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Investigation of the role of the plasminogen-binding group A streptococcal M-like protein (PAM) in the pathogenesis of Streptococcus pyogenes

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Investigation of the role of the plasminogen-binding group A streptococcal M-like protein (PAM) in the pathogenesis of Streptococcus pyogenes

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Declaration of Authenticity

This thesis is submitted in accordance with the regulations of the University of Wollongong in fulfillment of the degree of Doctor of Philosophy. It does not include any material previously published by another person except where due reference is made in the text. The experimental work described in this thesis is original, and has not been submitted for a degree to any other University.

Martina Louise Sanderson-Smith

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"Whereas in art nothing worth doing can be done without genius, in science, even a very moderate capacity can contribute to a supreme achievement"

Bertrand Russell 1872-1970

TABLE OF CONTENTS

List of Figures

List of Tables

List of Abbreviations

Ap	ampicillin
APS	ammonium persulfate
ARF	acute rheumatic fever
BSA	bovine serum albumin
CD	circular dichroism
CFU	colony forming units
DAB	diaminobenzidine
DIG	digoxigenin-11-ddUTP
EC_{50}	effective concentration of competitor required to decrease binding by 50%
ECM	extracellular matrix
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAS	group A streptococcus
GST	glutathione-S-transferase
6-His	hexahistidyl
HRP	horseradish peroxidase
K*d*	dissociation equilibrium constant
KLH	keyhole limpet hemocyanin
Km	kanamycin
LB	Luria Bertani
LBS	lysine-binding sites
NPBP	nephritogenic plasminogen-binding protein
OD	optical density
NTP	N-terminal peptide
PAGE	polyacrylamide gel electrophoresis
PAI-1	plasminogen activator inhibitor 1
PAI-2	plasminogen activator inhibitor 2
PAM	plasminogen-binding group A streptococcal M-like protein
PAS	plasminogen activation system
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGE	pulsed field electrophoresis
PMSF	phenyl-methyl-sulfonyl fluoride
SDS	sodium dodecyl sulphate
SEN	streptococcal surface enolase
Serpin	serine protease inhibitors
SIC	streptococcal inhibitor of complement- mediated lysis
Spe	streptococcal pyrogenic exotoxins
ST	sequence type
STSS	streptococcal toxic shock syndrome
TBS	tris-buffered saline
TEMED	N,N,N',N'-tetra-methylethylenediamine
THB	Todd Hewitt broth
THBN	Todd Hewitt broth 1% neopeptone
THBY	Todd Hewitt broth 1% yeast
tPA	tissue-type plasminogen activator
uPA	urokinase-type plasminogen activator
UV	ultra violet

List of Publications and Conference Presentations

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[*] See Chapter 9

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Summary

The Gram positive bacterium *Streptococcus pyogenes* (group A streptococcus; GAS) is the major etiological agent of a variety of skin and mucosal infections in humans. Whilst the majority of GAS infection results in only mild, uncomplicated disease, the migration of GAS from superficial to deep tissue sites can result in invasive infection. In recent years, there has been a resurgence in severe GAS disease, however, the details of GAS pathogenesis have yet to be fully elucidated. Increasingly, subversion of the host plasminogen activation system is being implicated in the virulence of *S. pyogenes*. GAS display receptors for the human zymogen plasminogen on the cell surface, one of which is the plasminogen-binding group A streptococcal M-like protein (PAM). PAM has been implicated in the pathogenesis of certain GAS isolates, but the mechanism of plasminogen binding by PAM, and the role of this interaction in the pathogenesis of GAS, requires further investigation. Thus, the focus of this thesis has been to characterise plasminogen binding by PAM and a number of naturally occurring PAM variants.

