Archives of Biochemistry and Biophysics
Vol. 384, No. 1, December 1, pp. 24–30, 2000
doi:10.1006/abbi.2000.2065, available online at http://www.idealibrary.com on IDEAL®

Identification of Residues in the Carboxy-Terminal Domain of *Clostridium perfringens* α-Toxin (Phospholipase C) Which Are Required for Its Biological Activities

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Received June 5, 2000, and in revised form August 21, 2000

A panel of random mutants within the DNA encoding the carboxy-terminal domain of *Clostridium perfringens* α-toxin was constructed. Three mutants were identified which encoded α-toxin variants (Lys330Glu, Asp305Gly, and Asp293Ser) with reduced hemolytic activity. These variants also had diminished phospholipase C activity toward aggregated egg yolk phospholipid and reduced cytotoxic and myotoxic activities. Asp305Gly showed a significantly increased enzymatic activity toward the monodisperse substrate ρNPPC, whereas Asp293Ser displayed a reduced activity toward this phospholipid analogue. In addition, Asp293Ser showed an increased dependence on calcium for enzymatic activity toward aggregated phospholipid and appeared calcium-depleted in PAGE band-shift assays. In contrast, neither Lys330Glu nor Asp305Gly showed altered dependence on calcium for enzymatic activity toward aggregated phospholipid. Asp305 is located in the interface between the amino- and carboxy-terminal domains, whereas Asp293 and Lys330 are surface exposed residues which may play a role in the recognition of membrane phospholipids.

Key Words: **bacterial toxin; *C. perfringens;* α-toxin; phospholipase; site-directed mutants.**

Clostridium perfringens is the etiological agent of gas gangrene (clostridial myonecrosis) in man, a serious disease which can occur after the entry of the bacterium into anoxic tissues (1). The bacterium is ubiquitous in the environment and traumatic injuries, with the associated ingress of soil or other organic matter containing the bacterium, are the events which most frequently lead to infection (1, 2). Gas gangrene is less frequently a consequence of surgical procedures, especially those involving the lower bowel which allow bacteria in the gut to infect host tissues (2). The spread of bacteria from the initial site of infection is a consequence of the production of toxins which damage the surrounding healthy tissues thus allowing bacterial invasion.

Although *C. perfringens* produces a wide range of protein exotoxins, the α-toxin, a zinc-metallophospholipase C, is the major virulence determinant in gas gangrene. It has been shown that α-toxin negative mutants of *C. perfringens* are no longer able to cause gas gangrene in mice (3), and immunization with an α-toxoid vaccine provides protection against disease (4). The α-toxin is hemolytic, cytotoxic and elicits a variety of effects on host tissues. The contraction of smooth muscle (5), especially that lining blood vessels (6), and the aggregation of platelets (7) might reduce the blood supply to tissues and promote the anoxic conditions required for bacterial growth. The mistrafficking of neutrophils such that they do not enter infected tissues appears to be a consequence of the upregulation of adhesion molecules in endothelial cells exposed to α-toxin (8, 9). The toxin might also cause generalized tissue damage by activating the arachidonic acid cascade leading to the production of inflam-

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0003-9861/00 $35.00

matory mediators (6, 10). The hemolytic, myotoxic and cytotoxic activities of α-toxin are believed to be a consequence of its interaction with host cell membranes and the subsequent hydrolysis of phospholipids. Therefore, the lysis of erythrocytes is often used as an *in vitro* measure of the ability of the α-toxin to hydrolyze phospholipids in cell membranes (11).

The crystal structure of the *C. perfringens* α-toxin (12) reveals an amino-terminal domain (residues 1–246) and a carboxy-terminal domain (residues 256–370) joined by a short hinge region (residues 247–255). We have previously shown that the phospholipase C active site is located in the amino-terminal domain of α-toxin (12, 13) and others have identified residues in this domain which are essential for enzymatic activity (14–16). In contrast, the function of the carboxy-terminal domain, which adopts a C2-like fold, is not completely understood. C2 domains in eukaryotic lipid metabolizing enzymes are often involved in phospholipid recognition and therefore the carboxy-terminal domain of α-toxin is believed to interact with phospholipids of the target membrane (12, 17). This is supported by our finding that removal of this domain abolished the hemolytic activity but not the enzymatic activity toward the water soluble substrate ρNPPC[2] (13).

