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Functional Complementation of Nontoxic Mutant Binary Toxins of *Bacillus sphaericus* 1593M Generated by Site-Directed Mutagenesis

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Alanine residues were substituted by site-directed mutagenesis at selected sites of the N- and C-terminal regions of the binary toxin (51- and 42-kDa peptides) of *B. sphaericus* 1593M, and the mutant toxins were cloned and expressed in *Escherichia coli*. Bioassays with mosquito larvae, using binary toxins derived from individual mutants, showed that the substitution of alanine at some sites in both the 51-kDa and the 42-kDa peptides resulted in a total loss of activity. Surprisingly, after mixing two nontoxic derivatives of the same peptide, i.e., one mutated at the N-terminal end and the other mutated at the C-terminal end of either the 51-kDa or the 42-kDa peptide, the toxicity was restored. This result indicates that the altered binary toxins can functionally complement each other by forming oligomers.

Mosquitoes transmit a number of vector-borne tropical diseases, such as malaria, filariasis, and viral encephalitis. They also have developed resistance to most of the chemical pesticides deployed for their control, making existing abatement programs ineffective. *Bacillus thuringiensis* subsp. *israelensis* and *Bacillus sphaericus* are the two potential biological alternatives identified for mosquito control programs (9). During sporulation, *B. sphaericus* 1593M synthesizes two mosquito larvicidal proteins, of 51 and 42 kDa. These two peptides act together as a binary toxin that has a major role in the overall efficacy of *B. sphaericus* (4). While the individual peptides of 51 and 42 kDa are nontoxic, when mixed together they exhibit toxicity (4). The genes coding for the binary toxins from a number of highly toxic strains of *B. sphaericus* have been identified and sequenced (2). A model for the mode of action of these binary toxins has been proposed on the basis of gene deletion and in vivo binding studies. In this model, it has been suggested that an N-terminal region of the 51-kDa peptide binds to the gut epithelial cells while its C-terminal region is essential for the interaction with the N-terminal region of the 42-kDa peptide. This apparently results in internalization of the toxin complex (8, 9). It is not known whether the binary toxin acts as a monomer, oligomer, or multimer.

Site-directed mutagenesis has been employed to generate deletion derivatives of the binary toxin to localize the regions essential for biological activity (2). Further, mutants with deletions of the binary-toxin genes have been generated to study the structure-function relationship of this toxin (8). However, the contribution of individual amino acid residues of the binary toxin to its biological activity is not yet understood. An understanding of the mechanism of action of the binary toxins will greatly aid in enhancing their efficacy as well as preventing the emergence of resistance to these biocontrol agents in mosquitoes (3, 5). In the present study, site-directed mutagenesis was used to further investigate the mode of action of the binary toxin. Alanine residues were substituted for other amino acids located in the N- and C-terminal regions of the binary-toxin subunits, and the effect of these changes on biological activity was assessed.

Bacterial strains and plasmids. *Escherichia coli* CJ236 and *E. coli* MV1190 were supplied with the Bio-Rad Muta-Gene M13 in vitro mutagenesis kit. *E. coli* BL21(DE3) containing a prophage carrying the T7 RNA polymerase gene under the control of the *lacUV* promoter and the phagemid pRSET A containing the T7 promoter were from Invitrogen. The parental recombinant plasmid (pAR5) containing the binary-toxin genes was from our laboratory (10).

Recloning of binary-toxin genes in the pRSET A vector. A 3.1-kb DNA fragment containing the coding sequences for the binary toxin (open reading frame 1 [ORF1], encoding the 51-kDa toxin of 448 amino acids, and ORF2, encoding the 42-kDa toxin comprising 370 amino acids) was restricted from the parental clone pAR5 with the enzymes *Kpn*I and *Hin*dIII. This 3.1-kb fragment was directionally cloned into pRSET A. Of the resulting transformants, one (pSV15) was characterized by restriction and PCR analysis for the presence of the insert (12).

Site-directed mutagenesis of binary-toxin genes. The EMBL accession numbers for the cloned genes encoding the 51- and 42-kDa peptides are XO7992 and Y00528, respectively. An analysis of the secondary structure of the binary toxin was performed for us with the Maxhorm multiple-alignment program (European Molecular Biology Laboratory). On the basis of the secondary-structure analysis and the published results of the deletion studies, we selected 13 sites for mutagenesis. The predicted secondary structures selected for disruption were the alpha helix, beta sheets, and loops. Oligonucleotides were synthesized by DNA Technologies, Lexington, Mass. The mutant oligonucleotide sequences complementary to the native sequences were as follows: 51N4, 5′ GTT ATA AAA TTT TTT TGA TAT TTC TGG 3′ (bp 104 to 131); 51C2, 5′ T AGG ATA CGA TTG TAT ACC TGC CAA 3′ (bp 1165 to 1190); 42N2, 5′ ATT TTC TCT GCT ACA GAT TTC TGTC 3′ (bp 128 to 152); and 42C2, 5′ GCC TGT ATA TCT AAC AGG AA 3′ (bp 925 to 945). The numbering of nucleotides starts from the initiation codon of the peptide. The underlined se-