Characterisation of PAM genes from 13 GAS isolates revealed that whilst these molecules are highly conserved in the c and d repeat domains, they display significant variation within the plasminogen binding repeat motifs (a1/a2). Percent identity to the prototype PAM a1/a2 repeat sequence ranged from 52% to 100% amongst the variants studied here. No correlation was seen between the presence of a PAM gene, or variation within the sequence of PAM, and site of GAS isolation. In order to determine the impact of sequence variation on protein function, recombinant proteins representing six naturally occurring variants of PAM, together with a recombinant M1 protein were expressed and purified. Equilibrium dissociation constants for the interaction of PAM

variants with biotinylated glu-plasminogen ranged from 1.58 nM to 7.58 nM. Effective concentrations of prototype PAM required for 50% inhibition of plasminogen binding to immobilised PAM variants ranged from 0.34 nM to 22.06 nM. These results suggest that while variation in the a1/a2 region of the PAM protein does affect the comparative affinity of PAM variants, the functional capacity to bind plasminogen at physiologically relevant concentrations is conserved. Additionally, a potential role for the a1 region of PAM in eliciting a protective immune response was investigated using a mouse model for GAS infection. The a1 region of PAM was found to protect immunised mice challenged with a homologous PAM-positive GAS strain. These data suggest a link between selective immune pressure against the plasminogen-binding repeats and the functional conservation of the binding domain in PAM variants.

Site-directed mutagenesis of full length PAM_{NS13} protein from an invasive GAS isolate was undertaken to assess the contribution of residues in the a1 and a2 repeat domains to plasminogen binding function. Mutagenesis to alanine of key plasminogen binding site lysine residues in the a1 and a2 repeats (Lys^{98} and Lys^{111}) did not abrogate plasminogen binding by PAM, nor did additional mutagenesis of Arg^{101}, His^{102} and Glu^{104}, which have previously been implicated in plasminogen binding by PAM. Plasminogen binding was only abolished with the additional mutagenesis of Arg^{114} and His^{115} to alanine. Furthermore, mutagenesis of both arginine (Arg^{101} and Arg^{114}) and histidine (His^{102} and His^{115}) residues abolished interaction with plasminogen despite the presence of Lys^{98} and Lys^{111} in the binding repeats. This study shows for the first time that residues Arg^{101}, Arg^{114}, His^{102} and His^{115} in both the a1 and a2 repeat domains of PAM can mediate high affinity plasminogen binding. These data suggest that highly conserved arginine and histidine residues may compensate for variation elsewhere in the a1 and a2

plasminogen binding repeats, and may explain the maintenance of high affinity plasminogen binding by naturally occurring variants of PAM.

Initial sequence characterisation of PAM variants in this study revealed a phylogenetically distinct PAM variant, $PAM_{NS88.2}$. This variant binds plasminogen with high affinity (K_d = 7.58 nM), despite displaying only 52% identity to the classical a1/a2 repeat domain of PAM. It was therefore of interest to characterise the putative plasminogen binding domain of $PAM_{NS88.2}$. Additionally, the association of GAS strain NS88.2, from which $PAM_{NS88.2}$ was isolated, with the invasive disease bacteraemia, makes it a candidate for virulence studies employing the recently developed human plasminogen transgenic mouse. Site-directed mutagenesis of the putative plasminogen binding site indicated that as with PAM_{NS13}, $PAM_{NS88.2}$ does not interact with plasminogen exclusively via lysine residues. Mutagenesis to alanine of lysine residues Lys^{96} and Lys^{101} reduced but did not abrogate plasminogen binding by $PAM_{NS88.2}$. Plasminogen binding was only abolished with the additional mutagenesis of Arg^{107} and His^{108} to alanine. Furthermore, mutagenesis of Arg^{107} and His^{108} abolished plasminogen binding by $PAM_{NS88.2}$ despite the presence of Lys^{96} and Lys^{101} in the binding site. Given that GAS strain NS88.2 is associated with the invasive disease bacteraemia, and is virulent in the humanised plasminogen transgenic mouse, the successful abrogation of plasminogen binding by $PAM_{NS88.2}$ may facilitate the development of a $PAM_{NS88.2}$ allelic replacement isogenic mutant for use in future studies involving this model.

This study examines in detail the interaction of PAM and PAM variants with the human zymogen plasminogen. The maintenance of plasminogen-binding function in spite of binding site sequence variation suggests that the ability to interact with plasminogen is

evolutionarily advantageous to a subset of GAS isolates. Additionally, this study provides previously unreported details of the ability of PAM to interact with plasminogen independently of binding site lysine residues. These findings have implications for both the future identification of novel plasminogen binding proteins, and may facilitate both the understanding of the role of PAM in GAS disease, and the development of therapeutics to assist in the treatment and prevention of streptococcal infection.