Crystallographic studies revealed that alpha-toxin might exist in two conformations: an open form with the active site accessible and a closed form with the active site covered by the loop encompassing residues 135 to 150 (18). The binding of the carboxy-terminal domain to the target membrane is proposed to position the active site facing toward the bilayer and to cause its uncovering, thus allowing turnover of the phospholipid substrate (18).

We have set out to identify residues in the carboxy-terminal domain which play a role in the biological activities of α-toxin. The results from this work provide new insights toward understanding the mechanism of action of this toxin at a molecular level.

MATERIALS AND METHODS

Chemicals and enzymes. Chemicals were obtained from Sigma (Poole, UK) or BDH (Poole, UK) unless otherwise stated. Materials for protein purification were obtained from Pharmacia Biotech Ltd. (St. Albans, UK). Materials for DNA purification were obtained from Boehringer Mannheim (Lewes, UK).

Random mutagenesis of the carboxy-terminal domain coding region. Plasmid pT2.2 (11) was digested with *Hin*dIII and *Nhe*I and the DNA fragment encoding amino acids 209 to 370 of α-toxin was isolated and cloned into plasmid pUC18 (Pharmacia Biotech Ltd.). This cloned fragment was used as the template DNA for random mutagenesis in a PCR (94°C, 7 min; 50°C, 1 min; 70°C, 4 min followed by 25 cycles of 94°C, 1 min; 50°C, 1 min; 70°C, 4 min). The PCR mixture (100 μl) contained 10 ng of template DNA, 25 pmol of pUC universal forward and reverse primers, 1 mM dGTP, dTTP, dCTP, and dITP and 200 μM dATP in 10 mM Tris–HCl, pH 8.3, containing 50 mM KCl, 0.5 mM $MnCl_2$, 6.1 mM $MgCl_2$, and 2.5 U of *Taq* polymerase (BCL Ltd., Lewes, UK). The PCR product was digested with *Eco*RI and *Hin*dIII and cloned into pUC18 (19). A pool of recombinant plasmids were digested with *Hin*dIII and *Nhe*I and the released DNA fragment isolated (19). Plasmid pT2.2 (11) was digested with *Hin*dIII and partially digested with *Nhe*I to release the *Nhe*I–*Hin*dIII fragment which encoded amino acids 209 to 370 of the α-toxin. The remainder of the plasmid and α-toxin encoding DNA was purified, ligated with the mutated *Hin*dIII–*Nhe*I DNA fragment and transformed (19) into *Escherichia coli* strain DH5α.

Construction of site-directed mutants. Site-directed mutants were constructed using an overlapping PCR method with plasmid pT2.2 (11) template DNA. For each mutant two PCRs were carried out using one primer containing the modified nucleotide sequence and one primer flanking the C-terminal *Nhe*I–*Hin*dIII DNA fragment (upstream flanking primer; 5′-GCAGAGGAAAGAAAAGA-3′: downstream flanking primer 5′-GTTTTCCCAGTCACGACGTTGTA-3′). The following primers were used: Lys330>Glu, 5′-TAGAAAAA-GAGAATATACAGC-3′ and 5′-GCTGTATATTCTCTTTTTCTA-3′; Asp293>Ser, 5-GGAAATGAGCAACCCAGGAA-3′ and 5′-TTCCT-GGGTTGCTCATTTCC-3′, where modified nucleotides are shown underlined.

The PCR products were used as template DNA for PCRs with the upstream and downstream primers. The PCR products were digested with *Nhe*I and *Hin*dIII, cloned into plasmid pT2.2 which had been digested with *Hin*dIII and partially digested with *Nhe*I and transformed into *E. coli* DH5α. Transformants were plated onto L-agar containing ampicillin (19) and 5% (v/v) mouse blood (11). After culture for 18 h at 37°C the diameter of the zone of hemolysis surrounding colonies was measured.

Nucleotide sequencing. Nucleotide sequencing was conducted using the Sequenase Version 2.0 DNA sequencing kit (Amersham Life Science, Amersham, UK) with [α-^{35}S]dCTP and conditions recommended by the supplier.

