Tyrosine 331 and phenylalanine 334 in *Clostridium perfringens* α-toxin are essential for cytotoxic activity

Marie Jepson[a], Helen L. Bullifent[a], Dennis Crane[b], Marietta Flores-Diaz[c,d], Alberto Alape-Giron[c,d], Pramukh Jayasekeera[a], Bryan Lingard[a], David Moss[e], Richard W. Titball[a,*]

[a] *Defence Evaluation Research Agency, CBD Porton Down, Salisbury, UK*
[b] *National Institute for Biological Standards and Control, Blanche Lane, South Mimms, UK*
[c] *Microbiology and Tumor Biology Center, Karolinska Institutet, Stockholm, Sweden*
[d] *Instituto Clodomiro Picado, Facultad de Microbiologia, Universidad de Costa Rica, San Jose, Costa Rica*
[e] *Department of Crystallography, Birkbeck College, Malet St., London WCIE 7HX, UK*

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Abstract Differences in the biological properties of the *Clostridium perfringens* phospholipase C (α-toxin) and the *C. bifermentans* phospholipase C (Cbp) have been attributed to differences in their carboxy-terminal domains. Three residues in the carboxy-terminal domain of α-toxin, which have been proposed to play a role in membrane recognition (D_{269}, Y_{331} and F_{334}), are not conserved in Cbp (Y, L and I respectively). We have characterised $D_{269}Y$, $Y_{331}L$ and $F_{334}I$ variant forms of α-toxin. Variant $D_{269}Y$ had reduced phospholipase C activity towards aggregated egg yolk phospholipid but increased haemolytic and cytotoxic activity. Variants $Y_{331}L$ and $F_{334}I$ showed a reduction in phospholipase C, haemolytic and cytotoxic activities indicating that these substitutions contribute to the reduced haemolytic and cytotoxic activity of Cbp. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Phospholipase C; Membrane binding; Site-directed mutant

1. Introduction

The zinc-metallophospholipases C are a group of related proteins produced by Gram-positive bacteria including *Clostridium perfringens*, *Clostridium novyi*, *Clostridium bifermentans*, *Bacillus cereus* and *Listeria monocytogenes*. Some of these enzymes play key roles in the pathogenesis of disease [1]. For example, the phospholipase C (α-toxin) produced by *C. perfringens* is the major virulence factor in gas gangrene [2,3]. The α-toxin hydrolyses phosphatidylcholine and sphingomyelin in both monodispersed and membrane-packed forms [4–6]. Unlike many zinc-metallophospholipases C, α-toxin is haemolytic, cytotoxic and myotoxic. Myotoxicity caused by α-toxin is complex, and depends not only on damage to the muscle fibres, but also on the effects of the toxin on the haemostatic system [7,8].

Many of these biological activities are a direct consequence of the hydrolysis of membrane phospholipids [6,9]. However, there is also evidence that haemolysis and cytotoxicity occur as a result of the activation of endogenous membrane phospholipases by α-toxin [10,11]. The mechanism by which these endogenous phospholipases are activated is not clear and could be mediated by the products of limited hydrolysis of membrane phospholipids [10,12] or by the binding of α-toxin to a cell surface GTP protein receptor [11].

Considerable insight into the mode of action of α-toxin has been provided by the crystal structure. This revealed an amino-terminal domain containing the active site and a carboxy-terminal domain which was structurally homologous to eukaryotic C2 domains, and allowed binding to membrane phospholipids [13,14]. This model explained why the isolated amino-terminal domain retained phospholipase C activity but was devoid of haemolytic, cytotoxic and myotoxic activities [7,15,16]. The recognition of membrane phospholipids by the carboxy-terminal of α-toxin is thought to involve both the insertion of surface-exposed hydrophobic residues into the lipid bilayer, and the Ca^{2+}-mediated recognition of phospholipid head groups [13,14]. Some amino acids proposed to play a key role in the recognition of membrane phospholipids (D_{269}, Y_{331} and F_{334}) are not conserved in the phospholipase C produced by *C. bifermentans* (Cbp), a structurally related enzyme which displays weak haemolytic, cytotoxic and myotoxic activities [7,16,17]. These differences might therefore provide a molecular explanation for the profound differences in the properties of these related enzymes. The aim of this work was to determine the roles of D_{269}, Y_{331} and F_{334} in the biological activities of α-toxin by replacing them individually with their counterparts in *C. bifermentans* Cbp.

