Binding of purified *Bacillus sphaericus* binary toxin and its deletion derivatives to *Culex quinquefasciatus* gut: elucidation of functional binding domains

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Highly larvicidal strains of *Bacillus sphaericus* produce a binary toxin composed of 51 and 42 kDa proteins which binds to sharply delineated regions of the gastric caecum and posterior midgut of susceptible larvae of the mosquito *Culex quinquefasciatus*. To investigate the role of the individual subunits and the organization of functional binding regions within the toxin, plasmids were constructed for the expression in *Escherichia coli* of the toxin proteins and their NH₂- and COOH-terminal deletion derivatives as fusions with glutathione *S*-transferase (GST). Toxin proteins were purified by affinity chromatography followed by cleavage from the GST carrier with thrombin. The LC₅₀ values for the purified toxin proteins and their deletion derivatives were determined. The binding patterns of fluorescently labelled toxin suggested that the 51 kDa protein is the primary binding component of the toxin and mediates the regional binding and internalization of the 42 kDa protein. Examination of the toxin deletion derivatives revealed that the NH₂-terminal region of the 51 kDa protein was required for binding to the larval gut, whilst the COOH-terminal region was responsible for interacting with the 42 kDa protein. Toxicity was strongly correlated with the subsequent internalization of the toxin, probably by endocytosis.

Introduction

Bacillus sphaericus is an aerobic, Gram-positive, spore-forming bacterium which is ubiquitously distributed in soil and aquatic environments. A number of *B. sphaericus* strains are toxic to mosquito larvae, notably *Culex*, *Anopheles* and *Mansonia* species (Yousten, 1984; Cheong & Yap, 1985). Larvicidal activity in highly toxic strains of *B. sphaericus* is principally associated with a parasporal crystal which contains several proteins, of which the 51 and 42 kDa proteins (designated P51 and P42 respectively in this study) are the best characterized

(Davidson, 1983; Baumann *et al.*, 1985; Berry *et al.*, 1991; Baumann *et al.*, 1991). When assayed against mosquito larvae, both proteins were found to be necessary for toxicity, indicating that the *B. sphaericus* larvicide may act as a binary toxin (Broadwell *et al.*, 1990*a, b*; Davidson *et al.*, 1990; Baumann *et al.*, 1991). Following ingestion of the toxin by larvae, the 51 and 42 kDa proteins are processed to lower molecular mass forms of approximately 44 and 39 kDa respectively (Broadwell & Baumann, 1987; Davidson *et al.*, 1987*a*, 1990; Aly *et al.*, 1989; Broadwell *et al.*, 1990*a*). Studies using binary toxin purified from the spores of *B. sphaericus*, a mixture of the 51 and 42 kDa proteins, have shown that toxin binding occurs in the gastric caecum and posterior midgut of *Culex* larvae (Davidson, 1988). However, purification of the individual proteins from *B. sphaericus* has proved to be problematic (Broadwell *et al.*, 1990*b*; Davidson *et al.*, 1990), and hence the respective roles of the 51 and 42 kDa proteins in toxin binding have remained unresolved.

In our previous work (Oei *et al.*, 1990), we constructed a series of deletion mutants in which varying numbers of amino acid residues had been deleted from both the

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Abbreviations: GST, glutathione *S*-transferase; P51, the 51 kDa component of the *B. sphaericus* binary toxin; P42, the 42 kDa component of the *B. sphaericus* binary toxin; LC₅₀, the concentration of toxin resulting in 50% mortality of larvae; AMG, anterior midgut; CMG, central midgut; PMG, posterior midgut; GC, gastric caecum; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; IPTG, isopropyl β -D-thiogalactoside.

NH₂- and COOH-termini of the 51 and 42 kDa proteins of *B. sphaericus* strain 2297. The recombinant proteins expressed in *Escherichia coli* were assayed for toxicity against *Culex quinquefasciatus* larvae. The minimal regions of both proteins necessary for toxicity have been identified in our laboratory and others (Oei *et al.*, 1990; Broadwell *et al.*, 1990c; Clark & Baumann, 1990; Sebo *et al.*, 1990).

The availability of purified preparations of the different classes of truncated proteins from the various deletion mutants presents the opportunity to compare *in vivo* toxicity with the patterns of the binding of the toxin to target cells in the larval gut and also to analyse precisely the toxicities of the different deletion proteins. Here we describe studies using the purified 51 and 42 kDa proteins and their deletion derivatives, labelled by direct coupling to isothiocyanate fluorochromes, to investigate the patterns of *in vivo* binding of the proteins in relation to their toxicity to *C. quinquefasciatus* larvae.

Methods

Materials. Restriction enzymes and DNA-modifying enzymes used in DNA manipulations were purchased from Boehringer Mannheim or New England Biolabs. Bovine thrombin, glutathione-agarose and the fluorochromes fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) were obtained from Sigma.