Purification of mutated proteins. Methods for the isolation of α-toxin from the periplasmic space of *E. coli* expressing the cloned α-toxin gene have previously been reported (11, 20). The extract was loaded onto a HiTrap Q column (Pharmacia Biotech Ltd.) and eluted with a 0 to 1 M NaCl gradient in 20 mM Tris–HCl, pH 8.0. Fractions containing α-toxin were dialyzed at 4°C against 20 mM Tris–HCl, pH 8.0, loaded onto a MonoQ HR10/10 column (Pharmacia Biotech Ltd.) and eluted with a 0 to 1 M NaCl gradient. Fractions containing α-toxin were concentrated and loaded onto a HiLoad 16/60 Superdex 200 column (Pharmacia Biotech Ltd.). The α-toxin was eluted with PBS (pH 7.2) and fractions containing purified toxin were stored at −70°C. The bicinchoninic acid (BCA) protein assay method [Pierce & Warriner (UK) Ltd., Chester, UK] was used to determine protein concentration.

SDS–PAGE. Protein samples were analyzed by SDS–PAGE using 10–15% Phast Gels (Pharmacia Biotech Ltd.) or 12% gels (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). The gels were stained with PhastGel Blue R and scanned using the Bio-Rad Gel Doc system (Bio-Rad Laboratories Ltd.). Toxin concentrations were adjusted according to their percentage purity, for activity assays.

Biological activities. The hemolytic activity, phospholipase C activity toward egg yolk phospholipid, and phospholipase C activity toward ρ-nitrophenylphosphorylcholine (ρNPPC) were determined as described (11, 13). Cytotoxicity was measured using Chinese hamster fibroblasts or the mutant cell line Don Q which were seeded in 96-well plates (500 to 1000 cells/well). When the cells had grown to 90% confluency they were exposed in three replicate samples to serial 10-fold dilutions of the toxins. After 24 h cell viability was measured using a neutral red assay; cells were incubated for 2 h with 200 μl/well of neutral red (50 mg/ml in culture medium). Incorpo-

[2] Abbreviations used: CK, creatine kinase; ρNPPC, *para*-nitrophenylphosphorylcholine; PBS, phosphate-buffered saline.

rated dye was extracted from the cells with 100 μl of acetic acid: ethanol:water (1:50:49), before reading at 540 nm in a microtiter plate reader. Results are expressed as percentages of neutral red uptake in controls wells incubated without toxin. Myotoxicity was measured as creatine kinase (CK) (EC 2.7.3.2) released to the plasma 3 h after injection of 1.5 μg of wild-type α-toxin or the mutant variants (in 50 μl of PBS) in the right gastrocnemius muscle in groups of eight Swiss–Webster mice. CK activity was determined using a kinetic assay (Sigma, CK-10).

Calcium binding assays. The ability of the wild-type and mutated toxins to bind calcium was determined using a calcium-dependant band shift method (21) A reaction mixture of 15 μl containing toxin at 250 μg/ml, 10 mM EDTA with or without 20 mM $CaCl_2$ was incubated for 5 min at room temperature prior to separation by native PAGE through an 8–25% Phast gel (Pharmacia Biotech Ltd.). The gel was stained with PhastGel Blue R.

Molecular modeling. The refined coordinates for the calcium-bound "closed" form of *C. perfringens* α-toxin, strain NCTC8237 (pdb code 1QM6) were used as the starting coordinates for structural analysis of the mutations. The amino-acid for each site-directed mutant was replaced in "O" (22), and then 200 steps of energy minimization were carried out with the X-PLOR refinement package (23).

RESULTS AND DISCUSSION

Random Mutation of the DNA Encoding the Carboxy-Terminal Domain of α-Toxin

The approach used here allowed random mutagenesis of the carboxy-terminal domain of α-toxin, while retaining the wild-type amino-terminal domain. The method involved isolation of a DNA fragment which encoded amino acids 209–370 of α-toxin and random mutagenesis using the PCR with dITP, limiting dATP and $MnCl_2$. When a representative sample of the cloned mutated DNA was sequenced, 65% of the cloned fragments contained mutations at an average frequency of 2 per 100 nucleotides. Approximately 1500 clones were screened for reduced hemolytic activity by measuring the size of the zone of hemolysis surrounding colonies on agar containing mouse blood. The screening method employed would have allowed the screening of over 6600 mutations within the carboxy-terminal domain coding region.