2. Materials and methods

2.1. Enzymes and chemicals

Unless stated enzymes were obtained from Sigma-Aldrich (Poole, UK). Materials for protein purification were obtained from Amersham-Pharmacia Biotech UK Ltd (Little Chalfont, UK). Materials for DNA purification were obtained from Roche Molecular Biochemicals (Lewes, UK).

2.2. DNA cloning and nucleotide sequencing

Oligonucleotides (α-toxinFWD 5′-GCTTGGGGCCCAAAATTAACGGGGGATATA-3′ or 5′-CTAGGAGCTCAGGGGAGATTAATACTTGA-3′ and α-toxinREV 5′-TGCTCTAGATTATTTTATATTATAAGTTGA-3′ or 5′-CTAGTCTAGATTATTTTATATTATAAGT-3′) were designed from the nucleotide sequence of the

*Corresponding author. Fax: (44)-1980-614307.
E-mail: rtitball@dera.gov.uk

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α-toxin gene of *C. perfringens* NCTC 8237 [18]. Unique restriction sites (*Xba*I, *Dra*II or *Sac*I) introduced are shown underlined. Other oligonucleotides contained nucleotide sequence changes to introduce single amino acid changes (D_{269}YFWD 5′-GGTGAGAAATATGCTGGAACAGATGAC-3′; D_{269}YREV 5′-GTCATCTGTTCCTGCATATTTCTCACC-3′; Y_{331}LFWD 5′-AGAAAAAGAAAATTAACAGCATTCTC-3′; Y_{331}LREV 5′-GAGAATGCTGTTAATTTTCTTTTTCT-3′; F_{334}IFWD 5′-ATACAGCAATATCAGATGCTTAT-3′; F_{334}IREV 5′-ATAAGCATCTGATATTGCTGTAT-3′). The oligonucleotides were used in the PCR (30 cycles of 94°C, 60 s; 45°C, 60 s; 72°C, 90 s) with plasmid pKSα3 [19] as template DNA. Two PCR products were generated, which encoded the mutated sequence and the region of the *cpa* gene 5′ and 3′ (respectively) to the mutation. These PCR products were used in a PCR reaction without the further addition of template DNA or oligonucleotide primers, to generate the mutated *cpa* gene. The D_{269}Y coding region was cloned into plasmid αPROM27 [17] and the Y_{331}L and F_{334}I coding regions were cloned into plasmid pTrc99a (Amersham-Pharmacia Biotech UK Ltd). The plasmids were transformed into *Escherichia coli* DH5α [20] and transformants containing recombinant plasmids identified using the PCR. Plasmid DNA was isolated and nucleotide sequenced using dithiothreitol chemistry, as detailed previously [17].

2.3. Expression and purification of α-toxin proteins

Wild-type α-toxin or variant D_{269}Y were expressed in *E. coli* and isolated from the periplasmic space [18,19]. Expression of variants Y_{331}L or F_{334}I was induced with 3 mM IPTG with further growth for 5 h. Protein was isolated from the periplasmic space of the recombinant *E. coli* [18,19].

The wild-type or variant proteins were purified using FPLC column chromatography [17] and analysed using SDS–PAGE (10–15% gels; (Amersham-Pharmacia Biotech UK Ltd PhastSystem) after staining with Coomassie blue R250. Protein concentrations were determined using a bicinchoninic acid assay (Pierce and Warriner UK Ltd, Chester, UK).

2.4. Mass spectrometry

The molecular weights of the α-toxin proteins were determined using on-line liquid chromatography-electrospray ionisation mass spectrometry. Purified protein (2 μg) was diluted to 0.4 mg/ml in 0.1% trifluoroacetic acid and loaded onto a HPLC column (Hewlett-Packard 1100, Waldbronn, Germany). The eluant was directed to the electrospray ionisation source of a Quattro II tandem quadrupole mass spectrometer (Micromass UK Ltd, Altrincham, UK). MaxEnt software (Micromass UK Ltd, Altrincham, UK) was used to calculate the molecular weights of the α-toxin proteins.

2.5. CD spectroscopy analysis

Secondary structures of the purified proteins were determined using circular dichroism spectroscopy (CD) in a Jasco J-720 spectropolarimeter at 25 ± 1°C as described previously [17].

