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Characterising the role of streptokinase polymorphism in the pathogenesis of streptococcus pyogenes

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Characterising the Role of Streptokinase Polymorphism in the Pathogenesis of *Streptococcus pyogenes*

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THESIS CERTIFICATION

I, Simon M. Cook, declare that this thesis, submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy, in the Department of Biological Sciences, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

Simon M. Cook

2014

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TABLE OF CONTENTS

THESIS CERTIFICATION ..... **i**
ACKNOWLEDGEMENTS ..... **ii**
LIST OF FIGURES ..... **vii**
LIST OF TABLES ..... **xi**
LIST OF ABBREVIATIONS ..... **xii**
CHAPTER PUBLICATIONS ..... **xv**
LIST OF CONFERENCE PRESENTATIONS ..... **xvii**
ABSTRACT ..... **xix**

Chapter 1: Literature Review **1**
Preface **2**
1.1 Overview **3**
1.2 Classification of Group A Streptococcus **4**
1.2.1 Lancefield Classification 5
1.2.2 M-serological Typing 5
1.2.3 *emm* Sequence Typing 6
1.3 Diseases and Sequelae Resulting from Group A Streptococcal Infection **6**
1.4 Epidemiology of GAS Disease **7**
1.5 GAS Virulence Factors **9**
1.6 The Plasminogen Activation System **13**
1.6.1 Plasminogen 13
1.6.2 Group A Streptococcus and the Plasminogen Activation System 18
1.6.3 GAS Plasmin(ogen) Cell Surface Receptors 19
1.6.3.1 Plg-binding Group A Streptococcal M-like Proteins (PAM and Prp) 19
1.6.3.2 Streptococcal Surface Enolase (SEN) 20
1.6.3.3 Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) 21
1.6.3.4 Indirect Interaction of GAS with Plg 21
1.7 Streptokinase **22**
1.7.1 Mechanism and Structure 22
1.7.4 Roles of α, β and γ Domains of Streptokinase in Plg Activation 27
1.7.2 Conjecture in the Molecular Mechanism that Streptokinase employs to Activate Plg 29
1.7.3 Variability of Streptokinase from GAS 32
1.8 Streptokinase as a Thrombolytic Therapeutic **36**
1.8.1 Overview 36
1.8.2 Comparison of Plg Activators Used in Thrombolytic Therapy 37
1.8.3 Enhancing Streptokinase for use as a Therapeutic 39
1.9 Summary and Aims of the Current Research **43**

Chapter 2: Cloning and Expression of Streptokinase Variants from *Streptococcus pyogenes* **45**
Preface **46**
2.2 Introduction **49**
2.3 Materials and Methods **52**

2.3.1 Bacterial strain, culture conditions and reagents....52
2.3.2 DNA cloning strategy for *ska* variants....52
2.3.3 Restriction enzyme digestion....54
2.3.4 Ligation of recombinant plasmids....54
2.3.5 Agarose gel electrophoresis....55
2.3.6 Preparation of electro-competent *E. coli* JM109 and M15/pREP4....55
2.3.7 Electro-transformation of electro-competent E. coli JM109 and M15/pREP4 56
2.3.8 DNA sequence analysis of pQE-30::*ska* variant constructs....56
2.3.9 Expression and purification of recombinant SK protein....58
2.3.10 Electrospray ionisation mass spectrometry (ESI-MS)....59
2.3.11 Anion-exchange fast protein liquid chromatography (FPLC)....60
2.3.12 Gel electrophoresis and Western blotting....60
2.4 Results....62
2.4.1 Amplification and cloning of *ska* alleles....62
2.4.2 Expression and purification of recombinant SK variants....63
2.4.3 Nano-electrospray ionisation mass spectrometry (Nano-ESI-MS)....66
2.4.4 Anion exchange chromatography....68
2.5 Discussion....70

Chapter 3: Streptokinase Variants from *Streptococcus pyogenes* Isolates Display Altered Plasminogen Activation Characteristics – Implications for Pathogenesis. 73
Preface....74
3.1 Abstract....75
3.2 Introduction....76
3.3 Materials and Methods....80
3.3.4 Far-UV circular dichroism (CD) spectroscopy....80
3.3.5 Non-proteolytic active site generation in Glu-Plg....80
3.3.6 Surface plasmon resonance....81
3.3.7 Glu-Plg activation assays....82
3.3.8 Inhibition of amidolysis by α_2-antiplasmin....82
3.3.9 Allelic exchange mutagenesis....83
....85
3.3.10 Growth comparison of *S. pyogenes* strains....86
3.3.11 Hyaluronic acid capsule determination....86
3.3.12 GAS strain streptokinase expression....86
3.3.13 Transgenic murine infection model....87
3.3.14 Statistical analyses....87
3.3.15 Ethics permissions....87
3.3.16 Accession numbers....88
3.4 Results....89
3.4.1 Expression and secondary structural analysis of recombinant SK proteins....89
3.4.2 Non-proteolytic active site generation in Glu-Plg by variant SK....91
3.4.3 Binding affinity of SK variants to human Glu-Plg and plasmin....93
3.3.4 Plasminogen activation by variant SK-plasmin activator complexes....93
3.3.5 Role of SK variation in GAS pathogenesis....95
3.5 Discussion....98

Chapter 4: Site Restricted Plasminogen Activation Mediated by Group A streptococcal Streptokinase Variants **104**
Preface **105**
4.1 Abstract **106**
4.2 Introduction **107**
4.3 Materials and Methods **111**
4.3.1 Bacterial strain, culture conditions and reagents 111
4.3.2 Cloning, expression and purification of recombinant proteins 111
4.3.3 Non-proteolytic active site generation in Glu-Plg 112
4.3.4 Plasminogen activation by variant SK-Plg complexes 113
4.3.5 Effect of fibrin on the amidolytic activity of variant SK-plasminogen complexes 114
4.3.6 Plasma clot lysis assay 115
4.3.7 Plasma fibrinogen depletion by SK variant plasminogen activation 116
4.3.8 Accession numbers 116
4.3.9 Ethics approval: 117
4.4 Results **118**
4.4.1 Non-proteolytic active site generation in Glu-Plg by variant SK 118
4.4.2 Effect of GAS cell surface Plg receptors on non-proteolytic active site generation in Glu-Plg by variant SK 120
4.4.3 Non-proteolytic active site generation in human plasma by variant SK 121
4.4.4 Regulation of soluble Plg activation activity of type-2b SK-Plg variants by host α_2-AP 123
4.4.5 The effect of fibrin on the activity of type-2b SK variants 125
4.5 Discussion **127**

Chapter 5: Elucidating the Molecular Mechanism of Streptokinase Function through the Construction of Chimeric Proteins from *Streptococcus pyogenes* Streptokinase Variants **134**
Preface **135**
5.1 Abstract **136**
5.2 Introduction **138**
5.3 Materials and Methods **140**
5.3.1 Bacterial strain, culture conditions and reagents 140
5.3.2 Cloning strategy for construction of chrimeic *ska* allele 140
5.3.3 Expression and purification of recombinant SK protein 143
5.3.4 Far-UV circular dichroism (CD) spectroscopy 143
5.3.5 Non-proteolytic active site generation in Glu-Plg 143
5.3.6 Plasminogen activation by variant SK-plasmin complexes 144
5.4 Results **145**
5.4.1 Determination of secondary structure by far-UV circular dichroism (CD) spectroscopy 146
5.4.2 Non-proteolytic active site generation in Glu-Plg by variant SK 147
5.4.3 Plasminogen activation by variant SK-plasmin-plasmin activator complexes 148
5.5 Discussion **150**

Chapter 6: Conclusion **155**

Appendices: **159**
Preface **160**
Appendix 1: DNA sequences of variant *ska* inserts in pQE-30::*ska* constructs **161**
Appendix 2: Expression and Purification of Recombinant SK Variants **164**
A.2.1 Expression and purification of SK using Ni^{2+}-NTA affinity chromatography **164**
A2.2 Factor Xa cleavage of SK variants **168**
A2.3 Additional anion exchange chromatography **170**
A2.4 Denaturing purification protocol for recombinant SK proteins **174**
Appendix 3: Enzyme Kinetic Analysis of SK variants **176**
A3.1 Steady state amidolytic kinetics analysis of SK-Plg variants **177**
A3.2 Steady state Plg activation assays **180**
A3.3 Calculation of plasmin amidase activity **184**
Appendix 4: Binding Interaction Analysis of SK Variants to Glu-Plg and plasmin **185**
A4.1 Solid state binding assays of SK variants to Plg **185**
A4.2 Surface plasmon resonance studies to determine affinity constants for variant SK interaction with Glu-Plg and plasmin **188**
Appendix 5: Effect of Fibrin on SK Amidolytic Activity and Clot Lysis Capacity **194**
A.5.1 Effect of fibrin on SK variant amidolytic activity **194**
A.5.2 Plasma clot lysis capacity of SK variants **195**
Appendix 6: Buffers, Media and Solutions **196**
Appendix 7: Plasmids Used in this Study **203**
Appendix 8: Construction of DNA Standard Curve **204**

References **206**

LIST OF FIGURES

Figure 1.1: Schematic of the mammalian plasminogen activation system 14
Figure 1.2: Schematic structure of Glu-Plg and Glu-plasmin..... 15
Figure 1.3: Diagrammatic representation of the closed and open conformations of plasminogen. 17
Figure 1.4: Crystal structure of streptokinase 23
Figure 1.5: Schematic representation of the steps involved in the activation of Plg by SK. 25
Figure 1.6: Mechanism for coupling of conformational and proteolytic activation of Plg by SK. 26
Figure 1.7: Phylogenetic tree for a 423 bp variable region encoding the β -domain of streptokinase. 33
F**igure 1.8:** Schematic diagram summarising the hypothesised pathways of cell surface plasmin acquisition by GAS strains expressing different alleles of SK..... 34
Figure 2.1: Exposure of the functional Ile_1 residue by limited proteolysis with Factor Xa 59
Figure 2.2: PCR amplification of *ska* from group A streptococcal cDNA 65
Figure 2.3: Restriction digestion of pQE-*ska* constructs 63
Figure 2.4: Expression and purification of native recombinant poly-histidine tagged fusion protein SK_{ALAB49} via FPLC Ni^{2+}-NTA affinity chromatography..... 62
Figure 2.5: Factor Xa cleavage of recombinant poly-histidine tagged SK_{ALAB49} fusion protein..... 66
Figure 2.6: A positive ion Nano-ESI-mass spectrum of purified SK_{ALAB49} in 10 mM ammonium acetate (pH 6.8) containing 0.1% formic acid after Factor Xa cleavage. 67
Figure 2.7: Anion-exchange fast protein liquid chromatography of SK_{ALAB49}..... 69
Figure 3.1: Schematic example of allelic exchange mutagenesis to create GAS strain 5448 isogenic mutants by replacement of the parental ska_{5448} with ska_{ALAB49}..... 85

Figure 3.2: ClustalW Multiple alignment of deduced amino acid sequences of the SK protein variants used in this study. 90
Figure 3.3: SDS-PAGE and Far-UV circular dichroism spectroscopy analysis of recombinant SK proteins used in this study 91
Figure 3.4: Non-proteolytic active site generation in Glu-Plg by SK variants and influence of Glu-Plg conformation on SK-Plg* generation ..... .92
Figure 3.5: Plg activation by SK-plasmin variant activator complexes and inhibition of variant SK-plasmin complexes by α_2-antiplasmin. 95
Figure 3.6: In vitro characterisation of wild type GAS strain 5448 and isogenic mutant strains 5448Δ*ska*, 5448::ska_{ALAB49} and 5448::ska_{NZ131} 96
Figure 3.7: Survival curves after subcutaneous infection of humanised Plg transgenic AlbPLG1 mice with GAS strain 5448 and isogenic mutants 97
Figure 4.1: Effect of Fg and Fg fragments on non-proteolytic active site generation in Glu-Plg and plasminogen activation by SK variants. 119
Figure 4.2: Effect of GAS cell surface Plg receptors on non-proteolytic active site generation in Glu-Plg and plasminogen activation by SK variants. 120
Figure 4.3: Non-proteolytic active site generation in Glu-Plg and plasminogen activation by SK variants in human plasma 122
Figure 4.4: Effect of Fg and PAM on inhibition of SK variant mediated Plg activation ability by α_2-AP 124
Figure 4.5: Generation of amidolytic activity and resistance to inhibition of variant SK-Plg complexes formed in the absence or presence of a fibrin clot 126
Figure 4.6: Induction of plasma clot lysis by SK variants and capacity to deplete plasma Fg 127
Figure 4.7: Schematic diagram summarising the site/surface restricted activation of Plg by type-2b SK expressing GAS strains. 132
Figure 5.1: Strategy for fragmentation and construction of domain swap chimeras between type-1 and type-2b variants of *ska*. 141
Figure 5.2: Schematic example of the cloning strategy used to create chimeric SK proteins. 142

Figure 5.3: SDS-PAGE analysis composite of expressed recombinant chimeric SK proteins used in this study. 145
Figure 5.4: Far-UV circular dichroism spectroscopy analysis of recombinant SK proteins used in this study. 147
Figure 5.5: Non-proteolytic active site generation in Glu-Plg by SK variants and influence of Glu-Plg conformation on SK-Plg* generation 148
Figure 5.6: Plasminogen activation by variant SK-plasmin activator complexes 149
Figure A1.1: DNA sequence of mature streptokinase data from pQE-30::*ska* variant constructs..... 161-163
Figure A2.1.1: Expression and purification of native recombinant poly-histidine tagged fusion protein SK_{NZ131} via FPLC Ni^{2+}-NTA affinity chromatography. 164
Figure A2.1.2: Expression and purification of native recombinant poly-histidine tagged fusion protein $SK_{\alpha T1}$ via FPLC Ni^{2+}-NTA affinity chromatography ..... .165
Figure A2.1.3: Expression and purification of native recombinant poly-histidine tagged fusion protein SKc via bench top column Ni^{2+}-NTA affinity chromatography. 166
Figure A2.1.4: Expression and purification of native recombinant poly-histidine tagged fusion protein SK_{5448} via bench top column Ni^{2+}-NTA affinity chromatography..... 166
Figure A2.1.5: Expression and purification of native recombinant poly-histidine tagged fusion protein SK_{NS696} via bench top column Ni^{2+}-NTA affinity chromatography..... 167
Figure A2.1.6: Expression and purification of native recombinant poly-histidine tagged fusion protein $SK_{NS88.2}$ via bench top column Ni^{2+}-NTA affinity chromatography..... 167
Figure A2.2: Factor Xa cleavage of recombinant poly-histidine tagged SK variant fusion proteins. 168-169
Figure A2.3: Anion-exchange fast protein liquid chromatography of SK variants 170-173

Figure A3.1: Mechaelis-Menten kinetic analysis of the amidase properties of SK variants complexed with Glu-Plg. 177-179
Figure A3.2: Mechaelis-Menten kinetic analysis of the plasminogen activation . 182-183
Figure A3.3: Mechaelis-Menten kinetic analysis of the amidase activity of plasmin . 184
Figure A4.1: Solid state binding analysis of SK variants to immobilised recombinant Glu-Plg. 186-187
Figure A4.2.1: Surface plasmon resonance analysis of the interaction between SK variants and immobilised Glu-Plg. 188-190
Figure A4.2.2: Surface plasmon resonance analysis of the interaction between SK variants and immobilised plasmin. 191-193
Figure A5.1: Generation of amidolytic activity and the effect of variant SK-Plg complexes formed in the presence of a fibrin clot 194
Figure A5.2: Induction of plasma clot lysis by SK variants ..... .195
Figure A7.1: Plasmid map of pHSGXceIS1 203
Figure A7.2: Plasmid map of pQE-30 expression vector. 203
Figure A8.1: Standard curve constructed using GeneRuler™ DNA Ladder 204
Figure A9.1: Standard curve constructed of PageRuler™ Unstained Protein Ladder. 205

LIST OF TABLES

Table 1.1: Common diseases caused by *S. pyogenes* 8

Table 1.2: Established or putative cell-associated virulence factors of group A Streptococcus compiled from recent reviews 11

Table 1.3: Established or putative secreted virulence factors of group A Streptococcus compiled from recent reviews 12

Table 1.4: Comparison of thrombolytic agents that currently approved or being developed for human use 40

Table 2.1: Oligonucleotides used for DNA sequencing of chimeric *ska* alleles 57

Table 3.1: Association (k_{on}) and dissociation (k_{off}) rate constants and apparent equilibrium dissociation (K_D) constants for the interaction of SK variants with immobilised Plg and plasmin by SPR 94

Table 6.1: Summary of the phenotypic differences observed for the SK variants investigated in this study ..... .157

Table A3.1: Kinetic constants for amidolysis by variant SK-Plg complexes 180

Table A3.2: Kinetic constants for activation of Plg by variant SK-Plg complexes 184

Table A8.1: Mobility of DNA markers GeneRuler™ DNA Ladder 204

Table A9.1: Mobility of PageRuler™ Unstained Protein Ladder 205

LIST OF ABBREVIATIONS

α_2-AP	α_2-antiplasmin
α_2-MG	α_2-macroglobulin
aa	Amino acid
ARF	Acute rheumatic fever
APSAC	Anisoylated plasminogen streptokinase activator complex (antistreplase)
APSGN	Acute post-streptococcal glomerulonephritis
bp	Base pairs
CD	Circular dichroism
CFU	Colony forming units
cm	Centimetre
Da	Dalton
DAB	3,3'-diaminobenzadine
EDTA	Eethylenediaminetetraacetic acid
ESI-MS	Electo-spray ionisation mass spectrometry
Fbp	Fibronectin-binding protein
FDA	US Food and Drug Administration
Fg	Fibrinogen
FgD	Fibrinogen fragment D
FgE	Fibrinogen fragment E
FgR	Fibrinogen receptor
FPLC	Fast protein liquid chromotography
g	9.8 ms^{-2}
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAS	Group A streptococcus
h	Hours
IPTG	Isopropyl-β-D-thiogalactopyranoside
K1-K5	Kringle domain 1 – kringle domain 5
k_a	Binding association constant
k_{cat}	Substrate turnover rate

k_d	Binding dissociation constant
k_{plg}	Catalytic rate constant of Plg activation
k_s	Catalytic rate constant of amidolysis
K_D	Equilibrium binding constant
Km	Michaelis-Menten constant
K_{plg}	Dissociation constant for the activation of Plg by SK
K_s	Dissociation constant for the hydrolysis of S-2251 by plasmin
kDa	KiloDalton
kV	Kilovolts
LB	Luria-Bertani
min	Minutes
mm	Millimetre
MUGB	4-methylumbelliferyl p-guanidinobenzoate
m/z	Mass to charge ratio
nm	Nanometer
nPA	Lanoteplase
OD	Optical density
PAI-1	Plasminogen activator inhibitor 1
PAI-2	Plasminogen activator inhibitor 2
PAM	Plasminogen-binding GAS M-like protein
PAP	Preactivation peptide
PAS	Plasminogen activation system
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline containing 0.05% Tween-20
PCR	Polymerase chain reaction
Plr	Plasminogen receptor
Plg	Plasminogen
Pln	Plasmin
PMSF	Phenylmethylsulphonyl fluoride
pNA	p-nitroanaline
PVP	Polyvinyl pyrrolidone

r-PA	Recombinant plasminogen activator (reteplase)
s	Seconds
SDH	Streptococcal surface dehydrogenase
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEN	Streptococcal surface enolase
SEM	Standard error of mean
SK	Streptokinase
SPD	Serine protease domain
STSS	Streptococcal toxic shock syndrome
TAE	Tris-Acetate-EDTA
TBS	Tris buffered saline
TBST	Tris buffered saline containing 0.05% Tween-20
TNK-tPA	Recombinant-tPA (tenecteplase)
Tris-base	Tris(hydroxymethyl)aminomethane
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
tPA	Tissue-type plasminogen activators
Tween-20	Polyoxyethylenesorbitan monolaurate
U	Units
UV	Ultraviolet
uPA	Urokinase plasminogen activator
V	Velocity
Vmax	Maximum enzyme velocity
v/v	Volume/volume
w/v	Weight/volume

CHAPTER PUBLICATIONS

This thesis includes chapters that have been written as the following journal articles:

Chapter 1: McArthur J. D., **Cook, S. M**., Venturini, C., and Walker, M. J., (2012) "The role of streptokinase as a virulence determinant of *Streptococcus pyogenes* - Potential for Therapeutic Targeting." *Current Drug Targets* **13**: 297-307.

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Chapter 3: **Cook, S. M**., Skora, A., Gillen, C. M., Walker, M. J. and McArthur, J. D., (2012) "Streptokinase variants from Streptococcus pyogenes isolates display altered plasminogen activation characteristics - implications for pathogenesis." *Molecular Microbiology* **86**: 1052-1062.

Chapter 4: **Cook, S. M**., Skora, A., Walker, M. J., Sanderson-Smith M.L. and McArthur, J. D., (2013) "Site restricted plasminogen activation mediated by streptokinase variants from *Streptococcus pyogenes." Biochemical Journal* Immediate Publication, doi:10.1042/BJ20131305

Chapter 5: **Cook, S. M**., Walker, M. J. and McArthur, J. D., (2014) "Construction of chimeric streptokinase from *Streptococcus pyogenes* to assist in the molecular elucidation of streptokinase phenotype" (*Manuscript prepared – brief communication)*

As the primary supervisor, I, Dr. Jason D. McArthur, declare that the greater part of the work in each article listed above is attributed to the candidate, Simon M. Cook. In each of the above manuscripts, Simon contributed to study design and was primarily responsible for data collection, data analysis and data interpretation. The first draft of each manuscript was written by the candidate and Simon was then responsible for responding to the editing suggestions of his co-authors. The co-author, Professor Mark Walker was Simon's co-supervisor that contributed to data interpretation and manuscript preparation. Amanda Skora was responsible for assisting in recombinant protein purification for use in Chapter 3 and 4 as well as construction of *ska* allelic exchange mutants used in Chapter 3. Dr. Christine Gillen developed the cloning method for allelic exchange mutagenesis employed in Chapter 3. Dr. Martina Sanderson-Smith contributed to experimental design and manuscript preparation in Chapter 4. Simon has been responsible for submitting each manuscript for publication to the relevant journals, and has been in charge of responding to reviewers' comments, with assistance from his co-authors.

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ADDITIONAL PUBLICATION

Cook, S. M., and McArthur J. D., (2013) "Developing *Galleria mellonella* as a model host for human pathogens." *Virulence* **4**: 350-353

LIST OF CONFERENCE PRESENTATIONS

Simon M. Cook, Amanda Groznik, Mark J. Walker and Jason D. McArthur 2013 Phenotypic Characterisation of streptokinase variants from *Streptococcus pyogenes*: implications for disease pathogenesis. Thompson Prize Final, Sydney Protein Group, University of Western Sydney, Macarthur, NSW, Australia. – *Oral presentation*

Simon M. Cook, Amanda Skora, Christine Gillen, Mark J. Walker and Jason D. McArthur 2012 Streptokinase variants from *Streptococcus pyogenes* isolates display altered plasminogen activation characteristics – implications for pathogenesis. Plasminogen Activation System in Pathology. Wollongong, NSW, Australia – *Oral presentation*

Simon M. Cook, Amanda Skora, Mark J. Walker and Jason D. McArthur 2012 Biochemical characterisation of plasminogen activators: tips, tricks and pitfalls using streptokinase variants from *S. pyogenes*. Plasminogen Activation System in Pathology. Wollongong, NSW, Australia – *Oral presentation*

David De Oliveira, **Simon M. Cook**, Ruby H. P. Law, James C. Whisstock, Jason D. McArthur, Martina L. Sanderson-Smith 2012 Human plasminogen glycoform variants display differences in binding affinity to the Group A Streptococcus cell surface receptor Plasminogen Binding M-like Protein (PAM) Plasminogen Activation System in Pathology. Wollongong, NSW, Australia – *Poster presentation*

Simon M. Cook, Amanda Groznik, Peta Bradbury, Mark J. Walker and Jason D. McArthur 2011 Phenotypic Characterisation of streptokinase variants from *Streptococcus pyogenes*: implications for disease pathogenesis. XVIII Lancefield International Symposium. Palermo, Sicily, Italy – *Oral presentation*

Simon M. Cook, Amanda Groznik, Mark J. Walker and Jason D. McArthur. 2010. Characterisation of Streptokinase Variants from Streptococcus pyogenes. NZMS and NZSBMB joint meeting, Auckland, New Zealand. – *Oral presentation*

Simon M. Cook, Peta Bradbury, Priya Shyam, Marie Ranson, Mark J. Walker and Jason D. McArthur. 2009. Streptokinase mediated plasminogen activation in *Streptococcus pyogenes.* BacPath 10 – The Molecular Biology of Bacterial Pathogens. Barossa Valley, SA, Australia. - *Oral presentation*

Simon M. Cook, Peta Bradbury, Priya Shyam, Marie Ranson, Mark J. Walker and Jason D. McArthur. 2009. Streptokinase variants from *Streptococcus pyogenes* display differing plasminogen activation characteristics which are the result of amino acid changes within the N-terminal domain. XIIth International Workshop on Molecular & Cellular Biology of Plasminogen Activation. Cold Spring Harbor, NY, USA. - *Oral presentation*

ABSTRACT

Streptococcus pyogenes (group A streptococcus, GAS) is a human specific pathogen responsible for a wide range of diseases. The majority of GAS infections give rise to uncomplicated disease, however, the migration of GAS from superficial to deep tissue sites can result in life-threatening invasive infections. GAS secrete streptokinase (SK), a potent plasminogen (Plg) activator which enables this bacterium to subvert the host Plg activation system to generate soluble and cell bound protease activity. Unlike the human Plg activators, u-PA and t-PA, which cleave the Plg activation bond (Arg_{561}-VaI_{562}) to generate the broad serine protease plasmin, SK lacks intrinsic protease activity. Instead, SK forms a stoichiometric complex with Plg and through non-proteolytic mechanisms, generates an active site in the bound Plg molecule to produce an activator complex with proteolytic activity. Among GAS isolates, SK gene (*ska*) sequences are polymorphic and can be grouped into two distinct sequence clusters (termed cluster type-1 and cluster type-2) with cluster type-2 being further divided into sub-clusters type-2a and type-2b. Allelic variants of SK produced by GAS isolates display unique Plg activation properties, however the biological significance of SK polymorphism among GAS isolates is yet to be elucidated.