Selection and Purification of Site-Directed Mutants

Three mutants with reduced hemolytic activity were selected for further study. D293S contained a single mutation (Asp293Ser). However, the plasmids isolated from the remaining two mutants (CTM34 and CTM206) contained several mutations (Table I). To determine which of these mutations was responsible for the reduced hemolytic activity it was necessary to construct genes each containing one of the mutations found in CTM34 and CTM206. These genes and the wild-type toxin were expressed in *E. coli* and periplasmic extracts from each clone assayed for hemolytic activity and protein content. The mutated proteins Lys330Glu and Asp305Gly were identified as having reduced hemolytic activity. The periplasmic extracts were then purified using a previously reported protocol by low-resolution ion-exchange chromatography, followed by Mono-Q ion-exchange chromatography. The yield of each purified mutated protein was between 1.2 and 16 mg/l of culture at a purity of greater than 70%.

TABLE I

Mutations Identified in Clones with Reduced Hemolytic Activity

Clone	Mutation(s) identified within clone
D293S	Asp293Ser
CTM 34	Glu245Gly
	Asp305Gly
	ILe321Met
	Asn358Ser
CTM206	Ser265Cys
	Lys317Arg
	Lys330Glu
	Lys335Glu
	Asp356Gly

Hemolytic, Cytotoxic, and Myotoxic Activities

The variants Asp293Ser, Lys330Glu, and Asp305Gly showed a reduced hemolytic activity toward murine erythrocytes (Fig. 1a). They also showed reduced cytotoxic and myotoxic activity (Figs. 1b and 1c). These findings are in agreement with previous studies showing that the carboxy-terminal domain is essential for hemolytic, cytotoxic and myotoxic activities of α-toxin (13, 24–26). Furthermore, they demonstrate the importance of the residues Lys330, Asp293, and Asp305 for the biological activities of this toxin.

Enzymatic Activity toward Egg Yolk Phospholipid and ρNPPC

Asp293Ser, Lys330Glu, and Asp305Gly showed reduced enzymatic activity toward egg yolk phospholipid (Fig. 1d). Only the variant Asp293Ser showed a significantly reduced activity toward ρNPPC, while the variant Asp305Gly displayed an increased activity toward this water soluble substrate (Fig. 1e). Previous studies have shown that the enzymatic activity of α-toxin toward egg yolk phospholipid is partially dependent on the carboxy-terminal domain (13) and our results reveal that Asp293, Lys330, and Asp305 play a role in the hydrolysis of this substrate. This finding might reflect the ability of egg yolk phospholipid to adopt a micellar structure which involves the packing of phospholipids in a form similar to that found in membranes.