2.6. Phospholipase C and haemolytic activity

Phospholipase C activity of the purified proteins was determined using 10% (v/v in 0.9% saline) egg yolk emulsion (Oxoid Ltd, Basingstoke, UK) or using *p*-nitrophenylphosphorylcholine (*p*NPPC) [17,18]. Membrane disrupting activity was determined using liposomes composed of sphingomyelin and cholesterol and containing carboxyfluorescein [17]. Haemolytic activity was measured using 5% (v/v) washed mouse erythrocytes [18].

2.7. Cytotoxicity and myotoxicity

Cytotoxicity towards the Chinese hamster fibroblast mutant cell line Don Q was measured as detailed previously [7]. Results are expressed as the percentages of neutral red uptake in control 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 the α-toxin proteins (in 50 μl of phosphate-buffered saline) in the right gastrocnemius muscle in groups of eight Swiss–Webster mice [7]. CK activity was determined using a kinetic assay (Sigma, CK-10).

2.8. Ca^{2+} dependence

The relative mobility of α-toxin and the variant proteins was determined in the absence and presence of Ca^{2+} as described previously [21]. A 0.5 μg/ml protein solution was prepared in 10 mM Tris–HCl pH 8.0 containing either 10 mM EDTA or 20 mM $CaCl_2$. The samples were incubated at room temperature for 5 min and analysed using native PAGE electrophoresis (8–25%, Amersham-Pharmacia

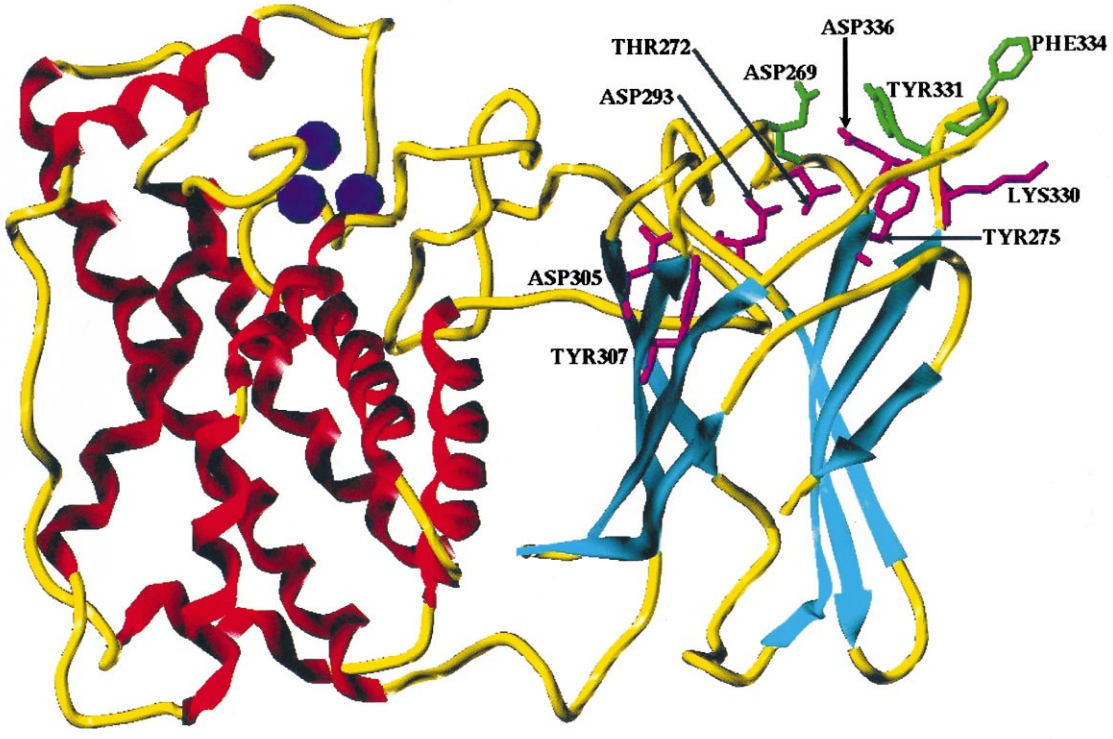

Fig. 1. Crystal structure of α-toxin with the putative membrane recognition interface shown uppermost. Residues which were modified in this study (D_{269}, Y_{331} and F_{334}) are highlighted in green. Residues previously shown to play a role in haemolytic, cytotoxic and myotoxic activities are shown in magenta.