This project describes the first comprehensive phenotypic study of GAS SK variants by characterising the structural, functional and biochemical differences displayed by SK molecules. Five distinct *ska* alleles representing examples from the three known phylogenetic sequence clusters, along with SKc from group C streptococcus were cloned and expressed as recombinant proteins. Structural analysis using far-UV circular dichroism spectroscopy indicated that all SK variants displayed similar secondary structure despite significant variation in amino acid sequence. Active site generation in Glu-Plg by SK variants was examined using the fluorescent active site titrant 4-methylumbelliferyl p-guanidinobenzoate. In these experiments, type-2b SK variants could not generate an active site in Glu-Plg through non-proteolytic mechanisms, while all other variants displayed this hallmark capacity of SK action. SKc, type-1 SK, and type-2a SK variants all bound human Glu-Plg with high affinity (K_D ranging from 62 – 88 nM) when analysed by surface plasmon resonance experiments In comparison type-

2b SK variants displayed a 29-35 fold reduction in affinity for Glu-Plg. All SK variants had increased affinity (69 - 347 fold) for plasmin relative to Glu-Plg and could activate substrate Glu-Plg when a SK-plasmin activator complex was pre-formed.

Ligands that bind to Glu-Plg can affect the conformation of the protein which in turn can influence Glu-Plg activation. Therefore bacterial and human Plg binding ligands were investigated for their ability to influence the activation of Glu-Plg by SK variants. Despite type-2b SK not possessing the ability to generate an active site in native Glu-Plg, when active site experiments were repeated in the presence of fibrinogen, Plg-binding group A streptococcal M protein (PAM) (a Plg receptor constrained to type-2b expressing GAS strains), fibrinogen fragment D and fibrin, type-2b SK could generate an active site in Glu-Plg. Additionally, in contrast to the inhibition resistant SKc, type-1 and type-2a SK activator complexes, type-2b SK activator complexes (both those formed with Pln and those formed with Glu-Plg in the presence of ligands) were inhibited by the plasmin specific inhibitor, α_2-antiplasmin (α_2-AP). Interestingly, when a combination of PAM and fibrinogen was present type-2b SK activator complexes were resistant to α_2-AP inhibition.

To determine if these observations could be translated into assays using more physiologically relevant conditions, activation assays were performed in pooled human plasma and in the presence of fibrin clots or whole cell GAS. As expected, in human plasma type-2b SK-Plg complexes were inhibited by the endogenous α_2-AP unless exogeneous PAM was added to the assay. Additionally, when these assays were conducted in the presence of whole cell GAS expressing PAM, type-2b SK could activate Glu-Plg but the complex was only protected from inhibition by α_2-AP when fibrinogen was also present. Similarly, when type -2b SK variants activated Glu-Plg that was bound to fibrin clots, these activator complexes were resistant to α_2-AP inhibition. Taken together, this distinct Plg activation/inhibition mechanism displayed by type-2b SK variants would restrict plasmin activity to specific micro-environments within the host such as fibrin deposits or the bacterial cell surface through the action of α_2-AP inhibition. As epidemiological studies have shown the type-2b streptokinase gene linage

to be largely restricted to *pam* positive GAS strains which have a strong tendency to cause skin infections, we speculate that phenotypic SK variation functionally underpins a pathogenic mechanism whereby SK variants differentially focus Plg activation, leading to specific niche adaption within the host.

When the *ska*$_{\text{type-2a}}$ from the M1T1 GAS strain 5448 was exchanged with *ska*$_{\text{type-2b}}$, the isogenic mutant displayed reduced virulence which was similar to that displayed by knockout mutant. Isogenic 5448 mutants that contained a *ska*$_{\text{type-1}}$ allele displayed increased virulence. These data indicate that the phenotypic differences displayed by GAS SK variants do influence the invasive pathogenesis of this organism.

These findings suggest that SK variants produced by GAS isolates utilise distinct Plg activation pathways which, in turn, directly affects the pathogenesis of this organism. These findings have implications for the future elucidation of the molecular mechanism of SK mediated Plg activation, the generation of enhanced thrombolytic therapeutics and may highlight a potential role for the differential regulation of Plg activation in the pathogenesis of GAS (or the bacterial species that interact with the Plg activation system).

Chapter 1:

Literature Review

Preface

The chapters of this thesis were written for publication. As a result, each chapter stands alone and is accompanied by an introductory section that addresses the overall theme for that portion of work. Consequently, this introduction aims to provide a general background to the thesis as a whole. Readers will therefore find that there are inevitable overlap of certain topics addressed in this chapter and the introductory sections of subsequent chapters.

Publication: McArthur J. D., **Cook, S. M.**, Venturini, C., and Walker, M. J., (2012) "The role of streptokinase as a virulence determinant of *Streptococcus pyogenes* - Potential for Therapeutic Targeting." *Current Drug Targets* **13**: 297-307.

1.1 Overview

Streptococcus pyogenes (group A streptococcus; GAS) is a Gram-positive, beta haemolytic, human-specific pathogen (Cunningham, 2000). GAS normally inhabit epidermal and pharyngeal tissue where they most frequently manifest as superficial cases of pharyngitis or impetigo. However, GAS is an opportunistic pathogen readily capable of infecting normally sterile deep tissue. Invasive GAS diseases are life-threatening and include streptococcal toxic shock-like syndrome and the "flesh-eating" disease necrotising fasciitis (Walker *et al.*, 2014). GAS remain sensitive to penicillin and the incidence of GAS disease has decreased over the major part of the 20th century through the use of antibiotics and the implementation of improved hygiene and sanitation (Weiss and Laverdiere, 1997). However, since the 1980s there has been an alarming increase in severe invasive infections caused by GAS. This has revived interest in understanding the pathogenic mechanisms by which this bacterium can progress from causing localised superficial diseases to severe invasive diseases and death (Kaplan, 1991). GAS diseases are underpinned by an extensive repertoire of virulence determinants that are differentially regulated in direct response to a succession of environmental signals within the host (Tart *et al.*, 2007, Musser and Shelburne, 2009, Olsen *et al.*, 2010, Cole *et al.*, 2011, Kreikemeyer *et al.*, 2003, Sumby *et al.*, 2006). One such virulence mechanism is the subversion of the host plasminogen activation system (PAS), whereby GAS are able to capture and activate the host protease, plasmin(ogen). The proteolytic activity of the generated broad serine protease plasmin is both soluble and cell-bound. Plasmin activity at the bacterial cell surface can circumvent host defence mechanisms and is capable of degrading host tissue barriers and is important in

allowing bacterial invasion of normally sterile tissues to produce severe systemic infections (McArthur *et al.*, 2008). To facilitate this process, GAS secrete streptokinase (SK), a potent plasminogen (Plg) activating protein. This chapter presents a review of the mechanisms that GAS employ to interact with the host PAS and detailed analysis of the bacterial Plg activator, SK.

1.2 Classification of Group A Streptococcus

The genus *Streptococcus* comprises 49 species of Gram-positive cocci that typically exist as commensal flora of warm-blooded animals, including humans (Patterson, 1996). For the past century, the serological reactivity of cell surface components has been established as the foundation of streptococcal classification, the most prominent being that based on immunological variations in cell wall polysaccharides, developed by Rebecca Lancefield in the early 20th century (Cunningham, 2000). As for several of the streptococcal species, Lancefield typing of GAS is not fully comprehensive. While GAS is a single species, variation that occurs within this organism is broad and the outcome in terms of virulence factor expression and pathogenic progress resulting from this diversity is extensive. Therefore, additional serological and (more recently) molecular techniques (M serological typing, T serological typing, *emm* sequence typing) have been developed for sub-typing within GAS (Beall *et al.*, 1996, Jones *et al.*, 1991, Lancefield, 1928).

1.2.1 Lancefield Classification

The discovery of streptococcal polysaccharides in the early 1900's allowed for the development of the first serological classification system for grouping the majority of streptococcus strains (Lancefield, 1928). The Lancefield serological grouping system for streptococci identification is a system based on the immunological differences in cell wall polysaccharides between streptococcal groups A, B, C, F and G (Cunningham, 2000). Confirmation and subsequent grouping of GAS cell wall carbohydrate was achieved by techniques such as Lancefield capillary precipitin and slide agglutination, which are highly accurate serological techniques that utilise standardised grouping anti-sera (Cunningham, 2000).

1.2.2 M-serological Typing

M proteins (encoded by *emm*) are dimeric, α-helical, coiled-coil protein molecules that are anchored to the GAS cell surface at the C-terminus. GAS strains are often classified by their M-serotype (Efstratiou, 2000). The N-terminal region of the protein is the basis for classification, as it is hyper-variable and contains sequences that are GAS type-specific (Fischetti, 1991). In this technique, M-protein is extracted from the cell surface of a given bacterial isolate and purified. Anti-sera is then raised against the M-protein and can be used for M-protein serotyping of GAS strains (Efstratiou, 2000). However, problems with this technique exist: the identification of strains with low-level expression of M-protein, strains that express novel M-protein and the difficulty associated with the preparation of type-specific anti-sera present technical problems with this procedure (Cunningham, 2000). While M- serological typing was once the

most commonly used techniques for GAS classification, it has largely been replaced by *emm* sequence typing (Walker *et al.*, 2014).

1.2.3 *emm* Sequence Typing

e*mm* sequence typing is conducted by determining the DNA sequence of the first 160 bp at the 5' end of *emm* which encodes the hyper-variable N-terminal region of M-protein (Beall *et al.*, 1996). *emm* sequence typing l7rgely reflects the serological grouping but has allowed for characterisation of isolates previously non-typeable by other serological methods. Over 200 *emm*-types have been identified (Beall *et al.*, 1996, Facklam *et al.*, 1999). *emm* pattern grouping is a related method of describing the arrangement of *emm* genes into one of five classes, designated A through E. The basis of *emm* pattern grouping is dependent on the number of *emm* genes present, the subfamily to which the *emm* genes belong and the relative arrangement of *emm* genes on the chromosome (Bessen *et al.*, 1999). An *emm* pattern A - C gene arrangement is generally considered to be associated with isolates from throat infections, while those from pattern D are related to skin infections. Isolates containing *emm* pattern E display a high affinity for both tissue types (Bessen *et al.*, 1999).

1.3 Diseases and Sequelae Resulting from Group A Streptococcal Infection

GAS is a specific human pathogen that is responsible for numerous diseases with diverse clinical manifestations (Carapetis *et al.*, 2005). GAS preferentially colonise the throat and skin, and are readily transmitted via aerosols and direct contact (Walker *et*

al., 2014). While, GAS infection frequently causes superficial infections, these organisms are opportunistic pathogens, capable of epithelial penetration, intravascular survival and secondary infection of deep normally sterile tissue, which results in invasive infections (Patterson, 1996). Furthermore, complications from repeated GAS infections can occur producing serious post-infection non-supporative sequelae that are often fatal if left untreated (Efstratiou, 2000). An outline of the most common GAS pathologies is given in Table 1.1.

1.4 Epidemiology of GAS Disease

On a global scale, GAS is a major cause of morbidity and mortality, primarily in less developed countries. However, there has been a global resurgence of severe invasive disease in developed countries since the mid-1980s (Carapetis *et al.*, 2005). The identification of highly virulent strains of distinct M-serotypes, most notably M1T1, isolated from severe invasive infections, has been pin-pointed as the main contributor to GAS related disease resurgence in the developed world. The M1T1 clone is the dominant clinical isolate within industrialised populations and is frequently associated with life-threatening cases of invasive disease (Johnston *et al.*, 1992, Cleary *et al.*, 1992, Musser *et al.*, 1993, Tart *et al.*, 2007). Furthermore, the epidemiology of GAS disease is not as well-described in developing countries as it has been for developed countries (Carapetis *et al.*, 2005). While no resurgence of GAS disease has been specifically reported in developing countries, GAS infection rates are at endemic levels in many of these regions and may be the result of increased transmission due to factors

Table 1.1 Common diseases caused by *S. pyogenes (Cole et al., 2011, Cunningham, 2000, Efstratiou, 2000)*

Category	Disease	Disease Site	Symptoms	Reference
Superficial	Pharyngitis	Pharynx	Sore throat, swollen lymph nodes and tonsillar exudates	(Choby, 2009)
	Impetigo	Epidermis	Superficial lesions, blisters and pustules	(Bisno and Stevens, 1996)
	Erysipelas	Epidermis	Superficial erythema and inflammation	(Bisno and Stevens, 1996)
Invasive	Cellulitis	Subcutaneous	Redness, inflammation and fever	(Bisno and Stevens, 1996)
	Streptococcal toxic shock syndrome	Various	High fever, rapid-onset hypotension and multiple-organ failure	(Lappin and Ferguson, 2009)
	Bacteraemia	Blood	Fever, nausea and vomiting	(Stevens, 1992)
	Puerperal sepsis	Genital Tract	Fever, pelvic pain and abnormal/odorous discharge. Specifically, maintained or recurrent fever in the 10-day period following childbirth	(van Dillen *et al.*, 2010)
	Septic arthritis	Joints	Fever and enlarged joints of the lower extremities	(van der Helm-van Mil, 2010)
	Necrotising fasciitis	Subcutaneous	Fever accompanying rapid destruction of fascia, muscle and adjacent tissue	(Stevens, 1992)
Non-suppurative Sequelae	Rheumatic heart disease	Heart	Mitral or aortic valve incompetence later developing into mitral stenosis	(Carapetis *et al.*, 2000)
	Acute rheumatic fever	Joints, heart, epidermis and nervous system	Tissue inflammation giving rise to carditis, valvulitis, arthritis, chorea, erythema marginatum and/or subcutaneous nodules	(Carapetis *et al.*, 2000)
	Acute poststreptococcal glomerulonephritis	Kidney	Rapid onset of gross haematuria, oedema and hypertension, usually following an episode of GAS-mediated pharyngitis or pyoderma	(Bisno, 1995))

such as overcrowded housing, limited medical intervention and inadequate implementation of hygiene and sanitation (Carapetis *et al.*, 1999). Current estimates on the burden of GAS disease, suggest that there are at least 517,000 deaths caused each year by severe GAS diseases (Carapetis *et al.*, 2005). The prevalence of GAS disease in the global population is currently at least 18.1 million cases, with as many as 1.78

million new cases each year (Carapetis *et al.*, 2005). The greatest toll on human health by GAS is due to rheumatic heart disease. Rheumatic heart disease has a prevalence of at least 15.6 million cases world-wide, with 282,000 new cases and 233,000 deaths annually. Furthermore, the burden of invasive GAS disease is high, with at least 663,000 new cases and 163,000 deaths annually. Overall, 19% of patients with invasive GAS disease die within 7 days of infection and the development of STSS further increases the mortality rate, as 44% of patients with STSS die within a week of developing the disease. In addition, there are more than 111 million cases of GAS pyoderma and 616 million incidents of GAS induced pharyngitis each year world-wide (Carapetis *et al.*, 2005).

The minimum estimate of over 500,000 deaths per year, places GAS among the major human pathogens in terms of overall burden on the world population. GAS is only exceeded by such severe pathogens as Human Immunodeficiency Virus, *Myobacterium tuberculosis*, *Plasmodium falciparum* and *Streptococcus pneumoniae* (Carapetis *et al.*, 2005). However, GAS causes more long term morbidity than most of these pathogens, and poor record keeping and documentation in developing countries, where the majority of GAS infections occur, suggest that the true impact of GAS disease is significantly underestimated (Carapetis *et al.*, 2005).

1.5 GAS Virulence Factors

In concurrence with the global burden caused by GAS disease, GAS has been a focus of resolute investigation for over a century. However, it has not been until the advent of

modern molecular biological techniques that it has been possible to research the myriad of GAS virulence factors that facilitate bacterial colonisation, evasion of the immune response and systemic dissemination, which enable GAS to cause a diverse range of diseases (Tart *et al.*, 2007, Musser and Shelburne, 2009, Olsen *et al.*, 2010, Cole *et al.*, 2011). Virulence factor expression is exquisitely controlled by 13 two-component regulatory systems and 30 transcriptional regulators allowing GAS to adapt to the dynamic physiological conditions encountered during the infection process (Kreikemeyer *et al.*, 2003, Sumby *et al.*, 2006). Many of the virulence factors produced by GAS interact specifically with human plasma proteins including fibrinogen, plasmin(ogen), IgG, fibronectin, α_2-macroglobulin and albumin and numerous complement factors (Walker *et al.*, 2005, Walker *et al.*, 2014). The ability of GAS to adhere, invade and colonise human skin and mucosal membranes in a variety of environments has been attributed to cell surface structures such as M proteins, the hyaluronic acid capsule and fibronectin-binding proteins (Bisno *et al.*, 2003). While, extracellular toxins secreted by GAS, including super-antigenic streptococcal pyrogenic exotoxins, hyaluronidase, cysteine protease (SpeB), DNases A - D and the Plg activator SK allow GAS to spread through tissue and are believed to initiate the cytokinase cascade responsible for necrotising facsitis and STSS progression (Hynes, 2004, Bisno *et al.*, 2003). There are numerous putative GAS cell-associated virulence factors which are summarised in Table 1.2 and a second class of secreted virulence factors are summarised in Table 1.3.

Table 1.2: Established or putative cell-associated virulence factors of group A S*treptococcus* compiled from recent reviews (Bisno et al., 2003; Cuningham, 2000; Hynes, 2004)(Walker *et al.*, 2014)

Virulence Factor or Molecular Class	Function(s)	References
Hyaluronic Acid Capsule	Aids in resistance to phagocytosis, infection and colonisation	(Russell and Facklam, 1975, Schrager *et al.*, 1998)
Plasminogen-binding proteins (GAPDH, SEN, PAM/Plr)	Bind plasmin(ogen) with high affinity	(Lottenberg *et al.*, 1992b, Pancholi and Fischetti, 1992)
Fibronectin-binding proteins (Fbp54, Pfbp, FbaA, FbaB, PrtF1/SfbI, PrtF2/PFBP, SOF/SfbII, protein F,SfbX)	Attachment of streptococcal cells to epithelia cells	(Falugi *et al.*, 2008, Hanski and Caparon, 1992, Talay *et al.*, 1994, Jaffe *et al.*, 1996, Kreikemeyer *et al.*, 1995, Rakonjac *et al.*, 1995, Delvecchio *et al.*, 2002, Terao *et al.*, 2002)
M protein superfamily	Antiphagocytic, immunoglobin- binding, collagen-binding, fibronectic binding, fibrinogen binding	(Fischetti, 1991)
Heme-binding protein **(**HtsA)	Iron appropriation	(Lei *et al.*, 2003)
HtrA protease	Chaperone and protease, required for thermal stability and resistance to oxidative stress	(Jones *et al.*, 2001)
Lipoprotein (Lsp)	Matrix glycoprotein interaction, transitional metal homeostasis	(Weston *et al.*, 2009, Elsner *et al.*, 2002)
Serum opacity factor	Serum opacification by cleavage of apoprotein A1, fibronectin/fibrinogen binding	(Kreikemeyer *et al.*, 2005, Timmer *et al.*, 2006)
CD15s-related antigen	Adhesion. Host immune system invasion	(Hirota *et al.*, 1995)
Protein GRAB	Sequestration of α_2-macroglobulin to cell surface	(Rasmussen *et al.*, 1999)
DNase (SpnA)	DNA degradation. Resistance to neutrophil extracellular traps	(Chang *et al.*, 2011)
Chemotaxis inhibitors (C5a peptidase, SpyCEP)	Inhibit C5a and IL-8 components, eliminating the chemostatic signal from the site of infection	(Edwards *et al.*, 2005, Chiappini *et al.*, 2012)
Laminin binding proteins (Lbp, Shr)	Adhesion to host cells	(Fisher *et al.*, 2008, Terao *et al.*, 2002)
Streptococcal collagen-like surface protein (Scl1, Scl2)	Adhesion to host cells	(Caswell *et al.*, 2008, Caswell *et al.*, 2010, Lukomski *et al.*, 2000, Whatmore, 2001)
AgI/II Related Family (AspA)	Adhesion to host cells, anti-phagocytosis	Franklin 2012(Franklin *et al.*, 2013)
Pili (Spy0130, Spy0128, Cpa)	Pilus structural proteins; collagen binding, gp340 binding, bacterial aggregation, biofilm formation, host immune ivasion	(Falugi *et al.*, 2008, Manetti *et al.*, 2007)

Table 1.3: Established or putative secreted virulence factors of group A *Streptococcus* compiled from recent reviews (Bisno et al., 2003; Cuningham, 2000; Hynes, 2004)(Walker *et al.*, 2014)

Virulence Factor or Molecular Class	Function(s)	References
Streptokinase	Binds and activates plasminogen to generate protease activity	(Tillett *et al.*, 1934, Malke, 1993)
Streptococcal inhibitor of complement	Bind to and inhibits C5bC7 complex to prevent formation of the membrane attrack complex. Also involved in adherence and colonization.	(Åkesson *et al.*, 1996, Pence *et al.*, 2010)
DNase (SdaI)	DNA degradation. Resistance to neutrophil extracellular traps	(Walker *et al.*, 2007)
Streptolysin O and S	Cytotoxins, lysins, pore forming toxins	(Timmer *et al.*, 2009, Nizet *et al.*, 2000)
Hyaluronidases	Organism/toxin spreading	(Hynes and Walton, 2000, Rivera Starr and Engleberg, 2006)
Immunoglobulin G-dergrading enzymes (IdeS, EndoS)	Cleavage of bound IgG, inhibition of opsonophagocytosis and killing by polymorphonuclear leukocytes	(Kawabata *et al.*, 2002, Collin and Olsén, 2001)
Streptococcal pyrogenic exotoxins (A-C, F-H, J-M, SSA, SMEZ, SMEZ-2)	Superantigens (aside from SpeB and SpeA), mitogens (mediate polyclonal T cell activation and division	(Commons *et al.*, 2014)
Cysteine protease (SpeB)	Proteolysis of GAS and host proteins. Strepadhesin, laminin-binding and cysteine protease activity	(Hytönen *et al.*, 2001, Svensson *et al.*, 2000, Cole *et al.*, 2006a)

The most important GAS virulence mechanism for the scope of this thesis is the interaction of GAS with the Plg activation system of the host. This interaction with Plg via the secretion of SK and expression of Plg binding proteins is critical for the pathogenic potential of this organism and is a diverse multi-faceted relationship (Sun *et al.*, 2004, Khil *et al.*, 2003, Walker *et al.*, 2005, Irigoyen *et al.*, 1999).

1.6 The Plasminogen Activation System

The PAS is a highly regulated *in vivo* proteolytic system for degrading intravascular fibrin clots, furthermore components of the PAS are involved in numerous physiological processes, such as cell migration, angiogenesis, wound healing, ovulation, remodeling and repair of both damaged and healthy tissue, trophoblast implantation, and embryonic development (Andreasen *et al.*, 1997, Coleman and Benach, 1999, Plow *et al.*, 1995b). However, subversion of the PAS components has become an increasingly recognised mechanism that contributes to the course of an infection directly through protease mediated degradation of tissue barriers or by affecting the immune response generated by the host by pathogenic bacteria (Berge and Sjobring, 1993, Sanderson-Smith *et al.*, 2012, McArthur *et al.*, 2012). Functionality of the PAS requires the involvement of several proteins. These include, Plg (the zymogen form of plasmin), specific mammalian Plg activators, urokinase (uPA) and tissue-type Plg activator (tPA), as well as Plg-activator inhibitors 1 and 2 (PAI-1/PAI-2) and plasmin specific inhibitors α_2-antiplasmin (α_2-AP) and α_2-macroglobulin (α_2-MG) (Figure 1.1) (Andreasen *et al.*, 2000).

