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**FOR THE RECORD**

# The C3 domain of *Pasteurella multocida* toxin is the minimal domain responsible for activation of G<sub>q</sub>-dependent calcium and mitogenic signaling

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LEILA R. AMINOVA, SHUHONG LUO, YUKA BANNAI, MENGFEI HO,  
AND BRENDA A. WILSON

Department of Microbiology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801, USA

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## Abstract

The large 1285-amino-acid protein toxin from *Pasteurella multocida* (PMT) is a multifunctional single-chain polypeptide that binds to and enters eukaryotic cells and acts intracellularly to promote G<sub>q</sub> and G<sub>12/13</sub> protein-dependent calcium and mitogenic signal transduction. Previous studies indicated that the intracellular activity domain responsible for PMT action was located within the C-terminal 600–700 amino acids. In this study, we have exogenously expressed a series of N- and C-terminal PMT fragments directly in mammalian cells and have used the dual luciferase reporter system to assay for toxin-mediated activation of calcium-calcineurin-NFAT signaling (NFAT-luciferase) and mitogenic serum response signaling (SRE-luciferase). Using this approach, we have defined the last 180 amino acids, which encompass the C3 domain in the crystal structure, as the minimum domain sufficient to activate both NFAT and SRE signaling pathways.

**Keywords:** dermonecrotic toxin; NFAT; SRE; calcium mobilization; G<sub>q</sub> protein

**Supplemental material:** see [www.proteinscience.org](http://www.proteinscience.org)

The protein toxin from *Pasteurella multocida* (PMT) experimentally induces all of the major symptoms of a number of economically important zoonotic diseases in wild animals and domestic livestock and pets (Foged 1992; Arashima and Kumasaka 2005; Harper et al. 2006; Wilson and Ho 2006), including moderate to severe progressive atrophic rhinitis, bite wound dermonecrosis and abscesses, chronic respiratory infection, and decreased overall stature and weight gain. PMT is a 1285-amino-

acid protein that binds to and enters mammalian cells via receptor-mediated endocytosis (Rozenfurt et al. 1990; Pettit et al. 1993; Dudet et al. 1996). PMT has dramatic and differential effects on differentiation and proliferation of fibroblasts, bone cells, epithelial cells, and cardiomyocytes (for review, see Wilson and Ho 2004, 2006). In fibroblasts and osteoblasts, PMT acts intracellularly to enhance calcium signaling pathways, including inositol phospholipid hydrolysis, intracellular Ca<sup>2+</sup> mobilization, increased protein kinase C (PKC)-dependent phosphorylation, and calcineurin-dependent nuclear factor of activated T cells (NFAT) activation (Staddon et al. 1990, 1991; Wilson et al. 1997; Sabri et al. 2002; Aminova and Wilson 2007; Luo et al. 2007), as well as mitogenic pathways, including increased tyrosine phosphorylation, MAPK (Erk1/2, p38) activation, and DNA synthesis (Rozenfurt et al. 1990; Dudet et al. 1996; Lacerda et al. 1996; Mullan and Lax 1996; Seo et al. 2000; Wilson et al. 2000).

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Reprint requests to: Brenda A. Wilson, Department of Microbiology, University of Illinois at Urbana-Champaign, 601 South Goodwin Avenue, B128 CLSL, Urbana, Illinois 61801, USA; e-mail: [bawilson@life.uiuc.edu](mailto:bawilson@life.uiuc.edu); fax: (217) 244-6697.

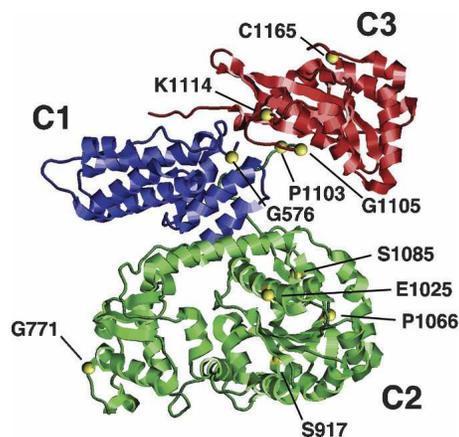
**Abbreviations:** rPMT, recombinant *Pasteurella multocida* toxin; NFAT, nuclear factor of activated T cells; SRE, serum response element; PLC, phospholipase C; PKC, protein kinase C.