Media, bacterial strains and plasmids. *E. coli* strain HB101 [F⁻, *hsdS20*(r_B⁻, m_B⁻), *recA13*, *supE44*, *ara-14*, *galK2*, *lacY1*, *proA2*, *rpsL20*(Str^r), *xyl-5*, *mtl-1*, λ⁻] was used for the propagation of plasmids and the expression of recombinant proteins. Plasmid pGEX-KG contains the glutathione S-transferase (GST) gene under the control of the *tac* promoter and has been described previously (Smith & Johnson, 1988; Guan & Dixon, 1991). Plasmids pRK42, pRK37 and pCC2297-F, encoding the deletion derivatives of the 51 kDa protein, and plasmids pME31 and pMN174, encoding the 42 kDa protein and its deletion derivative, have been described elsewhere (Oei *et al.*, 1990). Plasmid pRK24, containing a DNA fragment [nucleotides 509–1979 according to the published sequence of Baumann *et al.* (1988)], encoding the 51 kDa protein (minus the first four codons), was generated as part of the pRK series (Oei *et al.*, 1990). Plasmid pME23 contains a gene fragment (nucleotides 2023–3257) which encodes the 42 kDa protein (minus the first four codons) and was generated as part of the pME series (Oei *et al.*, 1990).

E. coli was cultured in Luria broth or 2 × TY liquid medium (10 g yeast extract, 16 g tryptone and 5 g NaCl in 1 litre) and supplemented with 60 µg ampicillin ml⁻¹ where necessary for plasmid maintenance. Isopropyl β-D-thiogalactoside (IPTG) at a final concentration of 0.2 mM was used to induce the expression of fusion proteins.

Mosquito larvae. The larvae of *Culex quinquefasciatus* were reared in our laboratory. *Aedes aegypti* larvae were obtained from the Insectarium of the Department of Microbiology, National University of Singapore.

Construction of GST-toxin gene fusions. For our plasmid constructions, we utilized pGEX-KG, a vector which allows the IPTG-inducible expression of a cloned gene as a fusion protein with GST (Smith & Johnson, 1988; Guan & Dixon, 1991). These GST-toxin

fusion proteins were purified by affinity chromatography on glutathione agarose under non-denaturing conditions. The vector also encodes a thrombin recognition site (L-V-P-R-G-S) which allows the separation of the GST and toxin polypeptides by endopeptidolytic cleavage with thrombin (Chang, 1985). The vector pGEX-KG is a derivative of the vector pGEX-2T (Pharmacia), which has a glycine-rich linker incorporated adjacent to the thrombin site (Guan & Dixon, 1991). This facilitated the cleavage of the fusion proteins while they were still attached to the glutathione-agarose and allowed a single-step purification of the recombinant toxin proteins.

(i) **GST-P51 gene fusions.** A 1.5 kb *NcoI*-*XmnI* fragment containing the gene encoding the 51 kDa protein (minus the first four codons) was excised from plasmid pRK24 and was made blunt-ended by treatment with Klenow polymerase. The DNA fragment was then ligated into the vector pGEX-KG which had previously been cut with *XbaI* and the ends made blunt-ended by treatment with Klenow polymerase. This yielded the plasmid pKG51 (Fig. 1a), which had the 5'-end of the gene encoding the 51 kDa protein inserted directly at the 3'-end of the GST gene, for the expression of an in-phase GST-P51 fusion protein.

The plasmids containing NH₂- and COOH-terminal deletions of the P51 gene, which are denoted as the pKG51ΔN and pKG51ΔC series respectively, are shown schematically in Fig. 2. To construct gene fusions in which NH₂-terminal deletions of P51 were coupled to GST, two different cloning procedures were utilized. Plasmid pKG51ΔN34 was constructed by subcloning a 1.4 kb *NcoI*-*XmnI* fragment (which had been treated with Klenow polymerase) from plasmid pRK42 (Oei *et al.*, 1990) into the *NcoI*-cut, Klenow-polymerase-treated vector pGEX-KG. A 1.4 kb DNA fragment was also isolated from plasmid pRK37 (Oei *et al.*, 1990) by digestion with *NcoI* and *XmnI* and filling in the ends with Klenow polymerase. This fragment was incubated overnight in ligation buffer with *NcoI*-cut pGEX-KG to allow the compatible *NcoI* ends to ligate. The DNA was then precipitated, subjected to phenol/chloroform extraction and treated with Klenow polymerase to blunt-end the unligated *NcoI* end. The plasmid DNA was then recircularized and the plasmid containing the insert in the desired orientation (designated pKG51ΔN45) was selected. These steps were necessary to ensure that the NH₂-terminal-deleted 51 kDa protein was produced in-phase with the GST protein.

To create GST-COOH-terminal deletions of P51 gene fusions, the fragment encoding the COOH-terminus of P51 was removed from pKG51 (Fig. 1a) and was replaced with fragments encoding 3' truncations of the gene as follows. Plasmid pKG51 was cut with *HindIII*, made blunt-ended with Klenow polymerase and restricted with either *AflIII* or *BglIII*. In both cases, the larger of the two fragments generated was isolated from low-melting-point agarose following electrophoresis. Plasmid pKG51ΔC52 was created by excising a 1.06 kb *AflIII*-*SmaI* fragment from plasmid pCC2297-F (Oei *et al.*, 1990) and ligating it to the *AflIII*/*HindIII*-cut (blunt-ended) pKG51. Similarly, to generate plasmid pKG51ΔC56, a 0.2 kb *BglIII*-*AccI* fragment was subcloned from pCC2297-F into pKG51 which had previously been restricted with *HindIII*, blunt-ended with Klenow polymerase and digested with *BglIII*.