1.6.1 Plasminogen

Plg, in its native form, is a 92 kDa, single chain glycoprotein zymogen of the broad spectrum serine protease plasmin (Berge and Sjobring, 1993, Ponting *et al.*, 1992). Synthesised in the liver and found in plasma and most extracellular fluids, Plg is maintained at a stable plasma concentration of approximately 2 µM (Ponting *et al.*, 1992). Plg consists of two polypeptide chains joined by disulphide bonds and the

functional Arg_{561}-Val_{562} peptide bond (Figure 1.2).The cleavage of the peptide bond

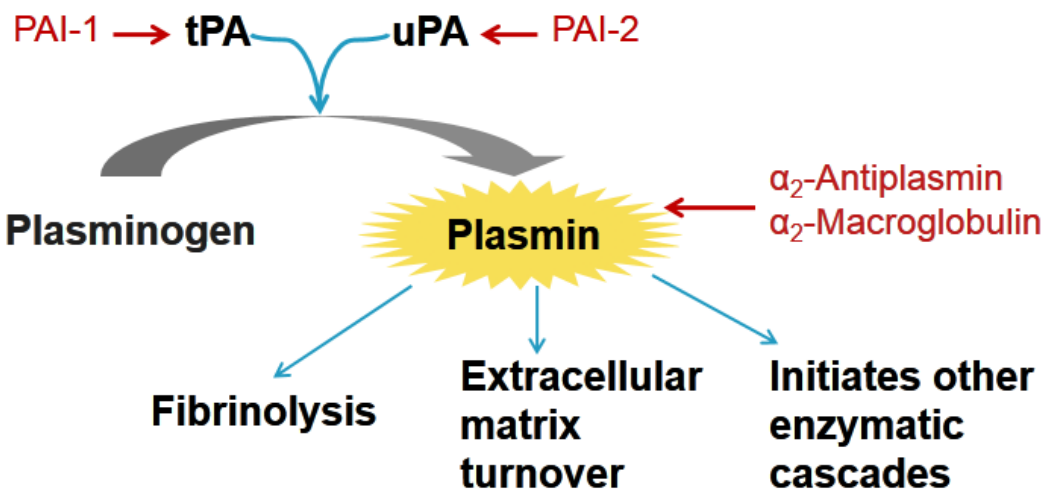

Figure 1.1: Schematic of the mammalian plasminogen activation system. The activation of plasminogen (Plg) to plasmin occurs via urokinase (uPA) or tissue-type Plg activators (tPA) and is inhibited by Plg activator inhibitors 1 and 2 (PAI-1/PAI-2). Plasmin can degrade fibrin or other extracellular membrane proteins and this process is inhibited by α_2-antiplasmin (α_2-AP) and α_2-macroglobulin (α_2-MG). Figure adapted from Andreasen, (2000).

between Arg_{560} and Val_{561} of Plg by Plg activators (uPA and tPA), results in the formation of broad serine protease plasmin (Nilsen *et al.*, 1999). The light 25 kDa polypeptide C-terminal chain B contains the serine active site, with His_{603}, Asp_{646} and Ser_{741} forming the catalytic triad, which is characteristic of serine proteases. The heavy 65 kDa polypeptide N-terminal chain A contains five homologous repeated 'kringle' domains (Figure 1.2) (Nilsen *et al.*, 1999). The kringle domains contain lysine binding sites that are responsible for interactions with fibrin and other lysine residues of tissue,

cell surfaces receptors and target molecules (Nilsen *et al.*, 1999, Ponting *et al.*, 1992, Law *et al.*, 2012). In particular kringles 1, 2, 4, and 5 contain lysine-binding sites comprised of a hydrophobic cleft formed by aromatic residues that most commonly bind C-terminal lysine residues and internal lysine residues of receptors (Marti *et al.*, 1997). Upon conversion to plasmin the heavy and light chains of the molecule remain connected by the two disulphide bonds. These bonds are important as they maintain the conformation of the serine activation loop that is responsible for the proteolytic activity of plasmin (Lahteenmaki *et al.*, 2001).

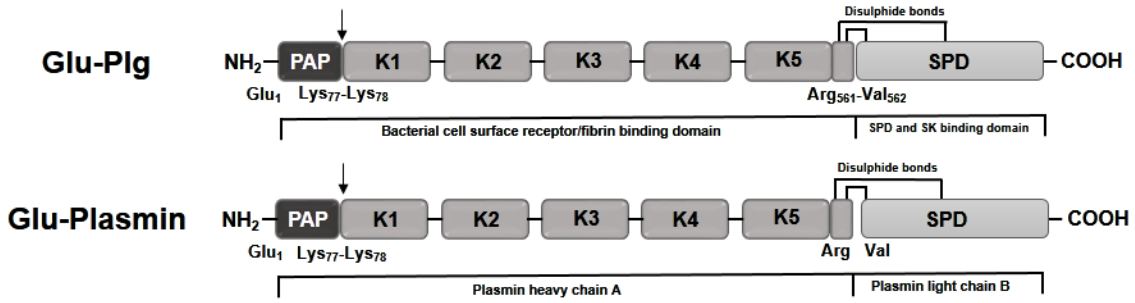

Figure 1.2: Schematic structure of Glu-Plg and Glu-Plasmin. Glu-Plg contains a preactivation peptide (PAP) that spans from Glu_1 to Lys_{77}, followed by 5 homologous kringle domains (K1–K5), followed by a peptidase S1 domain (SPD). PAP is generated by plasmin cleavage between Lys_{77}- Lys_{78} giving rise to Lys-Plg. The conversion of Glu-Plg or Lys-Plg to their respective plasmin forms occurs by hydrolysis of the Arg_{561}-Val_{562} peptide bond by either uPA or tPA. This yields chain A and the smaller chain B, which remain covalently associated by interchain disulfide bonds. Kringles 1, 2, 4, and 5 contain lysine-binding sites with affinity for free lysine and lysine-like compounds such as ω-aminocarboxylic ligands, cell surface receptors and fibrin in the in the following order of binding affinity K1 > K4 > K5 > K2. Kringle 3 shows no detectable binding to Lys or Lys-like compounds, due to sequence variation in the lysine binding site. SK (not shown) binds in a 1:1 complex with the SPD to generate an activator complex. Figure adapted from Sanderson-Smith *et al.* (2012)

Plg normally circulates as the full-length 791 amino acid native form termed Glu-Plg (Figure 1.2). Intramolecular binding between lysine residues and the lysine binding sites of these kringles maintains circulating Glu-Plg in a compact and internally rigid 'closed' conformation that is highly resistant to activation. Upon binding to specific cell surface receptors or ligands, such as fibrin, Glu-Plg adopts an 'open' conformation that becomes more susceptible to activation (Figure 1.3) (Castellino and Ploplis, 2005, Law *et al.*, 2012, Xue *et al.*, 2012). Additionally, the pre-activation peptide of Glu-Plg can be removed through plasmin mediated N-terminal cleavage between the residues Arg_{67} -Met_{68}, Lys_{76} -Lys_{77}, or Lys_{77} -Val_{78}, resulting in the production of Lys-Plg (Lahteenmaki *et al.*, 2001). Lys-Plg assumes the 'open' Plg conformation in circulation, which is internally flexible and displays distinct physiochemical and functional characteristics that enable stronger interactions with receptor and target molecules and enhanced activation (Figure 1.3) (McCance and Castellino, 1995, Claeys and Vermylen, 1974, Lahteenmaki *et al.*, 2001).

The primary *in vivo* function of plasmin(ogen) is to regulate vascular patency by degrading fibrin-containing thrombi, which form as both a reparative factor to tissue damage and a protective factor in reaction to inflammation caused by an immune response against an invading pathogen (Lewis *et al.*, 1984). However, the identification of Plg/plasmin receptors and the ability of plasmin to degrade matrix proteins other than fibrin, has implicated plasmin in other functions involving localised degradation of protein barriers and cell migration (Castellino and Ploplis, 2005). Diverse cell types promote plasmin generation through their expression of uPA, tPA and cell surface receptors (Cesarman-Maus and Hajjar, 2005). Endothelial cells, monocytes,

macrophages, neutrophils and some tumour cells, all bind Plg, as well as tPA and/or uPA. These receptors localise cell surface fibrinolytic activity and provide specialised environments that are protected from circulating inhibitors (Ellis and Whawell, 1997, Plow *et al.*, 1995b, Ponting *et al.*, 1992). The primary role of tPA is in the generation of plasmin for fibrinolysis in blood vessels and is known to be a poor Plg activator in the absence of fibrin (Andreasen *et al.*, 2000). While, uPA is generally agreed to be the enzyme primarily associated in tissue remodelling, wound healing and cell mediated degradation during inflammation (Andreasen *et al.*, 2000, Andreasen *et al.*, 1997).

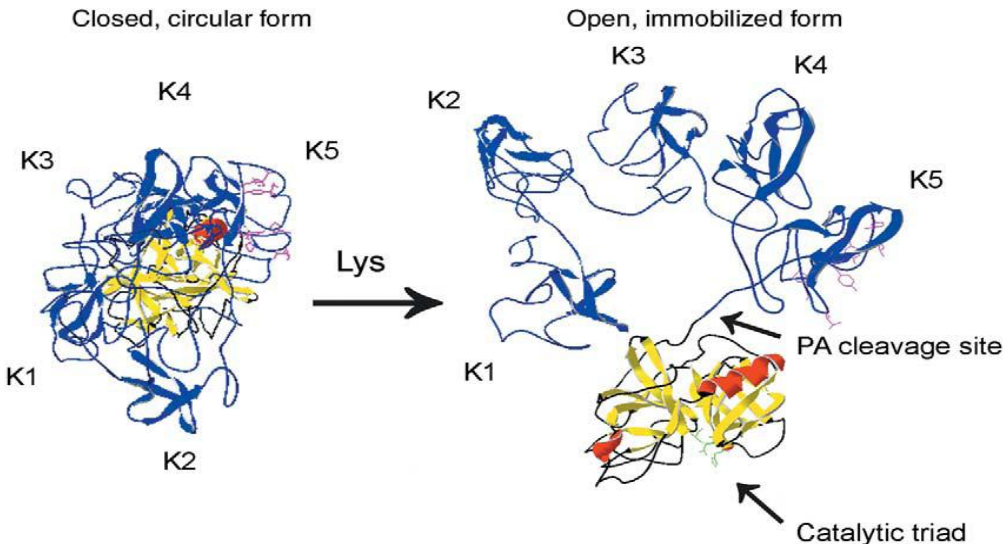

Figure 1.3: Diagrammatic representation of the closed and open conformations of plasminogen. (**A**) The circulating form of plasminogen is the closed conformation. The heavy chain consisting of five kringle domains and the light chain containing the catalytic domain are entwined. **(B)** When plasminogen binds to cells expressing plasminogen receptors or other cell surface proteins, the activation susceptible open conformation is induced. Physiological plasminogen activators cleave a peptide bond at the cleavage site that is more susceptible to cleavage in the open form, to yield plasmin. Figure from (Lahteenmaki *et al.*, 2001).

Due to the broad substrate specificity and proteolytic power of plasmin, the fibrinolytic system is tightly regulated and plasmin generation under normal physiological circumstances is generally restricted to specific foci. Failure to regulate the fibrinolytic system can result in excessive thrombi formation (via insufficient activation of the thrombolytic system or excessive inhibitors) or an increased bleeding tendency (via over stimulation of the thrombolytic system and deficiency of inhibitors) depending on the factor that is not regulated appropriately (El-Gengaihy *et al.*, 2007). Endothelial cells secrete PAI-1 and PAI-2, which circulate in plasma at a low concentration. PAI-1/PAI-2 inactivates uPA constitutively and acts on tPA when it is not in the presence of fibrin. In plasma, tPA and uPA also only have remarkably short half-lives of 5-8 min and 7-20 min respectively (Khan and Gowda, 2003, Fedan, 2004, Andreasen *et al.*, 2000). Circulating plasmin is inhibited by physiological serine protease inhibitors (α_2-AP and α_2-MG) that irreversibly bind and inactivate plasmin (Fedan, 2004). α_2-AP and α_2-MG have binding regions that interact specifically with the lysine binding sites of plasmin(ogen). This in the same region that makes the molecule specific for fibrin, thereby acting as competitive inhibitors (Aoki *et al.*, 1993). In addition to being resistant to α_2-AP and α_2-MG inhibition at the site of fibrin loci, plasmin(ogen) bound to the cell surface via these lysine residues are also resistant to inhibition (Wang *et al.*, 1995a).

1.6.2 Group A Streptococcus and the Plasminogen Activation System

The exploitation of host components to increase the chance of survival and spread within the human host is an essential pathogenic mechanism of GAS. Human Plg is

often used by invasive bacteria as a virulence factor and this process has been recognised as a critical step in GAS invasion. GAS can interact with PAS components via several mechanisms to produce extracellular proteolytic activity or acquire proteolytic activity at the cell surface (Cole *et al.*, 2006a, Walker *et al.*, 2005, D'Costa and Boyle, 1998). GAS can either bind host plasmin(ogen) directly to the cell surface, or indirectly by binding a tri-molecular complex consisting of Plg, SK and fibrinogen (Boyle and Lottenberg, 1997, McKay *et al.*, 2004, Wang *et al.*, 1995b). Currently three plasmin(ogen) cell surface receptors on GAS have been characterised; 1) Plg-binding GAS M-like proteins (PAM or Prp) (Berge and Sjobring, 1993, Sanderson-Smith *et al.*, 2007), 2) glyceraldehyde-3-phosphate dehydrogenase (GAPDH), also known as streptococcal surface dehydrogenase (SDH) or Plg receptor (Plr) (Lottenberg *et al.*, 1992a, Pancholi and Fischetti, 1992) and 3) streptococcal surface enolase (SEN) (Pancholi and Fischetti, 1998). Plg bound to the bacterial cell surface may also be converted into plasmin through the action of the host derived Plg activators uPA and tPA, or by SK (Lahteenmaki *et al.*, 2001).

1.6.3 GAS Plasmin(ogen) Cell Surface Receptors

1.6.3.1 Plg-binding Group A Streptococcal M-like Proteins (PAM and Prp)

PAM is a 43 kDa member of the M-like protein superfamily, encoded by *emm*-like genes (Berge and Sjobring, 1993). M-like proteins display an extensive array of binding activities for tissue and plasma proteins of the human host, which play a role in anti-phagocytic functions (Svensson *et al.*, 1999). PAM binds both Plg and plasmin with high affinity via a domain of 30 amino acids within the variable N-terminal region. This

domain consists of two lysine-containing thirteen amino acid repeated sequences, designated A1 and A2 (Wistedt *et al.*, 1998). These repeat sequences are identical in nine of the thirteen amino acid residues; non-identical residues possessing the same charge and polarity interact with the lysine-binding 2nd kringle domain of Plg (Wistedt *et al.*, 1998, Ringdahl and Sjobring, 2000). The conformational structure of Plg when bound to PAM is ordered in such a way that its catalytic domain is free to bind to endogenous SK and host Plg activators meaning that after this interaction, the streptococcal-bound Plg can be readily converted to plasmin (Ringdahl *et al.*, 1998, Wistedt *et al.*, 1998).

1.6.3.2 Streptococcal Surface Enolase (SEN)

SEN is a 45 kDa GAS surface protein found on most GAS serotypes (Pancholi and Fischetti, 1998). The contribution of two lysine residues (Lys_{434} and Lys_{435}) at the SEN C-terminus allow for high affinity binding to Lys-Plg and stabilise the conformation of Plg binding sites in SEN (Derbise *et al.*, 2004). SEN is functionally and structurally different to other GAS Plg binding proteins (SK, GAPDH or PAM), as it has significantly higher binding affinity for Plg than plasmin and binds to Lys-Plg with almost three times more affinity than to Glu-Plg (Derbise *et al.*, 2004, Lottenberg *et al.*, 1992b). The ubiquitous presence of SEN on the surface of almost all M types suggests that SEN may play a major role in the strong direct Lys-Plg-binding activity of GAS, including those serotypes that have been shown to bind Glu-Plg (Derbise *et al.*, 2004).

1.6.3.3 Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH)

GAPDH is a 41 kDa glycolytic enzyme that has been found to be present on the GAS cell surface of all isolates (Pancholi and Fischetti, 1992). GAPDH was first identified as a streptococcal receptor for plasmin and found to bind Glu-Plg very weakly. The localisation of GAPDH on the cell surface of GAS has been demonstrated by immunological and enzymatic methods (Pancholi and Fischetti, 1992, D'Costa and Boyle, 1998, Winram and Lottenberg, 1998). GAPDH preferentially binds to Lys-Plg and plasmin via interaction with the C-terminal Lys_{334}. GAPDH will also bind Glu-Plg, but at a relatively low affinity compared to SEN and PAM (Lottenberg *et al.*, 1987). GAPDH has also been shown to interact with fibronectin, lysozyme and other cytoskeletal proteins, and it is supposed that these additional interactions may play a role in GAS adherence to host tissue (Pancholi and Fischetti, 1992).

1.6.3.4 Indirect Interaction of GAS with Plg

In addition to the direct binding of plasmin(ogen), GAS can acquire plasmin activity on the bacterial cell surface via a second, more complex pathway. This pathway involves interactions between two host proteins, fibrinogen and Plg and two bacterial proteins, the secreted Plg activator SK and a surface expressed Plg or fibrinogen receptor (D'Costa and Boyle, 1998). The SK-Plg activator complex can bind to fibrinogen forming a tri-molecular complex, which maintains un-regulated plasmin activity and binds to the GAS cell surface via fibrinogen or Plg receptors (Wang *et al.*, 1995a). While the indirect pathway has not been shown to be related to invasive potential of GAS (Wang *et al.*, 1995b, Chibber *et al.*, 1985, Christner *et al.*, 1997), this pathway has

been proposed as a mechanism for PAM negative GAS isolates to acquire cell surface plasmin activity (Walker *et al.*, 2005).

1.7 Streptokinase

1.7.1 Mechanism and Structure

SK is a Plg activating protein secreted by group A, C and G streptococci (Marcum and Kline, 1983). The most studied forms of SK consist of 414 amino acids, are produced by streptococcal strains isolated from human hosts and display a high degree of homology (>85% identity at the amino acid level). While, Plg activators produced by group C and G isolates from animal hosts and *Streptococcus uberis* are smaller in size and display less homology with each other and human associated SK (20-40% identity at the amino acid level) (Ward and Leigh, 2002). GAS is a human specific pathogen and the SK secreted by GAS isolates is highly specific for human Plg and displays inefficient activation of Plg from other species. This also suggests that SK proteins have co-evolved with specific hosts and may contribute to the species-restricted infection (Marcum and Kline, 1983, Wohl *et al.*, 1983). Classical SK is composed of three distinct domains termed α, β and γ (Huang *et al.*, 1989). The three domains of SK are separated by two coiled-coils, and small regions at the N- and C- termini of the protein have disordered flexible structures (Wang *et al.*, 1998). As a 26 residue leader peptide is cleaved off the SK protein during secretion, the amino acid sequence positions of the domains span residues 27 – 173 for the α domain, residues 174 – 317 for the β domain and spans residues 318 – 440 for the γ domain (Kalia and Bessen, 2004, Parrado *et al.*, 1996, Wang *et al.*, 1998).

Crystallographic studies of SK bound to the catalytic domain of plasmin have deduced the three domains of SK appear in a complex of homologous folding. Domains α and β each contain a major β sheet of five mixed β strands and an α-helix, while the γ domain has only four β strands and contains a long coiled-coil region rather than an α-helix (Figure 1.4) (Wang *et al.*, 1998).

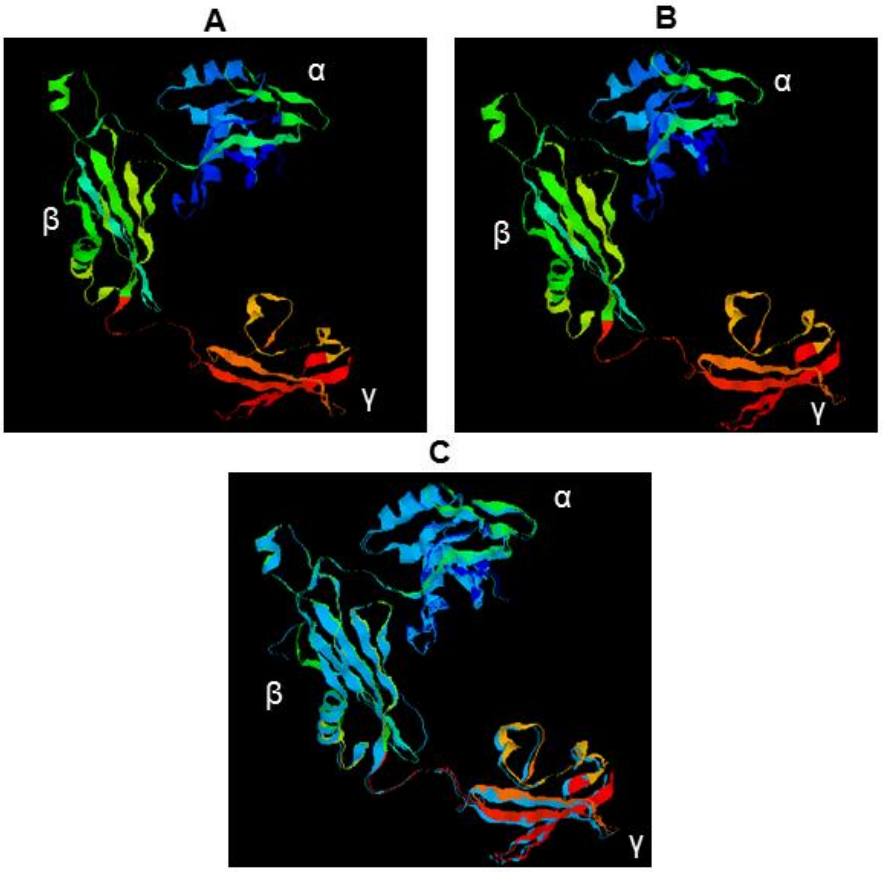

Figure 1.4: Crystal structure of streptokinase (A) The solved 3D model of SKc from Wang *et al.* (1998). **(B)** A 3D model for SK_{ALAB49} was constructed using an automated comparative protein modelling server, Swiss Model Workspace. **(C)** An overlay of (solved) SKc model and (predicted) SK_{ALAB49} model to highlight homology in the 3D model structures of group C SK and group A SK were deduced by comparison of protein residues to the crystallography data of SKc complexed with microplasmin by Wang *et al.* (1998). SK is composed of three distinct domains termed α, β and γ which are highlighted in the figure

SK has no intrinsic enzymatic activity and unlike other Plg activators, such as host uPA and tPA, it is not a protease (McClintock and Bell, 1971). Where host Plg activators activate Plg by limited proteolytic cleavage, SK binds via lysine dependant interactions between SK and the kringle/catalytic domains of Plg inducing conformational changes in the molecule that result in the formation of an active site and the production of an active complex, termed SK-Plg* (known as the 'Conformational Activation Pathway' or 'Pathway I') (Figures 1.5 and 1.6). The conformationally activated SK-Plg* complex can then sequester substrate molecules of Plg and proteolytically convert those to the broad serine protease plasmin via cleavage at the Arg_{561}-Val_{562} peptide bond (Boxrud *et al.*, 2000, Boxrud *et al.*, 2004). Plasmin, which has a higher affinity for SK than Plg, rapidly displaces Plg in the SK-Plg* complex to produce an activated SK-plasmin complex that is the main catalyst responsible for the full conversion of Plg to plasmin (known as 'Direct Proteolytic Activation Pathway' or 'Pathway II') (Figures 1.5 and 1.6) (Boxrud *et al.*, 2000, Boxrud *et al.*, 2004, McClintock and Bell, 1971, Reddy and Markus, 1972, Schick and Castellino, 1974)

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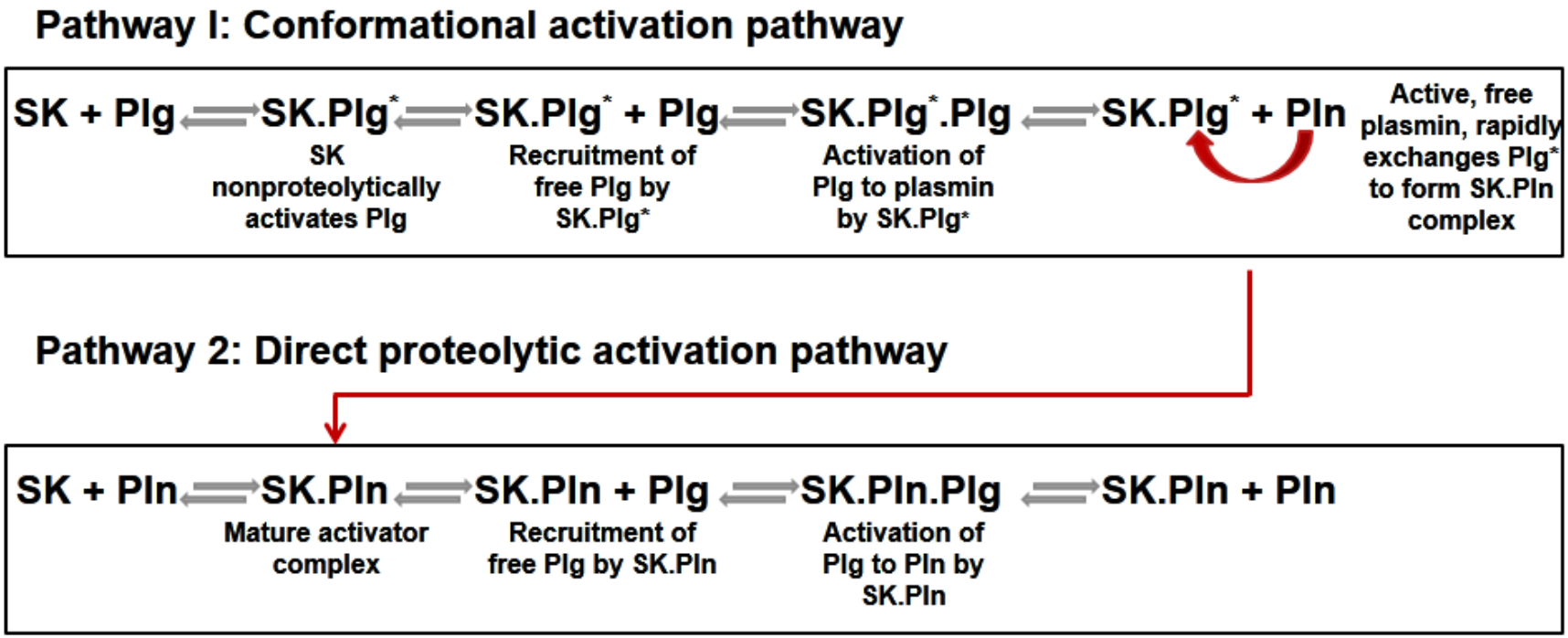

Figure 1.5: Schematic representation of the steps involved in the activation of Plg by SK. SK forms an equimolar complex with Glu-Plg (Plg) that leads to the generation of an active centre in the complex (SK.Plg*) without cleavage of Arg_{561}-Val_{562} scissile peptide bond in the 'partner' Glu-Plg (Pathway I). The SK-Plg complex recruits free Plg as substrate and proteolytically activates it to plasmin (Pln). Plasmin that is generated rapidly exchanges the Plg from SK.Plg* complex due to a much higher affinity for SK in comparison to Glu-Plg. Alternatively, SK can directly combine with Pln to form the mature SK.Pln activator complex (Pathway II). The SK-Pln activator complex catalytically acts on 'substrate' Plg molecules and converts them into Pln. Schmatic information adapted from Aneja *et al.* (2009)