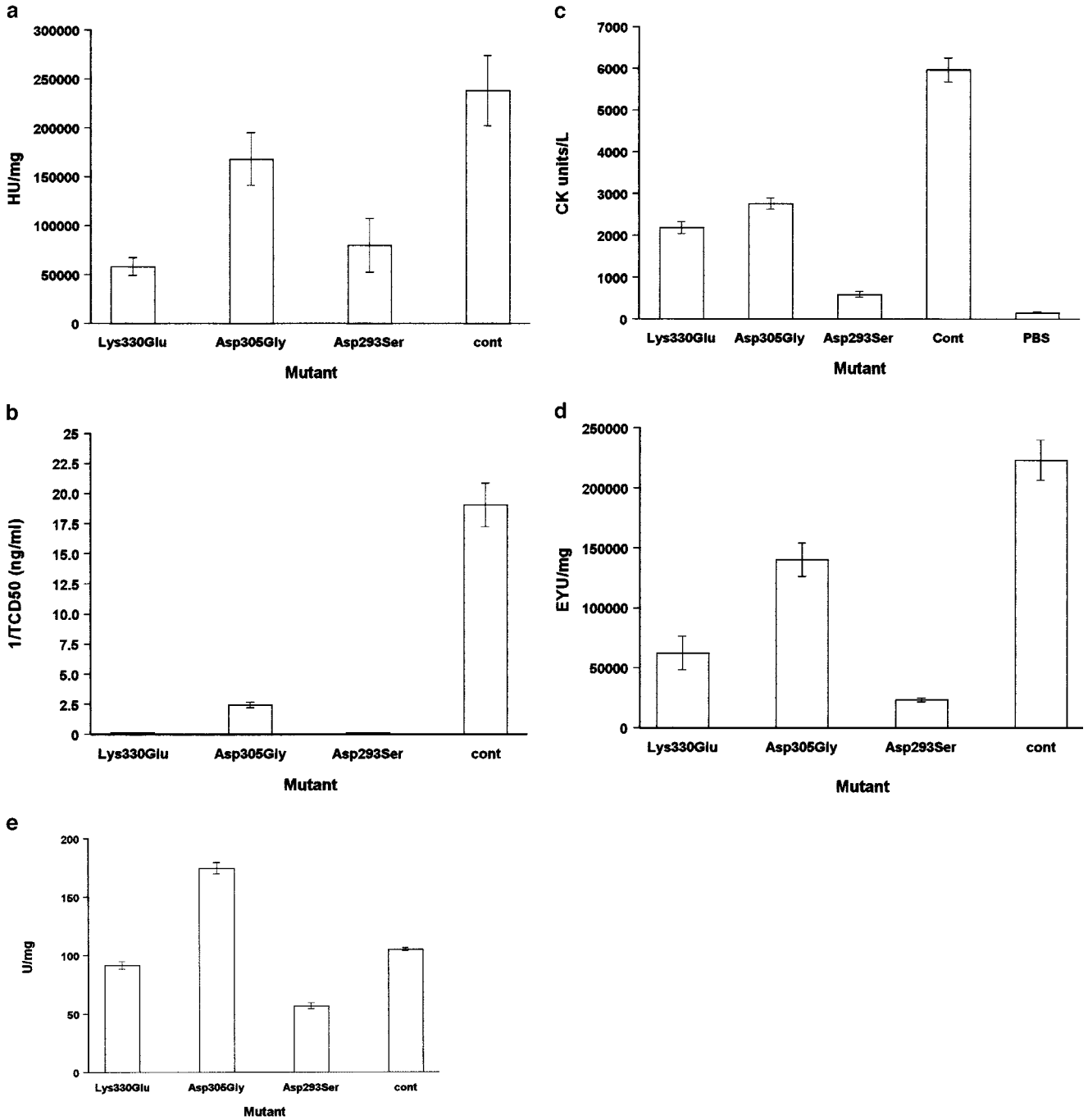

FIG. 1. Biological activities of wild-type α-toxin and variants with single amino acid substitutions in the carboxy-terminal domain. Results shown are the mean of at least three independent determinations with standard error bars shown. Hemolytic activity toward murine erythrocytes (a), cytotoxic activity toward a Chinese hamster fibroblast cell line (b), myotoxic activity after intramuscular injection in Swiss–Webster mice (c), and enzymatic activity toward egg yolk phospholipid (d) or enzymatic activity toward ρNPPC (e).

Substitution of Aspartate 293 with Serine

The carboxy-terminal domain of α-toxin is believed to play a key role in the calcium-mediated recognition of phospholipids (18). Crystallographic studies (18) indicate that three distinct calcium ion binding sites (Ca1, Ca2, and Ca3) are present in the carboxy-terminal domain of α-toxin (Fig. 2). The significance of Ca1 and Ca3 for the biological activities of α-toxin has been already demonstrated (17, 18). The side chains of the aspartic acid residues at 269 and 336 are involved in