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The primary intracellular target of PMT responsible for activation of the phospholipase C  $\beta$ 1 (PLC $\beta$ 1) pathway is the free, monomeric  $\alpha$  subunit of the G $_q$  protein (Wilson et al. 1997; Zywiec et al. 2001; Wilson and Ho 2004). PMT-induced stimulation of PLC $\beta$ 1 by G $\alpha_q$  leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate and diacylglycerol. Accordingly, the release of these second messengers stimulates Ca $^{2+}$  mobilization and activates PKC-dependent phosphorylation and calcineurin-dependent NFAT activation. PMT stimulation of mitogen-activated protein kinase occurs upstream via G $_{q/11}$ -dependent transactivation of the epidermal growth factor receptor (Seo et al. 2000). PMT also initiates cytoskeletal rearrangements, including focal adhesion assembly and stress fiber development (Dudet et al. 1996; Lacerda et al. 1996; Ohnishi et al. 1998). At least part of the mitogenic effect of PMT occurs through a G $_q$ -independent pathway (Zywiec et al. 2001). Rho activation by PMT has been reported to be the result of indirect activation through G $_{12/13}$  (Orth et al. 2005) or through tyrosine kinases (Sagi et al. 2001). Our recent study revealed two additional pathways affected by PMT, namely, down-regulation of Notch1 and stabilization of  $\beta$ -catenin during adipocyte differentiation (Aminova and Wilson 2007).

There is now strong evidence that the G $_q$ -activating domain of PMT is localized to the C terminus, since site-specific mutations at H1205, H1223, and C1165 abolish intracellular activity when the mutant proteins are introduced into cells via microinjection (Pullinger et al. 2001; Baldwin et al. 2004) or electroporation (Busch et al. 2001; Orth et al. 2003). The recently reported crystal structure of the C-terminal 575–1285 residues revealed three subdomains (see Fig. 1), designated as C1, C2, and C3 domains (Kitadokoro et al. 2007). The shortest active fragment in this study was identified as residues 569–1285. According to this study, the C1 domain (residues 575–719) may be responsible for membrane targeting, the C2 domain (residues 720–1104) has no obvious function, and the C3 domain (residues 1105–1285) possesses a putative Cys-His-Asp catalytic triad, which when the C1165 is released from a disulfide bond (C1159-C1165) through mutation of C1159 to Ser sterically aligns to that of a papain or cysteine protease-like triad. However, the authors were not able to demonstrate any proteolytic activity.

The minimal domain of PMT responsible for intracellular activation of G $_q$ -dependent signaling has been the subject of some debate. One group reported that a C-terminal fragment of PMT consisting of residues 720–1285, when microinjected into cells, induced cellular morphology changes similar to full-length PMT, while a smaller fragment consisting of residues 849–1285 showed no activity (Pullinger et al. 2001). Consistent with the shortest active fragment of 569–1285 reported in the



**Figure 1.** Structure of C-PMT. Shown is a ribbon diagram of the C terminus of PMT (C-PMT, residues 575–1285): C1 (blue, 575–719), C2 (green, 720–1104), and C3 (red, 1105–1285). Lines indicate location of the starting residue (yellow balls) of the truncation mutants in this study, as well as the active site catalytic Cys residue C1165. Image was generated with RasMol using the PDB data file 2EBF (C-PMT).

crystallographic study (Kitadokoro et al. 2007), another group found that a fragment of PMT consisting of residues 581–1285, when electroporated into cells, increased intracellular inositol phosphate levels, but a fragment consisting of residues 701–1285 was not biologically active (Busch et al. 2001). However, none of these studies demonstrated intracellular activity in smaller fragments consisting of only the C3 domain.