(ii) **GST-P42 gene fusions.** To construct plasmid pKG42 (Fig. 1b), which contains the gene encoding a GST-P42 fusion protein, the following DNA manipulations were performed. Plasmid pME23, which contains the gene encoding the 42 kDa protein (minus the first four amino acids), was first digested with *SmaI* and subsequently partially digested with *XbaI*. A 1.23 kb *XbaI*-*SmaI* fragment was then isolated and ligated to the *XbaI*-cut vector pGEX-KG. After 12 h incubation at 15 °C to allow the compatible *XbaI* ends to ligate, the DNA fragments were precipitated and subjected to phenol/chloroform extraction. The ends were then filled in with Klenow polymerase and the plasmid containing the insert was recircularized.

The plasmids containing the genes encoding NH₂- and COOH-terminal deletions of the 42 kDa protein (denoted as the pKG42ΔN

Fig. 1. Structure of plasmids (a) pKG51 and (b) pKG42 containing GST-toxin translational fusions. The GST gene segment is represented by the black box and the genes encoding the 51 and 42 kDa proteins are represented by the open boxes. The nucleotide and amino acid sequences are shown for the linker connecting the GST and toxin gene fragments. The boxed amino acids designate the thrombin recognition sequence; the amino acids underlined indicate the start of the toxin protein sequences. The location of genes for ampicillin resistance (Ap^r) and the *lac* repressor (*lacI*) are denoted by arrows in shaded and open boxes respectively.

Fig. 2. Schematic representation of GST-toxin deletion mutants. The genes encoding the GST-toxin fusion proteins are shown with the black and open regions representing GST and *B. sphaericus* toxin segments, respectively. Below P51 and P42 (the full-length toxin proteins), the NH₂- and COOH-terminal deletion constructs are shown schematically. The names of the toxin deletion derivatives indicate the number of amino acids deleted from either the NH₂- or the COOH-terminus. The amino acids of the toxin sequences (e.g. 4-448) which are encoded are listed. The toxic activity of each recombinant protein (in combination with the other component of the binary toxin) is also indicated.

and pKG42ΔC series respectively) are shown schematically in Fig. 2. Plasmid pKG42ΔN34 was formed by ligating a 0.26 kb *Xba*I fragment from pMN174 (Oei *et al.*, 1990) into the larger fragment isolated from the *Xba*I digestion of pKG42. To construct pKG42ΔN17, the plasmid pKG42 was digested with *Xba*I, the ends were filled in with Klenow polymerase and then a second digestion with *Cla*I was performed. The larger fragment was purified and ligated to a 0.48 kb *Rsa*I-*Cla*I fragment from pME31 (Oei *et al.*, 1990).

To construct GST-COOH-terminal deletions of P42 gene fusions, the fragment encoding the COOH-terminus of P42 was removed from pKG42 (Fig. 1b) and was replaced with fragments encoding 3' truncations of the gene as follows. pKG42 was digested with *Hind*III, the ends filled in with Klenow polymerase and then digested with *Cla*I. The larger 5.5 kb fragment was subsequently gel purified. Plasmid pKG42ΔC12 was created by ligating a 0.55 kb *Cla*I-*Eco*NI (blunt-ended) fragment from pME31 with the purified 5.5 kb fragment of pKG42. pCO3 was created by the ligation of a blunt-ended *Sal*I-*Eco*RV gene fragment (nucleotides 2009-3066) encoding the 42 kDa protein with a COOH-terminal deletion of 21 amino acids into the *Sma*I site of pKK223-3. pKG42ΔC21 was subsequently constructed by ligating a 0.52 kb *Cla*I-*Bam*HI (blunt-ended) fragment from pCO3 to the 5.5 kb DNA fragment from pKG42 described above.

Dideoxynucleotide sequencing of the pKG plasmids confirmed that the GST and toxin protein coding sequences were translationally in frame with each other and could therefore express the predicted GST-toxin fusion proteins.

Expression, purification and thrombin cleavage of the fusion proteins. The recombinant plasmids were introduced into *E. coli* HB101 to express the GST-toxin fusion proteins. The expression of the fusion proteins was achieved as previously described (Guan & Dixon, 1991)

except that the cells were grown at 30 °C. After harvesting by centrifugation, cells were sonicated (W-380 Sonicator, Heats Systems-Ultrasonics) for 2 min on ice at a 50% pulse and centrifuged for 10 min at 10000 *g* to remove the cell debris. Then 10 ml of the supernatant was mixed with 10 ml 50% (v/v) glutathione-agarose beads for 1 h at 4 °C with gentle agitation. The beads were transferred to a 20 ml polypropylene column (Bio-Rad) and washed with PBST [150 mM-NaCl, 20 mM-sodium phosphate pH 7.3 1% (v/v) Triton X-100] until the *A*₂₈₀ of the eluate was less than 0.5 (approximately 250 ml of wash buffer). The agarose beads were equilibrated by washing with 10 ml thrombin cleavage buffer (50 mM-Tris/HCl pH 8.0, 150 mM-NaCl, 2.5 mM-CaCl₂, 0.1% β-mercaptoethanol) (Guan & Dixon, 1991). The beads in the column were subsequently resuspended in 5 ml of the thrombin cleavage buffer containing 2 μg of bovine thrombin and incubated at room temperature with gentle shaking for 30 min. After this time the column was allowed to drain and the proteins released by thrombin cleavage were collected in the buffer mixture that drained from the column.