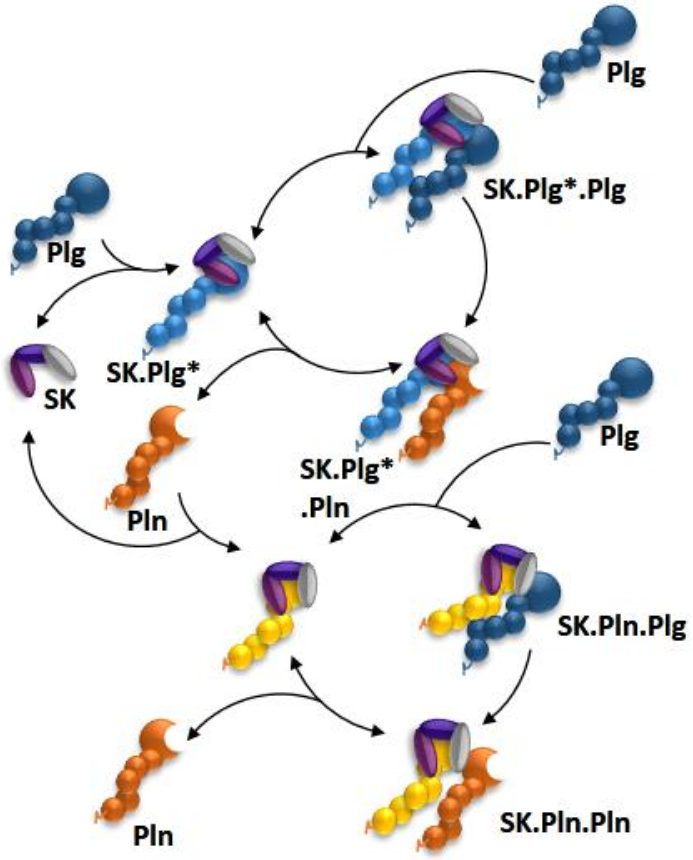

Figure 1.6: Mechanism for coupling of conformational and proteolytic activation of plasminogen by SK. A unified hypothesis for the mechanism of SK-induced conformational and proteolytic activation of Plg is illustrated. Free Plg is represented as the five kringle domains and the proteinase domain in blue. This has been done to simplify the scheme and is not meant to be restricted to this form of Glu-Plg but to represent both conformational forms of Glu-Plg and Lys-Plg. The reaction proceeds through the indicated complexes. Proteolytic activation catalytic cycle (Pathway I) is initiated by SK binding to Plg and conformational activation to generate SK.Plg* (light blue). This complex interacts with substrate Plg forming the ternary Michaelis complex, SK-Plg*.Plg, which result in the generation of plasmin (Pln) (orange) by proteolysis of the Arg_{561}-Val_{562} scissile peptide. Pln generated by SK.Plg* can displace Plg from the SK-Plg* complex or interact with free SK, forming SK.Pln (yellow). The tightly bound SK.Pln complex initiates the lower catalytic cycle of direct proteolytic activation (Pathway II), binding free substrate Plg to form SK.Pln,Plg, from which free Pln is generated. Figure adapted from Boxrud and Bock (2004)

1.7.4 Roles of α, β and γ Domains of Streptokinase in Plg Activation

The exact manner that each domain contributes to the high rates of Plg activation by full length SK remains to be identified. However, the incredibly high catalytic turnover by the SK.plasmin(ogen) activator complex has been attributed to the three-domain architecture of SK. Selective deletions of α, β and γ domains, or parts thereof, result in a drastic reduction in Plg activation activity, indicating that all three domains of SK are essential in the functioning of the molecule (Parrado *et al.*, 1996, Loy *et al.*, 2001, Conjero-Lara *et al.*, 1998, Chaudhary *et al.*, 1999). When individually isolated, each domain retains significant amounts of native-like structure and significant capability to bind with Glu-Plg, but cannot activate Plg (Parrado *et al.*, 1996, Loy *et al.*, 2001, Conjero-Lara *et al.*, 1998). While it is clear that the three domains are required to function in concert for full activity, the exact inter-domain mechanism responsible for interactions with partner plasmin(ogen), the formed SK-plasmin(ogen) activator complex with substrate Plg and those responsible for post-docking and/or post-cleavage steps such as product expulsion, are little understood at present (Yadav and Sahni, 2010).

Considering the large surfaces involved in the protein–protein interactions between SK and plasmin(ogen), it seems very likely that there are a number of sites in the SK molecule participate in the catalytic process. The crystal structure of SK alone, or in complex with Glu-Plg is yet to be solved, however, the crystal structure of the catalytic domain of human plasmin (sans kringle domains) complexed with SK, gives an indication to the extent of interactions displayed by each SK domain. The level of these interactions descends in order from α, γ, to β (Wang *et al.*, 1998). In the current solved

structure, the α domain binds to plasmin mainly through interactions between the $^{\alpha}\beta_1$ and $^{\alpha}\beta_2$ strands of SK and a loop region (residues 713 to 721) and also interacts with catalytic domain near the catalytic triad residues His_{603} and Asp_{646}. The γ domain binds near the activation cleavage site of Plg in the "calcium-binding loop" (residues 622 to 628) and the "autolysis loop" (residues 692 to 695), via extensive charged and hydrophobic interactions with the major coiled coil region and the strands $^{\alpha}\beta_1$ and $^{\alpha}\beta_2$ of the domain. Lastly, the interaction β domain with the catalytic domain of plasmin appears to be relatively limited (Wang *et al.*, 1998).

Without structural data for SK interaction with native Glu-Plg, the focus for the elucidation of the rest of the binding determinants and key residues in the molecular mechanism of SK mediated Plg activation has been shifted to amino acid deletion, domain truncation, site directed mutagenesis and molecular modelling approaches. Subsequently, while not interacting with the catalytic domain, binding of the β domain to the 5^{th} kringle domain of Plg has been predicted. A distinct hairpin loop structure (residues 250-264) links the two β sheets of the domain and in this region, residues Lys_{256} and Lys_{257} are critical for Plg identification and subsequent high affinity binding, however, this interaction alone is incapable of causing Plg activation (Chaudhary *et al.*, 1999, Wang *et al.*, 1999b, Lin *et al.*, 1996). Thus, it appears from the current data that the α and γ domains provide most of the substrate recognition sites with the plasmin(ogen) moiety and exhibit a synergistic effect of plasmin activation by full length SK (Wang *et al.*, 1998). While, the β domain provides no direct contact site with the plasmin active site, it is required to bind to Plg and instigates the conformation change of the Plg molecule (Chaudhary *et al.*, 1999, Lizano and Johnston, 2005). This

initial interaction of the β domain of SK with circulating native Glu-Plg results in the conversion of Plg from the physiological 'closed' conformation to the more flexible 'open' conformation (Figure 1.6) (Boxrud *et al.*, 2000). When in the 'open' conformation, Plg is more internally flexible, and exposes other lysine residues present in the kringle domains making them available for further interactions with the α and γ domains (Conjero-Lara *et al.*, 1998, Loy *et al.*, 2001).

1.7.2 Conjecture in the Molecular Mechanism that Streptokinase employs to Activate Plg

Plg is usually activated by the proteolysis of the Arg_{561} - Val_{562} peptide bond. This new N-terminal amino group (Val_{562}) is believed to form a salt-bridge with the Plg carboxylate group (Asp_{740}) (Wang *et al.*, 2000). This is analogous to the conversion of trypsinogen to trypsin, a serine protease found in the digestive system of mammals, where Ile_{16} forms a salt-bridge with Asp_{194} to induce active site development. Formation of this salt bridge, in both cases, triggers a conformational change that generates active protease (Wang *et al.*, 2000, Wang *et al.*, 1999a). However, SK binds to Plg and causes it to assume an active conformation. As this occurs without the regular cleavage of the activation peptide bond, the salt-bridge between Val_{562}-Asp_{740} cannot form in the SK:Plg complex. It is therefore likely that a residue within the SK moiety is able to provide a counter-ion for Asp_{740}, however this process is poorly defined and two hypotheses have been proposed (Wang *et al.*, 2000).

The first mechanism proposed, termed 'molecular sexuality hypothesis', involves the insertion of the N-terminus of SK (Ile_1-Asp_2-Gly_3) into the activation pocket of Plg,

facilitating the formation of a salt bridge between Ile_1 and Asp_{740} of Plg (Bode and Huber, 1976, Parrado *et al.*, 1996, Wang *et al.*, 1999a). The mutation or removal of this Ile_1 residue from SK by Wang *et al.*, (2000) decreased the formation of the active site in the SK:Plg complex by 100 fold. However, none of the N-terminal mutations that have been conducted (Ile_1 to Ala_1, Gly_1, Val_1, Trp_1 or Lys_1) perturb the binding affinity of SK to Plg, which suggest that Ile_1 is not essential for the formation of the SK:Plg complex, but is required for the formation of the active site in the molecule (Loy *et al.*, 2001, Mundada *et al.*, 2003). Furthermore, removal of the N-terminal Ile_1-Lys_{59} peptide alters the mechanism of SK mediated Plg activation, resulting in a fibrin-dependent mode of action, similar to that of tPA. Whereby, SK cannot activate circulating substrate Glu-Plg and requires Plg to be in an 'open' conformation bound to fibrin or the presence of plasmin to generate proteolytic activity (discussed further in the introduction to Chapter 5) (Reed *et al.*, 1999).

An alternative to the 'molecular sexuality' theory involves the hypothesised interaction of the γ domain of SK in the formation of the active SK:Plg complex (Wang *et al.*, 1998, Young *et al.*, 1998, Wu *et al.*, 1990, Zhai *et al.*, 2002). Known as 'contact activation' (Zhai *et al.*, 2002) or 'binding activation' (Wu *et al.*, 2001), this theory suggests that the γ domain binds to Plg inducing a conformational change in the Plg molecule that exposing Lys_{698} to Asp_{740} of Plg. Lys_{698} acts as the important counter-ion to form the crucial salt-bridge, triggering the conformational change of the Plg moiety to its active state (Wu *et al.*, 2001). By analysing the crystallography data presented by Wang *et al.*, (1998) it can be seen, as previously mentioned, that the SK γ domain binds in close proximity to the activation cleavage site of micro-plasmin. Extensive charged

and hydrophobic interactions are seen between the 'serine active loop' (residues 558 - 566) and the 'calcium-binding loop' (residues 622-628) of Plg and the coiled-coil region of the SK γ domain (Wang *et al.*, 1998). It is the coiled-coil region of the SK γ domain (residues 314 - 342), specifically amino acid residues Arg_{324}, Asp_{325}, Lys_{332} and Lys_{334} that are potentially critical for formation of the activator complex and conformational modification of Plg (Wu *et al.*, 2001).

Whilst there is evidence to support both theories, most conflict is inferred as to which residue acts as the crucial counter-ion for Asp_{740}. Experiments conducted by Wang *et al.*, (1999, 2000) have used mutational characterisation studies to test these two hypotheses. They found that mutation of the Ile_1 residue blocked formation of the active SK:Plg complex but had no effect on the affinity of SK for Plg, while, mutation of Lys_{698} impaired the initial formation of the activator complex and the affinity of SK for Plg (Wang *et al.*, 2000). In light of this, it is perhaps more likely, as suggested by Loy et al., 2001, that active site formation occurs via two simultaneous interactions of SK α and γ domains with Plg that are synergistic to the generation of an active site in the molceule (Loy *et al.*, 2001). In summary, whilst the exact function of each SK domain in Plg activation is not fully defined, it is possible to conclude that complex formation is initiated by the rapid and requisite binding of the SK β domain to the 5^{th} kringle of Plg (Wang *et al.*, 1999a). This interaction triggers a conformational change in Plg from the 'closed' to 'open' conformation, followed by the binding of all three domains of SK to the catalytic domain of Plg (Loy *et al.*, 2001). Active site formation is cooperatively induced by the SK α and γ domains, while substrate Plg is recognised by the activator

complex through interactions predominately mediated by the SK α domain (Loy *et al.*, 2001).

1.7.3 Variability of Streptokinase from GAS

Although S. *pyogenes* is restricted to a singular human host, SK alleles from distinct clinical isolates exhibit considerable genetic diversity and variability of SK occurs across all three domains (Kapur *et al.*, 1995). The α and γ domains show 23% and 17% amino acid divergence, respectively, while 54% amino acid divergence occurs in the β domain. (Figure 1.4) (Kalia and Bessen, 2004). Through phylogenetic analysis of the β domain from the 80 *ska* variants, two distinct phylogenetic lineages have been identified, termed cluster type-1 and cluster type-2, with further evidence of sub clusters in type 2 (cluster type-2a and 2b) (Figure 1.7) (Johnson *et al.*, 1992, Kalia and Bessen, 2004).

SK variation has been linked to strains associated with acute post streptococcal glomerulonephritis (Malke, 1993, Peake *et al.*, 1991) and to strains that exhibit skin tissue tropism (Kalia and Bessen, 2004). However, the full biological significance of SK polymorphism in streptococcal pathogenesis has not been determined. It has recently been shown that variant SK proteins produced by distinct clinical S. *pyogenes* isolates display differing Plg activation capacities (McArthur *et al.*, 2008). SK encoded by cluster 1 *ska* alleles readily formed an active complex with soluble Plg while cluster 2 SK required the formation of a tri-molecular complex with Plg and fibrinogen to display Plg-activating capability (McArthur *et al.*, 2008). The phenotypic differences displayed by SK variants suggest that these proteins may have differing roles in streptococcal

pathogenesis. Despite such phenotypic differences, mechanisms have been proposed by which all S. *pyogenes* strains with differing *ska* alleles can acquire plasmin activity onto the cell surface. Plg receptors interact with different regions of the Plg protein. PAM binds to Plg via interaction with K2 (Wistedt *et al.*, 1998), whereas

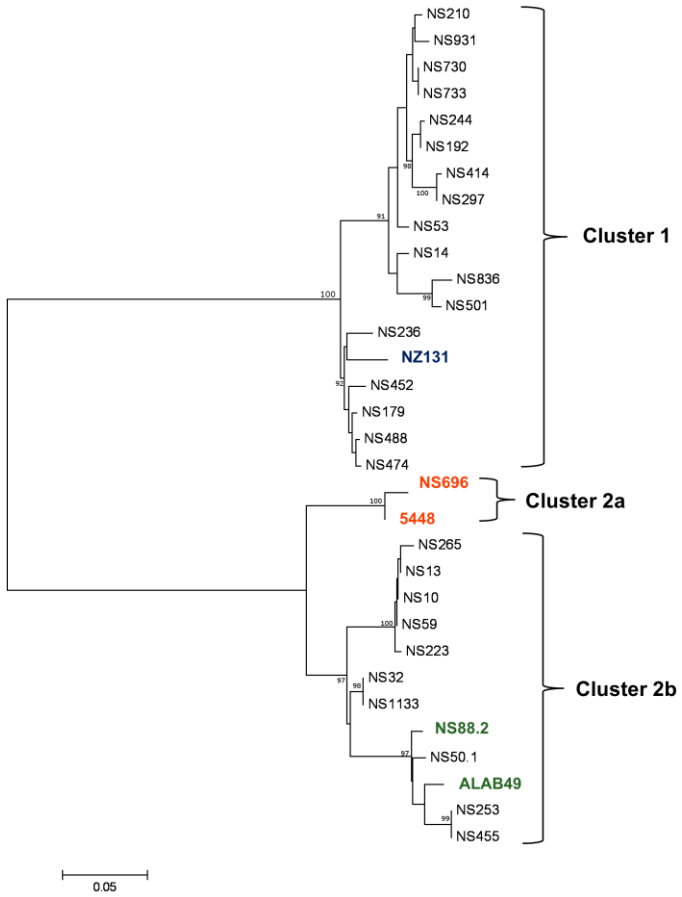

Figure 1.7: Phylogenetic tree for a 423 bp variable region encoding the β -domain of streptokinase. Bootstrap values of >90% (500 replicates) are indicated. Scale bar = 0.05 substitution per site. *ska* alleles used in this study as representative SK molecules for each cluster type are highlighted in colour. Figure adapted from McArthur *et al.*, 2008.

fibrinogen and other streptococcal Plg receptors (SEN and GAPDH) are believed to bind via lysine-dependent interactions with K1, K4, and K5 (Ponting *et al.*, 1992). Therefore, in strains that express cluster 2 type SK, the active tri-molecular complex can only be bound to the cell surface via PAM or fibrinogen-binding receptors (FgR) such

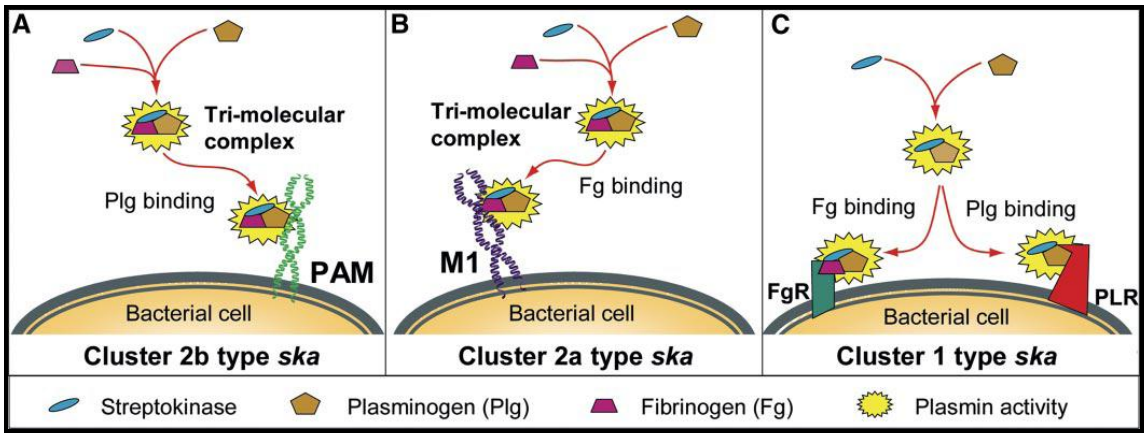

Figure 1.8 Schematic diagram summarising the hypothesised pathways of cell surface plasmin acquisition by GAS strains expressing different alleles of SK. **A)** Cluster type-2b SK must combine with Plg and fibrinogen to form a trimolecular complex that exhibits plasmin activity. The trimolecular complex is bound to the cell surface *via* the interaction between PAM and kringle 2 of Plg. Other GAS Plg-binding proteins such as SEN cannot bind this trimolecular complex, because the domains K1, K4, and K5 required for interaction are involved in the interaction with fibrinogen. **B)** Cluster type-2a SK is believed to require Plg and fibrinogen to form a trimolecular complex that exhibits plasmin activity. The trimolecular complex is bound to the cell surface *via* the interaction between fibrinogen-binding receptors (FgR) such as M1 protein and fibrinogen. Fibrinogen can also initially bind to FgR and recruit SK-Plg complexed to the cell surface to form the tri-molecular complex **C)** Cluster type-1 SK will combine with Plg to form a complex with plasmin activity. This complex can be bound directly to the bacterial cell surface *via* Plg receptors (PLRs) or through an interaction with fibrinogen and fibrinogen receptors. Figure from McArthur *et al.*, 2008.

as Ml (Figure 1.8). Alternatively, strains that express cluster 1 type SK can bind the activator complex directly to the bacterial cell surface via Plg receptors (PLRs) or through an interaction with fibrinogen and FgR (Figure 1.8) (McArthur *et al.*, 2008).

Interestingly, a sub-cluster of highly related *ska* alleles is found in strong linkage disequilibrium with the *pam* gene, which clusters amongst the skin-tropic *emm* pattern D isolates (Kalia and Bessen, 2004, Svensson *et al.*, 1999). PAM and SK cooperate in GAS plasmin acquisition *in vitro*, and both genes are required for full virulence in an *in vivo* model of impetigo (Ringdahl *et al.*, 1998, Svensson *et al.*, 2002). Together, these data support an involvement of *ska* allelic subtypes in tissue tropism at the skin (Kalia and Bessen, 2004). Further analyses of the cluster groups have determined that cluster-1 *ska* alleles are identifiable from all three different disease related *emm* pattern groups (pattern A-C, pattern D and pattern E). Interestingly all type-1 SK D pattern isolates lack PAM (Kalia and Bessen, 2004). Isolates containing type-2a SK were determined to come from *emm* pattern groups A-C and D. Type-2a SK isolates also lack PAM (Kalia and Bessen, 2004). Variants from sub-cluster type-2b harbour D *emm* patterns, and are PAM positive. These results have led to the hypothesis that type-2b *ska* alleles and *pam* have been co-inherited and that PAM may play a vital role in harnessing plasmin activity to the bacterial cell surface and disease manifestation GAS isolates containing a cluster type-2b SK allele (Kalia and Bessen, 2004). Further research investigating the biological significance of *ska* gene polymorphisms is required.

1.8 Streptokinase as a Thrombolytic Therapeutic

1.8.1 Overview

Occlusion of blood vessels by thrombus (blood clot) is an essential function of haemostasis. Under healthy conditions thrombus formation is suppressed, but reacts extensively in the event of vascular injury to prevent blood loss. Thrombus formation is limited to the site of vessel injury and is regulated to prevent clot dissemination through normal healthy vessels. In the event of pathological conditions and/or failure to maintain haemostasis, several cardiovascular disease states can arise and are divided into three main categories: atherosclerotic heart disease (acute myocardial infarction), cerebrovascular disease (stroke) and venous thromboembolism (deep-vein thrombosis and pulmonary embolism) (Collen *et al.*, 1988, Collen, 1990, Francis and Marder, 1991). Cardiovascular disease has increased significantly over the past few years and is a major cause of disability and is the most common cause of mortality, accounting for approximately 50% of deaths in individuals over the age of 50 (Lloyd-Jones *et al.*, 2009, Collen *et al.*, 1988).

Pathological thrombosis formation is currently treated using thrombolytic therapy to dissolve clots, resulting in reperfusion of the occluded blood vessels and improved outcomes from resulting disease states (Collen *et al.*, 1988). Thrombolytic therapy involves intravenous infusion of Plg activators and has been in practice for almost 50 years (Fletcher *et al.*, 1958, El-Gengaihy *et al.*, 2007). The relatively recent realisation that early administration (under 3 h) of thrombolytics can distinctly reduce mortality rates, along with the rise of recombinant DNA technology has rekindled interest in

thrombolytic therapy as a preventative measure against the burden of cardiovascular disease (Banerjee *et al.*, 2004). Consequently, tPA and SK became established as a lifesaving treatment against acute myocardial infarction and were approved by the US Food and Drug Administration (FDA) in the mid-1980s (Collen *et al.*, 1988, El-Gengaihy *et al.*, 2007).

1.8.2 Comparison of Plg Activators Used in Thrombolytic Therapy

There is currently several existing thrombolytic therapeutics that are approved for use in clinical settings. However, each agent differs in efficacy of thrombolysis, fibrin specificity, half-life, activation potential, immunogenicity and associated bleeding complications; as such, preference has not been assigned to one kind. The relatively non-fibrin-specific agents include SK, anistreplase (anisoylated Plg SK activator complex, APSAC), and uPA. The newer (or second-generation) Plg activators include tPA (alteplase), staphylokinase and several variants of tissue-type Plg activator: reteplase (r-PA), tenecteplase (TNK-tPA), and lanoteplase (n-PA). However, the limitations of current Plg activators used as therapeutics continue to drive research for improved agents.

SK is the least expensive fibrinolytic agent and is the most widely used thrombolytic therapeutic, particularly in developing countries. Studies comparing thrombolytic agents have shown that SK to be as good as that of tPA in terms of both efficacy and safety (Boland *et al.*, 2003, Dundar *et al.*, 2003). However, the use of SK as a therapeutic has been limited by immunogenicity, short half-life and lack of fibrin specificity. These drawbacks have seen a decline in the use of SK in most developed nations (Sikri and

Bardia, 2007). Short half-life and higher incidence of clinically significant haemorrhagic complications associated with other Plg activator use has also driven the continuation of therapeutic SK use over other thrombolytics. SK is the least expensive, but most immunogenic Plg activator and is not fibrin specific. Fibrin bound plasmin generated by the SK-Plg complex causes direct fibrinolysis of thrombus, however, the explosive generation of unbound plasmin away from the site of thrombus leads to systemic fibrinolysis and a hypocoagulable state, which in turn, can lead to non-specific bleeding events. This is due to depletion of circulating fibrinogen, Plg and factors V and VIII and an increase in bradykinin production (which lowers blood pressure) (Blann *et al.*, 2002, Khan and Gowda, 2003, Ohman *et al.*, 2001). SK is eliminated from the body via the liver and can be inactivated in part by anti-streptococcal antibodies resulting from previous streptococcal infection or SK therapy (Bruserud *et al.*, 1992). Plasmin produced by SK-mediated activation of Plg also degrades SK. Consequently, SK has two half-lives: rapid (11-13 mins) due to circulating antibodies and slow (23-29 mins) due to loss of activity by plasmin degradation (Fedan, 2004, Wu *et al.*, 1998).

Both uPA and tPA are trypsin like serine proteases which activate Plg directly and are non-immunogenic (Table 1.4). uPA activates both circulating and fibrin bound Plg, but does not bind to fibrin itself. uPA has a half-life of 15-20 min which results in less systemic fibrinolysis when compared to SK (Fedan, 2004). In developed countries, tPA is the most common fibrinolytic agent used for treatment of coronary artery thrombosis, pulmnary embolism and acute stroke (El-Gengaihy *et al.*, 2007). tPA is a fibrin specific Plg activator which means plasmin production is confined to the area of thrombus. Circulating tPA is rapidly cleared from plasma (initial half-life of 4-10 min) resulting in

high rates of re-thrombosis (Khan and Gowda, 2003). As a result, for greatest efficacy tPA administration is via an initial bolus injection, followed by short continuous infusion (Khan and Gowda, 2003). Such large doses can produce systemic fibrinolysis and unwanted bleeding episodes, side effects similar to those seen with SK treatment. Therefore, much research has been aimed at improving the efficacy of current Plg activators or identifying novel activators with more favourable Plg activation characteristics, some of which have been summarised in Table 1.4.