The methods used in previous studies to define the minimal intracellular activity domain relied on expression and purification of the recombinant truncated proteins from *Escherichia coli*, which assumed that these mutant proteins folded properly in *E. coli* without proteolytic degradation and were stable enough to withstand subsequent purification steps. In each study, the choice of truncation site was arbitrary and only a few relatively large truncation mutants were examined. In addition, these studies also assumed that the techniques used to introduce the truncated proteins into the cells (i.e., microinjection or electroporation) would not perturb the functional readout of the assay, such as cytoskeletal changes, calcium, or other signaling.

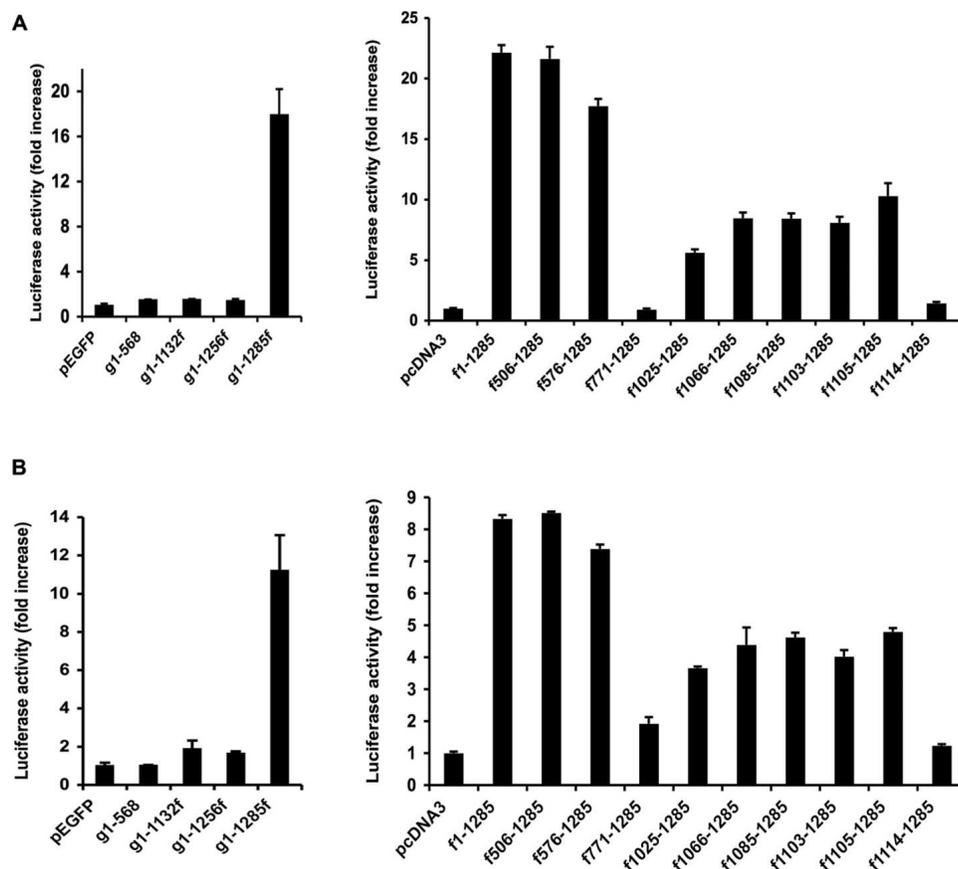
Our approach reported here circumvented these disadvantages by expressing a series of N- and C-terminal toxin fragments (the locations of the truncation sites are indicated in Fig. 1) and directly expressing them in mammalian cells using mammalian expression vectors. To determine the intracellular activity of these proteins, we used two different downstream luciferase reporter systems: one based on PMT activation of calcium signaling (Wilson et al. 1997; Aminova and Wilson 2007; Luo et al. 2007), leading to stimulation of calcineurin and NFAT (Crabtree and Olson 2002; Im and Rao 2004;