SDS-PAGE and quantification of protein. SDS-PAGE was performed by the method of Laemmli (1970). Protein concentration was determined by the method of Bradford (1976), using bovine gamma globulin (Bio-Rad) as a standard.

Toxicity bioassays. Second-instar larvae of *C. quinquefasciatus* were used to determine the concentration of the toxin needed to cause 50% mortality (LC₅₀). Each concentration of the 51 and 42 kDa proteins and their deletion derivatives was tested in duplicate. Ten larvae were introduced into 3 ml water containing recombinant toxin proteins in a 3.5 cm Petri dish. Yeast was added to a final concentration of 1% (w/v). Controls received yeast only. The surviving larvae were counted after

24 h. Previous results showed that maximum toxicity occurred when the 51 and 42 kDa proteins were present in approximately equal concentrations (Broadwell *et al.*, 1990*b*; Davidson *et al.*, 1990). Based on this observation, the bioassays were designed such that the 51 kDa protein and its deletion derivatives were fed to the larvae in the presence of an equivalent concentration of 42 kDa protein and vice versa.

Isothiocyanate labelling of proteins. The purified 51 kDa protein and its deletion derivatives were labelled with FITC, while the purified 42 kDa protein and its deletion derivatives were labelled with TRITC. The toxins were labelled by incubation in the dark at 4 °C for 8 h with either FITC or TRITC in 0.1 M-Na₂CO₃ (pH 9.0) as described by Harlow & Lane (1988). Unbound fluorochrome was separated from the conjugated protein by gel filtration using Sephadex G-50 (Pharmacia). The labelled proteins were stored at -20 °C.

In vivo binding of the fluorescently labelled toxins. Third- or fourth-instar larvae were placed in solutions containing 10 µg ml⁻¹ of FITC- or TRITC-labelled proteins alone, or in combination with unlabelled proteins (Tables 2, 3, and 4). After 4–5 h of feeding, guts were dissected from the larvae and fixed on poly-L-lysine-coated glass slides (Davidson *et al.*, 1987*b*; Davidson, 1988). Coverslips were then mounted onto the slides after the addition of Permafluor aqueous mounting medium (Lipshaw). The midgut specimens were observed using a Zeiss fluorescence microscope.

Results

Purification and cleavage of the fusion proteins

The constructs encoding P51, P42 and their deletion derivatives yielded approximately 0.5–1.5 mg protein per litre of cells. *E. coli* cells containing plasmids pKG51 and pKG42 produced GST-toxin fusion proteins of 77 and 68 kDa respectively (26 kDa of GST and 51 or 42 kDa corresponding to each component of the binary toxin) (Fig. 3, lanes 2 and 4). Thrombin cleavage of the fusion proteins GST-P51 and GST-P42 resulted in two recombinant toxin proteins of approximately 51 and 42 kDa (Fig. 3, lanes 3 and 5), leaving the GST protein bound to the agarose beads (Fig. 3, lane 6). Both recombinant proteins lack four NH₂-terminal residues (M-C-D-S for the 51 kDa protein and M-R-N-L for the 42 kDa protein) compared to the primary translation products of the toxin genes (Baumann *et al.*, 1988). Following thrombin cleavage, 13 amino acid residues (G-S-P-G-I-S-G-G-G-G-I-L) found in the 'glycine kinker' (Guan & Dixon, 1991) and three additional amino acid residues remain attached to the NH₂-terminus of the toxin proteins (Fig. 1).

The GST-toxin deletion derivatives were also effectively purified by the same method, yielding fusion proteins of the molecular masses predicted from the nucleotide sequences (results not shown). After cleavage of the toxin deletion derivatives from the GST by thrombin, the deletion mutants were purified to homogeneity as shown by SDS-PAGE (Fig. 4). Unexpectedly,

Fig. 3. Affinity-purified GST-P51 and GST-P42 fusion proteins and thrombin cleavage products. Purified GST-toxin fusion proteins were incubated with thrombin (for details, see Methods), and the samples were resolved by electrophoresis on a 0.1% SDS/10% polyacrylamide gel, followed by staining with Coomassie blue. The sizes (kDa) and positions of the molecular mass markers are indicated in lane 1. Lanes 2 and 4 contain the fusion proteins GST-P51 and GST-P42 respectively, while lanes 3 and 5 contain the toxin proteins after cleavage from the GST carrier with thrombin. Lane 6 contains the GST moiety of the fusion proteins remaining on the glutathione affinity column after thrombin cleavage.

the deletion derivative P42ΔN34 migrated more slowly than P42ΔN17 on SDS-PAGE (Fig. 4, lanes 8 and 9). As mentioned above, all the pKG plasmid constructs obtained in this study were sequenced across the junctions linking GST and the toxin proteins. The anomalous migration of the P42ΔN34 deletion derivative could have arisen from a change in conformation of the modified protein, as a result of a deletion of 34 amino acids from the NH₂ terminus. The change in the three-dimensional folding pattern of P42ΔN34 could result in reduced binding of SDS, and hence the retarded migration on SDS-PAGE. The anomalous migration of modified *B. sphaericus* toxin proteins has been observed previously (Clark & Baumann, 1990).