Biotech UK Ltd PhastSystem). One gel was stained with Coomassie blue whilst the second was transferred onto a PVDF membrane and a Western blot performed using a polyclonal antibody (mouse anti-α) and detected using a goat anti-mouse IgG secondary antibody (AuroProbe, Amersham-Pharmacia Biotech UK Ltd). The Western blot was developed using an IntenSE kit (Amersham-Pharmacia Biotech UK Ltd) according to the manufacturer's instructions.

3. Results and discussion

3.1. Construction, purification and characterisation of site-directed mutants

We have previously shown that differences in the carboxy-terminal domains of *C. bifermentans* Cbp and *C. perfringens* α-toxin play a key role in determining the haemolytic and cytotoxic activities of these phospholipases C [16,17]. To further identify amino acid residues in the carboxy-terminal domains which modulated haemolytic and cytolytic activities, three site-directed mutants were constructed which would encode proteins in which the amino acid in α-toxin (D_{269}, Y_{331} or F_{334}; Fig. 1) was replaced with its counterpart from *C. bifermentans* Cbp (Y, L and I respectively). The authenticity of the mutated genes was verified by nucleotide sequencing of the entire gene. The mutated genes were expressed in *E. coli*, and the corresponding proteins isolated from the periplasmic space of the bacteria and purified using ion exchange chromatography.

Using electrospray mass spectrometry the molecular weights of the wild-type and variant proteins were shown to be similar to those predicted from the amino acid sequence (α-toxin, 42 522 Da; α-toxin $D_{269}Y$, 42 579 Da; α-toxin $Y_{331}L$, 42 473 Da; α-toxin $F_{334}I$, 42 493 Da). Far-UV (180–260 nm) CD spectra demonstrated that the secondary structures were similar for the mutants $Y_{331}L$ and $F_{334}I$. However, the variant $D_{269}Y$ showed lower intensities in both the positive and negative bands indicating that this substitution may cause a destabilising effect on the structure (Fig. 2).

3.2. Calcium binding properties of α-toxin variants

The residue D_{269} in α-toxin is proposed to play a role in Ca^{2+}-mediated recognition of phospholipids. Its replacement by Tyr, the counterpart from Cbp, would be expected to modify the ability of the protein to bind Ca^{2+} ions. Hammarberg et al. [21] have previously shown that native gel electrophoresis can be used to determine Ca^{2+} binding by proteins, since in the presence of Ca^{2+} they migrate with greater mobility. We have previously replaced another Ca^{2+}-co-ordinating residue (D_{293}) in the carboxy-terminal domain of α-toxin with serine and found that the variant $D_{293}S$ has a similar mobility in the presence or absence of Ca^{2+} ions [22]. The relative mobility of the α-toxin variants generated in this study in the presence of Ca^{2+} was compared with mobility in the presence of excess EDTA, to chelate Ca^{2+} ions. The mobility of wild-type α-toxin or the variants $Y_{331}L$ and $F_{334}I$ was greater in the presence of Ca^{2+}. In contrast, the variant $D_{269}Y$ migrated at a similar rate in the presence or absence of Ca^{2+} ions (Fig. 3), demonstrating that this amino acid substitution alters the ability of α-toxin to bind Ca^{2+} ions.

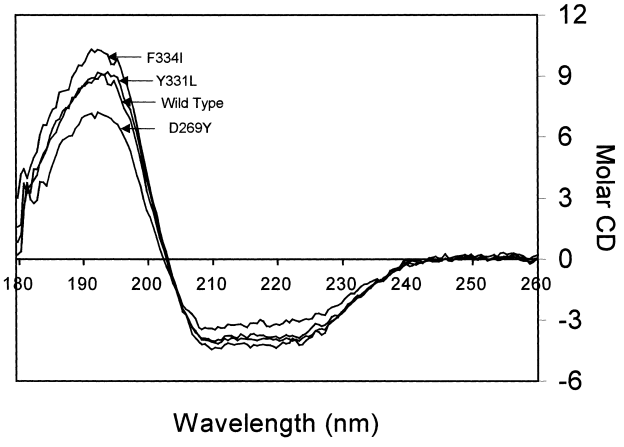

Fig. 2. Far-UV CD analysis of α-toxin, variant $D_{269}Y$, variant $Y_{331}L$ and variant $F_{334}I$. Data were collected at 180–260 nm and compared with wild-type α-toxin.