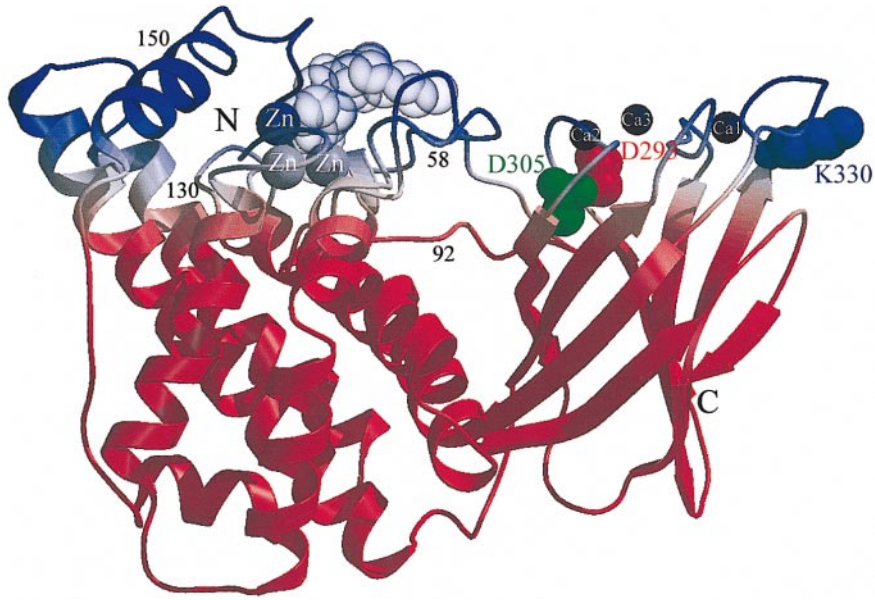

FIG. 2. Secondary structure representation of α-toxin. Shaded from red for residues predicted to be 5 Å or more outside the membrane to blue for residues predicted to be 5 Å inside the membrane. The amino-terminal domain (N) contains three zinc ions within the active site cleft. A modeled phospholipid is shown bound to the active site in a semitransparent representation. The three calcium ions (Ca1, Ca2, and Ca3), bound to the carboxy-terminal domain (C) are shown in dark blue. The start and end points of the lid covering the active site cleft and connecting loops are labeled. Residues mutated in this paper (Asp293, Asp305, and Lys330) are highlighted and labeled.

the coordination of Ca1 (18). The substitution of these residues with asparagine significantly reduced the hemolytic, cytotoxic and myotoxic activities (24). The mutant variants Asp269Asn and Asp336Asn display an altered calcium dependency in the enzymatic activity toward aggregated phosphatidylcholine (17). The main chain of Thr272 is involved in the coordination of Ca3 (18). The mutant variant Thr272Pro had dramatically reduced hemolytic and platelet aggregating activities and displayed reduced enzymatic activity toward aggregated phosphatidylcholine (17).

Asp293 is one of the proposed calcium coordinating residues in the Ca2 site (18). The possibility that Asp293Ser had an altered affinity for calcium ions was investigated by measuring the calcium dependency of this variant for the hydrolysis of egg yolk phospholipid and by monitoring changes in mobility of calcium free and calcium-bound proteins (band-shifting) after native PAGE.

The rate of hydrolysis of egg yolk phospholipid by wild-type and mutated α-toxins in the presence of 1.25 or 6.25 mM calcium ions was determined as previously described (17). The enzymatic activity of wild-type α-toxin (Fig. 3) was not affected by altering the calcium ion concentration. In contrast, the activity of Asp293Ser was markedly stimulated by increasing the calcium ion concentration from 1.25 to 6.25 mM (Fig. 3). A similar response of the enzymatic activity to increases in calcium concentration occurs in the variants Asp269Asn and Asp336Asn (17) which also harbor substitutions in calcium-binding residues (18).

To confirm that Asp293Ser had reduced affinity for calcium ions, the ability of the protein to bind calcium was determined using a calcium-dependent band shift method. The results showed that wild-type protein underwent a band shift when free calcium ions were present (Fig. 4). In contrast, the mobility of Asp293Ser was not affected by the presence of calcium ions.

Our findings that the variant Asp293Ser had reduced hemolytic, cytotoxic and myotoxic activities as well as an altered calcium dependency for enzymatic activity demonstrate the biological significance of the

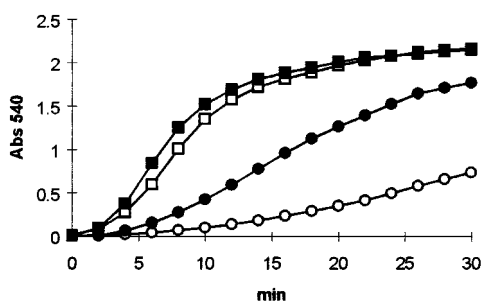

FIG. 3. Comparison of the effect of calcium ions on the phospholipase C activity toward egg yolk phospholipid of the wild-type and mutant Asp293Ser toxins. □, wild-type toxin + 1.25 mM Ca^{2+}; ■, wild-type toxin + 6.25 mM Ca^{2+}; ○, Asp293Ser + 1.25 mM Ca^{2+}; ●, Asp293Ser + 6.25 mM Ca^{2+}.

