Characterisation of a Novel Subtilase Cytotoxin from Shiga Toxigenic *Escherichia coli*

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A thesis submitted in fulfilment of the requirements
for the degree of Doctor of Philosophy

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October 2008

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Abstract

Subtilase cytotoxin (SubAB) is the prototype of a novel class of AB_5 cytotoxins produced by Shiga-toxigenic *Escherichia coli* (STEC). The A subunit (SubA) is a serine protease that cleaves the ER chaperone BiP causing cell death by a previously-undetermined mechanism. The B subunits of AB_5 toxins typically recognise host cell glycan receptors and direct the subcellular transport of the A subunit. Although the function of SubA and its intracellular substrate have been elucidated, the B subunit (SubB) is relatively uncharacterised.

The subcellular trafficking pathway of SubAB was initially examined. SubAB conjugated to Oregon Green 488 (SubAB-OG) was internalised by Vero cells by 5 min, and co-localised with its ER target BiP within 30 min. When Vero cells were incubated with SubAB-OG and either Alexa Fluor 594-conjugated Cholera toxin B subunit (CtxB-AF594) or Texas Red-conjugated Shiga toxin B subunit (StxB-TR), individual cells exhibited differential toxin uptake. This was shown to be cell cycle-dependent, in which, SubAB-OG was preferentially internalised by cells migrating through G1 and early S phases. In contrast, CtxB-AF594 was taken up by cells in S through M phases and by a majority of cells in G1, while StxB-TR endocytosis occurred in cells traversing G1. Fluorescent SubAB co-localised with the clathrin marker transferrin, but not with Caveolin-1 (a marker for cholesterol-associated caveolae) and was subsequently trafficked via a retrograde pathway to the TGN, Golgi and ER. The clathrin inhibitor phenylarsine oxide prevented SubAB entry and BiP cleavage in SubAB-treated Vero, HeLa and N2A cells, while cholesterol depletion did not, demonstrating that, unlike either Stx or Ctx, SubAB internalisation is exclusively clathrin-dependent.

Identification of the SubB receptor was initially approached using toxin overlay assays in which Vero cell glycolipid extracts were separated by thin-layer chromatography and overlaid with SubAB. SubAB exhibited a high affinity for particular acidic species in the ganglioside fraction. However, none co-migrated with commercial glycolipid standards. SubAB-OG also exhibited an affinity for the oligosaccharide structures of chimeric LPS from GM_2 and GM_3 bacterial receptor mimic constructs in an LPS toxin overlay assay. Glycan array analysis revealed that SubB possessed a unique affinity for carbohydrate receptors with a terminal Neu5Gcα(2→3)Galβ disaccharide. Monovalent receptor analogues with distal Neu5Gc or Neu5Gcα(2→3)Galβ and highly-sialylated α_1-AGP did not prevent endocytosis of SubAB-OG, BiP cleavage or cytotoxicity in Vero cells. This indicated that SubAB has a greater affinity for the host cell receptors than the receptor analogues and may engage multiple receptors displayed on a lipid bilayer.

In addition to mediating toxin binding and subcellular trafficking, CtxB and StxB can also potentiate the immune response to co-administered antigen. Accordingly, the systemic immunomodulatory properties of SubB administered by the i.p. route were assessed in mice. Using $SubA_{A272}$ as a bystander antigen, SubB significantly increased mouse anti-$SubA_{A272}$ titres to levels that were comparable to those obtained using Alum adjuvant. However, when admixed with structurally-unrelated OVA, SubB did not significantly affect anti-OVA titres whereas Alum and CtxB did. This indicated that SubB may function as a systemic carrier protein (rather than an adjuvant) for particular antigens.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university of tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