Aminova and Wilson 2007), and the other based on PMT activation of mitogenic signaling (Seo et al. 2000; Wilson et al. 2000; Sagi et al. 2001; Orth et al. 2005), leading to stimulation of serum response element (SRE) through several mitogenic signaling pathways, including those regulated by the Ras, Rho, G<sub>q</sub>, and G<sub>12/13</sub> families of GTPases (Hill et al. 1995; Fromm et al. 1997; Seo et al. 2000; Sagi et al. 2001).

## Results and Discussion

Inclusion of GFP- or FLAG-tags as markers for visualization allowed us to determine via Western blot analysis the level of expression and stability of the expected full-length and mutant toxin proteins exogenously expressed in the mammalian cells (see Supplemental material). Full-length PMT intracellularly expressed in CHO-K1 cells caused a 20-fold increase in NFAT-luciferase reporter activity and a 10-fold increase in SRE-luciferase reporter

activity (Fig. 2). Whereas truncations in the C terminus of PMT (1–568g, g1–1132f, and g1–1256f) showed no activation of these two signaling pathways (Fig. 2, left plots), two larger N-terminal deletion fragments f506–1285 and f576–1285, encompassing the putative catalytic regions reported previously (Busch et al. 2001; Pullinger et al. 2001; Kitadokoro et al. 2007), possessed NFAT-luciferase and SRE-luciferase activities comparable to that of full-length toxin (f1–1285 or g1–1285f) (Fig. 2, right plots). In addition to these fragments, we tried to express mutants starting from residues 710, 720, 771, 917, and 1005 (Supplemental Fig. 1); however, all of these constructs showed significant degradation, except fragment f1005–1285, which expressed poorly, and all of these constructs exhibited little or no NFAT or SRE activity (only data for f771–1285 and f1025–1285 are shown in Fig. 2). Interestingly, while a bacterially expressed fragment encompassing residues 701–1285 showed no activity by electroporation (Busch et al. 2001), a smaller bacterially



**Figure 2.** Luciferase-reporter assays for PMT activity. Shown are plots of NFAT-luciferase (A) and SRE-luciferase (B) activities after transient transfection with dual NFAT-luciferase or SRE-luciferase reporter plasmids and plasmids expressing full-length PMT on PMT mutants. Fold increase determined as the ratio of activity units for toxin-containing plasmids versus empty control plasmid (pEGFP or pcDNA3-FLAG). (Left plots) Full-length (g1–1285f) or N-terminal fragments of PMT (g1–568, g1–1132f, and g1–1256f). (Right plots) Full-length (f1–1285) or C-terminal fragments of PMT (f506–1285, f576–1285, f771–1285, f1025–1285, f1066–1285, f1085–1285, f1103–1285, f1105–1285, and f1114–1285).

expressed fragment starting from residue 720 exhibited mitogenic activity upon microinjection into cells (Pullinger et al. 2001). However, an analogous fragment f720–1285 expressed in mammalian cells was unstable and showed little or no intracellular activity (data not shown).

Truncated mutants starting from residues 1025, 1066, 1085, 1103, or 1105 to the C terminus were expressed as stable proteins (Supplemental Fig. 1) and showed ~50% of NFAT or SRE-luciferase activity compared with that of full-length toxin (Fig. 2, right plots). As shown in Figure 1, the smallest active domain of PMT (f1105–1285) corresponded to the C3 domain defined in the crystal structure (Kitadokoro et al. 2007). Any further deletion into the C3 domain resulted in loss of activity (Fig. 2, right plots) even though protein was expressed (Supplemental Fig. 1). Thus, this C-terminal 180 amino acid fragment was sufficient to activate both NFAT and SRE signaling pathways, strongly supporting that the biological activity of PMT responsible for activation of both of these signaling pathways is confined primarily to this region. Activation of the NFAT and SRE signaling pathways was specific to PMT activity, since point mutations previously shown to abolish PMT activity, such as C1165S, likewise ablated the activity of the toxin proteins in both assays (Supplemental Fig. 2). The partial activity exhibited by the C3 domain (residues 1105–1295), compared with the full activity of the larger fragments containing domains C1–C3 (residues 576–1285), suggests that the entire C1–C3 domain is important for full toxin activity and that although C3 possesses the minimal intracellular biological activity, the C1 and C2 domains may contribute to proper intracellular localization, interaction with other potential protein cofactors, and/or protein conformation necessary for full activity.