1.8.3 Enhancing Streptokinase for use as a Therapeutic

SK is an immunogenic protein and as humans are frequently exposed to streptococcal infections, the presence of circulating anti-SK antibodies can be detected in most individuals (Tillett *et al.*, 1934). Upon exposure to purified SK protein during thrombolytic therapy, high titres of anti-SK antibodies are generated and can be long lasting (up to 54 months) (Lee *et al.*, 1993). High levels of circulating anti-SK antibodies reduce the effectiveness of repeat SK therapy by rapidly neutralising SK upon administration or by causing numerous allergic complications (Jalihal and Morris, 1990, Mcgrath and Patterson, 1984) A number of antigenic regions have been identified within the SK protein by using murine monoclonal antibodies or patient sera (Parhami-Seren *et al.*, 2003, Reed *et al.*, 1993, Torrens *et al.*, 1999, Coffey *et al.*, 2001). Targeting these regions with deletion or site directed mutagenesis has been proposed as possible mechanisms by which the immunogenicity of SK can be reduced. An obvious requirement with this approach is to retain the Plg activating properties of native SK. Parhami-Seren *et al.* (2003) used naturally occurring variants of SK from different strains of group G and group A streptococcus to determine how amino acid sequence

Table 1.4: Comparison of thrombolytic agents that currently approved or being developed for human use.

	Streptokinase*	Antistreplase (APSAC)*	uPA*	Pro-uPA	tPA (Alteplase)*	rtPA (Reteplase)*	TNK-PA (Tenecteplase)*	nPA	Staphylokinase
Molecular Weight (kDa)	47	131	32/55	49	68	39.5	65	53.5	16.5
Source	*Streptococcus equisimilis*	*Streptococcus equisimilis*, plasminogen anisoylated	Recombinant, human fetal, kidney	Recombinant, *E. coli* or, mammalian cells	Recombinant, human melanoma cell line	Recombinant, human mutant tPA	Recombinant plus mutation	Recombinant plus mutation	*Staphyloccocus aureus*
Plasminogen Activation	Indirect	Indirect	Direct	Direct	Direct	Direct	Direct	Direct	Indirect
Half-life (min)	20	100	<5	9	5	14	25	57	6
Fibrin Specificity	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes
PAI-1 Resistance	N/A	N/A	No	No	No	No	Yes	No	N/A
Elimination	Hepatic	Hepatic	Renal	Hepatic	Hepatic	Renal	Renal	Hepatic	Hepatic
Antigenic	Yes	Yes	No	No	No	No	No	No	Yes
Dosing (AMI treatment)	1 h infusion	10 min single bolus	1 h infusion	Bolus, 1 hr infusion	Bolus, 90 min infusion	Double bolus	Single bolus	Single bolus	Double bolus over 30 min
Hospital Cost/Dose ($US)	$300	$1,800	$2,000	>$2,000	$2,200	$2,200	$2,200	$2,200	N/A

Abbreviations: kDa = KiloDalton, PAI-1 = Plasminogen Activator Inhibitor-1, uPA = urokinase, tPA = tissue type plasminogen activator, N/A = Data Not Available, AMI = acute myocardial infarction, * = FDA approved. (Ross, 1999, Banerjee *et al.*, 2004, Baruah *et al.*, 2006, Fedan, 2004, El-Gengaihy *et al.*, 2007, Ohman *et al.*, 2001, Khan and Gowda, 2003)

variation in previously identified antigenic epitope regions affected antibody binding and SK function.

In this study, variant SK proteins which contained a $Ser_{138}Lys$ substitution were not recognised by an anti-SK monoclonal antibody but displayed normal Plg activation activity. When this amino acid change was introduced into therapeutic SK, the mutant protein displayed unchanged Plg activation activity but was not bound by the anti-SK mAb (Parhami-Seren *et al.*, 2003). SK variation in GAS has evolved through the interaction of this pathogen with the human fibrinolytic and immune systems. Therefore, careful analysis of novel variants in future studies will assist in identifying regions that can be mutated for immunogenic purposes without disrupting Plg activation function.

Attempts to increase the *in vivo* half-life of SK have utilised chemical modifications and site directed mutagenesis. Plasmin will initially cleave SK at Lys_{59} and Lys_{386} producing a 37 kDa intermediate which retains only 16% of the Plg activation activity of native SK (Shi *et al.*, 1994). A $Lys_{59}Glu$ SK mutant maintained Plg activation activity that was comparable to native SK but displayed increased resistance to plasmin proteolysis indicating that this variant may have a longer functional half-life in therapeutic applications (Wu *et al.*, 1998). Similarly, PEGylated SK proteins also maintain good activator function but are protected from proteolytic degradation and display decreased antigenicity due to increased steric interference (Koide *et al.*, 1982, Rajagopalan *et al.*, 1985). Currently, the only modified SK approved for human use is acylated Plg-SK activator complex (APSAC) (Khan and Gowda, 2003). This therapeutic consists of an

inactive complex of SK and Lys-Plg containing an acylated catalytic centre. Upon injection, APSAC undergoes a controlled deacylation before displaying thrombolytic activity which serves to increase the half-life of this therapeutic (Crabbe *et al.*, 1990). This allows APSAC to be administered as a rapid, bolus injection which is more favourable when compared with the long infusions required with native SK. However, the higher costs associated with this therapy has limited the use of APSAC in clinical settings (Khan and Gowda, 2003) (Table 1.4)

Modifying the Plg activation characteristics of SK has also been attempted to improve the therapeutic potential of this molecule. Fibrin-independent Plg activation by the SK-Plg activator complex is thought to be due to the insensitivity of the activator complex to α_2-AP inhibition and due to the ability of the activator complex to activate circulating Glu-Plg in the absence of fibrin (Sazonova *et al.*, 2009). Critical for these characteristics is the a-domain of SK. SK mutants harbouring an N-terminal, 59 amino acid deletion (SKΔ59) of the α-domain will only form an activator complex with plasmin which is susceptible to α_2-AP inhibition and is a poor activator of circulating Glu-Plg (Reed *et al.*, 1999, Sazonova *et al.*, 2004). However, the SKΔ59-plasmin complex will readily activate unfolded forms of Plg such as Glu-Plg in the presence of fibrin (Reed *et al.*, 1999).When compared to tPA, this mutated form of SK displays less fibrinogen degradation in human plasma and\has a greater capacity to degrade blood clots *in vivo* using a murine model of human thrombosis (Sazonova *et al.*, 2009).

SK from GAS are polymorphic and these variants posses differing Plg activation characteristics. These differences are a result of divergence of GAS SK away from

group C SK during a long co-evolution with the human host. This divergence could naturally confer some of the characteristics that have been introduced previously into the group C SK alleles via recombinant DNA technologies. Sequence modifications have been the focus of attempts at increasing half-life, reducing or eliminating immunogenicity and generally improving the activation potential of SK. However, the genetic diversity displayed by *ska* alleles from GAS isolates represent an existing reservoir of SK proteins that have become attuned to evading the host immune system and interacting with the human fibrinolytic system.

1.9 Summary and Aims of the Current Research

The increasing incidence of invasive GAS infection over the past two decades has re-ignited interest in the study of the invasive pathogenesis of the bacterium, *Streptococcus pyogenes.* The subversion of the host Plg activation system by GAS facilitates the capture of cell-surface proteolytic activity which is used by the bacterium for invasion into normally sterile areas of the host. SK has been identified as one of the key factors in PAS subversion. Allelic variants of SK produced by GAS isolates display unique Plg activation properties, however the biological significance of SK polymorphism among GAS isolates is yet to be elucidated. Therefore, the underlying hypothesis of this project is that phenotypic differences resulting from *ska* polymorphism have an effect on the pathogenesis of this organism. In this project, we propose to undertake part of the first comprehensive phenotypic study of SK variants produced by GAS by characterising the structural, functional and biochemical differences displayed by SK variants. Investigating the role of SK polymorphism and

effect on GAS virulence will further our understanding of PAS specific pathogenic mechanisms employed by GAS and will assist in the rational design of recombinant SK molecules that can be used as second generation thrombolytic therapeutics with improved efficacy and safety, as well as potentially assisting in the development of novel strategies to treat invasive disease caused by *S. pyogenes*..

Specific aims

(i) Develop a system to express and purify recombinant forms of SK variants from GAS.

(ii) Undertake structural, functional and biochemical characterisation of recombinant SK variants

(iii) Investigate the effect of host and bacterial plasminogen co-factors on SK mediated activation of Plg

(iv) Assess the effect of phenotypic differences displayed by SK on GAS pathogenesis.

Chapter 2:

Cloning and Expression of Streptokinase Variants from *Streptococcus pyogenes*

Preface

Current SK expression protocols have been developed to produce large quantities of SK for therapeutic purposes, however many of these protocols produce SK protein of poor quality or low yield of full length active SK. Thus, to examine the subtle structural and functional differences displayed by GAS SK variants, a recombinant expression system that results in the production of pure, full-length active protein was crucial. The work in this chapter highlights the development of a SK expression system which produced high quality protein that could be used in numerous downstream characterisation processes to elucidate the molecular basis for the phenotypic differences displayed by GAS SK variants.

Publication: **Cook, S. M**., Walker, M. J. and McArthur, J. D., (2014) "Cloning and expression of streptokinase variants from *Streptococcus pyogenes* to assist in the characterisation of streptokinase mediated plasminogen activation." (*Manuscript prepared*).

2.1 Abstract

Streptokinase (SK) is a bacterial plasminogen activator, secreted by β-hemolytic group A, C and G strains of the genus *Streptococcus*. Among group A streptococcal (GAS) isolates, SK gene sequences (*ska*) are polymorphic and can be grouped into two distinct sequence clusters (termed cluster type-1 and cluster type-2) with cluster type-2 being further divided into sub-clusters type-2a and type-2b. In this study, SK from 5 different GAS isolates, which represented examples from the three known phylogenetic sequence clusters of SK were purified. SK from the group C streptococcal strain H46A was also expressed for use as a control, as it is the most widely studied SK variant due to its use as a thrombolytic therapeutic. Polymerase chain reaction with *ska* variant specific 5' and 3' primers was used on template *Streptococcus spp.* genomic DNA. This resulted in the amplification of DNA fragments of ~1250 bp, which correlated to the expected size of *ska* (without the region encoding the 26-amino acid signal peptide) with restriction enzyme (5' and 3' ends) and Factor Xa protease recognition sequences (5' end) incorporated. These fragments were cloned into pQE-30 for expression as N-terminal poly-histidine fusion proteins which could be purified via nickel affinity chromatography. SK fusion proteins had an estimated molecular mass of ~49 kDa which correlates to the theoretical molecular weight (49028 Da) and were regularly purified at yields between 25 and 35 $mg.L^{-1}$ of culture. The poly-histidine tag was removed from purified SK proteins using Factor Xa digestion which also produced an unwanted cleavage event at Arg_{401}-Tyr_{402} in the C-terminus of the protein. Anion exchange chromatography was used to separate full length SK from the truncated

protein fragments and from Factor Xa. Recombinant SK proteins were subsequently used in further structural and biochemical analyses as detailed in chapters 3, 4 and 5.

2.2 Introduction

Streptokinase (SK) is a potent plasminogen (Plg) activator of bacterial origin, secreted by β-haemolytic *Streptococcal* A, C and G isolates. SK consists of 414 amino acid and is composed of three distinct domains; α (aa 1-150), β (aa 151-287) and γ (aa 288-414) (Wang *et al.*, 1998). The three domains of SK are separated by two coiled-coil regions while the N- and C- termini of the protein have disordered flexible structures (Wang *et al.*, 1998). Despite extensive research, the exact role each domain plays in the activation of human Glu-Plg is not completely understood, nor is the molecular mechanism of SK mediated Plg activation (Boxrud and Bock, 2004).

SK from the group C streptococcal isolate H46A is the most frequently characterised SK variant (Christensen, 1945). To date, the majority of expression, purification, structural and functional characterisation studies have been conducted on group C SK due to its extensive use as a thrombolytic therapeutic (McArthur *et al.*, 2012). Group A streptococcus (GAS; *Streptococcus pyogenes*) secrete SK as a virulence determinant to generate soluble protease activity and to assist in the acquisition of plasmin activity to the bacterial cell surface, allowing invasion into normally sterile tissue of the host (McArthur *et al.*, 2012). While SK from group C streptococcal isolates display minimal genetic diversity, SK from human specific GAS isolates display considerable genetic variability (Kalia and Bessen, 2004, McArthur *et al.*, 2008, Kapur *et al.*, 1995, Zhang *et al.*, 2012, Cook *et al.*, 2012). Phylogenetic studies of the most divergent *ska* sequences have revealed two main sequence clusters (cluster type-1 and 2) with evidence of

smaller sub-clusters observed in cluster type-2 sequences (cluster type-2a and 2b) (Kalia and Bessen, 2004, McArthur *et al.*, 2008). To examine the specific phenotypic differences displayed by SK variants, a recombinant expression system that results in the retrieval of pure, full-length active protein is crucial. Current SK expression protocols are designed to produce large amounts of SK for therapeutic purposes, however many of these protocols produce SK protein of poor quality (Longstaff *et al.*, 2005). Common contaminants present in recombinant SK preparations include proteins containing unwanted N-terminal methionine residues or proteins containing C-terminal truncations. Both modifications are known to reduce the ability of SK to activate Glu-Plg (Wang *et al.*, 1999a, Zhai *et al.*, 2002). In this study, we developed a protocol to produce full-length recombinant SK protein (1-414 amino acids), possessing a functional Ile_1 residue, which is separated from C-terminal truncation products and other contaminating protein. This technique was used to produce phylogenetically distinct recombinant SK proteins to assess the phenotypic differences displayed by these variants (Cook *et al.*, 2012, Russell and Facklam, 1975).

The *ska* alleles used in this study were amplified from genomic DNA from five different GAS isolates of both Australian and International origins that represented type-1, type-2a and type-2b *ska* (highlighted in Figure 1.7). These clinical strains were isolated from patients suffering from a range of disease states encompassing invasive, superficial skin and throat infections as well as a patient presenting with a case of GAS induced non-suppurative sequelae. Type-1 SK came from strain NZ131 which was isolated from a case of acute post streptococcal glomerulonephritis; type-2a SK representatives came from strains 5448 and NS696 which were isolated from patients with invasive

(necrotising fasciitis) and uncomplicated (throat) infections, respectively; and type-2b SK representatives came from strains ALAB49 and NS88.2 which were isolated from an impetigo lesion and from the blood blood of infected patients respectively (Simon and Ferretti, 1991, Svensson *et al.*, 2000, McKay *et al.*, 2004, Aziz *et al.*, 2004). SK from human associated isolate of group C streptococcus (H46A) was also expressed for use as a control (Christensen, 1945).

The cloning, expression and purification system developed in this chapter produced high quality recombinant SK proteins that were used in subsequent experiments elucidating the molecular basis for the phenotypic differences displayed by these variants.

2.3 Materials and Methods

2.3.1 Bacterial strain, culture conditions and reagents

GAS isolates NZ131 (Simon and Ferretti, 1991), ALAB49 (Svensson *et al.*, 2000), NS88.2 (McKay *et al.*, 2004), NS696 (McKay *et al.*, 2004), 5448 (Aziz *et al.*, 2004) and *S. dysgalactiae* subsp. *equisimilis* strain H46A (Christensen, 1945) were used in this study. Streptococci strains were routinely cultured at 37°C on horse-blood agar (Biomerieux, Sydney, NSW, Australia) or in static liquid cultures of Todd-Hewitt broth (BD, Sydney, NSW, Australia) supplemented with 1% (w/v) yeast extract (Oxoid, Adelaide, SA, Australia) (THY medium). *E. coli* strains JM109 and M15[pREP4], were used as hosts for plasmid construction and protein expression respectively, and were cultured at 37°C in Luria-Bertani broth (Sambrook *et al.*, 1989). Where appropriate, antibiotics were used for selection at the following concentrations: chloramphenicol, 100 $\mu g.mL^{-1}$; kanamycin, 50 $\mu g.mL^{-1}$; and ampicillin, 100 $\mu g.mL^{-1}$. Factor Xa was purchased from Sigma-Aldrich, Sydney, NSW, Australia.

2.3.2 DNA cloning strategy for *ska* variants

Genomic DNA from each GAS strain was extracted using a Wizard® Genomic DNA Purification Kit as per manufactures instructions (Promega, Madison, WI, USA). The SK encoding gene (*ska*) from each GAS strain without the 26-amino acid signal peptide, was amplified from genomic DNA by polymerase chain reaction (PCR). PCR primers were designed to incorporate *Bam*HI and *Pst*I restriction sites at the 5' and 3' ends of the fragment respectively. This allowed cloning into pQE-30 (Qiagen, Valencia, CA,

USA) for expression of recombinant SK as a poly-histidine tagged fusion protein. A Factor Xa recognition site was incorporated at the 5' end of sense primers to facilitate removal of the poly-histidine tag after purification and expose the functional Ile_1 N-terminal residue of SK. The primers used for amplification *ska* are as follows, a solid underline indicates a *Bam*HI recogntion sequence, double underline indicates a *Pst*I recognition sequence and a dashed underline indicates the sequence coding for the Factor Xa recognition sequence: Type-1, type-2a and type-2b *ska* sense (5'-GTGGATCCATCGAGGGAAGGATTGCTGGGTATGAATGGCTG-3'). H46A *ska* sense (5'-GTGGATCCATCGAGGGAAGGATTGCTGGACCTGAGTGGCTG-3'). Type-1, type-2a and type-2b (NS88.2) *ska* antisense (5'-TGCTGCAG TTATTTGTCTTTAGGGTTATC-3'). H46A and type-2b (ALAB49) *ska* antisense (5'-TGCTGCAGTTATTTGTCGTTAGGGTTATC-3') (Sigma-Aldrich, Sydney, NSW, Australia). Each PCR mix contained 0.25 μL of PfuUltra II DNA polymerase (Agilent Technoligies, Santa Clara, CA, USA), in 1X Pfu reaction buffer, 1 μL of a 1:40 dilution of GAS cDNA as template, forward and reverser primers (1 μM) (Sigma-Aldrich, Sydney, NSW, Australia), 200 μM dNTPs (Bioline, NSW, Australia) and dH_2O to a total reaction volume of 20 μL. PCR were performed in a Thermocycler (Eppendorf, Hamburg, Germany) using 1 cycle of 95°C for 1 min, 35 cycles of 95°C for 20 s, 55°C for 30 s and 72°C for 1 min, followed by a final extension period of 10 min at 72°C. Once completed reactions were assessed for DNA amplification by agarose gel electrophoresis, before restriction digestion and ligation into linearised pQE-30 vector. Each ligation mixture was electo-transformed into *E. coli* JM109 and positive clones screened by either colony PCR using the same PCR parameters as above with Taq polymerase (Bioline, Sydney, NSW, Australia) and/or crude alkaline lysis. Positive

clones were confirmed by repeat restriction digestion to release the *ska* gene (~1200 bp) from the linearised vector (~3400 bp). Upon completion of restriction digestion analysis suspected positive clones were confirmed by DNA sequence analysis.

2.3.3 Restriction enzyme digestion

Restriction enzyme digestion of DNA was performed using *Bam*H1 and *Pst*I (Fermentas, Pittsburgh, PA, USA). To prepare pQE-30 and full length *ska* for cloning, purified plasmid or insert *ska* DNA (~1 μg) was double digested with 20 U of *Pst*I (Fermentas, USA) and 10 U of *Bam*H1 (Fermentas, Pittsburgh, PA, USA) in a 20 μL reaction volume containing 1X *Bam*H1 buffer (Fermentas, Pittsburgh, PA, USA). Digestions were incubated at 37ºC for 2 h and restriction enzymes were heat deactivated by incubation at 80°C for 20 min. Agarose gel electrophoresis (see section 2.3.5) was used to visualise and separate the digested DNA fragments, which were subsequently extracted from the gel via Wizard® SV Gel and PCR Clean-up System (Promega, Madison, WI, USA) according to manufacturer specifications.

2.3.4 Ligation of recombinant plasmids

Ligations were performed using T4 ligase (Fermentas, Pittsburgh, PA, USA). Each ligation contained ~100 ng of digested plasmid DNA, ~500 ng of insert DNA and 20 U of T4 ligase (Fermentas, Pittsburgh, PA, USA) in a final reaction volume of 30 μL containing 1X T4 ligase buffer (Fermentas, Pittsburgh, PA, USA). Ligations were incubated at 15°C over-night, followed by T4 ligase inactivation by incubation at 65°C for 15 min. Ligations were purified for transformation via Wizard® SV Gel and PCR

Clean-up System (Promega, Madison, WI, USA) according to manufacturer specifications.

2.3.5 Agarose gel electrophoresis

Agarose gel electrophoresis was performed with a 0.85% agarose gels in 1X TAE buffer. Electrophoresis was conducted at 90 V for 1 h in a MiniSub Cell GT electrophoresis tank (BioRad, Hercules, CA, USA). DNA was visualised using a Novaline Gel Documentation System (Novex, St Lucia, QLD, Australia) under ultraviolet light (UVP, Upland, CA, USA) after staining in ethidium bromide solution (1 $\mu g.mL^{-1}$) for 20 min and de-staining in dH_20 for 10 min. Approximate DNA fragment size and concentration was determined by comparison to the migration of GeneRuler™ 1kb DNA Ladder (Fermentas, Pittsburgh, PA, USA) (Appendix 8).

2.3.6 Preparation of electro-competent *E. coli* JM109 and M15/pREP4

To prepare electro-competent *E. coli* JM109 and M15/pREP4 for transformation with recombinant plasmid DNA, a single colony of each bacterial strain was grown in 3 mL of LB broth overnight at 37°C with shaking. The culture was used to inoculate 400 mL of LB broth and grown at 37°C with shaking to an optical density (OD_{600}) of 0.6. The culture was chilled on ice for 20 min before centrifugation at 4,000 x *g* for 15 min at 4°C to harvest the cells. The cells were re-suspended in 400 mL of cold MilliQ water (Millipore, Sydney, NSW, Australia) and re-centrifuged. The pellet was washed by re-suspension in 200 mL of cold MilliQ water (Millipore, Sydney, NSW, Australia) and centrifuged again. Cells were re-suspended in 4 mL of chilled 10% glycerol and

centrifuged under the same conditions, before final re-suspension in 600 µL of chilled 10% glycerol. Cells were distributed into 40 µL aliquots for storage at -80°C.

2.3.7 Electro-transformation of electro-competent E. coli JM109 and M15/pREP4

Transformation of *E. coli* JM109 or M15/pREP4 was performed by adding 1 µL (~200 ng) of plasmid DNA to a 40 µL aliquot of electro-competent cells. The transformation mixture was transferred to a cold 1 mL, 2 mm gap, electro-transformation cuvette (Eppendorf, Hamburg, Germany) and transformed using MircoPulser™ (Bio-Rad, Hercules, CA, USA) using settings defined by the manufacturer for bacterial electroporation (2.5 µF capacitance, 2.5 kV single pulse, 20 Ω in series, 200 Ω in parallel). Following transformation, 500 µL of LB broth was added to the transformation mixture which was then transferred to a 1.5 mL microcentrifuge tube for incubation at 37°C for 1 h. To select for successfully transformed bacteria, dilution series of the transformation culture were plated onto LB agar containing appropriate antibiotics and incubated over-night at 37°C.

2.3.8 DNA sequence analysis of pQE-30::*ska* variant constructs

DNA sequencing to confirm the construction of hybrid ska alleles was conducted using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Perkin-Elmer, USA). Each sequencing reaction contained 0.5 µL of Big Dye Terminator Ready Reaction Mix (RR-100) (Perkin-Elmer, USA), in 1X Sequencing Buffer, ~500 ng (5 µL) of template DNA, 3.2 pmol sequencing primer (2 µL) (Table 2.1) and 0.5 µL of dH_20 to a total reaction volume of 10 µL. Sequencing reactions were performed using a Thermocycler (Eppendorf, Hamburg, Germany) over 25 cycles of 96°C for 10 sec, 50°C for 5 sec and

60°C for 4 min followed by a final extension period of 10 min at 60°C. Following sequencing PCR reactions, DNA was purified via ethanol precipitation by adding 3 µL of 2 M sodium acetate (pH 4.5) and 35 µL of cold 95% (v/v) ethanol to the reaction mix. The solution was incubated on ice for 10 min and centrifuged at 14,000 x g for 20 min to precipitate the DNA. The supernatant was removed and the DNA pellet re-suspended in 250 µL of cold 70% (v/v) ethanol. The re-suspended pellet was centrifuged at 14,000 x g for 20 min and the supernatant removed. The pellet was allowed to air dry away from light and stored at -20°C until sequencing analysis was performed. DNA sequencing was performed by Margret Phillips (Biological Sciences, University of Wollongong, Australia) using a 3130xl Genetic Analyzer (Applied Biosystems, USA), as per the manufacturer's instructions.

Table 2.1: Oligonucleotides used for DNA sequencing of chimeric *ska* alleles.