Damien Christopher Chen Sau Chong

Acknowledgements

I was once told that if you want to go exploring, you have to move out of your comfort zone. Indeed, such a situation was aptly encapsulated by Dorothy in *The Wizard of Oz*: "I've got a feeling we're not in Kansas anymore". However, despite the often esoteric nature of research, the pursuit of an answer is rarely achieved alone. While the following pages list the numerous persons to whom I am grateful for their assistance, I'd particularly like to thank James and Adrienne Paton for their enthusiasm as supervisors and unrelenting challenges. It was through their questioning which they granted me the intellectual freedom one desires when undertaking a truly enviable project. My gratitude extends to those who have assisted in the development and adaptation of new techniques including, but not limited to, Stephen Gregory, Peter Sharp (Women's and Children's Hospital), Ursula Talbot, Cheleste Thorpe (Tufts-New England Medical Center), Lyn Waterhouse (Adelaide Microscopy) and Jo White.

My veneration goes to Renato Morona and Uwe Ströher who have donated countless hours and suggestions to address the differential with their perspicacity, ensuring that my aforementioned freedom was not limited by my inexperience. As always, those of the Paton and Morona labs receive thanks for creating a highly professional yet social environment where every logistical quandary was analysed and *every* conversation topic breached. Due to poor timing of my lunch with such conversations, you have inadvertently imbued me with iron guts and I have learnt to appreciate my meals, untainted, all the more. Thankyou to the chocoholics and alcoholics (i.e. students from the Discipline) who were forever willing to indulge my impieties and deepest regrets for the resulting pancreatic, brain and liver damage. To the

shooters, cinephiles, café connoisseurs and dwellers of The Apothecary: thanks for the great times and for reminding me of a world outside the lab. With the completion of this thesis and the subsequent abating of encephalitic stress, the following is a list of those who have helped during this journey into unfamiliar realms and, most importantly, nurtured my interest in research as they, too, simply wanted to pursue an answer to a question. If I have omitted any names, please accept my apologies; I blame the aforementioned brain damage.

Supervisors
Adrienne Paton
James Paton

Paton Laboratory
James Byrne
Jan Cook
Tony Focareta
Emma Gordon
Marcin Grabowicz
Rikki Graham
Richard Harvey
Sylvia Herold
Kim LeMessurier
Layla Mahdi
Kerrie May
Lauren McAllister
David Miller
Judy Morona
David Ogunniyi
Maggie Papadopoulos
Bec Pinyon
Trish Rogers
Katie Spackman
Alistair Standish
Uwe Ströher
Ursula Talbot
Hui Wang

Morona laboratory

Receptor Isolation and Identification
Grant Booker
John Bowie (Chemistry, University of Adelaide)

Tony Focareta
Peter Hoffmann (Adelaide Proteomics Centre, University of Adelaide)
Dan Peet
Peter Sharp (Chemical Pathology, Women's and Children's Hospital, Adelaide)
David Smith (Consortium for Functional Glycomics, The Scripps Research Institute, La Jolla, CA)

Toxins and Fluorescence
Luisa van den Bosch
Jocelyn Darby (Institute of Medical and Veterinary Science, Adelaide)
Rob Moyer
Ursula Talbot
Cheleste Thorpe (Division of Geographic Medicine and Infectious Diseases, Tufts-New England Medical Center, Boston, MA)

Intracellular Trafficking
Lesley Crocker
Nick Eyre
Sandy Macintyre (Institute of Medical and Veterinary Science, Adelaide)
Patty Tam (Medicine and Pathobiology, University of Toronto, Toronto, Canada)

Cell Cycle
Stephen Gregory (CMGD, University of Adelaide)
Jo White

Microscopy
Renato Morona
Meredith Wallwork (Adelaide Microscopy, Adelaide)
Lyn Waterhouse (Adelaide Microscopy, Adelaide)
Ruth Williams (Adelaide Microscopy, Adelaide)

Adjuvanticity Assessment
Jan Cook
Ursula Talbot

With Special Thanks
Jan Cook
Renato Morona
Uwe Ströher
Ursula Talbot

Finally, thankyou to my closest friends and family for your support over the years and for enduring my incommunicado. Although I leave most of you behind at the close of this expedition, know that I could not have succeeded without the friendship, love and memories that have kept me sane for so long. It's time to see what's out there. It's time to go exploring once again.