In summary, we have used exogenous expression of a series of PMT truncations and substitution mutants in mammalian cells to define the minimal intracellular activity domain of PMT. Our results demonstrated that the last 180 amino acids, the C3 domain in the crystal structure (Fig. 1), define the minimal activity domain sufficient to stimulate both calcium-dependent (calcineurin-NFAT) and mitogenic (SRE) signaling pathways. With this information, it may now be possible to more easily ascertain the as yet elusive biological activity of PMT.

## Materials and Methods

### Plasmid constructs

Deletion mutants were constructed by fragment exchange or PCR cloning using previously described PMT constructs in pET21 vectors (Novagen) (Wilson et al. 1999). Two mammalian expression vectors were used for cloning: pEGFP-C1 (Clontech) or pcDNA3 (Invitrogen) with a 3×FLAG sequence inserted into KpnI/BamHI restriction sites. Corresponding PMT fragments

were amplified by PCR using PCR master mix (ABgene) and specific primers (IDT) and cloned into pEGFP or pcDNA3 vectors. The single mutation C1165S was introduced into wild-type PMT fragment (1005–1285) by PCR-based site-directed mutagenesis using published primers (Ward et al. 1998; Orth et al. 2003). After sequence verification, the corresponding mutations were introduced into the indicated toxin constructs by fragment exchange using standard methods.

### Cell culture

CHO-K1 cells (ATCC, CCL-61) were cultured at 37°C and 5% CO<sub>2</sub> in F-12 medium, consisting of F-12 Nutrient Mixture (GIBCO) supplemented with 10% heat-inactivated bovine growth serum (BGS, HyClone), pH 7.4, 100 units/mL penicillin G, and 100 µg/mL streptomycin. For transient transfection, cells were plated into 24-well plates at 70%–80% confluence 24 h prior to transfection.

### Luciferase assay

The effect of external treatment with rPMT on calcium signaling was assayed using a stable CHO-K1 cell line containing NFAT-luciferase reporter (Aminova and Wilson 2007). To study the effect of expression of PMT or PMT deletion mutants on NFAT-luciferase or SRE-luciferase activity in mammalian cells, CHO-K1 cells were transiently transfected with a combination of pNFAT-*luc* and pRenilla-TK vectors (pGL 7.4 hRluc/TK, Promega) or pSRE-*luc* (Stratagene) and pRenilla-TK vectors in a ratio of 10:1 and vector expressing recombinant protein (50 ng/well) using Lipofectamine 2000 reagent (Invitrogen). Cell extracts were prepared and analyzed 24 h after transfection using the Dual Luciferase Assay System (Promega), according to manufacturer's protocol. Luminescence was measured using a Synergy-HT multi-detection microplate reader (BioTek), and results were reported as relative light units. All cell extracts used for luciferase assay were analyzed by Western blot to verify expression of protein of interest. In all experiments reported here, data are expressed as the mean ± SD of results from at least three independent experiments performed in triplicate.

## Electronic supplemental material

The Supplemental materials provide additional details on the following: (1) results of Western blots of CHO-K1 cells showing expression levels of toxin mutant proteins (Supplemental Fig. 1), (2) methods used for determination of mammalian expression of toxin proteins using immunoblotting, and (3) results of point mutations previously shown to abolish PMT activity on NFAT and SRE luciferase activity (Supplemental Fig. 2).

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