Toxicity bioassays against *C. quinquefasciatus* larvae

Table 1 shows the LC₅₀ values of purified P51, P42 and their deletion derivatives for the larvae of *C. quinquefasciatus*. Consistent with previous results (Broadwell *et al.*, 1990*a, b*; Davidson *et al.*, 1990; Oei *et al.*, 1990), when P51 or P42 were fed alone to larvae they were not toxic, even at a concentration of 250 µg ml⁻¹. A combination of the two proteins was toxic to larvae, confirming that the

Fig. 4. SDS-PAGE of purified P51, P42 and their NH₂- and COOH-terminal deletion derivatives expressed as GST fusion proteins. Purification and subsequent thrombin cleavage of the fusion proteins were as described in Methods. Purified recombinant proteins were analysed by electrophoresis through a 0.1% (w/v) SDS/12% (w/v) polyacrylamide gel, followed by staining with Coomassie blue. Lanes 2–6 contain purified P51 and its deletion derivatives, while purified P42 and its deletion mutants are shown in lanes 7–11. The position and sizes (kDa) of molecular weight markers are indicated in lane 1.

Table 1. Larvicidal activities of purified recombinant 51 and 42 kDa toxin proteins and their deletion derivatives towards *C. quinquefasciatus* larvae

Protein	LC ₅₀ * (ng ml ⁻¹)
P51	30†
P51ΔN34	144†
P51ΔN45	>10000†
P51ΔC52	784†
P51ΔC56	>10000†
P42	30‡
P42ΔN17	436‡
P42ΔN34	>10000‡
P42ΔC12	142‡
P42ΔC21	>10000‡

* Concentration needed to kill 50% of *C. quinquefasciatus* larvae in a 24 h assay.

† Assayed in the presence of an equivalent amount of the 42 kDa protein.

‡ Assayed in the presence of an equivalent amount of the 51 kDa protein.

larvicidal proteins may act as a binary toxin (Broadwell *et al.*, 1990b; Davidson *et al.*, 1990). Similarly, the deletion derivatives of P51 and P42 when fed alone to larvae were not toxic. The LC₅₀ value of the combination

of equal amounts of P51 and P42 was determined to be 30 ng ml⁻¹ of each protein.

Compared to P51, the toxic NH₂-terminal deletion derivative of the 51 kDa protein P51ΔN34 exhibited a fivefold decrease in toxicity (LC₅₀ 144 ng ml⁻¹), whereas a COOH-terminal deletion of 52 amino acids showed a greater decrease in toxicity (LC₅₀ 784 ng ml⁻¹) when they were fed in combination with P42 (Table 1). In the case of the 42 kDa protein, an NH₂-terminal deletion of 17 amino acids (P51ΔN17) led to a 15-fold reduction in toxicity (LC₅₀ 436 ng ml⁻¹) whilst the COOH-terminal deletion derivative, P42ΔC12 showed only a fivefold reduction in toxicity (LC₅₀ 142 ng ml⁻¹) when compared to the toxicity of P42. The other four truncated proteins were non-toxic at the highest concentration tested (10 µg ml⁻¹).

Binding of FITC-labelled 51 kDa protein and its deletion derivatives to the gut of *C. quinquefasciatus* larvae

The 51 kDa protein and its deletion derivatives were assayed for their ability to bind to the midgut of *C. quinquefasciatus* larvae. When FITC-labelled P51 was fed to the larvae, intense fluorescence was observed at

Fig. 5. Gut of *C. quinquefasciatus* larvae fed FITC-labelled P51 toxin. Fluorescence is very intense on the gastric caecum (GC; *a*) and on the posterior midgut (PMG; *b*). Bars, 20 μ m.

Table 2. Binding patterns of FITC-labelled P51 and its deletion derivatives on the midgut of *C. quinquefasciatus* larvae

Protein	Regional binding to gastric caecum and posterior midgut	Binding to anterior and central midgut
P51	+	—
P51 Δ N34	+	—
P51 Δ N45	—	—
P51 Δ C52	+	—
P51 Δ C56	+	—

Fig. 6. Posterior midgut cells of *C. quinquefasciatus* larvae fed FITC-labelled P51 and unlabelled P42. Internalization of the fluorescently labelled toxin as small, bright vesicles within the cells is apparent. Bar, 5 μ m.

the gastric caecum (GC) and the posterior midgut (PMG) (Fig. 5). This pattern of binding is referred to as regional binding. The binding patterns of the deletion derivatives of P51 are summarized in Table 2. The NH₂-terminal deletion derivative which was still toxic, P51 Δ N34, exhibited a similar regional binding pattern to P51. However, the non-toxic NH₂-terminal deletion protein, P51 Δ N45, showed no binding to the larval gut (not shown). In contrast, the two COOH-terminal deletions of the 51 kDa protein, P51 Δ C52 and P51 Δ C56, exhibited the regional binding pattern found for P51. In order to differentiate between surface binding and internalization of the fluorescently-labelled toxin, the guts were examined at higher magnification. This revealed that binding of the FITC-labelled P51 or its deletion derivatives occurred at the cell surface with no evidence of internalization (results not shown). In the presence of unlabelled P42, although no change in the regional binding of FITC-labelled P51 occurred, observation at a higher magnification showed internalization of P51 into the cytosol of cells of the gut (Fig. 6).