A forward and reverse sequence was produced for each gene to make consensus sequences and confirm domain switching. For sequencing of the mature SK encoding region a combination of primers listed in this table were used depending on *ska* variant type

Primer	Primer Sequence (5'- 3')
pQEF	CGGATAACAATTTCACACAG
pQER	GTTCTGAGGTCATTACTGG
*ska*seqFOR	GATCCCTTTGATCGCAGTCAC
*ska*seqREV1	TATGAAGTGAGCTTTGTCTCC
*ska*seqREV2	ATATACTGTACAGTTTACTCC

2.3.9 Expression and purification of recombinant SK protein

Recombinant SK protein expression in transformed M15[pREP4] was induced by addition of isopropyl-1-thio-b-D-galactopyranoside to log-phase cells at a final concentration of 1 mM. Three hours later, cells were harvested by centrifugation, resuspended in native lysis buffer (1 $mg.mL^{-1}$ Lysozyme, 0.1 mM PMSF, 10 mM $MgCl_2$, 5 $\mu g.mL^{-1}$ DNase I, 0.1% (v/v) Triton X-100, 10 mM Imidazole, 50 mM NaH_2P0_4, 300 mM NaCl, pH 7.4), lysed using an EmulsiFlex-C5 (Avestin Inc., Ottawa, ON, Canada) and purified under native conditions using nickel-nitriloacetic acid affinity chromatography. Purification of protein from cleared lysate was performed using an ÄKTAprime plus fast protein liquid chromatography (FPLC) system (GE Healthcare Life Sciences, Piscataway,NJ, USA) with two 1 mL HisTrap HP columns in series (QE Scientific, USA). The resin was equilibrated with 10 column volumes of binding buffer (50 mM NaH_2P0_4, 300 mM NaCl, 10 mM Imidazole, pH 8) and once equilibrated, 50 mL of 0.22 μm syringe filtered lysate was loaded onto the column. The column was washed with binding buffer until the A_{280} trace returned to a constant pre-sample injection baseline. The column was then washed with 10 column volumes of washing buffer (50 mM NaH_2P0_4, 300 mM NaCl, 20 mM Imidazole, pH 8) to remove non-specifically bound contaminants. Column bound recombinant SK was eluted from the column by flowing 20 column volumes of elution buffer (50 mM NaH_2P0_4, 300 mM NaCl, 250 mM Imidazole, pH 8) over the column resin and collected in 1mL elution fractions. The native recombinant SK proteins were cleaved from the poly-histidine tag by buffer exchange into QB cleavage buffer (50 mM Tris-HCl, 50 mM NaCl, 1 mM $CaCl_2$, pH 6.5) and incubation with $2U.mg^{-1}$ SK of Factor Xa (Sigma-Aldrich, Sydney, NSW, Australia) for 12-36 h at 4°C (Figure 2.1). Post Factor Xa treatment a secondary

truncation product was observed for all SK preparations during sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

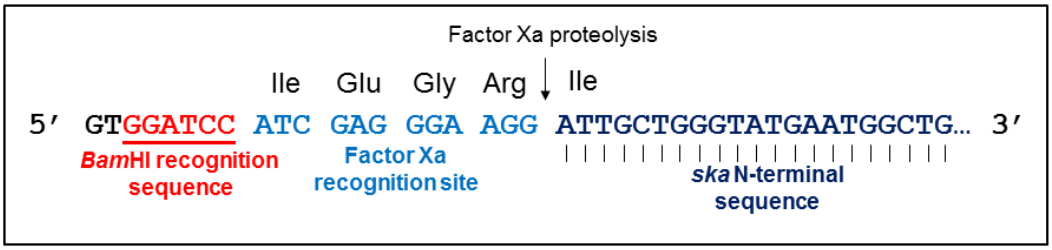

Figure 2.1: Exposure of the functional Ile_1 residue by limited proteolysis with Factor Xa. A schematic outlining the process by which recombinant poly-histidine tagged SK variants were cleaved with Factor Xa to expose the functional Ile_1 residue. Recombinant SK was incubated with Factor Xa which cleaves at the C-terminal side of the recognition sequence, as indicated, resulting in the removal of poly-histidine tags from full-length SK

2.3.10 Electrospray ionisation mass spectrometry (ESI-MS)

A positive ion mass spectrum of expressed and purified SK_{ALAB49} and SK_{NZ131} was acquired on a quadrupole time of flight spectrometer (Q-TOF-MS) (Micromass Q-TOF Ultima, Waters, Elstree, HRT, UK) fitted with a Z-spray ionisation source. SK_{ALAB49} and SK_{NZ131} were exchanged into 10 mM ammonium acetate buffer (pH 6.8) containing 0.1% formic acid and made up to a final concentration of 10 µM. The protein was then injected into the Q-TOF Ultima mass spectrometer (20 µL) and the mass spectrum acquired with a capillary voltage of 2.6 kV, cone voltage of 50 V, source block temperature of 40°C, and a resolution power of 5000 Hz. Ceasium iodide was used for external calibration. The mass spectrum data is presented as raw data, on an *m*/*z* scale. Mass was calculated using MassLynx MS software (Waters, Elstree, HRT, UK).

2.3.11 Anion-exchange fast protein liquid chromatography (FPLC)

To separate full-length recombinant SK from truncation products, a secondary purification step using anion exchange chromatography was undertaken using a Mono Q 5/50 GL column attached to an ÄKTA explorer FPLC (GE Healthcare and Lifesciences, Piscataway, NJ, USA). The resin was pre-equilibrated with 10 column volumes of exchange buffer A (20 mM Bis-Tris, pH 6.5) and each protein mixture (~5 mg) was subsequently loaded onto the column at a flow rate of 0.5 $mL.min^{-1}$, after being dialysed into exchange buffer A to remove salt and impart a net negative charge on each of the protein species. The bound protein was washed with ~10 column volumes of exchange buffer A to maintain a constant A_{280} trace baseline before a NaCl elution gradient up to 25% of exchange buffer B (20 mM Bis-Tris, 1 M NaCl, pH 6.5) was applied to the resin over 35 column volumes, at a flow rate of 0.4 $mL.min^{-1}$. These conditions resulted in optimal separation resolution between each protein species. Fractions of each A_{280} peak were collected in 500 µL aliquots and analysed by SDS-PAGE.

2.3.12 Gel electrophoresis and Western blotting

Expression and purification of SK was monitored by SDS-PAGE and Western blotting. Aliquots of the SK purification procedure were added to SDS-PAGE sample buffer containing 1% SDS and 5% β-mercaptoethanol at 100°C. Samples were separated using 10% SDS-PAGE before being transferred to PVDF membrane (Millipore, Sydney, NSW, Australia) via Western transfer. For detection of polyhistine tagged SK PVDF membranes were blotted using Ni^{2+}-NTA-HRP conjugate (Qiagen, Valencia, CA,

USA). To directly detect SK a polyclonal rabbit-anti-SK antibody (Jason McArthur, University of Wollongong, Wollongong, AUS) was used in combination with a secondary goat-anti-rabbit-HRP antibody conjugate (Pierce Biotechnology, Rockford, IL, USA). To visualise target proteins, 3,3'-diaminobenzidine at 0.05% (w/v) and hydrogen peroxide at 15% (v/v) diluted in 100 mM Tris, pH 7.4 was applied to the membrane.

2.4 Results

2.4.1 Amplification and cloning of *ska* alleles

To construct plasmid vectors for the expression of recombinant SK variants, *ska* was amplified from streptococcal genomic DNA using the primers described in section 2.3.2. The reaction product was analysed by agarose gel electrophoresis to determine if amplification was successful (Figure 2.2). A single band at approximately 1250 base pairs was observed corresponding to the expected size of *ska* with restriction enzyme and N-terminal Factor Xa protease recognition sequences incorporated (Appendix 1). The DNA fragments were purified from the gel, restriction digested with *Bam*HI and *Pst*I and ligated into pQE-30 with T4 ligase.

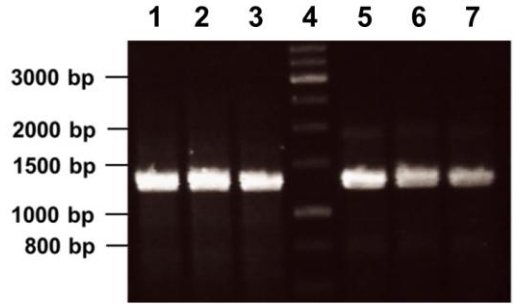

Figure 2.2: PCR amplification of *ska* from group A streptococcal cDNA. s*ka* was amplified using primers that incorporated a Factor Xa recognition site and *Bam*HI restriction digestion recognition sequence at the 5' end and a *Pst*I restriction digestion recognition sequence at the 3' end. Full length *ska* variants appear as a DNA band at ~1250 bp in a 0.85% agarose gel ska_{H46A} (lane 1), ska_{NZ131} (lane 2), ska_{NS696} (lane 3), ska_{5448} (lane 5), ska_{ALAB49} (lane 6) and ska_{NS696} (lane 6).

The ligation reactions were transformed into *E. coli* JM109 and screened by colony PCR and/or alkaline lysis (Sambrook *et al.*, 1989) (data not shown). Successful plasmid constructs for each variant were confirmed by extracting plasmid DNA from *E. coli* JM109 transformants and subsequent restriction enzyme analysis with *Bam*HI and *Pst*I. DNA fragments of ~3400 and ~1250 bp were observed for all constructs, which corresponds to the predicted sizes of pQE-30 (~3400 bp) and full length *ska* (~1250 bp) (Figure 2.3).

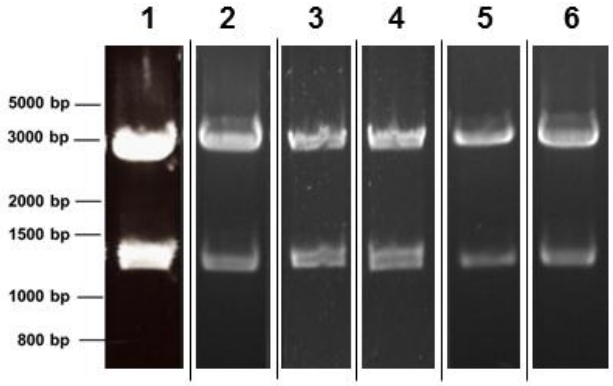

Figure 2.3: Restriction digestion of pQE-*ska* constructs. Composite figure of insert positive pQE-30 constructs identified via colony PCR or crude alkaline lysis that were extracted using a Wizard® Plus Mini-prep DNA purification system and digested with 10U of *Bam*H1 and 20U of *Pst*I for 2 hours at 37°C. pQE-30::*ska* constructs were deemed positive if a DNA band was released at ~1250 bp from the linearised pQE-30 vector (~3400 bp) on 0.85% agarose gel. Positive constructs were DNA sequenced to confirm the correct *ska* sequence was present in the correct coding frame with no errors. pQE-SK_{H46A} (lane 1), pQE-SK_{NZ131} (lane 2), pQE-SK_{NS696} (lane 3), pQE-SK_{5448} (lane 4), pQE-SK_{ALAB49} (lane 5) and pQE-SK_{NS696} (lane 6).

2.4.2 Expression and purification of recombinant SK variants

Recombinant SK proteins were expressed as N-terminal, poly-histidine fusion proteins and were purified via Ni^{2+}-NTA affinity chromatography using an ÄKTAprime plus

FPLC (GE Healthcare Life Sciences, Piscataway, NJ, USA) (Figure 2.4A). SDS-PAGE analysis indicated the purified recombinant protein had a molecular mass of ~49 kDa (Figure 2.4B), which corresponds closely to the theoretical molecular weight of fusion tagged SK (49028 Da). Additionally, the recombinant protein also reacted with rabbit polyclonal antisera specific for SK from group C streptococcus (McArthur *et al.*, 2008) (data not shown). The expression and purification of recombinant SK proteins using this method regularly achieved yields between 25 and 35 $mg.L^{-1}$ of culture. The purification of SK_{ALAB49} is given as a representative, while all other SK variant expression profiles appear in Appendix 2.

N-terminal poly-histidine tags were removed from SK fusion proteins via proteolytic cleavage with Factor Xa. This treatment effectively removed all vector encoded histidine amino acid residues producing a recombinant SK protein possessing an N-terminal Ile_1 residue. The Ile_1 is present on the secreted wild type form of SK and is critical for the proper functioning of this protein (Wang *et al.*, 2000). Cleavage of poly-histidine tags from recombinant SK protein was assessed by SDS-PAGE and Western blot analysis using a Ni^{2+}-NTA-HRP conjugate that specifically binds to poly-histidine tags. A representative of this process is given for SK_{ALAB49} (Figure 2.5). Digestion of SK_{ALAB49} with Factor Xa resulted in the production of two truncated protein species with a molecular mass of 47 kDa and 45.9 kDa respectively (Figure 2.5A; lane 3). This cleavage pattern is the result of the action of Factor Xa, as SK_{ALAB49} incubated under the same reaction conditions, but in the absence of Factor Xa, maintained an intact molecular mass of 49 kDa (Figure 2.5A; lane 2). Western blot analysis using the Ni^{2+}-NTA-HRP conjugate indicated that Factor Xa digestion was successful in the removal

of the poly-histidine tag residues from the recombinant fusion protein (Figure 2.5B; lane 3).

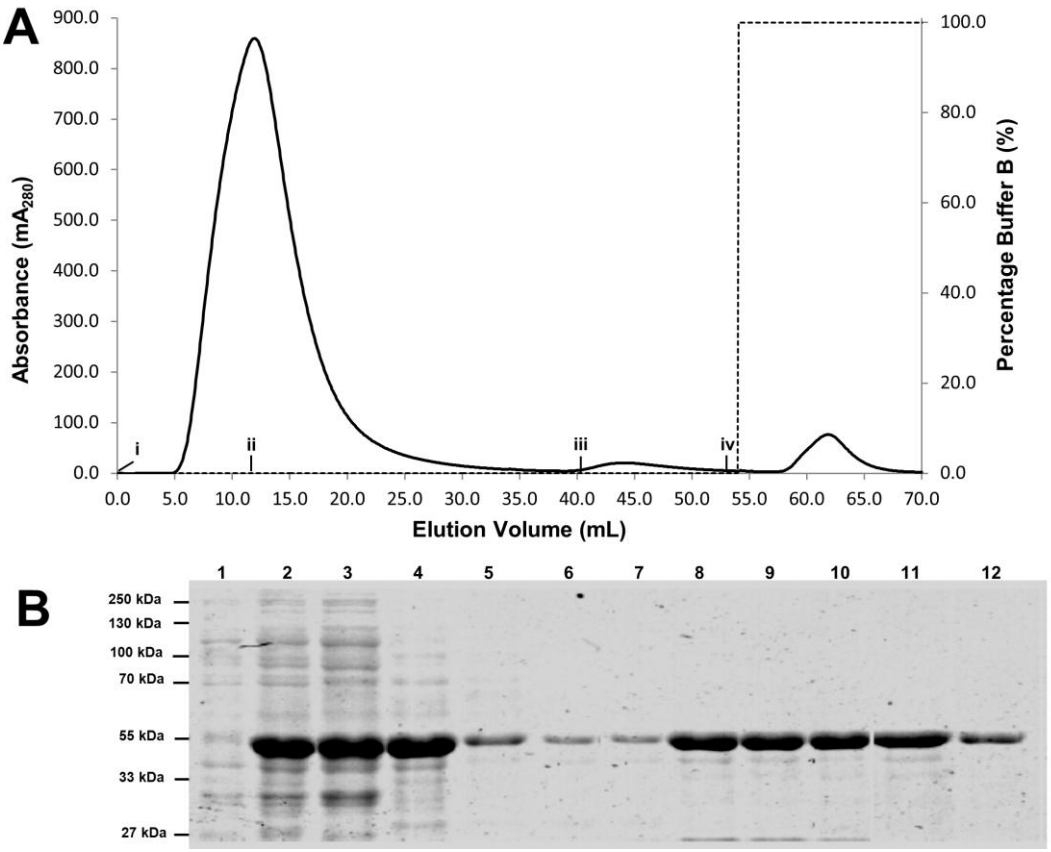

Figure 2.4: Expression and purification of native recombinant poly-histidine tagged fusion protein SK_{ALAB49} via FPLC Ni^{2+}-NTA affinity chromatography. (**A**) Elution profile from ÄKTAprime plus FPLC showing A_{280} trace (solid line) and percentage elution buffer (dashed line) for $SK_{ALAB49.}$ Point *i* represents injection of cleared lysate. Point *ii* represents injection of binding buffer. Point *iii* represents injection of wash buffer and point *iv* represents injection of elution buffer. (**B**) Coomassie stained 10% SDS-PAGE of SK_{ALAB49} purification process. Un-induced *E. coli* whole cell lysate (lane 1), 2 h post induction *E. coli* whole cell lysate (lane 2) native lysis pellet containing insoluble protein (lane 3), lysate (lane 4) Ni^{2+}-NTA column flow through (lane 5), wash buffer Ni^{2+}-NTA column flow through (lane 6) and Ni^{2+}-NTA column elution fractions (lanes 7-12). Figures relating to the expression and purification of all other SK variants used in this study are given in Appendix 2.1.

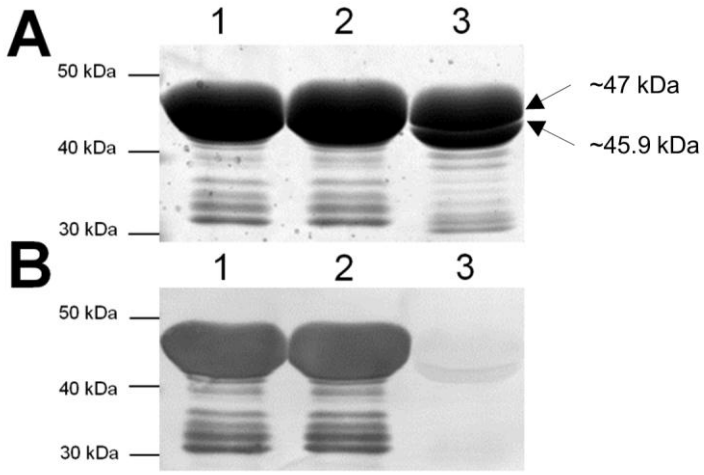

Figure 2.5: Factor Xa cleavage of recombinant poly-histidine tagged SK_{ALAB49} fusion protein (A) Coomassie stained 10% SDS-PAGE of SK_{ALAB49} cleavage. SK_{ALAB49} T = 0 h (lane 1), SK_{ALAB49} T = 36 h without Factor Xa (lane 2) and SK_{ALAB49} T = 36 h Factor Xa added (lane 3) **(B)** Western blot of the duplicate samples using Ni^{2+}-NTA-HRP conjugate to detect poly-histidine tags. Figures relating to Factor Xa cleavage of all other SK variants used in this study are given in Appendix 2.2.

2.4.3 Nano-electrospray ionisation mass spectrometry (Nano-ESI-MS)

Nano-ESI-MS was used to determine the composition of SK truncation products in the sample after treatment with Factor Xa. The representative Nano-ESI-MS spectrum of SK_{ALAB49} (Figure 2.6) shows a pattern of multiply charged ions in the *m*/*z* range of 950–1170 consistent for the 47 kDa species (Figure 2.6B). The calculated molecular weight of 47469.75 Da was in agreement with the theoretical average molecular weight of 47465.21 Da (Figure 2.5A). In addition, two further charge states from *m/z* 1,500 – 1,600 representing a product of 45946.97 Da were detected (Figure 2.5A and B). This lower molecular weight product was the result of a second proteolytic cleavage

occurring at Arg_{401}-Tyr_{402} in the C-terminus of the recombinant SK (theoretical average molecular weight of 45943.60 Da) (Figure 2.6A).

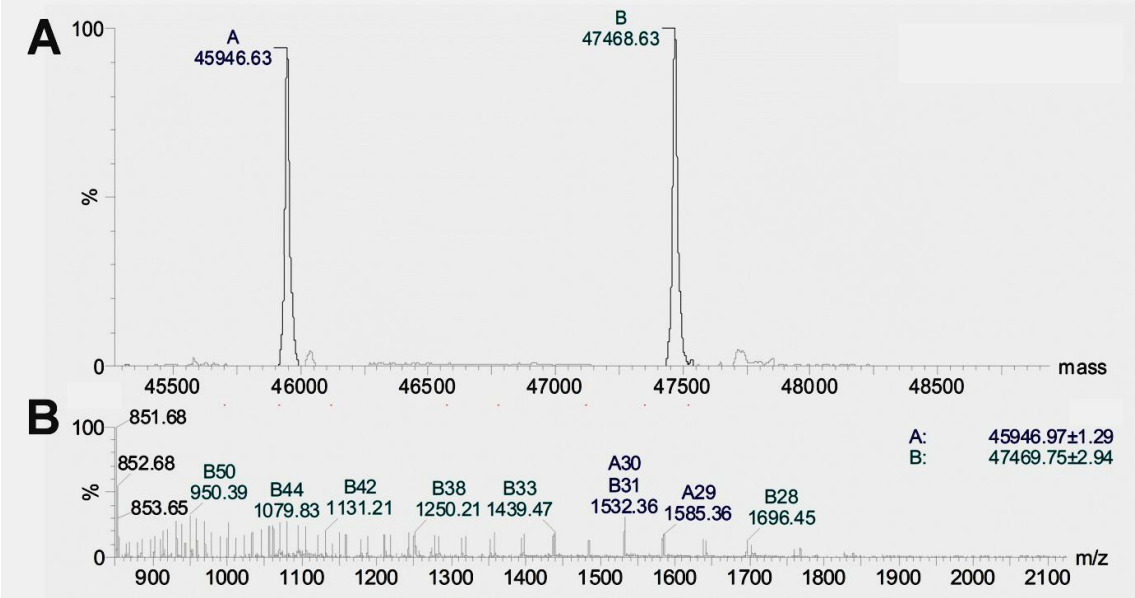

Figure 2.6: A positive ion Nano-ESI-mass spectrum of purified SK_{ALAB49} in 10 mM ammonium acetate (pH 6.8) containing 0.1% formic acid after Factor Xa cleavage. (**A**) The two SK species observed during MS analysis were calculated to be 47469.75 Da and 45946.97 Da SK. **(B)** The *m/z* spectrum shows a pattern of multiply charged ions in the range of 950–1170 consistent for 47469.75 Da the and two further charge states from *m/z* 1,500 – 1,600 representing the 45946.97 Da SK truncation product.

2.4.4 Anion exchange chromatography

To separate full-length recombinant SK from the C-terminal Arg_{401} truncation product, a secondary purification step using anion exchange chromatography was undertaken. The theoretical pI of full length SK and C-terminal truncated SK were determined to be 5.41 and 5.48, respectively. This allowed a difference in negative charge to be imparted on the SK species at pH 6.5. Chromatography was conducted using an ÄKTA explorer FPLC (GE Healthcare Life Sciences, Piscataway, NJ, USA) and monitored by measuring relative change in A_{280}. The A_{280} profile for the purification of SK_{ALAB49} is given as a representative (Figure 2.7). The first peak of ~49 mA_{280} units on the trace correlates to the elution of a ~45.9 kDa protein, which is the expected molecular weight of the SK C-terminal Arg_{401} truncation product (Figure 2.7A and B, i). Peak ii of the A_{280} trace (~86 mA_{280} units) was determined to be a protein of ~47 kDa (Figure 2.7A and B, ii), which correlates to the expected molecular weight of full length SK. Following this a small ~ 11 mA_{280} unit co-elution of full length and truncated SK is exhibited in peak iii of the trace (Figure. 2.7A and B, iii). Fractions corresponding to peak ii containing full length SK protein free from all other contaminating protein were pooled for use in subsequent experiments.

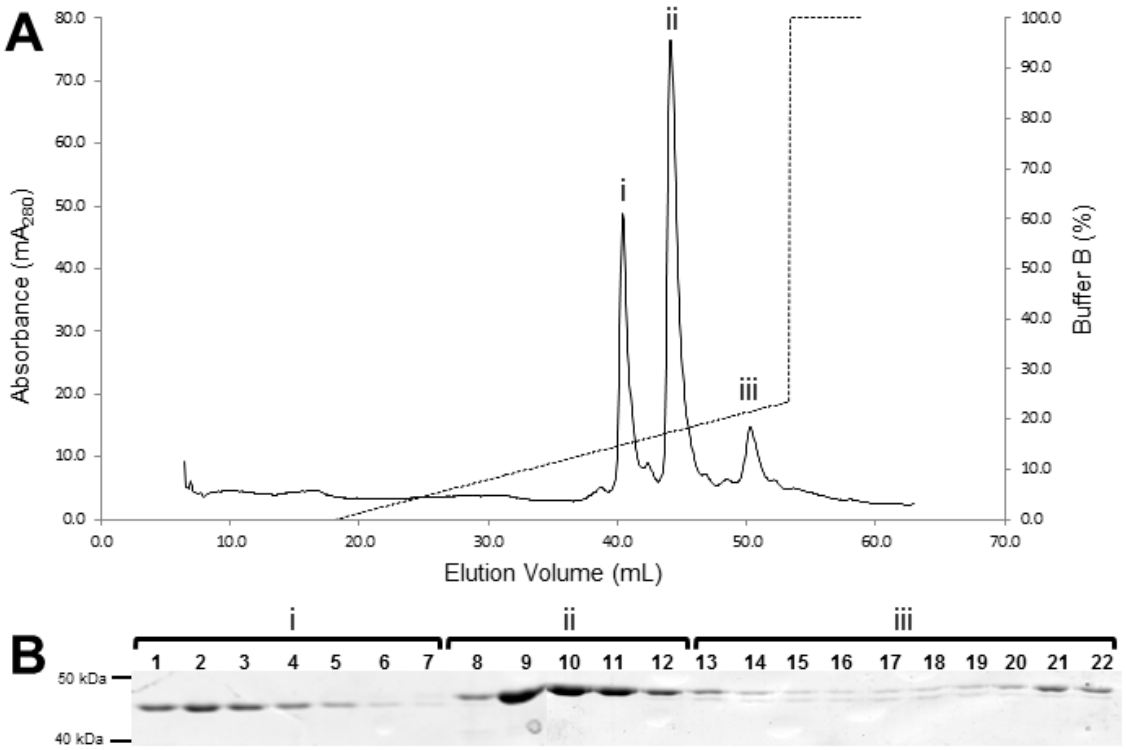

Figure 2.7: Anion-exchange fast protein liquid chromatography of SK_{ALAB49}. (A) Elution profile from an ÄKTA explorer FPLC showing A_{280} trace (solid line) and percentage of Buffer B (0 -1 M NaCl) (dashed line). Three peaks were resolved in the A_{280} trace correlating to truncated SK_{ALAB49} (peak i), full length 47 kDa SK (peak ii) and a co-elution of both species (peak iii). **(B)** Coomassie stained 10% SDS-PAGE of anion exchange elution peaks. Elution peak I (lanes 1-7) correlated to truncated $SK_{ALAB49,}$ elution peak ii (lanes 8-12) correlated to full length ~47 kDa SK and elution peak iii (lanes 13-22) appeared to be a co-elution of both species. Each lane corresponds to an elution volume of 0.5 mL. Figures relating to anion exchange chromatography of all other SK variants used in this study are given in Appendix 2.3, each SDS-PAGE has been truncated due to the absence of other protein species.