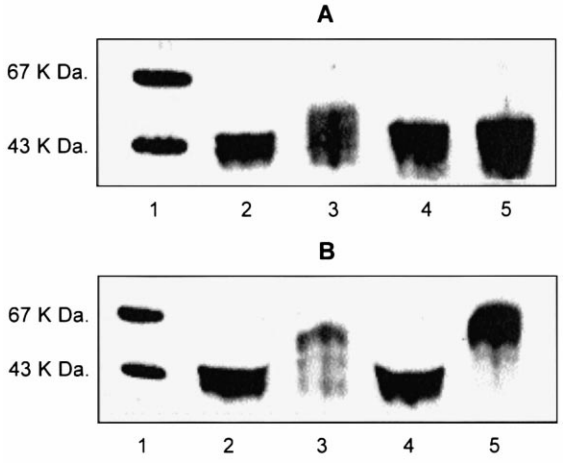

Fig. 3. Determination of Ca^{2+} dependence of α-toxin mutants $D_{269}Y$, $Y_{331}L$ and $F_{334}I$ using native gel electrophoresis. Samples were incubated with EDTA in the absence or presence of 20 mM Ca^{2+} and relative mobility determined. A: Lane 1 = molecular weight markers, lane 2 = α-toxin+EDTA, lane 3 = α-toxin+EDTA+20 mM Ca^{2+}, lane 4 = variant $D_{269}Y$+EDTA, lane 5 = variant $D_{269}Y$+EDTA+20 mM Ca^{2+}. B: Lane 1 = molecular weight markers, lane 2 = variant $Y_{331}L$+EDTA, lane 3 = variant $Y_{331}L$+EDTA+20 mM Ca^{2+}, lane 4 = variant $F_{334}I$+EDTA, lane 5 = variant $F_{334}I$+EDTA+20 mM Ca^{2+}.

3.3. Replacement of aspartate 269 with tyrosine

Although the ability of the variant $D_{269}Y$ to bind Ca^{2+} was reduced, the variant protein showed only a small reduction in phospholipase C activity towards aggregated egg yolk phospholipid (Fig. 4A) or *p*NPPC (Fig. 4B). Furthermore, its membrane-disrupting activity towards sphingomyelin liposomes and its myotoxic activity were affected only slightly (Fig. 4D,E). Surprisingly, variant $D_{269}Y$ showed enhanced haemolytic and cytotoxic activities (Fig. 4C,F). This result contrasts with the finding that replacement of D_{269} with asparagine resulted in a marked reduction of haemolytic and cytotoxic activity [7]. The crystal structure of α-toxin reveals that D_{269} is located on the proposed membrane binding face (Fig. 1), and it is predicted to play a role in Ca^{2+}-mediated recognition of the phospholipid head group [13]. Tyrosine residues are known to play key roles in the interfacial binding of other protein to membranes [23–26]. It is possible that the substitution of D_{269} with tyrosine would enhance the interaction of the variant protein with the target cell membrane.