Binding patterns of TRITC-labelled 42 kDa protein and its deletion derivatives

When TRITC-labelled P42 was fed alone to larvae, fluorescence was observed over the entire larval gut, including the GC, the PMG and the anterior midgut (AMG) (Fig. 7). The same distribution of fluorescence was also observed with both the toxic and non-toxic TRITC-labelled NH₂- and COOH-terminal deletion derivatives of P42 (results not shown). Internalization of the TRITC-labelled P42 or its deletion derivatives was

Fig. 7. Midgut of *C. quinquefasciatus* larvae fed TRITC-labelled P42. Strong fluorescence is present over the entire midgut and the gastric caecum. Bar, 20 μ m.

Fig. 8. Gut of *C. quinquefasciatus* larvae fed a combination of TRITC-labelled P42 and unlabelled P51. Note the strong regional binding of labelled P42 to the gastric caecum (GC) and posterior midgut (PMG) in the presence of P51. Bar, 50 μ m.

not observed. When TRITC-labelled P42 was fed to larvae in the presence of unlabelled P51, binding of labelled P42 became strongly localized to the PMG and GC (Fig. 8). This regional binding was different from the

non-specific binding observed when P42 alone was fed to larvae (compare Figs. 7 and 8) and seemed to be directed by P51, as the regional binding pattern was identical to that observed when larvae were fed FITC-labelled P51

Fig. 9. Posterior midgut cells of *C. quinquefasciatus* larvae fed TRITC-labelled P42 and unlabelled P51. Internalization of the fluorescent labelled toxin as small, bright vesicles within the cells is apparent. Bar, 5 μ m.

alone (Fig. 5). The labelled P42 also appeared to become internalized as small, bright vesicles in the cytoplasm of the midgut cells (Fig. 9) (as reported previously with mixed labelled toxins: Davidson, 1988). No vesicles were observed in the nucleus (Fig. 9).

Table 3 summarizes the binding patterns of the TRITC-labelled NH_2 - and COOH -terminal deletion derivatives of P42 in the presence of unlabelled P51. A less toxic derivative of the 42 kDa protein, P42 Δ N17 (15-fold reduction in toxicity), showed regional binding to the PMG and GC, although binding to the AMG and central midgut (CMG) region of the larvae was occasionally observed. There was no evidence of internalization of the labelled protein in the AMG or CMG. With the non-toxic derivatives P42 Δ N34 and P42 Δ C21, regional binding was completely abolished. The toxic COOH -terminal deletion derivative P42 Δ C12 had a similar binding pattern to that of full-length P42. Only P42 and the toxic deletion derivatives P42 Δ N17 and P42 Δ C12 were internalized by cells of the GC and PMG (Table 3).

Table 3. Binding patterns of TRITC-labelled P42 and its deletion derivatives in the presence of unlabelled P51* on the midgut of *C. quinquefasciatus* larvae

Protein (labelled)	Binding to gastric caecum and posterior midgut	Binding to anterior and central midgut	Internalization of the labelled protein
P42	+	—	+
P42 Δ N17	+	$\pm$ †	+
P42 Δ N34	+	+	—
P42 Δ C12	+	—	+
P42 Δ C21	+	+	—

* Larvae were fed with 10 μ g of TRITC-labelled test protein ml^{-1} and 10 μ g of unlabelled P51 ml^{-1} .

† Occasional binding to this region.

Table 4. Binding patterns of TRITC-labelled P42 in the presence of unlabelled P51* or its deletion derivatives on the midgut of *C. quinquefasciatus* larvae

Protein (unlabelled)	Binding to gastric caecum and posterior midgut	Binding to anterior and central midgut	Internalization of labelled P42
P51	+	—	+
P51 Δ N34	+	—	+
P51 Δ N45	+	+	—
P51 Δ C52	+	$\pm$ †	+
P51 Δ C56	+	+	—

* Larvae were fed with 10 μ g of TRITC-labelled P42 ml^{-1} and 10 μ g of unlabelled P51 or its deletion derivatives ml^{-1} .

† Occasional binding to this region.

Effect of truncations of unlabelled 51 kDa protein on the binding of TRITC-labelled 42 kDa protein

As described above, the specific regional binding of TRITC-labelled P42 only occurred in the presence of P51. It was therefore of interest to study the interaction of the 51 and 42 kDa proteins in the larval gut, as well as the effect of NH_2 - or COOH -terminal deletions of P51 on the binding patterns of the TRITC-labelled P42. The results of these experiments are summarized in Table 4. Labelled P42, in the presence of a toxic derivative of the 51 kDa protein P51 Δ N34, showed distinct regional binding to the GC and PMG. However, in the presence of the non-toxic derivatives P51 Δ N45 and P51 Δ C56, labelled P42 bound non-specifically over the entire larval gut. When labelled P42 was fed in combination with an unlabelled P51 derivative, P51 Δ C52, which shows

reduced toxicity (Table 1), there was strong fluorescence in the GC and PMG, but binding to the AMG and CMG regions was occasionally observed as well (Table 4). In the presence of the toxic derivatives P51 Δ N34 and P51 Δ C52, internalization of the labelled P42 was observed in the GC and PMG regions.

