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Mutation of a putative ADP-ribosylation motif in the *Pasteurella multocida* toxin does not affect mitogenic activity

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Abstract

Pasteurella multocida toxin (PMT) is a potent mitogen for Swiss 3T3 fibroblasts and cytotoxic to embryonic bovine lung cells. Site-directed mutagenesis was used to investigate the functional significance of a three amino acid motif in PMT that is present in five other bacterial protein toxins which exhibit ADP-ribosyl transferase activity. Crude lysates of mutant clones were fully cytotoxic for embryonic bovine lung cells. Purified mutant toxin was also as effective at stimulating inositol phosphate turnover and nucleic acid synthesis as wild type toxin. We conclude that this motif has no functional significance in *Pasteurella multocida* toxin.

Key words: Mitogen; Toxin; ADP-ribosylation; *Pasteurella multocida*; Site-directed mutagenesis

1. Introduction

The osteolytic toxin of *Pasteurella multocida* (PMT), has been identified as a potent mitogen for cultured fibroblasts [1]. PMT stimulates inositol phosphate accumulation, calcium mobilization [2], and phosphorylation by protein kinase C [3], and is mitogenic at picomolar concentrations [1]. It has also been shown to induce anchorage independence in Rat1 cells [4]. Various lines of evidence suggest that the toxin acts intracellularly, but the molecular mode of action of the toxin remains unclear.

A His-Glu-Trp amino acid motif has been identified in a number of bacterial protein toxins which exhibit ADP-ribosylation activity. The histidine residue is spaced 125 amino acids from the glutamic acid residue, which in turn precedes the tryptophan residue by four amino acids [5]. Site-specific mutations of this putative ADP-ribosyl transferase motif have been used to attribute functional significance to these amino acids in the case of *Pseudomonas aeruginosa* exotoxin A (ETA) [5,6]. This was achieved primarily through the identification of His^{426} as a conformationally significant residue [7,5], and Glu^{553} as an active site residue involved in NAD^+ binding [8]. A structural model of ETA was used to locate His^{426} within an α-helix in the carboxyl-terminal domain III of the enzyme [7], to which catalytic activity had been assigned [9,10]. A range of His^{426} substitutions were used [6] to suggest that this residue was crucial to the three dimensional structure of the catalytic site of ETA.

In the case of diphtheria toxin (DT), photoaffinity labelling was used to locate Glu^{148} in an important catalytic region in DT [11], and subsequent substitution of Glu^{148} with aspartic acid reduced activity by at least 100-fold [12].

In addition, the deletion of Glu^{148} has been shown to reduce DT activity considerably [13]. More recently, His^{21} of DT has been linked with NAD^+ binding [14,15].

This specific His-Glu-Trp motif occurs once in the PMT sequence, located towards the N-terminus [16]. Computer analysis of the amino acid sequence of PMT also located His^{29} in an α-helix, and predicted the peptide was likely to be flexible in this region (Ward P.N., unpublished observations). On this basis it seemed plausible that similar functions might be assigned to this motif in PMT.

We have investigated the potential role of the His-Glu-Trp motif in PMT, by making single and double mutations in this motif and testing for changes in the activity of the toxin.

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Abbreviations: PMT, *Pasteurella multocida* toxin; rPMT, recombinant *Pasteurella multocida* toxin; G protein, guanine nucleotide binding regulatory protein; EBL, embryonic bovine lung.

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2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Full length recombinant toxin is expressed from its own promoter in *Escherichia coli* hosts at much higher levels than that observed in *P. multocida*, and for this reason all manipulations using infectious material were carried out under Category 3^+ regulations as defined by the UK Advisory Committee for Genetic Manipulation (ACGM).

E. coli strain XL1-Blue (Stratagene) was used as a general cloning host. *E. coli* strain GM242 (dam^-) was used to isolate unmethylated DNA for subsequent restriction enzyme digestion. *E. coli* strain CJ236 (Boehringer) was used to produce uracil-rich single stranded (uss) DNA for use as a mutagenesis template as directed by the manufacturer.

The full coding sequence of PMT was subcloned from pAJL13 [17], into pBluescript II SK- (Stratagene) and named pTox3.

Culture of *E.coli* containing pTox3 derived clones was in L-broth [18], supplemented with ampicillin at 50 μg/ml and tetracycline at 10 μg/ml, both from Sigma, with shaking at 37°C.

2.2. Reagents

Unless stated, all chemicals were from BDH. DNA modifying enzymes were purchased from Boehringer Mannheim and used in accordance with the manufacturer's instructions. Sequence determination of mutants was performed on single stranded (ss) template DNA using a Sequenase Version 2.0 kit (USB) in accordance with the instructions supplied.