As always, thanks to everyone in the building for putting up with my ugly face.

ABBREVIATIONS

Abbreviations accepted by the American Society for Microbiology are used in this thesis without definition. Additional abbreviations (listed below) are defined when first used.

$A_{[\#]}$	Absorbance at [wavelength in nm]
A/E	Attaching-effacing
AF[#]	Alexa Fluor [fluorochrome wavelength in nm]
Alum	Aluminium hydroxide
AP	Alkaline phosphatase
APC	Antigen presenting cell
Amp	Ampicillin
aGM_1	Asialo-GM_1
BCIP	5-bromo-4-chloro-3-indoyl-phosphate (X-phosphate)
BFA	Brefeldin A
BSA	Bovine serum albumin
Cmah	CMP-Neu5Ac hydroxylase
CPK	Creatine phosphokinase
CPZ	Chlorpromazine
Ctx	Cholera toxin
CtxA	Cholera toxin A subunit
CtxB	Cholera toxin B subunit
CV	Coefficient of variation
DAB	Diaminobenzidine
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DIG	Digoxigenin
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
FCS	Foetal calf serum
g	Gravity units
G418	Geneticin
Gb_3	Globotriaosyl ceramide
Gb_4	Globotetraosyl ceramide
GD_{1a}	Disialo-ganglioside 1a
GD_{1b}	Disialo-ganglioside 1b
GGS	Ganglioside
GM_1	Monosialo-ganglioside 1
GM_2	Monosialo-ganglioside 2
GM_3	Monosialo-ganglioside 3
GSL	Neutral glycosphingolipid
HCT-8	Human colonic epithelial cells
HRP	Horseradish peroxidise
HUS	Haemolytic uraemic syndrome
i.n	Intranasal
i.p.	Intraperitoneal
IPTG	Isopropyl-beta-D-thiogalactoside

Kan	Kanamycin
LB	Luria Bertani broth
LD_{50}	50% lethal dose
LEE	Locus for enterocyte effacement
LPS	Lipopolysaccharide
LT	Heat labile enterotoxin
MβCD	Methyl-β-cyclodextrin
MALDI	Matrix-assisted laser desorption/ionisation
MQ	Milli Q
MR	Molar ratio (dye:protein)
MS	Mass spectroscopy
OG	Oregon Green 488
ORF	Open reading frame
OVA	Ovalbumin
PAO	Phenylarsine oxide
PBS	Phosphate-buffered saline
Pen-Strep	Penicillin and streptomycin
PIBM	Polyisobutylmethacrylate
PH3	Phospho-Histone PH3
POD	Peroxidase
PVDF	Polyvinylidene difluoride
^{R}	Resistant
R_f	Retardation fraction
RFU	Relative fluorescence units
RMC	Receptor mimic construct
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error about the mean
ST	Heat-stable toxin
STDEV	Standard deviation
STEC	Shiga toxigenic *Escherichia coli*
Stx	Shiga toxin
StxB	Shiga toxin B subunit
SubA	Subtilase cytotoxin A subunit
SubAB	Subtilase cytotoxin
SubB	Subtilase cytotoxin B subunit
TBS	Tris-buffered saline
TE	Tris-EDTA buffer
TEM	Transmission electron microscopy
TEMED	N,N,N'N'-tetramethyl-ethylene-diamine
TGN	*Trans*-Golgi network
TLC	Thin-layer chromatography
TR	Texas Red
TSA	Tris-saline azide
TTBS	Tween-Tris-buffered saline
WGA	Wheat germ agglutinin
VP-SFM	Virus production serum-free medium
VT	Vero cytotoxin