Binding of fluorescently labelled 51 and 42 kDa proteins to the gut of Aedes aegypti larvae

It has previously been shown that *A. aegypti* larvae do not bind the FITC-labelled mixed toxin proteins isolated from the spores of *B. sphaericus* (Davidson, 1988). Similarly in this study, no binding was observed when *A. aegypti* was fed with only FITC-labelled P51 (results not shown). When TRITC-labelled P42 was fed alone or in combination with unlabelled P51, binding of the labelled P42 over the entire midgut was observed (results not shown). The labelled 42 kDa protein appeared to bind to the lumen edge of the midgut cells, but was not internalized in vesicles.

Discussion

To investigate the individual roles of the *B. sphaericus* 51 and 42 kDa toxin proteins in mediating toxicity in the mosquito larval gut, it was necessary to obtain purified protein preparations. In this study, we used the GST fusion protein purification system for the expression and rapid purification of milligram quantities of the 51 and 42 kDa proteins as well as their NH₂- and COOH-terminal deletion derivatives. The 51 and 42 kDa proteins produced in this study retained toxin activity and were easily purified with yields of up to 1.5 mg protein per litre of culture.

The effects of the various deletions of the 51 and 42 kDa proteins on the LC₅₀ of the toxins assayed against *C. quinquefasciatus* larvae (Table 1) paralleled results of previous studies in which deletion mutants were produced (Oei *et al.*, 1990; Broadwell *et al.*, 1990c; Clark & Baumann, 1990). However, in these earlier studies, LC₅₀ values were given in terms of concentrations of bacterial cells expressing, or amorphous inclusions containing, the proteins (Broadwell *et al.*, 1990c; Clark & Baumann, 1990). A limitation of using bacterial cells for the determination of LC₅₀ is that changes in the level of expression of the recombinant proteins are indistinguishable from changes in the intrinsic potency of the toxins produced. By assaying the toxicity of the purified proteins instead of bacterial cells expressing the proteins, we can be confident that our results (Table 1) reflect the true toxicity of our recombinant toxin proteins.

In this study we have investigated the *in vivo* binding of the *B. sphaericus* toxin proteins to the gut of *C. quinquefasciatus* larvae in order to examine the initial binding events leading to intoxication and cell death. It has been previously demonstrated that FITC-labelled toxin isolated from the spores of *B. sphaericus* bound to discrete regions of the PMG and GC of the susceptible larvae of *C. quinquefasciatus* (Davidson, 1988, 1989). Internalization of the toxin as small, bright vesicles was also observed. At the time it was believed that the regional binding pattern of the FITC-labelled toxin was solely due to the action of the 42 kDa protein (Davidson, 1988). However recent studies have shown that the 42 kDa protein preparations used were contaminated with lower-molecular-mass derivatives of the 51 kDa protein (Broadwell *et al.*, 1990b; Davidson *et al.*, 1990; Baumann *et al.*, 1991) so that the reported binding patterns of the FITC-labelled proteins were due to a mixture of the 51 and 42 kDa proteins. In this work, we have separately cloned, expressed and purified the 51 and 42 kDa proteins as well as their NH₂- and COOH-terminal deletion derivatives. Using the GST fusion protein system, there was no possibility of cross-contamination between the two proteins (or their deletion derivatives) during their preparation and purification.

In this study, using individually purified toxin proteins, FITC-labelled P51 bound regionally to the PMG and GC when it was fed alone to *C. quinquefasciatus* larvae, and no internalization of the labelled protein was observed. In the presence of P51, the binding pattern of TRITC-labelled P42 changed from a non-specific binding to a regional P51-directed binding. Internalization of P42 was observed in the presence of P51 but did not occur with P42 alone. P51 was also internalized only in the presence of P42. The results indicate that the presence of P51 was necessary for the regional binding and internalization of P42. We suggest that the 51 kDa protein may function as the binding component of the binary toxin, and directs the binding of the 42 kDa protein to the sites in the larval gut where toxicity occurs, i.e. the GC and PMG.

Davidson *et al.* (1990) have shown that the 51 kDa protein can remain associated with the midgut tissue for up to 24 h after feeding *E. coli* cells containing the protein to larvae and that this protein remains available to interact with the 42 kDa protein to cause mortality. In contrast, larvae fed cells containing the 42 kDa protein were not killed when they were given cells containing the 51 kDa protein 24 h later. Therefore from the results of our work (Davidson *et al.*, 1990; this study), we suggest that the regional binding of P51 to the GC and PMG is a specific and tight binding whereas the binding of P42 in the absence of P51 is relatively weak and non-specific.

We propose that the tight binding of P51 serves to direct the subsequent binding of P42 due to a strong interaction between the two proteins. Previous work (Narasu & Gopinathan, 1986; Sgarella & Szulmajster, 1987) has shown that the 51 and 42 kDa proteins associate very strongly with one another, and they were copurified by several standard protein purification techniques (Davidson *et al.*, 1990). It has been suggested that tight binding by the binding component of a binary toxin serves to increase the local concentration of the active component at the membrane surface, and so increase the possibility of penetration of the active component (van Heyningen, 1982). In the case of *B. sphaericus* binary toxin, the function of P51 in localizing P42 to the GC and PMG, which are the sites of cytopathogenicity, is consistent with the proposed role of the 51 kDa protein as the binding component.