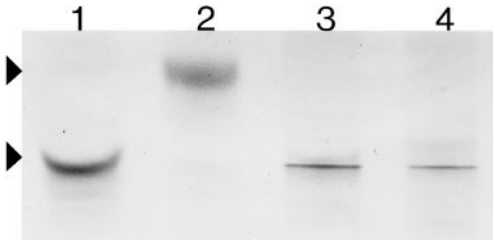

FIG. 4. Band shift of wild-type and Asp293Ser toxins in the presence of calcium ions. Calcium depleted (lanes 1 and 3) and calcium bound forms (lanes 2 and 4) of the wild-type (lanes 1 and 2) and Asp293Ser (lanes 3 and 4) toxins were analyzed by native gel electrophoresis. The positions of protein bands visible after staining with PhastGel Blue R are indicated by arrowheads.

Ca2 site. The variant Asp293Ser also had reduced activity toward ρNPPC, which lacks the hydrocarbon tail groups of phospholipids, suggesting that the calcium bound to the Ca2 site plays a role in the recognition of the head group of the phospholipid substrate.

Substitution of Lysine 330 with Glutamic Acid

The substitution of lysine 330 with glutamic acid markedly reduced the hemolytic activity and the egg yolk phospholipid hydrolyzing activities without affecting phospholipase C activity toward ρNPPC. These findings show that this substitution affects the interaction of α-toxin with aggregated but not monodisperse substrates. Lysine 330 is one of a number of positively charged residues on the proposed membrane binding face of the carboxy-terminal domain (also including Lys328 and Arg329; Fig. 2). The Lys330Glu protein did not show altered dependency on calcium ions for activity toward egg yolk phospholipid (data not shown), indicating that Lys330 does not play a role in calcium binding. Lysine-rich regions in the C2 domains of eukaryotic proteins such as synaptotagmin have been shown to play a role in the recognition of the phospholipid head group (27, 28) and in the binding to liposomes (28). The mutation of residues within this lysine-rich region reduced the ability of synaptotagmin to bind phospholipids (29, 30). Although there is no significant amino acid sequence identity between the C2 domains of synaptotagmin and the carboxy-terminal domain of α-toxin, our previous work has shown that there is significant structural similarity between these domains (12). Our findings that mutation of Lys330 reduced the hemolytic, cytotoxic and myotoxic activities and enzymatic activity toward egg yolk phospholipid suggest that the lysine rich region of α-toxin might play a similar role to the C2 domain of synaptotagmin in phospholipid recognition.

Substitution of Aspartate 305 with Glycine

The variant Asp305Gly showed reduced hemolytic, cytotoxic, and myotoxic activities. Furthermore it also displayed reduced enzymatic activity toward egg yolk phospholipid. Analysis of the 3D crystal structure of the wild-type protein revealed that this residue is not located on the membrane binding face (Fig. 2). Although Asp305 is located close to the Ca2 binding site, the Asp305Gly protein did not show altered dependency on calcium ions for activity toward egg yolk phosphatidylcholine (data not shown), indicating that Asp305 does not play a role in calcium binding. Molecular modeling studies predicted that the substitution would not cause local changes in the structure of the carboxy-terminal domain. Surprisingly, the mutated protein was more active toward ρNPPC suggesting that the active site in the amino-terminal domain of α-toxin was more accessible to substrate.

Asp305 is located in the loop involved in hydrogen bonding between the amino- and the carboxy-terminal domains (Fig. 2). Since there are few direct contacts between the two domains, these hydrogen bonds are likely important in communicating membrane binding to the amino-terminal domain and causing the active site to open. The Asp305Gly substitution could destabilize the interaction between the two domains hence opening the lid and making the active site more accessible. This would explain the increased enzymatic activity of the variant Asp305Gly toward the monodisperse substrate ρNPPC.

In conclusion, we have identified three mutated forms of α-toxin with reduced biological activities. Two mutations affected residues located in regions of the carboxy-terminal domain which are believed to be involved in membrane phospholipid recognition and the third affected a residue located in the interface between the amino- and carboxy-terminal domains. Further structural analysis of these mutants could help to clarify the exact role of the mutated residues in the interaction of α-toxin with eukaryotic cell membranes.

ACKNOWLEDGMENTS

This work was partially supported by the Swedish Medical Research Council (Grant 05969), by the Swedish Cancer Society (Grant 3826-B96-01XAB), by the Royal Society, by the Biotechnology and Biological Sciences Research Council, and by CONCIT. We acknowledge the technical assistance of Patricia White and Marie Fernie.

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