led to no toxicity. It was, therefore, suggested that the interaction of 20- and 45-kDa fragment was crucial for the toxicity against *C. pipiens* larvae.

## 4. DISCUSSION

In this study, we investigated the function of the 20- and 45-kDa fragments of activated Cry4A in the light of the role of α-helices of domain I. The 20-kDa fragment of Cry4A is the segment spanning from $Gly^{58}$ to $Arg^{235}$ of Cry4A, and contains helices α1, 2, 3, 4, and 5, and the 45-kDa fragment has $Gln^{236}$ at its N-terminus, and includes helices α6 and α7 of domain I in the N-terminal region. To perform the functional analysis of these two fragments, deletion mutants were constructed, and expressed as fusion proteins. In the bioassay for the insecticidal activity against *C. pipiens* larvae, all GST-Cry4A fusion proteins constructed in the present study were not toxic when each of them was given separately to the larvae, but the combination of GST-20 del30 and GST-45 in addition to that of GST-20 and GST-45 exhibited the significant mosquitocidal activity. This implied that the C-terminal 30 amino acids of 20-kDa fragment of Cry4A, which corresponded to the putative helix α5, were not essential for the insecticidal activity against *C. pipiens* larvae. Apparently this result seem inconsistent with the generally accepted idea that the helix α5 is involved in the channel formation of the toxin molecule. To perform further investigation, another mutant GST-20 del67 devoid of putative α4 and α5 helices was constructed. Combination of GST-20 del67 and GST-45 did not exhibit the toxicity against *C. pipiens* larvae. It was, therefore, suggested that the α4 and α5 helices were crucial for the insecticidal activity. As mentioned above, it was confirmed that the lack of N-terminal 67 amino acids of 20-kDa fragment did not affect the association of two fragments, i.e. an inactive complex was formed. Thus, we concluded the loss of the toxicity was due to the lack of the C-terminal region of the 20-kDa fragment that corresponded to the predicted α4 and α5 helices.

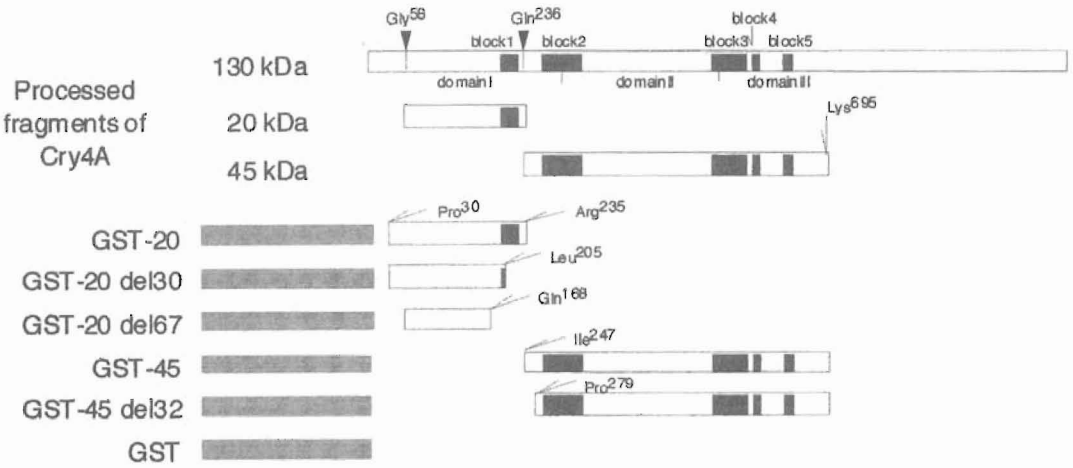


**Fig. 3 Structures of fusion proteins of the fragments from Cry4A**

Schematic representation of the fusion protein structures is shown. The arrowheads indicate the cleavage sites with gut extract. The putative domains and blocks are also depicted. GST-20 is the fusion protein of GST and the $Pro^{30}$~$Arg^{235}$ region of Cry4A. GST-20 del30 is the fusion protein of GST linked to the segment spanning from $Pro^{30}$ to $Leu^{205}$ of Cry4A. GST-20 del67 is the fusion protein of GST linked to the segment spanning from $Gly^{58}$ to $Gln^{168}$ of Cry4A. In GST-45, the $Ile^{247}$~$Lys^{695}$ segment of Cry4A is fused to the C-terminus of GST. GST-45 del32 is the fusion protein of GST linked to the segment spanning from $Pro^{279}$ to $Lys^{695}$ of Cry4A.

In Cry1Ac, only mutations in the helix α5 resulted in no or low toxicity probably because of either the lack of insertion into the membrane or the inability to form an ion channel [17, 18]. Synthetic peptide corresponding to the helix α5 of Cry3A was able to span artificial membranes and self-assemble in a parallel manner [9]. These results and the structural basis that the helix α5 is centered in the domain I helical bundle in Cry1Aa and Cry3A have led to the consensus that the helix α5 is implicated in channel formation and crucial for the insecticidal activity of δ-endotoxins.

