Structural and Biophysical Characterization of the Binding/Translocation Domain of *Pasteurella multocida* Toxin and Related Toxins

Michael C. Brothers∏*, Mengfei Ho‡, Ram Maharjan, Chad M. Rienstra* (PI) and Brenda A. Wilson‡ (PI)
Department of Homeland Security Research Fellow∏
Department of Chemistry* and Department of Microbiology‡, University of Illinois at Urbana-Champaign

Research Area: Biological Threats and Countermeasures

Introduction

Pasteurella multocida is a zoonotic bacterium that causes significant economic damage each year. It is transmitted readily to humans via animal bites and/or respiratory exposure. *P. multocida* toxin (PMT) is its primary virulence factor and is one of the most potent known mitogens of the biological toxins characterized. Nasal inoculation with the toxin alone causes atrophic rhinitis (Figure 1) and injection into the skin causes dermonecrosis

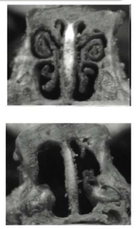

Figure 1 Mild (top) and severe (bottom) atrophic rhinitis observed in swine caused by PMT.

It also is a model toxin for other related toxins including the cytotoxic necrotizing factors from *E. coli* and *Yersinia* and the dermonecrotic toxin from *Bordetella*. While we are beginning to understand how these toxins act intracellularly, far less is known about their mechanism of binding and translocation.

Our goal is to understand and define the mechanism of toxin binding to host cells using cellular biology, biochemical and biophysical techniques, including thin-layer chromatography (TLC), surface plasmon resonance (SPR), and other analytical tools. The scope of this study focuses on the various components that enhance binding to the cell surface and how they affect not only the thermodynamic binding properties, but also the kinetic binding properties of PMT.

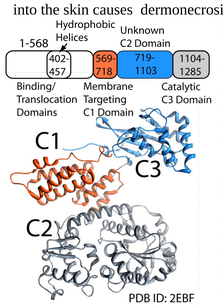

Figure 2 Schematic of current information known about PMT, including crystal structure of C-terminus

Methods

A. Recombinant PMT and PMT fragments were expressed and purified using previously published protocols (Wilson et al., **1999**).

B. Thin-Layer Chromatography Experiments were performed as follows:

1. Membrane components (e.g., as in Figure 4) were isolated by extraction with chloroform/methanol.
2. TLC plates were run with membrane components in organic solvents and then stained using cupric sulfate to quantify or $Na^{125}I$ labeled PMT and imaged using a scanner or a phosphoimager

C. Lipid vesicles were prepared as follows:

1. Synthetic vesicles were prepared by sonication, and then extrusion to generate small unilamellar vesicles (SUV's, ~200 kDa).
2. Natural membrane vesicles were made from HEK-293 cells using gradient sucrose centrifugation (Boone, 1969).

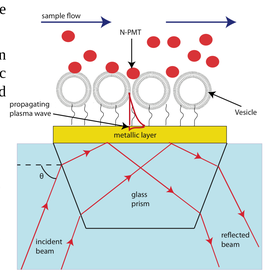

Figure 3 Illustration of how SPR was used to interrogate binding to membranes bound to the L1 chip.

D. Circular dichroism experiments were performed as follows:

1. PMT-N in PBS buffer with or without lipids (10 x CMC) was diluted to 0.015 mg/mL in pH 7.4 citrate-phosphate buffer.
2. Sample was pH adjusted by pulsed phosphate-citrate buffer.

E. Surface plasmon resonance (SPR) experiments: (**Figure 3**):

1. SUVs or native membranes were loaded onto the L1 chip before treatment with trypsin, lipases and/or injections of PMT-N.
2. Increasing concentrations (ranging from 5 nM to 15 μM) of PMT-N were injected over the chip for 5 minute intervals (5 μL/min).
3. Kinetic analysis was performed using least squares to fit kinetics and thermodynamics (right). Error analysis determined using bootstrap methodology.