2.5 Discussion

In 1984, *ska* from *Streptococcus equisimilis* H46A was first cloned and expressed in *E. coli* (Malke and Ferretti, 1984). Since then, several methods of recovery and purification of SK have been described for this therapeutically important protein. These include purification of SK from the parental wild-type streptococcus strain H46A, as well as numerous recombinant SK production systems utilising several Gram-negative and Gram-positive bacterial hosts (Yazdani and Mukherjee, 1998, Zhang *et al.*, 1999, Estrada *et al.*, 1992, Avilan *et al.*, 1997, Perez *et al.*, 1998, Yazdani and Mukherjee, 2002, Babu *et al.*, 2008, Reed *et al.*, 1995, Gladysheva *et al.*, 2007, Mundada *et al.*, 2003, Dhar *et al.*, 2002, Chaudhary *et al.*, 1999, Yadav and Sahni, 2010, Wu *et al.*, 2001, Malke and Ferretti, 1984, Malke *et al.*, 1984).

Mature wild type SK is naturally secreted by streptococcal species. After cleavage of the signal sequence during secretion, the mature protein contains an N-terminal Ile_1 residue that is critical for the proper functioning of this protein as it forms a salt bridge with Asp_{740} of Glu-Plg during active site formation (Wang *et al*., 2000). Therefore, it is essential that mature 414 amino acid recombinant SK proteins also possess an N-terminal Ile_1 residue. To achieve this, recombinant SK (1- 414 amino acid) proteins in this study were produced as fusion proteins containing an N-terminal poly-histidine tag followed by a Factor Xa protease recognition sequence for affinity tag removal and Ile_1. One difficulty encountered using this strategy was the generation of an unwanted C-terminal truncation product during Factor Xa digestion of SK-fusion proteins. Analysis of this truncation product found it to be the result of proteolytic cleavage between

Arg_{401} and Tyr_{402} in the C-terminal region of SK. This highly flexible region is known to be susceptible to proteolytic activity and similar truncation products have been observed for recombinant group C SK expression systems (Figure 2.6) (Shi *et al.*, 1994, Zhai *et al.*, 2002). SK proteins with C-terminal truncations have been shown to display reduced biological activity, therefore it is important to remove this common truncated species from protein preparations prior to subsequent experimentation (Zhai *et al.*, 2002). To remove these contaminating species we employed a secondary purification system utilising anion exchange chromatography, which was successful in allowing the retrieval of full length SK of high quality and purity (Figure 2.7 and Figure A2.2.2).

Several groups have attempted to produce large quantities of recombinant SKc using *E. coli* as a host (Yazdani and Mukherjee, 1998, Zhang *et al.*, 1999, Estrada *et al.*, 1992, Avilan *et al.*, 1997, Perez *et al.*, 1998, Yazdani and Mukherjee, 2002, Babu *et al.*, 2008). In *E. coli*, the N-terminal methionine residue is removed from many proteins by endogenous methionine aminopeptidase enzymes (Ben-Bassat *et al.*, 1987). Therefore it may be possible to produce Ile_1-SK in *E. coli* without the need to remove N-terminal residues using exogenous proteases such as Factor Xa. However, previous studies investigating this strategy have found the N-terminal Met residue is removed from recombinant SK expressed in *E. coli* with only low efficiency. This may be due to the large side chain residing on the penultimate Ile residue inhibiting the activity of methionine aminopeptidase (Walker *et al.*, 2014, Jones *et al.*, 1991). Due to the importance of the N-terminal Ile_1 residue and a non-truncated C-terminus biochemical and functional data generated in studies that have failed to purify SK proteins that are free from N-terminal affinity tags, methionine residues and contaminating proteins

cannot be used in direct comparison with data generated using full length protein (Dhar *et al.*, 2002, Chaudhary *et al.*, 1999, Yadav and Sahni, 2010, Wu *et al.*, 2001).

Other studies utilising SK expression strategies have employed several combinations of affinity tags and cleavage proteases to ensure recombinant SK proteins contain the N-terminal Ile_1 residue. However, it is not made clear if these expression and purification systems were hindered by similar unwanted proteolytic events (Reed *et al.*, 1995, Gladysheva *et al.*, 2007, Mundada *et al.*, 2003, Zhang *et al.*, 2012). Here it was observed that GAS SK is more susceptible to C-terminal truncation than SKc and the extent of C-terminal degradation differed between these variants (Figure 2.7, Figure A2.3). The reason for this phenomenon was not elucidated, but the system we employed here ensures the retrieval of full length SK at a purity that is suitable for comparative biochemical analyses and can be applied to all SK variants. Nevertheless, the observation of variant specific differences in C-terminal degradation suggests GAS SK variants may exhibit structural differences when not bound to Plg.

Here, we successfully implemented a method to purify full-length recombinant SK in *E. coli*. Further biochemical characterisation of these proteins will increase our understanding of the phenotypic differences displayed by these novel SK variants from GAS.

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Chapter 3:

Streptokinase Variants from *Streptococcus pyogenes* Isolates Display Altered Plasminogen Activation Characteristics – Implications for Pathogenesis.

Preface

The subversion of the human plasminogen activation system by GAS is critical for the pathogenesis of this organism. The work in this chapter aimed to characterise the functional differences displayed by allelic variants of SK secreted by *S. pyogenes*, with a view to assess the implications of the observed biochemical and functional differences on pathogenesis. Our findings clearly demonstrate that variation in the molecular mechanism of SK-mediated Plg activation influences the pathogenesis of *S. pyogenes* and may contribute to the varied disease spectrum associated with this pathogen.

Publication: **Cook, S. M**., A. Skora, C. M. Gillen, M. J. Walker and J. D. McArthur, (2012) "Streptokinase variants from Streptococcus pyogenes isolates display altered plasminogen activation characteristics - implications for pathogenesis." *Molecular Microbiology* **86**: 1052-1062.

3.1 Abstract

Streptococcus pyogenes (group A streptococcus, GAS) secretes streptokinase (SK), a potent Plg activating protein. Among GAS isolates, SK gene sequences (*ska*) are polymorphic and can be grouped into two distinct sequence clusters (termed cluster type-1 and cluster type-2) with cluster type-2 being further divided into sub-clusters type-2a and type-2b. In this study, far-UV circular dichroism spectroscopy indicated that purified SK variants of each type displayed similar secondary structure. Type-2b SK variants could not generate an active site in Glu-Plg through non-proteolytic mechanisms while all other variants had this capability. Furthermore, when compared to other SK variants, type-2b variants displayed a 29-35 fold reduction in affinity for Glu-Plg. All SK variants could activate Glu-Plg when an activator complex was preformed with plasmin; however type-2b and type-1 complexes were inhibited by α_2-antiplasmin. Exchanging $ska_{\text{type-2a}}$ in the M1T1 GAS strain 5448 with $ska_{\text{type-2b}}$ caused a reduction in virulence while exchanging $ska_{\text{type-2a}}$ with $ska_{\text{type-1}}$ into 5448 produced an increase in virulence when using a mouse model of invasive disease. These findings suggest that SK variants produced by GAS isolates utilise distinct Plg activation pathways which directly affects the pathogenesis of this organism.

3.2 Introduction

Group A streptococcus (GAS; *Streptococcus pyogenes*) is a human specific pathogen responsible for a diverse range of diseases which have a major impact on global morbidity and mortality rates (Carapetis *et al.*, 2005). GAS readily colonise skin and pharyngeal tissue producing mild superficial infections such as pyoderma, impetigo and pharyngitis. However, GAS may also produce life-threatening systemic (streptococcal toxic shock syndrome) and invasive infections (necrotising fasciitis). Additionally, post-infection sequelae can occur, which include post streptococcal glomerulonephritis and acute rheumatic fever (Walker *et al.*, 2014).

To cause this diverse range of diseases, GAS employ a broad range of virulence factors that facilitate bacterial colonisation, evasion of the immune response and systemic dissemination (Tart *et al.*, 2007, Musser and Shelburne, 2009, Olsen *et al.*, 2010, Cole *et al.*, 2011). Virulence factor expression is exquisitely controlled by 13 two-component regulatory systems and 30 transcriptional regulators allowing GAS to adapt to the dynamic physiological conditions encountered during the infection process (Kreikemeyer *et al.*, 2003, Sumby *et al.*, 2006). Many of the virulence factors produced by GAS interact specifically with human plasma proteins including fibrinogen, plasmin(ogen), IgG, α_2-macroglobulin, albumin and numerous complement factors (Cunningham, 2000, Walker *et al.*, 2005). GAS can encounter plasma proteins during invasive systemic dissemination through the vasculature, but may also be exposed to plasma constituents at the site of infection through vascular leakage produced during the inflammatory response induced in the host (Herwald *et al.*, 2004).

The interaction of GAS with the Plg activation system of the host is a virulence mechanism critical for the invasive pathogenesis of this organism (Sun *et al.*, 2004, Khil *et al.*, 2003, Walker *et al.*, 2005, Irigoyen *et al.*, 1999). Plasminogen (Plg), a single-chain glycoprotein zymogen of the serine protease plasmin, is a key component of the fibrinolytic system and is found in plasma and extracellular fluids. GAS can bind plasmin(ogen) to the cell surface via numerous cell wall associated proteins such as M proteins (PAM, Prp), glyceraldehyde-3-phosphate (GAPDH) and streptococcal enolase (SEN) (Lahteenmaki *et al.*, 2001, Walker *et al.*, 2005, Castellino and Ploplis, 2005). GAS also secrete a Plg activating protein, designated streptokinase (SK), which facilitates the production of both soluble and cell-bound plasmin activity. The generation of plasmin activity at the site of infection may result in the activation of host matrix metalloproteinases, degradation of extracellular matrix and/or tissue barriers and degradation of fibrin networks produced by the host to confine the initial infection (Walker *et al.*, 2005). These processes allow bacteria to spread to other, normally sterile, sites of the body.

Unlike mammalian Plg activators that activate Plg by limited proteolytic cleavage, SK binds to Plg inducing conformational changes in the molecule that results in the formation of an active site and the production of an enzymatically active complex, termed SK-Plg* (known as the 'Conformational Activation Pathway' or 'Pathway I'). The conformationally activated SK-Plg* complex can then sequester substrate molecules of Plg and proteolytically convert those to plasmin (Boxrud *et al.*, 2000, Boxrud *et al.*, 2004). Plasmin (which has a higher affinity for SK than Plg) rapidly displaces Plg in the SK-Plg* complex to produce an irreversibly activated SK-plasmin

complex that is the main catalyst responsible for the full conversion of Plg to plasmin. (known as 'Direct Proteolytic Activation Pathway' or 'Pathway II') (Boxrud *et al.*, 2000, Boxrud *et al.*, 2004). These SK-plasmin activator complexes are also resistant to inhibition by host plasma inhibitors (α_2-antiplasmin (α_2-AP) and α_2-macroglobulin), thereby allowing complexes to sequester and activate substrate Plg while bypassing host protease regulation mechanisms (Parry *et al.*, 2000).

SK is a single chain, 414 amino acid protein, composed of three distinct domains; α (aa 1-150), β (aa 151-287) and γ (aa 288-414) (Wang *et al.*, 1998). The three domains of SK are separated by two coiled-coil regions while the N- and C- termini of the protein have disordered flexible structures (Wang *et al.*, 1998). Despite extensive research, the exact role each domain plays in the activation of human Glu-plasminogen (Glu-Plg) is still not completely understood. While the majority of structural and functional studies conducted to date have used the therapeutic form of SK (originally isolated from the group C streptococcal isolate H46A) (Christensen, 1945), SK proteins from group A streptococcal isolates display considerable variability and have not been well characterised (Kalia and Bessen, 2004, McArthur *et al.*, 2008, Kapur *et al.*, 1995). Phylogenetic studies of the most divergent *ska* sequences have revealed two main sequence clusters (cluster type-1 and 2) with evidence of smaller sub-clusters observed in cluster type-2 sequences (cluster type-2a and 2b) (Kalia and Bessen, 2004, McArthur *et al.*, 2008). While some phenotypic differences displayed by GAS type-1 and type-2 SK variants have been identified (McArthur *et al.*, 2008), further research is required to determine if these variants play differing roles in pathogenesis. In this study, we found

that SK variants produced by GAS isolates display different mechanisms of Plg activation and that this process directly affects GAS pathogenesis.

3.3 Materials and Methods

3.3.4 Far-UV circular dichroism (CD) spectroscopy

CD spectroscopy was performed using a J-810 spectropolarimeter (Jasco, Tokyo, Japan) at room temperature. Samples were prepared in 10 mM phosphate buffer (pH 7.4) to a final concentration of 100-300 µg mL^{-1}. Spectra representing the average of six scans were collected from 190 nm to 250 nm at 1 nm intervals, with a path length of 1 mm. Molar residue ellipticity (Θ) was calculated using the following formula: $[\Theta] = \Theta$ x 100 x molecular weight (kDa)/concentration (mg mL^{-1}) x path length x number of amino acids.

3.3.5 Non-proteolytic active site generation in Glu-Plg

Non-proteolytic active site generation in Glu-Plg by SK variants was examined using the fluorescent active site titrant 4-methylumbelliferyl p-guanidinobenzoate (MUGB) (Marker Gene Technologies, Inc., Eugene, OR, USA) in a POLARstar Omega fluorescence spectrophotometer (BMG LABTECH, Ortenberg, HE, GER). Glu-Plg (Haematologic Technologies, Essex Junction, VT, USA) (200 nM) was added to a black 96-well micro-plate containing MUGB (1 µM) in assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) at 37 °C. To initiate the reaction, SK was added to a final concentration of 400 nM in a total volume of 100 µL and the development of fluorescence was monitored continuously with excitation at 355 nm and emission at 460 nm. Data was normalised by subtracting a control reaction of 200 nM Glu-Plg and 1 µM MUGB. This accounted for intrinsic fluorescence associated with buffer and protein species, as well as non-specific hydrolysis of MUGB over the course of reactions.

3.3.6 Surface plasmon resonance

Binding of SK variants to Glu-Plg and plasmin were examined *via* Biacore T200 (Biacore AB, Uppsala, Sweden) at 25ºC. Ligand Glu-Plg and plasmin were immobilised on a Series S Sensor Chip CM4 (Biacore AB, Uppsala, Sweden) via primary amino acids using an amine coupling kit according to the manufacturer's instructions (Biacore AB, Uppsala, Sweden). Briefly, the chip was activated with a 1:1 mixture of 0.2 M *N*-ethyl-*N*'-(3-dimethylaminopropyl)carbodiimide and 0.05 M *N*-hydroxysuccimide. Glu-Plg and plasmin were coated onto the chip at 40 μg mL^{-1} in 10 mM sodium acetate (pH 4) to a level of ~1500 and ~250 response units respectively. Unoccupied binding sites were blocked using 1 M ethanolamine, pH 8.5. A blank immobilised CM4 cell was used as a reference. Analytes were diluted into running buffer (10 mM HEPES, 150 mM NaCl, 0.005% P-20, pH 7.4) and kinetic assays were performed by injecting recombinant SK proteins at varying concentrations (0-1600 nM), for 200-300 s at a flow rate of 20 μL min^{-1} with a 600 s dissociation period. Regeneration of the ligand surface was achieved with 6 M urea in running buffer for 15 s at 20 μL min^{-1}. To test ligand binding efficacy and reproducibility on the sensor chip, control group C SK was injected at a known concentration and response was monitored. The response to SKc binding post burst 6M urea treatment was the same on all flow cells (tested over several sensor chips) indicating that Glu-Plg was folding correctly. Glu-Plg interaction biosensorgram data were prepared for analysis using Scrubber2 (BioLogic Software, Campbell, ACT, Australia) and data was analysed manually using a two-component heterogeneous surface model with data curves fit using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). For Glu-Plg interactions with each SK variant, one

binding component accounting for ~30-90% of the total response showed relatively fast association rate constants (k_a values) and dissociation rate constants (k_d values), with k_a showing a linear dependence on [SK], while the other showed slow on and off rates independent of [SK]; we chose to ignore this second non-specific component and determined equilibrium binding constants (K_D) from the ratio of k_d and k_a for the specific binding component. For plasmin interactions, k_a, k_d and K_D were calculated from sensorgrams by non-linear fitting of the association and dissociation curves according to a 1:1 Langmuir binding model using the Biacore T200 evaluation software supplied by the manufacturer (Biacore AB, Uppsala, Sweden).

3.3.7 Glu-Plg activation assays

The Plg activation potential of SK variants were studied by the addition of stoichiometric SK-plasmin activator complexes (final concentration 5 nM) that had been preformed for 5 min at 37°C, to assay buffer (10 mM HEPES, 150 mM NaCl, 0.01% Tween-20, pH 7.4) containing Glu-Plg (500 nM) and S-2251 (Chromogenix, Mölndal, Sweden) (500 µM) in a total volume of 100 µL. The exponential generation of plasmin was monitored by change in absorbance at 405 nm and measured for 30 min using a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 37°C.

3.3.8 Inhibition of amidolysis by α_2-antiplasmin

Stoichiometric complexes of SK-plasmin were formed by mixing SK (400 nM) and plasmin (Haematologic Technologies, Essex Junction, VT, USA) (200 nM) for 5min at 37°C. Complexes were diluted to 20 nM in assay buffer in the presence of increasing

α_2-AP (Haematologic Technologies, Essex Junction, VT, USA) concentration (0-400 nM) and incubated at 37°C for 15 min. The reactions were initiated by the addition of S-2251 (final concentration 500 µM) and change in absorbance at 405 nm was measured at 37°C. IC_{50} values were determined by plotting percentage of residual activity (V_{max}) versus log α_2-AP concentration and fit to a sigmoidal dose response curve using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA)

3.3.9 Allelic exchange mutagenesis

Isogenic mutants of GAS strain 5448 were produced by replacing the parental ska_{5448} with either ska_{ALAB49}, ska_{NZ131} or with the chloramphenicol acetyltransferase (*cat*) gene via precise, allelic replacement using a modified protocol to Buchanan *et al.* (2006). To construct plasmids for allelic exchange, ska_{ALAB49} and ska_{NZ131} were amplified from genomic DNA by PCR using sense primer: (5'-TTCTTCC TGTCTGTTTATGTACCCGCAGCTACTTGATACC-3') and antisense primer: (5'-TTGTCCTCTTCTGTTTTGGCTACCAAGAACGCTTGATTG-3') (Sigma-Aldrich, Sydney, NSW, Australia), to include GAS chromosomal flanking regions 834 bp upstream and 853 bp downstream of *ska,* in addition to regions homologous to the temperature sensitive, erythromycin resistant shuttle vector pHY304-LIC {Buchanan, 2006 #518}. For knockout plasmid construct p5448Δska, upstream (369 bp) and downstream (538 bp) *ska* DNA fragments containing regions homologous to pHY304-LIC and *cat* were amplified using sense primer (5'-TTCTTCCTGTCTGTTTAGATGAGGGCCTACTTGCATC-3') and antisense (5'-GTGGCTTTTTTCTCCATACGGTCTGGTAGCCATCCAT-3') for the upstream homology region and sense primer (5'-GTGGCTGGGCGG

GGCGTAAAAGCTTACAGCTACCTGCGT-3') and antisense (5'-TTGTC CTCTTCTGTTTCGGACCAATGGCTAAGAAAG-3') for the downstream homology region. The *cat* gene was amplified using the sense primer (5'-GGAGAAAAAAGCCACTGGATATACCACC-3') and antisense primer (5'-ACGCCCCGCCCAGCCACTCATCGCAATACTGTT-3'). Single-strand overhangs were created on all PCR products and *Pme*I pHY304-LIC shuttle vector by T4 DNA polymerase treatment at 22°C for 30 min. Treated pHY304-LIC was combined with equal concentrations of *ska* upstream/downstream regions and the *cat* gene to create the Δ*ska* knock-out construct, or with either ska_{ALAB49} or ska_{NZ131} gene fragments to create allelic exchange constructs (Figure 3.1). Complementary sequences were allowed to anneal on ice for 30 min before transformation into chemically competent *E. coli*. The purified plasmid constructs were confirmed by DNA sequencing analysis and transformed into GAS strain 5448. Erythromycin resistant transformants were grown at the permissive temperature for plasmid replication (30°C). Single-crossover chromosomal insertions were selected by shifting to the non-permissive temperature (37°C) while maintaining erythromycin selection. Single-crossover mutants were incubated overnight at 30°C to allow for looping out of the inserted plasmid and then patched onto both THY agar and THY agar containing erythromycin and incubated at 37°C. This allowed selection of double crossover mutants encoding in-frame allelic exchanges and was confirmed using DNA sequence analysis (Figure 3.1). The allelic exchange mutant strains were designated 5448::ska_{ALAB49}, 5448::ska_{NZ131} and 5448Δ*ska*.

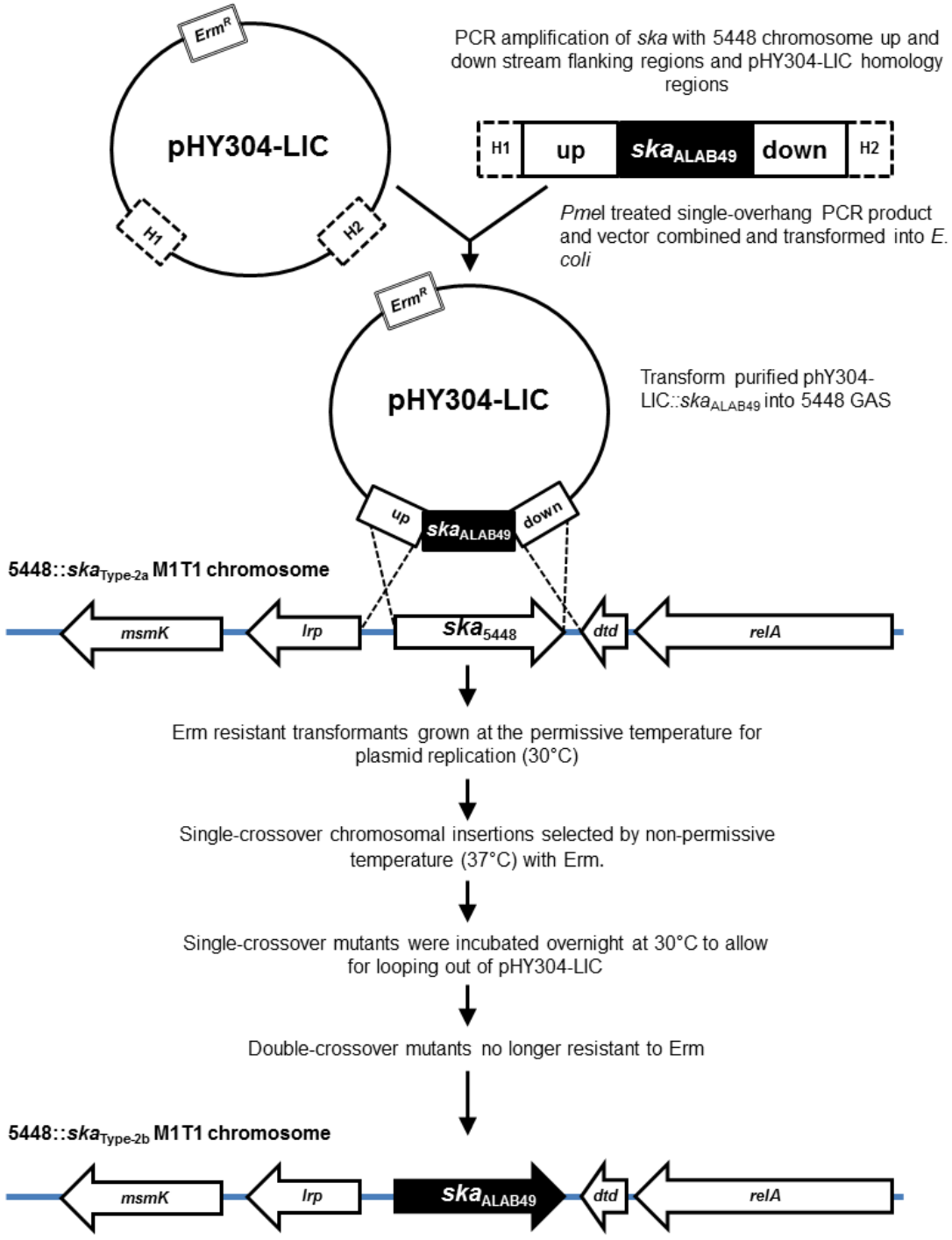

Figure 3.1: Schematic example of allelic exchange mutagenesis to create GAS strain 5448 isogenic mutants by replacement of the parental *ska*$_{5448}$ with *ska*$_{ALAB49}$. Isogenic mutants were constructed via precise allelic replacement using a modified protocol to Buchanan et al. (2006).

3.3.10 Growth comparison of *S. pyogenes* strains

Overnight cultures of *S. pyogenes* were diluted 1:10 in fresh THY media and grown to stationary phase. Optical density (OD) of the strains at 600 nm (OD_{600}) was documented at 30 min increments to determine relative growth.

3.3.11 Hyaluronic acid capsule determination

Overnight cultures of *S. pyogenes* were diluted 1:10 in fresh THY media and grown to an OD_{600} of 0.5– 0.6. The hyaluronic acid capsule was extracted and quantified by the stains-all method as described previously (Cole *et al.*, 2006a).