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TABLE 1. Biological activity of mutant binary toxins against mosquito larvae

Mutant	Amino acid(s) replaced with alanine	Mutated site in binary toxin		Larvicidal effect	LC_{50}[a] for second-instar larvae of:	
		51-kDa peptide	42-kDa peptide		*C. quinquefasciatus*	*A. stephensi*
pSV15	None (wild-type toxin)	N C	N C	Toxic	20	100
51N4	$S_{38}KK_{40}$	↓		Nontoxic	—[b]	—
51C2	$I_{392}Q_{393}$	↓		Nontoxic	—	—
42N2	C_{47}		↓	Nontoxic	—	—
42C2	R_{312}		↓	Nontoxic	—	—
51N4		↓		Toxic	80	200
51C2		↓				
42N2			↓	Toxic	140	300
42C2			↓			

[a] Protein concentration, in nanograms per milliliter of the assay medium, required to kill 50% of the larvae in 24 h.
[b] —, not toxic at up to 10 μg of protein per ml.

quences were targeted for alanine (CGC) substitution. Site-directed mutagenesis (Muta-Gene M13 in vitro mutagenesis kit; Bio-Rad) was carried out as detailed in the manufacturer's manual. In brief, after complementary-strand synthesis with mutant primers, the DNA mixtures were transformed into *E. coli* MV1190. The single-stranded M13 DNA from the transformants was extracted and used for DNA sequencing to identify mutant clones. In the regions where mutations were introduced, about 100 to 150 bp upstream of the mutated site was sequenced to confirm the presence of the mutation. DNA sequencing was carried out by the dideoxy method of Sanger et al. (11) in accordance with the manufacturer's instructions (United States Biochemicals, Cleveland, Ohio). The specific primers used for DNA sequencing were 5′ CCGAATCAAG AATCGAGG 3′ for 51N4, 5′ GTTAATTTTAGGTATTAA TTC 3′ for 51C2, 5′ GTTTAAAGCAACCCATGGGAT 3′ for 42C2, and 5′ GATATCTGATACTACACTTGTGGC 3′ for 42N2.

Purification of inclusion bodies from *E. coli*. The wild-type plasmid (pSV15) and the plasmid carrying the mutant genes were transformed into *E. coli* BL21(DE3) for expression. The inclusion bodies were purified as described previously (1) and solubilized in 50 mM Na_2CO_3 buffer, pH 9.5. The concentrations of the solubilized proteins were determined by the method of Lowry et al. (7). Solubilized wild-type and mutant toxins (10 μg of protein containing both the 51- and 42-kDa peptides) were used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. The antibodies used for Western blot analysis were raised in rabbits against purified crystals of *B. sphaericus* 1593M.

Bioassay against mosquito larvae. The solubilized wild-type and mutant toxins were assessed for their toxicity against the second-instar larvae of *Culex quinquefasciatus* and *Anopheles stephensi* in accordance with a published protocol (6). Briefly, 20 mosquito larvae were added to disposable cups containing 30 ml of sterile distilled water to which different concentrations of proteins derived from different samples were added. Wild-type and mutant toxins were tested individually and in combination. For in vivo complementation studies, larvae were fed with one mutant toxin, and after 4 h, transferred to another cup containing distilled water to which another mutant toxin was added. The mortality rate was calculated after 24 h. Bioassays were carried out in triplicate and repeated more than five times. The 50% lethal concentration was defined and is expressed as the concentration of protein per milliliter of the assay medium that was required to kill 50% of the larval population in 24 h.

In this study, an attempt was made to explore the mechanism of action of the binary toxin of *B. sphaericus* by site-directed mutagenesis. Since the three-dimensional structure of the binary toxin is not known, the sites for mutagenesis were selected on the basis of secondary-structure analysis and previously published deletion studies. In the absence of any previous report on the functional role of the amino acids selected for mutagenesis located in these targets, we decided to replace these amino acids with alanine. Wild-type and mutant toxins, which accumulated as inclusion bodies in *E. coli*, were purified and solubilized. Alkali-solubilized binary toxin from each mutant (consisting of one mutant peptide and one wild-type peptide) was separately assayed against mosquito larvae. Alanine substitution in some of the sites of the toxin affected the biological activity when compared to the wild type (unpublished data). Among the defective mutants, 51N4 ($S_{38}KK_{40}$ to $A_{38}AA_{40}$), 51C2 ($I_{392}Q_{393}$ to $A_{392}A_{393}$), 42N2 (C_{47} to A_{47}), and 42C2 (R_{312} to A_{312}) showed a total loss of biological activity. These four mutants were chosen for further studies. It should be noted that in 51N4 and 51C2, the mutations were made only in the gene encoding the 51-kDa protein; the gene coding for the 42-kDa peptide was intact. Similarly, in mutants 42N2 and 42C2, mutations were located only in the 42-kDa