Kinetics

Association

$R=R_E*(1-e^{-(k_{on})}t)$

$R_E = R_{max}*k_{on}*C/k_{obs}$

$k_{obs} = k_{on}*C+k_{off}$

Dissociation

$R=(R_{max}*(e^{-(k_{off})}*t))$

Thermodynamics

$$B = B_{max}\frac{K_bF}{1+K_bF}$$

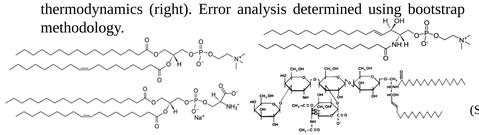

Figure 4 Phosphatidyl choline (PC), phosphatidyl serine (PS), sphingomyelin (SP), and ganglioside (GM1)

Results

TLC Studies to Determine Non-Protein Binding Partners

Previous studies suggested the receptors of PMT consisted of gangliosides (Dudet et al, **1996**; Pettit et al, **1993**). If this is true, then cell extracts and purified compounds of GM1 immobilized on a TLC plate should bind to PMT as observed with other toxins (Brothers et al, submitted).

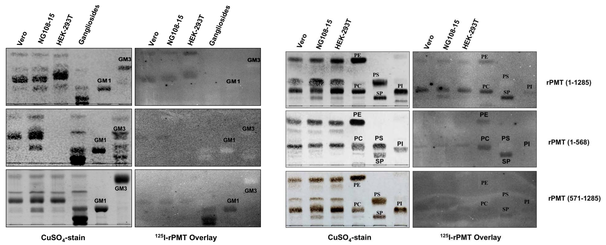

Figure 5 Pattern of binding of PMT and PMT fragments to membrane components. Only the C-terminal catalytic fragment of PMT (PMT-C, 571-1285) binds gangliosides (GM1 or GM3). The N-terminal fragment of PMT (PMT-N, 1-568) and full-length PMT bound to components of the phospholipid membrane, primarily sphingomyelin (SP) and phosphatidyl choline (PC).

A Bi-Phasic Model Can be Used to Deconvolute Kinetic Traces

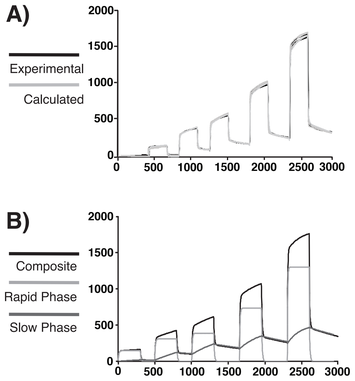

Figure 6 A) Calculated versus experimental trace of increasing injections of PMT onto a HEK-293T ghost. B) Using a bi-phasic fit, the trace can be separated into a rapid phase and a slow phased, where the slow phase is believed to be indicative of productive binding. The equations below defines the interactions, where association is fit to:

$R(t) = R_{E1}*(1-e^{-kobs2}) + R_{E2}*(1-e^{-kobs2})$ and dissociation is fit to:

$R(t) = R^1_{max}(e^{-koff1*t}) + R^2_{max}(e^{-koff2*t})$

Where 1 signifies the slow phase and 2 signifies the rapid phase. R_{max} is the increase in resonance units above baseline attributed to either the rapid or slow phase

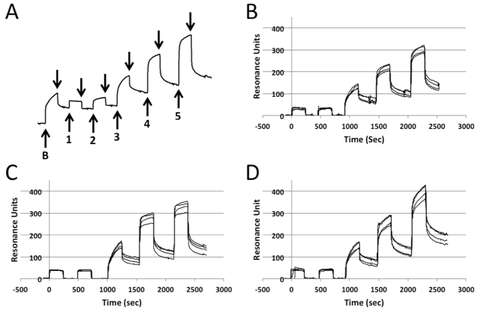

Figure 7 A) Figure depicting how BSA (B), then increasing concentrations of PMT-N (1) 7.5 nM, (2) 75 nM, (3) 750 nM, (4) 3.75 μM, (5) 7.5 μM) were injected over the synthetic vesicles. B) PC vesicles showed an intermediate binding compared to C) repressed slow phase binding to PC/PS and D) increased slow phase binding to PC/SP vesicles. PMT-N is shown to have preference for SP, especially in the slow phase.