2.3. DNA preparation

Plasmid DNA was isolated by alkaline lysis [18]. Single stranded DNA was prepared from XL1-Blue and CJ236 hosts transformed with pTox3 or its derivatives, using the VCSM13 helper bacteriophage (Stratagene), according to the manufacturer's instructions.

2.4. Mutagenesis

Oligonucleotide-directed mutagenesis of His^{29} (H29) was carried out using sense strand wild type ss-template DNA, following the protocol supplied by Stratagene. The products of the mutagenesis reactions were transformed into *E. coli* strain GM242 (dam^-) and plasmid DNA isolated for screening by restriction enzyme digestion with *BclI* (Boehringer). H29 mutant clones were identified by the loss of the *BclI* recognition sequence at His^{29}, and the mutation was verified by DNA sequencing.

A site-directed mutagenesis procedure [19,20] was adopted to make Glu^{155} (E155) mutants, and the candidate clones screened by DNA sequencing.

The oligonucleotide used for in vitro mutagenesis of His^{29} to alanine (5′-dTTGCTTGTCAGGAGCATCAGTACACAATC) contained two base changes (underlined), whereas the oligonucleotides used to mutate Glu^{155} to alanine (5′-dGGTTATATGCTGGACCAT), and Glu^{155} to aspartic acid (5′-dGGGTTATAATCTGGACC) contained single base changes (underlined).

2.5. Toxin purification

The initial stages of toxin purification were performed at ACGM category 3^+ where potentially infectious material was being handled. Later stages of the procedure were performed within a Class II biological safety cabinet.

Wild type and mutant toxin was prepared using an adaptation of a previously published method [21]. Overnight cultures (800 ml) were grown as described above. Cell pellets were resuspended in 150 ml lysis buffer (5 mM EDTA, 50 mM Tris-Cl (pH 7.2), 100 mM NaCl, 0.1% toluene and 50 μg/ml lysozyme), and incubated without stirring at room temperature for 2 h. The lysate was cleared by centrifugation at $1,000 \times g$ for 20 min, and nucleic acid removed by addition of DNase I and RNase A (both from Sigma) each to final concentrations of 10 μg/ml with incubation at 37°C for 1 h. The cleared lysate was filtered through 0.22 μm filters (Schleicher and Schuell) and protease inhibitors benzamidine and phenylmethylsulphonylfluoride (Sigma) were added to final concentrations of 1 mM and 0.1 mM, respectively. The filtrate was fractionated by DEAE-Sephacel (Pharmacia) chromatography and preparative polyacrylamide gel electrophoresis as previously described [21]. Protein concentration was estimated using the Pierce BCA protein assay kit.

Cleared crude cell lysates were prepared from the mutant clones using a modified version of the purification procedure described. Five millilitre bacterial cultures were resuspended in 2 ml of lysis buffer with the addition of DNase I and RNase A, each to final concentrations of 20 μg/ml, with incubation at room temperature for 30 min. Lysates were then filtered through 0.22 μm filters (Schleicher and Schuell).

Glycerol was added to samples of crude lysate and purified toxin to a final concentration of 50% before storage at −20°C.

2.6. Embryonic bovine lung (EBL) cell assay

The cytotoxicity of toxin preparations was assessed on standard 96-well microtitre plates using a previously published method [22], with the following modifications. 10 μl of cleared crude cell lysate was serially diluted ten-fold with 90 μl Eagle's minimum essential medium (Flow), containing 10% fetal calf serum, and 90 μl of a suspension of EBL cells at 3×10^5/ml added to each well.

2.7. Swiss 3T3 fibroblast assays

Incorporation of [^{3}H]thymidine into DNA was assessed [23]. Confluent, quiescent cultures of Swiss 3T3 cells were washed and incubated at 37°C in 2 ml of Dulbecco's modified Eagles medium (DMEM)/Waymouths medium 1:1 (v/v) containing 1 μCi of [^{3}H]thymidine per ml and various concentrations of rPMT or mutants of rPMT. After 40 h DNA synthesis was assessed by measuring the level of [^{3}H]thymidine incorporated into the acid precipitable material.