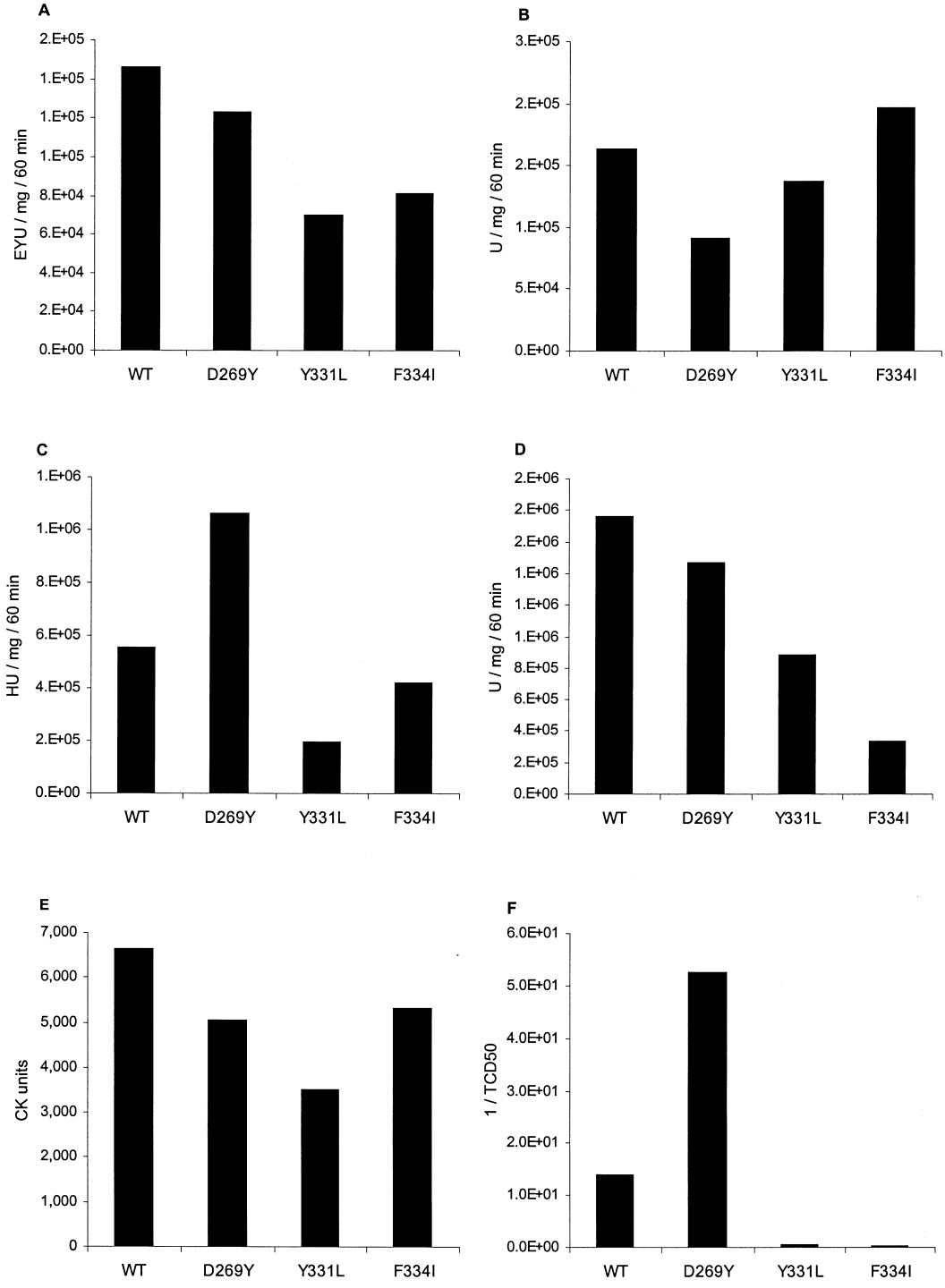

Fig. 4. Activity of purified wild-type α-toxin (WT) and variant $D_{269}Y$, variant $Y_{331}L$ or variant $F_{334}I$; phospholipase C activity towards egg yolk phospholipid (A) or *p*NPPC (B), haemolytic activity towards mouse erythrocytes (C), release of carboxyfluorescein from sphingomyelin liposomes (D), myotoxicity in mice (E) or cytotoxicity towards Chinese hamster fibroblast cells (F). Values shown are the mean of at least three determinations (S.D. ±0.1%).

Therefore, the reduced ability of variant $D_{269}Y$ to bind Ca^{2+} may have been offset by the enhanced ability of this protein to interact with other regions of the membrane.

3.4. Replacement of tyrosine 331 with leucine

In comparison with the wild-type toxin, the replacement of Y_{331} with leucine resulted in a two-fold reduction of activity towards aggregated egg yolk phosphatidylcholine (Fig. 4A) or towards sphingomyelin liposomes (Fig. 4D), but only a slight reduction in activity towards *p*NPPC (Fig. 4B). However, in comparison with the wild-type toxin, variant $Y_{331}L$ showed a significant reduction in haemolytic, myotoxic and cytotoxic activities (Fig. 4C,E,F). These findings demonstrate the role of Y_{331} in the biological activities of α-toxin and indicate that its substitution with leucine in Cbp contributes to the reduced activities of this enzyme. In α-toxin, residue Y_{331} is located on

the proposed membrane binding face and its phenolic group is surface-exposed [13]. The phenolic side chains of tyrosine residues are known to play key roles in the anchoring of proteins and peptides into phospholipid bilayers [25,26]. In the case of lipase and phospholipase A_2, tyrosine residues located on the membrane recognition interface have been shown to be critical for interfacial binding [23,24]. It is possible that Y_{331} in α-toxin also plays a important role in the interaction with the target membrane.