SPR Studies to Understand Roles of SM, PC, and Protein in Binding

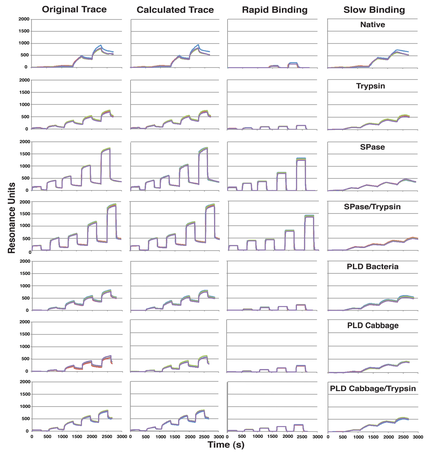

Figure 8 Plots depicting how various treatments affect binding of increasing concentrations of PMT-N to membrane surfaces (75 nM, 750 nM, 3.75 μM, 7.5 μM, and 15 μM). Native trace lacks 15 μM treatment. Calculated traces mirror the actual traces, indicating high fidelity of model fit to data. Results show that the removal of sphingomyelin shows a visible increase of the rapid phase while treatment with any of the lipases decreases the slow phase. Of note is that treatment with any enzyme outside of PLD Cabbage caused an increase at lower concentrations of PMT. Calculated curves were used to calculate kinetic values in **Table 1**.

	Rapid Component			Slow Component				
	$*B_{max}$ (RU)	$*K_D x10^6$ (M)	k_{off} $x10^2$ (s^{-1})	B_{max} (RU)	K_D $x10^6$ (M)	k_{off} $x10^4$ (s^{-1})	k_{on} $x10^{-2}$ ($M^{-1}s^{-1}$)	N (x4)
Reconstituted membranes								
PC	243±2	3.78±0.12	29.6±24.3	165±30	0.54±0.10	8.46±2.77	16.1±6.2	3
PC/PS	221±1	*1.57±0.03*	24.4±15.6	185±30	*0.41±0.11*	5.58±0.75	14.0±3.0	3
PC/SP	264±3	2.80±0.13	26.6±15.5	*256±42*	**0.84±0.21**	6.57±0.51	**8.22±1.93**	3
HEK-293T cell membranes								
Native	530±29	12.4±1.1	4.9±1.0	802±193	0.68±0.24	5.7±1.1	9.3±2.8	4
Trypsin	**201±1**	*3.07±0.15*	5.3±0.7	**572±52**	0.60±0.07	6.4±0.3	10.8±1.3	2
SPase	*4785±99*	**38.3±1.4**	**15.7±6.6**	**525±82**	**1.60±0.76**	5.2±2.4	**3.3±0.4**	2
SPase/T	*5516±64*	**48.5±0.9**	**14.0±5.5**	**616±54**	**1.46±0.63**	5.2±1.7	**3.7±0.5**	3
PLDCab	423±3	14.3±0.3	**12.9±3.3**	**423±83**	0.91±0.32	8.6±1.3	10.2±2.8	3
PLDCab/T	416±3	*9.44±0.24*	**11.1±0.3**	**548±37**	0.79±0.19	7.5±0.8	10.2±3.0	2
PLDSc	**313±8**	*7.18±0.32*	**11.6±4.6**	**518±48**	0.68±0.13	6.8±1.1	10.2±1.7	3

Table 1 Thermodynamic statistics indicate that for the slow phase believed to be important for productive binding, protein, PC, and SP are all important. However, only removal of SP causes a significant decrease in the kinetics of binding affinity for membrane extracts. It also causes an independent significant increase in the total number of looser binding sites. The importance of SP is confirmed in native vesicles where doping PC vesicles with SP increases the maximal binding at a slight cost to binding kinetics.

CD Studies to Understand pH Dependence of Interaction

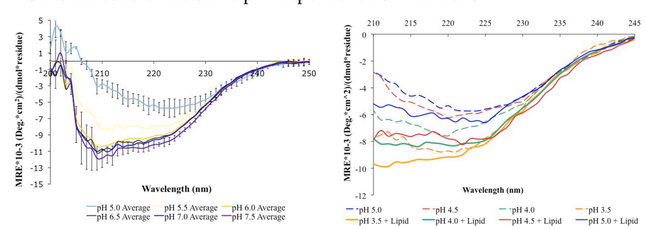

Figure 9 Circular dichroism of PMT-N shows a denaturation of the protein as pH decreases from physiological to endosomal pH (left). Spiking the solution with PC vesicles reduced the loss of alpha helical structure (seen at 210 nm), indicating that the PMT-N interacts with PC vesicles in a stabilizing manner under conditions similar to the late endosome.