The ability of toxin preparations to stimulate inositol phosphate production was determined [2]. Confluent quiescent cultures of Swiss 3T3 cells in 30 mm dishes were prelabeled with 10 μCi/ml [2-^{3}H]inositol for 16–18 h. rPMT mutant toxin preparations were then added at a concentration of 10 ng/ml and the cultures were incubated at 37°C for 4.5 h. LiCl was then added to a final concentration of 20 mM. After a further 30 min the reaction was terminated by replacing the medium with 1 ml of ice-cold $HClO_4$. After 20 min at 4°C the extract was neutralized with 0.5 M KOH containing 25 mM HEPES, 5 mM EDTA, and 0.01% Phenol red. Precipitated $KClO_4$ was removed by centrifugation at $1,000 \times g$ for 5 min. Analysis of total inositol phosphates was by anion exchange chromatography. Samples were diluted to 10 ml with water and then loaded onto 1 ml of Dowex AG1-X8 (100–200 mesh, $HCOO^-$ form) in Bio-Rad Econo-columns. After washes with 3×10 ml of water and 2×10 ml of 60 mM NH_4COOH, 5 mM $Na_2B_4O_7$ the inositol phosphates were eluted with 7 ml of 1 M NH_4COOH, 0.1 M HCOOH. One millilitre of eluate was counted in 10 ml of Picofluor.

3. Results

3.1. Oligonucleotide-directed mutagenesis

The oligonucleotides used for in vitro mutagenesis were designed to substitute alanine for histidine at position 29 of PMT, and alanine or aspartic acid for the glutamic acid residue at position 155 of the toxin. The rationale for the substitution of alanine was to introduce a residue that was quite different in nature but considered biochemically unreactive. The substitution of aspartic acid for glutamic acid was to maintain the characteristics of the side group while subtly altering the location of the charged moiety. Such a substitution has previously been shown to decrease the ADP-ribosyltransferase activity of diphtheria toxin by a factor of at least 100 [12].

A *BclI* restriction site encompassing His^{29} was lost by mutation and enabled rapid screening to be carried for the His^{29} to alanine mutation. One of ten randomly selected *E. coli* GM242 transformants from the His^{29} mutagenesis reactions exhibited a different restriction endonuclease digestion pattern with *BclI*. The altered DNA fragment sizes were consistent with those expected following the alteration of the *BclI* recognition sequence at

amino acids 27–29. DNA sequence analysis confirmed that the desired His^{29} to alanine substitution had taken place. Since there was not a suitable restriction site at Glu^{155}, a more efficient mutagenesis strategy [19,20] was used to obtain mutations at this site. DNA isolated from pTox3 and pTox3/H29A was used to prepare uracil-rich template DNA for subsequent in vitro mutagenesis reactions. The improved efficiency of mutagenesis enabled candidate mutants to be screened directly by sequence analysis. The combination of single and double substitutions achieved using this technique are summarised in Table 1.

3.2. Properties of mutant PMT

Toxin was purified from each of the mutant clones. Each toxin preparation behaved identically when subjected to anion exchange chromatography, or native PAGE and migrated to the same position on SDS-PAGE.

The cytopathic effect of crude lysates of the mutant clones was compared to that of the pTox3-wild type clone, using the EBL cell assay. In all cases the preparations of mutant toxin showed levels of cytotoxicity comparable to that of wild-type toxin (Table 1). We ascribe the apparent increases of cytotoxicity observed in some cases to slight variations in the toxin content of crude lysates.

The ability of purified mutant toxins to initiate DNA synthesis in Swiss 3T3 fibroblasts was assessed (Fig. 1). There were no differences in either the relative potency of mutated toxins or their ability to stimulate DNA synthesis equivalent to that induced by 10% serum. Inositol phosphate accumulation was also measured. In all cases the purified mutant toxin preparations exhibited comparable levels of activity to that of wild-type toxin (Fig. 2). Purified H29A mutant toxin also stimulated DNA synthesis and inositol phosphate turnover in similar amounts to that of wild type toxin (data not shown).

Table 1
Site directed mutants of the His-Glu-Trp motif and their EBL cytotoxicity

Mutants	His^{29}	Glu^{155}	Trp^{160}	EBL toxicity (end point dilution factor)
H29A	Ala	–	–	1.3×10^7
E155D	–	Asp	–	1.0×10^7
H29A E155D	Ala	Asp	–	5.5×10^6
H29A E155A	Ala	Ala	–	1.3×10^8
pTox3 (Wt)	–	–	–	5.5×10^6

Combinations of point mutations were made in the rPMT gene as described in section 2. The cytotoxicity of crude lysate preparations of the mutant clones was determined as described, and expressed as the mean of duplicate determinations of the cytotoxic end point dilution factor.