3.5. Replacement of phenylalanine 334 with isoleucine

The substitution of F_{334} with isoleucine resulted in a reduction of the enzymatic activity towards aggregated egg yolk phosphatidylcholine (Fig. 4A), but a slight enhancement in activity towards *p*NPPC, in comparison with the wild-type α-toxin (Fig. 4B). Furthermore, the variant F_{334}I showed a reduced membrane-disrupting activity towards sphingomyelin liposomes (Fig. 4D), decreased haemolytic and myotoxic activities (Fig. 4C,E) and a marked reduction (88-fold) in cytotoxic activity (Fig. 4F), in comparison with the wild-type α-toxin. These findings show that F_{334} in α-toxin plays a key role in conferring full toxicity and also demonstrate that its substitution by isoleucine, which occurs naturally in the *C. bifermentans* Cbp, contributes to the reduced haemolytic, cytotoxic and myotoxic activity of the latter enzyme. Like Y_{331}, residue F_{334} is located on the proposed membrane binding face of α-toxin [13]. In proteins, such as pancreatic lipase and snake venom phospholipases A_2, phenylalanine residues located on the interfacial binding surface play key roles in membrane interaction because their hydrophobic side chains become buried in the core of the lipid bilayer [23–25]. Therefore, it is possible that the substitution of F_{334} by isoleucine in α-toxin results in a reduced ability of the protein to interact with the hydrocarbon tail groups of phospholipids, due to the loss of this surface-exposed aromatic side chain.

3.6. Interactions with synthetic versus cellular membranes

In general, the patterns of activity of the variant proteins towards murine erythrocytes and Chinese hamster fibroblasts were similar, although they did not correlate exactly with the activity towards sphingomyelin liposomes. This finding may reflect differences in the membrane recognition event in the different assays. There is evidence that the presence of gangliosides in the target membrane modulates the interaction with α-toxin [27,28]. Sphingomyelin liposomes lack these glycosphingolipids and therefore the interaction of α-toxin with these artificial membranes might be different from that with cellular membranes. Also, endogenous membrane phospholipases, which are activated in eukaryotic cells exposed to α-toxin, are thought to make a significant contribution to membrane damage [10–12] amplifying any effects due to the action of α-toxin. These endogenous enzymes would not be present in liposomes which might also explain their reduced sensitivity to the action of α-toxin. The myotoxic activity of the variant proteins showed only a partial correlation with their haemolytic and cytotoxic activities. This could be explained because the pathogenesis of the myotoxicity depends not only on the direct destruction of the muscle fibre membranes, but also on the ability of the toxin to induce the formation of intravascular thrombi, with a resultant reduction in blood flow and increased tissue anoxia [7,8].

The results presented here have shown that the substitution of a single amino acid residue within the carboxy-terminal domain of α-toxin was not sufficient to completely abolish the haemolytic or cytotoxic activity. However, the results clearly demonstrate that residues Y_{331} and F_{334} play key roles in conferring these activities on α-toxin. Furthermore, the data also indicate that the substitutions of these residues, which occur in Cbp, contribute to explain the reduced haemolytic and cytotoxic activity of this enzyme.

The variants investigated in this study add significantly to the previous studies in which 14 other residues in the carboxy-terminal domain of α-toxin have been substituted [7,22,29,30]. These studies indicate that mutations which modulate the haemolytic, cytotoxic or myotoxic activities of α-toxin are located primarily on the putative membrane binding face of the carboxy-terminal domain (Fig. 1). The exceptions to this are residue T_{272}, where the mutation to proline is likely to cause a gross structural destabilisation [30], and residue D_{305}, which is thought to play a role in communication of the amino- and carboxy-terminal domains [22]. Overall these findings support the model that surface-exposed hydrophobic amino acids and calcium-mediated phospholipid binding are both essential for binding of the carboxy-terminal domain of α-toxin to cell membranes [13,14].

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