Future Work

- Optimize Isolation of Binding Domain from PMT-N
- Optimize purification of putative translocation domain of PMT-N
- Use SSNMR to characterize binding pocket of PMT-N to binding domain
- Identify protein via GST-pull-down assay and confirm role it plays in entry into the cell.
- Generate atomic resolution structures via NMR of the binding domain.

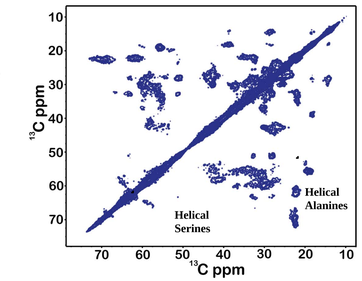

Figure 10 CC-2D spectra of PMT-N precipitated in PEG shows the structure is maintained in the solid state and that atomic resolution structures of the smaller fragments can be solved using current techniques.

Conclusions

- Data suggests that the binding domain of PMT does not bind gangliosides as previously reported and instead interacts with choline headgroups present on the cell surface.
- SM plays a receptor role that may be analogous to the role of gangliosides as a co-receptor for botulinum neurotoxins *in vivo*.
- PC and protein also appear to play a role, but the role of a specific protein receptor is yet undefined.
- Once in the endosome, low pH triggers PMT-N to embed itself within the membrane. In the full-length toxin, this is followed by subsequent translocation of the catalytic domain PMT-C.
- Fragmentation of PMT-N is allowing for atomic resolution structural interrogation of these interactions.

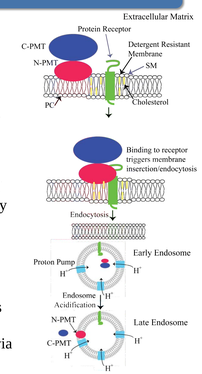

Figure 11 Figure of the proposed model for how PMT-N binds to choline on the cell surface, is funneled towards SM enriched regions, binds irreversibly to its protein partner, enters the cell via endocytosis, and then mediates escape of the catalytic domain.

References

•Boone, C. W., Ford, L. E., et al. (1969). "Isolation of Plasma Membrane Fragments from Hela Cells." *Journal of Cell Biology* **41**(2): 378-390.

•Brothers, M. C. and Ho, M. et al. Submitted, FEBS Journal, 2011.

•Dudet, L.I., Chailler, P., et al. (1996). "*Pasteurella multocida* toxin stimulates mitogenesis and cytoskeleton reorganization in Swiss 3T3 cells." *Journal of Biological Chemistry* **271**(1): 439-445.

•Pettit, R.K.; Rimler, R.B.; Ackermann, M.R. (1993a). "Receptor -mediated bnding of *Pasteurella multocida* dermonectrotic toxin to canine osteosarcoma and monkey keidne(vero) cells." *Laboratory Investigations* **69**: 94-100

•Wilson, B.A.; Ho, M.. "*Pasteurella multocida* toxin." *The Comprehensive Sourcebook of Bacterial Protein Toxins*. Alouf and Popoff. Elsevier, 2006. p. 430-447.

•Wilson, B.A.; Ponferrada, V.G.; Vallance, J.E.; Ho, M. (1998). "Localization of the intracellular activity domain of *Pasterurella multocida* toxin to the n terminus." *Infection and Immunity* **67** (1): 80-87

Acknowledgments

This project was funded through the fellowship program from the Department of Homeland Security, Science and Technology Directorate, Office of University Programs.

This project was also funded through the following sources:

NIH/NIAID grant AI038396 (to B.A.W).

University of Illinois Chemical Biology Training Grant.

For Further Information

Please contact brother3@illinois.edu. More information on this and related projects can be obtained on http://www.scs.uiuc.edu/~rienstra/ and http://mcb.illinois.edu/faculty/profile/1203