Davidson (1989) examined the binding patterns of FITC-labelled toxin isolated from *B. sphaericus* spores to the midgut cells of the non-susceptible larvae of *A. aegypti* and found that the lack of toxicity of the larvicide to this species was due to the absence of binding of the toxin. In this study, we found no binding of P51 to the midgut of *A. aegypti*, and a non-regional distribution of P42 binding in the presence or absence of P51 (a distribution comparable to that seen in the absence of P51 in guts of *C. quinquefasciatus*). However, *A. aegypti* larvae could still be killed when the *B. sphaericus* toxin was present at a very high dose (100–1000-fold greater than required for *C. quinquefasciatus*) (Lacy & Undeen, 1986). The increase in the amount of the toxin could result in sufficient toxin being present at the susceptible sites of the gut to cause mortality. We propose that binding of P51 to a larval gut receptor is likely to be the crucial first step leading to toxicity and that the host-range specificity of the toxin is attributable to the receptor affinity of P51.

The precise role of the 42 kDa protein is unresolved but analogies with other binary toxins would suggest that this protein might be the active component following internalization (Simpson *et al.*, 1987; Singh *et al.*, 1989). Schroeder *et al.* (1989) have demonstrated that in a toxin-resistant cell line of *C. quinquefasciatus*, binding and internalization of the toxin may occur without resulting in cell death. They concluded that the primary lethal action of the toxin occurred after initial interactions with the cell membrane and internalization. This conclusion is consistent with the proposed role of the 42 kDa protein as the active component of the *B. sphaericus* binary toxin.

The finding that the 51 kDa protein was the binding component of the binary toxin of *B. sphaericus* led us to investigate the relationship of binding to toxicity for truncated P51 derivatives. The toxic mutant protein P51ΔN34 exhibited regional binding to the GC and

PMG but there was a total loss of binding for the non-toxic deletion mutant P51ΔN45. We suggest therefore that the NH₂-terminal region located between amino acid residues 34 and 45 may be part of the receptor-binding domain of the 51 kDa protein. Deletions of the COOH-terminus of P51 (the toxic P51ΔC52 and the non-toxic P51ΔC56) had no effect on the regional binding of the 51 kDa protein, indicating that the COOH-terminus of P51 is not involved in the binding step. However, these deletions resulted in a loss of the ability (P51ΔC56) or a decrease in the ability (P51ΔC52) of these proteins to direct the regional binding of P42. In addition, internalization of P42 was observed when this protein was fed together with the toxic COOH-terminal deletion protein (P51ΔC52) but not with the non-toxic P51 deletion mutant (P51ΔC56), suggesting that toxin internalization is important for activity. These results strongly suggest that the COOH-terminal region of P51 is likely to be involved in binding and interacting with P42 prior to the internalization event. Evidence that P51 and P42 associate very strongly has been presented elsewhere (Davidson *et al.*, 1990), and we propose that the binding of P42 to P51 or to a P51–receptor complex is mediated via the COOH-terminal region of the P51 protein. The 26-fold decrease in toxicity of P51ΔC52 compared to P51 could be explained by a decrease in affinity of the mutant protein towards P42 as a result of the deletion, while loss of a further four amino acids totally abolished the binding of P51ΔC56 to P42. The observation that labelled P51 was only internalized in the presence of unlabelled P42 suggests that internalization of the P51–P42 complex or the P51–P42–receptor complex occurred, possibly by receptor-mediated endocytosis as previously described (Davidson *et al.*, 1987*b*; Davidson, 1988).

Loss of toxicity in both NH₂- and COOH-terminally deleted P42 derivatives paralleled their loss of binding to P51 or to the P51–receptor complex. This suggests that both the NH₂- and COOH-termini of P42 are involved in this interaction. However, in a deletion derivative of the 42 kDa protein showing a 15-fold decrease in toxicity (P42ΔN17), occasional binding to the AMG and CMG in addition to regional binding to the PMG and GC was observed, possibly due to a decrease in binding affinity for the 51 kDa protein.

In conclusion, we propose here a scheme for the initial events of binding and interaction of the binary toxin of *B. sphaericus*. Our studies show that P51 (or its gut-activated form) binds specifically to the GC and PMG of the larval gut of *C. quinquefasciatus*, and that this binding is mediated via the NH₂-terminal region of the protein. The bound P51 contains a site at its COOH-terminus with which P42 (or its gut-activated form) interacts, and subsequently the P51–P42 complex or possibly a P51–

P42-receptor complex is internalized via endocytosis. In this study we have elucidated the initial sequence of interactions of the 51 and 42 kDa proteins with the target cells in the insect gut and have suggested a possible role for the 51 kDa protein in toxicity. Our work has also revealed a strong correlation between toxicity and internalization of the toxin. Further studies on the *B. sphaericus* toxin should concentrate on the mechanism of action by which the toxin causes cell death after binding and internalization.

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