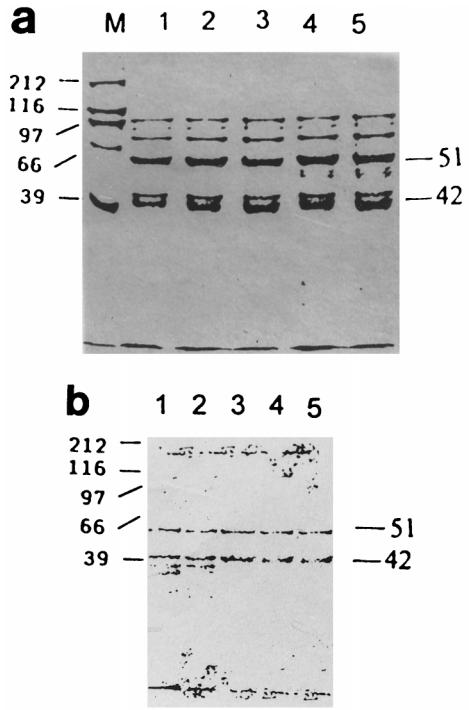

FIG. 1. (a) SDS-PAGE analysis of wild-type and mutant binary toxins. Each lane was loaded with 10 μg of protein containing both the 51- and 42-kDa peptides. M, Molecular weight markers. (b) Western blot analysis of wild-type and mutant binary toxins. Binary-toxin peptides (51 and 42 kDa) were detected by immunoblotting with polyclonal antibodies. Lanes: 1, mutant 51N4; 2, mutant 51C2; 3, mutant 42N2; 4, mutant 42C2; 5, wild type.

toxins; in each case, the gene coding for the 51-kDa toxin was intact. The locations of the replaced amino acids and the corresponding biological activities of these four nontoxic mutant proteins are presented in Table 1.

The intactness of the genes encoding the 51- and 42-kDa proteins in the nontoxic mutant clone was confirmed by PCR. The sizes of the PCR-amplified products (1.3 and 1.1 kb for the 51- and 42-kDa coding sequences, respectively) were similar to those of the parental and mutant plasmids (data not shown). The inclusion bodies containing the binary toxin were isolated, subjected to SDS-PAGE, and analyzed by Western blotting (Fig. 1). The profiles of the mutant binary toxins in SDS-PAGE and Western blot analyses were similar to the sizes of the toxins isolated from the parental clone. These results imply that the loss of biological activity in these mutants was not due to loss of expression or to structural deletions but rather was due to mutations in specific sites of the mutant toxin that result in the formation of inactive peptides. It is known that N- and C-terminal deletions in genes coding for the 51- and 42-kDa proteins reduce or abolish the toxicity (2, 8); our results are in agreement with these reports. In addition, this result clearly shows that immunological identity of the mutant toxins to the wild type is not sufficient to ensure biological activity.

Since site-directed mutations could affect inter- or intramolecular interactions, we wanted to investigate whether individual nontoxic proteins could associate and give rise to functional toxins. The combined effect of different mutant toxins on biological activity was determined by mixing two solubilized nontoxic-mutant derivatives of the same toxin. For example, the mutant 51N4 was mixed with 51C2 and tested for toxicity. Surprisingly, this combination of nontoxic peptides resulted in restoration of the toxicity against *C. quinquefasciatus* as well as *A. stephensi* larvae (Table 1). The toxicity levels obtained were only fourfold lower against *Culex* and twofold lower against *Anopheles* larvae than those of the wild-type toxin. We further extended this finding with respect to the 42-kDa peptide. The binary toxins from the two nontoxic mutants of the 42-kDa protein, 42N2 and 42C2, were mixed and tested for toxicity. This combination also resulted in restoration of the biological activity, although the toxicity levels were seven- and threefold lower against *Culex* and *Anopheles* larvae, respectively, than those of the wild-type toxin (Table 1). It is known that *B. sphaericus* toxins are generally less effective against *Anopheles* species. It is probable that changes in the active domain can enhance the toxic effect against species of mosquito larvae, which are less sensitive to the wild-type toxin (3).

Since the mixing of the mutant toxins restored the biological function of these peptides in vitro, it was of interest to know whether this phenomenon could also occur in vivo. To explore this possibility, mosquito larvae were fed with the solubilized toxin from the 51N4 mutant and, after 4 h, with the toxin from mutant 51C2, or vice versa. This treatment also restored the toxicity to the same levels seen after mixing the mutant toxins in vitro. A similar result was obtained with toxins derived from the 42-kDa protein. These results clearly established that complementation occurs also under in vivo conditions, i.e., inside the mosquito larva gut. The mutant toxic-peptide monomers perhaps complement each other by their molecular association in situ or ex situ, which results in the availability of a functional domain(s) of the toxins.

In conclusion, we suggest that the mutant binary toxins act as dimers or multimers and that this may be true with the wild-type toxins as well. Further, we have demonstrated that the complementation can occur in vivo. Certain amino acids important for toxicity have been identified; to gain an understanding of their functional significance, further manipulations at these sites are in progress.

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