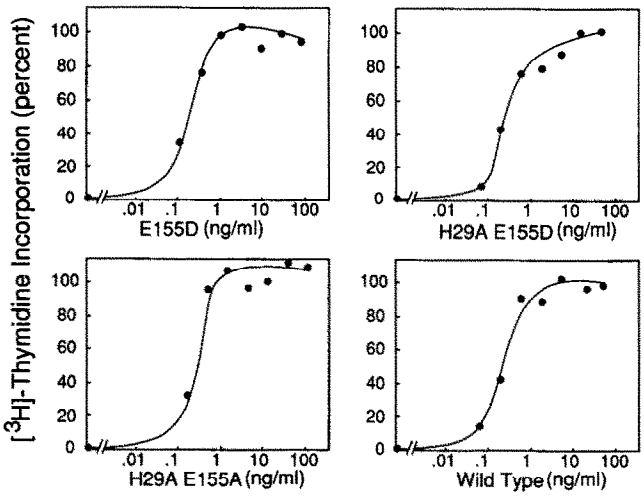

Fig. 1. Dose response curves for the stimulation of DNA synthesis by rPMT or mutants of rPMT. Each point is the mean of two determinations and is expressed as a percentage of the incorporation given by 10% fetal bovine serum (27.3×10^4 cpm).

4. Discussion

Investigation of the mode of action of bacterial toxins has helped identify components of eukaryotic signal transduction pathways [24,25]. Several of these toxins (notably pertussis toxin and cholera toxin) are known to modify G proteins [26,27]. Since *P. multocida* toxin is mitogenic for cultured cells at extremely low concentrations it is likely to have an enzymatic mode of action on an as yet unidentified target. By analogy with other toxins it was tempting to speculate that its intracellular target might be a G protein. Its role in stimulating inositol phosphate release would suggest that the recently identified Gq [28,29] could be a possible target.

We have shown that the His-Glu-Trp motif is not functional in PMT. Mutation of His^{29} to alanine did not affect any of the properties of PMT. Since the histidine residue in the motif is remote from the other two residues, and PMT contains 18 additional histidine residues which might be functional, the Glu^{155} residue was also mutated either to alanine or to aspartic acid since a similar substitution has been shown to decrease the activity of diphtheria toxin severely [12]. The single and double mutants were fully active in inducing both [^{3}H]thymidine incorporation and inositol phosphate accumulation.

This result is consistent with the failure to detect ADP-ribosylation using a novel method for the direct measurement of toxin-catalyzed ADP-ribosylation using whole cells [30]. However, it was possible in view of the extreme potency of PMT that the cellular target was present at very low levels and could not be identified using this method. Our results make it unlikely that PMT has an ADP-ribosylating function.

Sequence analysis of PMT shows no significant ho-

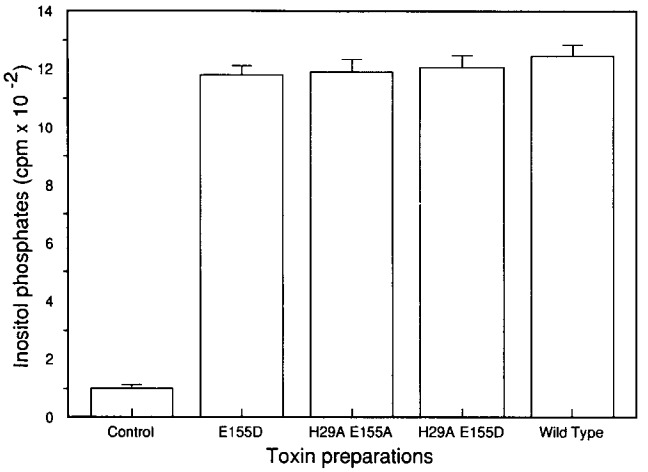

Fig. 2. rPMT mutants stimulate inositol phosphate production in Swiss 3T3 cells. Analysis of total inositol phosphates in rPMT and mutant rPMT treated Swiss 3T3 cells was as described in section 2.

mology to either DNA or protein databases with the exception of Cytotoxic Necrotizing Factor 1 (CNF1) from *Escherichia coli* [31], which displays homology with the putative membrane associated central hydrophobic domain of PMT [16], but does not contain the His-Glu-Trp motif. The absence of any further similarities between the PMT sequence and those in the databases is a further indication of the novelty of this molecule and of its mode of action. Recent experiments show that PMT potentiates stimulation of inositol phosphate by various mitogenic neuropeptides, which strongly suggests that PMT facilitates G protein coupling to phosphoinositol-phospholipase C [32]. The results presented here suggest that it is likely that PMT will have a novel molecular mode of action.

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