More recently, however, the role of helix α4 rather than helix α5 in channel formation has been drawing the attention. Mutagenic analysis revealed that replacement of tyrosine153, which is in the loop between α4 and α5 of Cry1Ab, by aspartic acid resulted in a great loss of toxicity [19]. Gazit *et al.* reported that helices α4 and α5 (not α5 only) inserted into the membrane as a helical hairpin [10]. Replacement of a glutamine residue by a helix-breaking proline in the center of α4 helix destroyed the toxicity of Cry4B against *Aedes aegypti* larvae [20]. Mutagenic studies with Cry1Ac suggested that the helix α4 have a more direct role in channel formation than α5, and the helix α5 is important for aggregation of the toxin molecules and has other function for the toxicity [21]. Finally, Masson *et al.* reported that the helix α4 of Cry1Aa lines the lumen of the ion channel [22]. According to their refined umbrella model, the α4 helix faces the hydrophilic lumen of the channel while α5 helix faces the lipid surface of the membrane. Thus, it is strongly suggested that the helix α4 rather than α5 is directly involved in channel formation. In the umbrella model, the α4-α5 hairpin is inserted into the

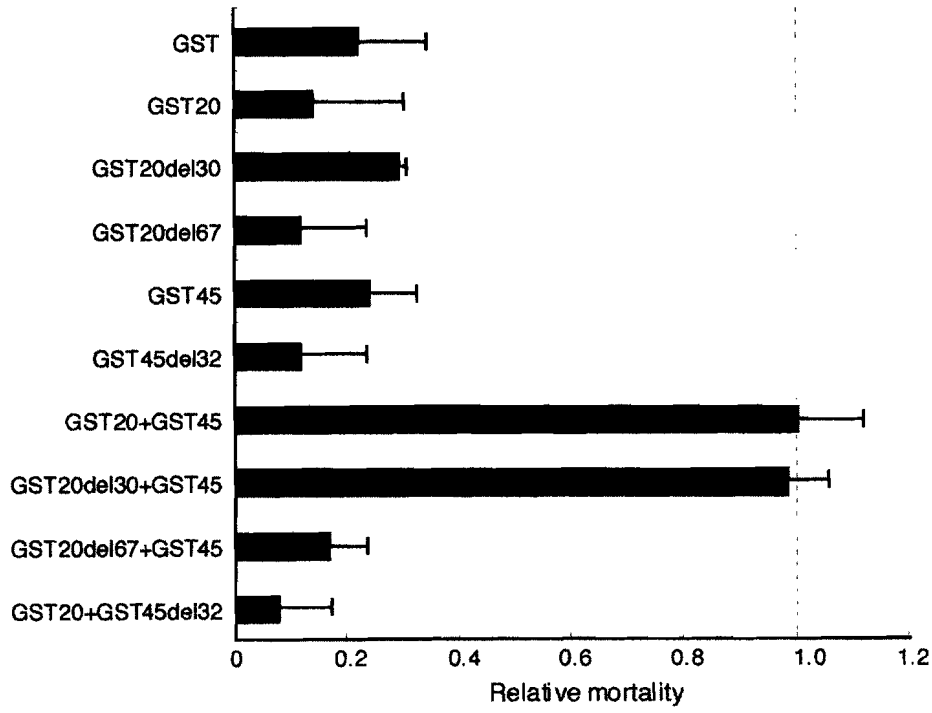


**Fig. 4 Insecticidal activity of GST-Cry4A fusion proteins**

The GST-Cry4A fusion proteins were adsorbed to the latex beads to give a final protein concentration of 10 μg/ml. The mosquitocidal activities were assayed on the 4th instar larvae of *C. pipiens*. Before the assays, each larva was transferred to 200 μl of distilled water in each well of 96-well plate. After 8 h, the GST-Cry4A fusion proteins adsorbed to latex beads were added. In each experiment, 96 larvae were tested at the protein concentration of 0.5 μg/ml and more than 3 times of assay were performed. The mortality of mosquito larvae in the presence of each GST fusion protein was scored after 12-h incubation at 25°C by subtracting the mortality with latex beads alone. The relative mortalities with GST-Cry4A fusion proteins are expressed as a proportion of the mortality with the combination of GST20 and GST45. The error bars denote standard errors of the mean. The toxicity of the combination of GST-20 del30 and GST-45 was as high as that of GST-20 and GST-45. However the combination of GST-20 del67 and GST-45 was not toxic.

membrane followed by the subsequent spreading of the remaining helices of domain I on the membrane, and the functional role of the α4-α5 hairpin seems essential. Since GST-20 del30 holds the α4-α5 hairpin (Fig. 3), this mutant may be able to perform the membrane insertion.

We note that all experiments about functional analysis of helices of domain I have been performed with lepidopteran-specific Cry1A toxins or the coleopteran-specific Cry3A toxin, and few data have been obtained to date for a dipteran-specific δ-endotoxin, Cry4A. Our present data suggested that, unlike in Cry1A, helix α5 was not necessarily important in Cry4A for its insecticidal activity. Further investigation is needed to answer the questions about what the function of α5 helix or other helices is in Cry4A.

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