3.3.12 GAS strain streptokinase expression

For analysis of SK expression, aliquots of overnight GAS cultures were washed twice by centrifugation, followed by subsequent resuspension with equal volumes of fresh THY before being used to inoculate new cultures. When GAS cultures had reached midlog phase (OD_{600} = 0.6), supernatants were harvested by centrifugation, filtered using a 0.2 µm polyvinylidene difluoride (PVDF) syringe filter (Millipore, Sydney, NSW, Australia). GAS culture supernatants were then concentrated 18-fold by 10% trichloroacetic acid precipitation before SDS-PAGE and western transfer to PVDF membrane (Millipore, Sydney, NSW, Australia). For detection of SK, the membranes were immunoblotted using rabbit immune serum raised against commercial group C SK (Sigma-Aldrich, Sydney, NSW, Australia) as described previously (Cole *et al.*, 2006a).

3.3.13 Transgenic murine infection model

Humanised Plg transgenic *AlbPLG1* mice, heterozygous for the human Plg gene (Sun *et al.*, 2004), were used as the animal model for determining GAS invasive potential as previously described (Walker *et al.*, 2007). Briefly, GAS isolates were grown in THY medium at 37°C to logarithmic phase (OD_{600} = 0.6), washed with sterile 0.7% (w/v) NaCl and appropriately diluted to prepare the inoculum. Cohorts of 9-10 mice were infected with a 100 µL intradermal injection containing 5448 (Maamary *et al.*, 2010) (3.9 x 10^7 CFU/dose), 5448::Δ*ska* (3.7 x 10^7 CFU/dose), 5448::ska_{ALAB49} (4.6 x 10^7 CFU/dose), or 5448::ska_{NZ131} (3.7 x 10^7 CFU/dose) and mortality was recorded over a 10-day period.

3.3.14 Statistical analyses

Differences in survival of humanised Plg transgenic mice infected with GAS strains 5448, 5448::Δ*ska*, 5448::ska_{ALAB49}, or 5448::ska_{NZ131} were determined by the log-rank test. Differences were considered statistically significant at $P < 0.05$. All statistical tests were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

3.3.15 Ethics permissions

Permission to undertake animal experiments was obtained from the University of Wollongong Animal Ethics Committee.

3.3.16 Accession numbers

The nucleotide sequences of the *ska* genes used in this study are deposited in the GenBank database and shown in Appenidix A1.2: H46A: K02986.1, NZ131: CP000829.1, 5448: JQ650489, NS696: JQ650488, ALAB49: AY234134.1 and NS88.2: JQ650490. Details of data submission can be found at: GenBank: www.ncbi.nlm.nih.gov.

3.4 Results

3.4.1 Expression and secondary structural analysis of recombinant SK proteins

To characterise the phenotypic differences displayed by SK proteins from different group A streptococcal isolates, a cluster type-1 *ska* allele (SK_{NZ131}), two cluster type-2a *ska* allele (SK_{5448} and SK_{NS696}) and two cluster type-2b *ska* allele (SK_{ALAB49} and $SK_{NS88.2}$) were cloned, sequenced and expressed as recombinant proteins. SK from the group C streptococcal isolate H46A (SKc) was also produced as a recombinant protein using the same methodology for use as a positive control. From the alignment of the deduced amino acid sequences (Figure 3.2), the type-1 SK protein had the most divergent β domain sequence (66% identity to SKc). The β domains from type-2b SK proteins and type-2a SK were more conserved displaying 85% and 91% identity respectively. The type-1 SK protein also had the most conserved α and γ domains (94% and 97% identity) while these domains in type-2a and type-2b proteins were less conserved with identities ranging from 83% to 87% for α domains and 88% to 90% for γ domains.

Recombinant SK proteins were analysed for size and purity by SDS-PAGE (Figure. 3.3A). Proteins ranged in size from 44 to 49 kDa, which is similar to the sizes observed for native SK proteins present in GAS culture supernatants (McArthur *et al.*, 2008). All protein preparations were free from contaminating proteins. Far-UV circular dichroism spectroscopy was utilised to compare the secondary structure of all recombinant SK proteins. Despite significant differences in amino acid composition, variant SK proteins

had similar molar residue ellipticity spectra across the full range of wavelengths measured. The output spectra observed was typical of that expected for an amalgamation of α helical (minima at ~ 210 nm maxima at ~190 nm) and anti-parallel β sheet (minima at ~215 nm and maxima at ~195 nm) spectra indicative of the known structure of SK (Wang *et al.*, 1998) (Figure 3.3B).

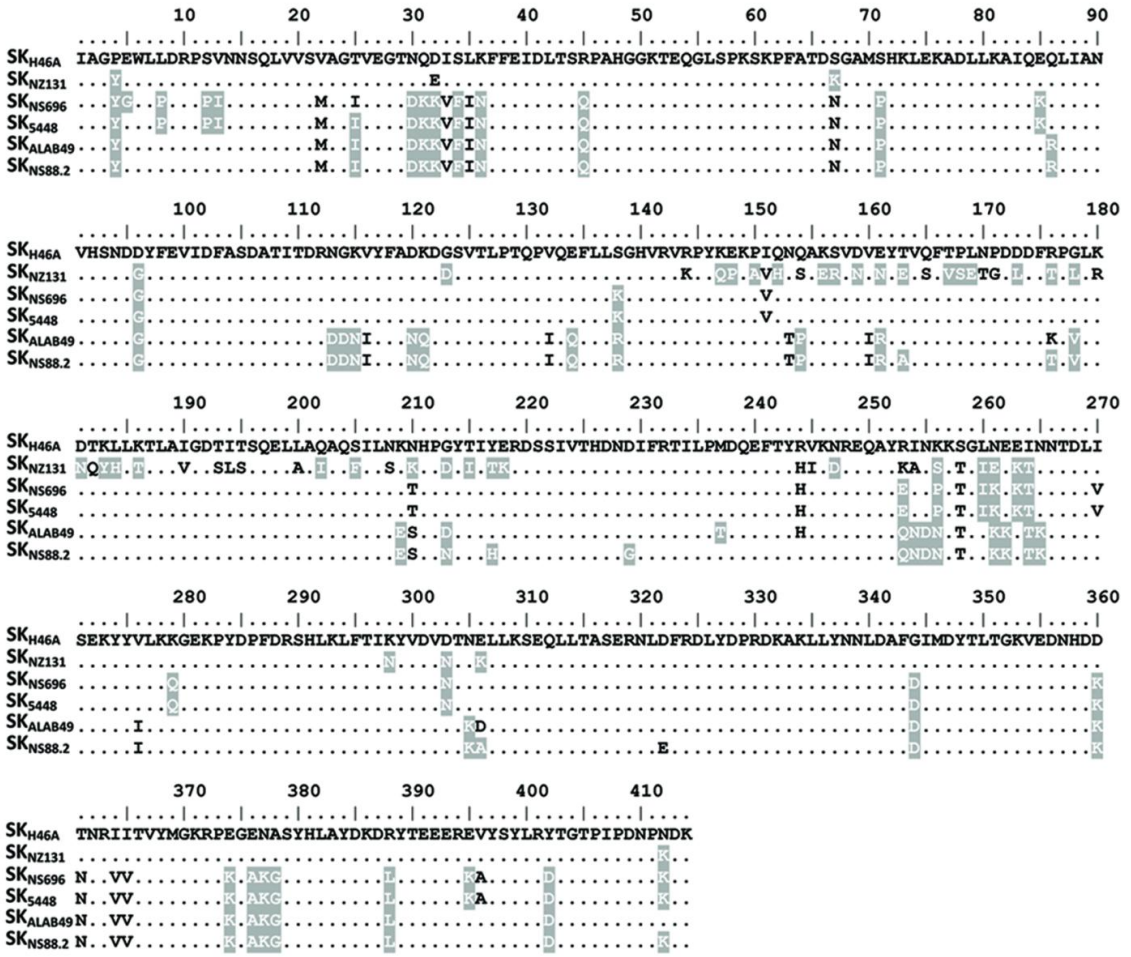

Figure 3.2: ClustalW Multiple alignment of deduced amino acid sequences of the SK protein variants used in this study. Amino acid residues identical to those in SK_{H46A} (SKc) are indicated with a dot while amino acid changes have been indicated. Non-conserved amino acid changes are highlighted in grey. The SK sequence positions of the three domains span residues 1 – 147 for the α domain, residues 148 – 291 for the β domain and spans residues 292 – 414 for the γ domain (Kalia and Bessen, 2004, Parrado *et al.*, 1996, Wang *et al.*, 1998)

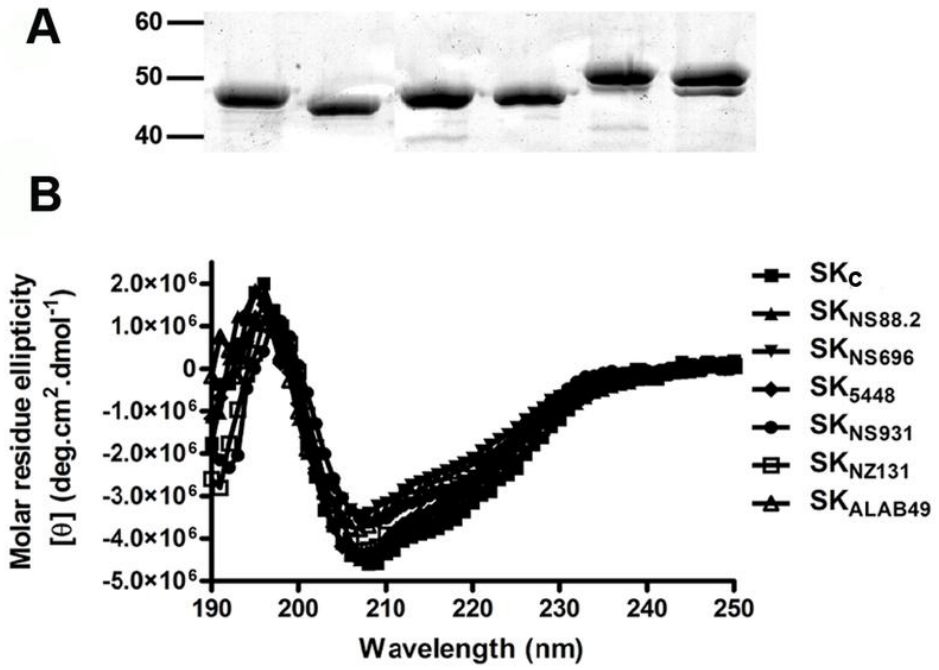

Figure 3.3: SDS-PAGE and Far-UV circular dichroism spectroscopy analysis of recombinant SK proteins used in this study. (**A**) Variant SKc (lane 1), SK_{NZ131} (lane 2), SK_{5448} (lane 3), SK_{NS696} (lane 4), SK_{ALAB49} (lane 5) and $SK_{NS88.2}$ (lane 6) were separated by 10% SDS-PAGE under reducing conditions. Molecular weight standards in kDa are indicated. (**B**) All SK variants have similar molar residue ellipticity values and CD out-put spectrum structure across the full range of wavelengths indicating no differences in secondary structure.

3.4.2 Non-proteolytic active site generation in Glu-Plg by variant SK

Active site generation in Glu-Plg by SK variants (SK-Plg*) was examined using the fluorescent active site titrant 4-methylumbelliferyl p-guanidinobenzoate (MUGB). This allowed generation of SK-Plg* (conformational activation, Pathway I) to be measured directly. SK_{NZ131} (Type 1) displayed the fastest rate of conformational activation of Glu-Plg, followed by SKc (group C) (Figure 3.4A). SK_{NS696} and SK_{5448} (type-2a variants) both displayed very slow rates of Glu-Plg activation while SK_{ALAB49} and $SK_{NS88.2}$ (type-

2b variants) failed to induce an active site in Glu-Plg (Figure 3.4A). As SK mediated Glu-Plg activation is known to be affected by the conformation of Plg, experiments were conducted in the absence of Cl^- ions to compare the effect of 'open' Plg conformation on non-proteolytic active site generation (McCance and Castellino, 1995). Under these conditions SK_{NZ131} and SKc displayed increased rates of active site generation that were very similar (Figure 3.4B). The rate of active site generation by type-2a variants (SK_{NS696} and SK_{5448}) was also significantly enhanced, but was less than that observed for SK_{NZ131} and SKc (Figure 3.4B). Interestingly, type-2b variants (SK_{ALAB49} and $SK_{NS88.2}$) both failed to generate an active site in open Glu-Plg (Figure 3.4B). Taken together, these data indicate that type-2b SK variants cannot conformationally induce an active site in open or closed forms of Glu-Plg.

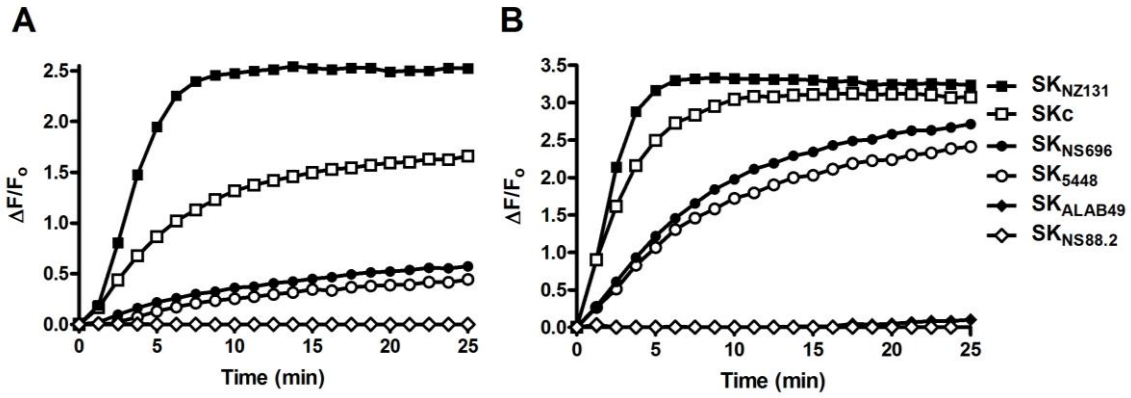

Figure 3.4: Non-proteolytic active site generation in Glu-Plg by SK variants and influence of Glu-Plg conformation on SK-Plg* generation. Glu-Plg (200 nM) was added to 1 µM MUGB in (**A**) Cl^- present assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) or (**B**) chloride Cl^- absent buffer (50 mM Tris pH 7.4) at 37 °C. To initiate the reaction, SK was added to a final concentration of 400 nM in a total volume of 100 µL and the development of fluorescence was monitored continuously with excitation at 355 nm and emission at 460 nm. Data shown represent minimum of two independent experiments (n = 3). Error less than 10% are not shown for clarity of presentation.

3.4.3 Binding affinity of SK variants to human Glu-Plg and plasmin

To determine if SK variants display differences in affinity for human Glu-Plg and plasmin, the binding of each SK variant to immobilised Plg and plasmin was assessed using surface plasmon resonance (SPR) analysis (Appendix A4.2). Group C SK (SKc), type-1 SK (SK_{NZ131}) and type-2a SK (SK_{5448} and SK_{NS696}) variants all displayed high affinity for human Glu-Plg (K_D ranging from 62 – 88 nM). In contrast, type-2b SK variants had lower affinities for human Glu-Plg with SK_{ALAB49} and $SK_{NS88.2}$ displaying a 29 and 35-fold reduction respectively (Table 3.1). All SK variants had increased (69 - 347 fold) affinity for plasmin over Glu-Plg. Group C SK (SKc), type-1 SK (SK_{NZ131}) and type-2a SK (SK_{5448} and SK_{NS696}) had K_D ranging from 0.37 – 1.03 nM, while type-2b (SK_{ALAB49} and $SK_{NS88.2}$) displayed slightly lower affinities for plasmin with K_D values of 11.5 nM and 6.2 nM respectively (Table 3.1).

3.3.4 Plasminogen activation by variant SK-plasmin activator complexes

The ability of variant SK-plasmin complexes to sequester and activate substrate Plg was determined by mixing preformed, stoichiometric SK-plasmin activator complexes (5 nM) with an excess of substrate Glu-Plg and monitoring the generation of plasmin activity using the chromogenic substrate, S-2251. All variant SK-plasmin complexes examined in this study were capable of efficient substrate Plg activation (Figure 3.5A). The plasmin activity displayed by the plasmin-SK activator complex is known to be resistant to the major physiological plasmin inhibitor α_2-AP (Cederholm-Williams, 1979). In this study, we observed that complexes of plasmin with SKc or type-2a SK (SK_{5448} and SK_{NS696}) variants were also resistant to inhibition by α_2-AP (Figure 3.5B). Interestingly, complexes of plasmin with type-2b SK variants (SK_{ALAB49} and $SK_{NS88.2}$)

Table 3.1. Association (k_{on}) and dissociation (k_{off}) rate constants and apparent equilibrium dissociation (K_D) constants for the interaction of SK variants with immobilised Plg and plasmin by SPR.

Analyte		Ligand Plg			Ligand Plasmin		
SK Variant Type		k_a ($M^{-1} s^{-1}$)	k_d (s^{-1})	K_D (nM)	k_a ($M^{-1} s^{-1}$)	k_d (s^{-1})	K_D (nM)
Group C	SKc	$2.9 \pm 0.5 \times 10^{4}$	$1.8 \pm 0.3 \times 10^{-3}$	62 ± 19	$8.6 \pm 0.2 \times 10^{5}$	$3.1 \pm 0.5 \times 10^{-4}$	0.37 ± 0.04
Type-1	SK_{NZ131}	$2.8 \pm 0.7 \times 10^{3}$	$2.5 \pm 0.6 \times 10^{-4}$	88 ± 12	$1.1 \pm 0.8 \times 10^{6}$	$1.1 \pm 0.4 \times 10^{-3}$	0.76 ± 0.09
Type-2a	SK_{5448}	$1.1 \pm 0.2 \times 10^{6}$	$72.6 \pm 1.4 \times 10^{-3}$	66 ± 7	$1.0 \pm 0.6 \times 10^{6}$	$8.3 \pm 0.7 \times 10^{-4}$	0.96 ± 0.39
	SK_{NS696}	$1.2 \pm 0.2 \times 10^{6}$	$84.1 \pm 3.4 \times 10^{-3}$	70 ± 17	$1.2 \pm 0.8 \times 10^{6}$	$9.4 \pm 0.1 \times 10^{-4}$	1.03 ± 0.47
Type-2b	SK_{ALAB49}	$4.6 \pm 0.4 \times 10^{4}$	$81.3 \pm 0.8 \times 10^{-3}$	1767 ± 199	$4.2 \pm 0.4 \times 10^{5}$	$4.3 \pm 0.3 \times 10^{-3}$	11.55 ± 3.03
	$SK_{NS88.2}$	$7.9 \pm 0.5 \times 10^{4}$	$170.0 \pm 3.0 \times 10^{-3}$	2153 ± 512	$4.4 \pm 0.3 \times 10^{5}$	$2.2 \pm 0.6 \times 10^{-3}$	6.22 ± 2.70

Glu- Plg binding data was prepared for analysis using Srubber2 (BioLogic Software, Campbell, ACT, Australia) and analysed by fitting data to a heterogeneous ligand population model using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) as described under 'Experimental Procedures'. Values represent the mean ± SD. Plasmin binding data were calculated by non-linear fitting of the association and dissociation curves according to a 1:1 Langmuir binding model using the Biacore T200 evaluation software (Biacore AB, Uppsala, Sweden)

or type-1 SK (SK_{NZ131}) were susceptible to inhibition by α_2-AP, displaying IC_{50} values of 20 nM, 35 nM and 7 nM respectively (Figure 3.5B).

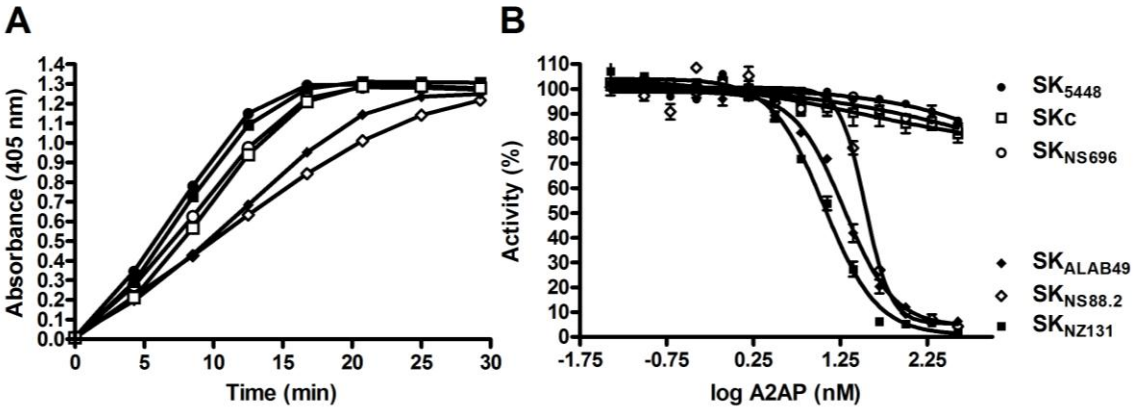

Figure 3.5: Plg activation by SK-plasmin variant activator complexes and inhibition of variant SK-plasmin complexes by α_2-antiplasmin. (**A**) SK was preformed with plasmin for 5 min at 37°C (5 nM), then added to assay buffer (10 mM HEPES, 150 mM NaCl, 0.01% Tween-20, pH 7.4) containing Glu-Plg (500 nM) and S-2251 (500 µM). The generation of Plg activation activity was monitored at an absorbance of 405 nm. (**B)** Stoichiometric complexes of SK-plasmin (20 nM) were preformed for 5 min at 37°C in assay buffer (10 mM HEPES, 150 mM NaCl, 0.01 % Tween-20, pH 7.4) and subsequently incubated for 15 min at 37°C with α_2-antiplasmin (final concentration 0 – 400 nM). The reactions were initiated by addition of S-2251 (500 µM) to complex mixtures and the percentage of residual activity of the SK-plasmin complexes was determined by measuring the absorbance at 405 nm.

3.3.5 Role of SK variation in GAS pathogenesis

In vitro characterisation of the isogenic mutants 5448Δ*ska*, 5448::ska_{ALAB49} and 5448::ska_{NZ131} indicated all strains maintained similar growth rates and expressed similar amounts of hyaluronic capsule (Figure 3.6A-B). Additionally, 5448Δ*ska* did not produce SK while 5448::ska_{ALAB49} and 5448::ska_{NZ131} both secreted the exchanged variant of SK (Figure 3.6C). Utilising the humanised Plg transgenic mouse line

AlbPLG1, the virulence of the wild-type 5448 and the isogenic mutant GAS strains, 5448Δ*ska*, 5448::*ska*$_{ALAB49}$ and 5448::*ska*$_{NZ131}$ was assessed. The virulence of GAS strain 5448 has been well characterised and has previously been shown to be virulent

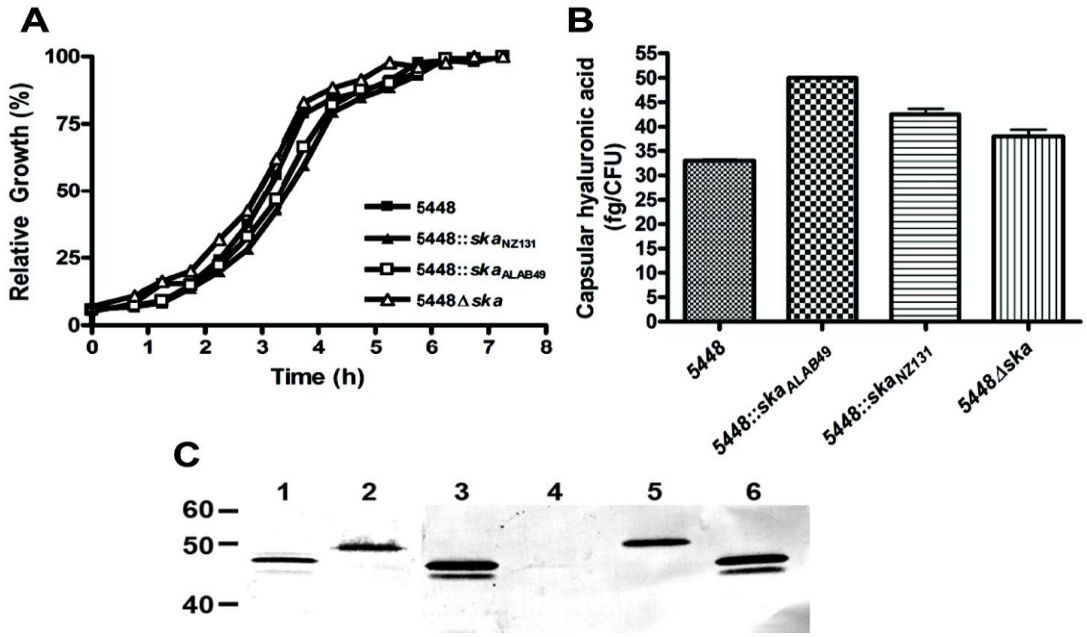

Figure 3.6: *In vitro* characterisation of wild type GAS strain 5448 and isogenic mutant strains 5448Δ*ska*, 5448::*ska*$_{ALAB49}$ and 5448::*ska*$_{NZ131}$ **(A)** 5448 and the isogenic mutants display comparative growth at 37°C in THY medium. **(B)** 5448 and the isogenic mutants express capsular hyaluronic acid (mean ± SD) and **(C)** Composite anti-SK probed Western blot showing SK expression by wild type GAS strains 5448, ALAB49, NZ131 (Lanes 1-3) and similar expression by isogenic mutants 5448::*ska*$_{ALAB49}$ and 5448::*ska*$_{NZ131}$ (Lanes 5-6), while 5448Δska does not express SK (Lane 4).

in this mouse model (Figure 3.7) (Maamary *et al.*, 2010, Walker *et al.*, 2007). In comparison to the wild-type 5448 strain, the virulence of 5448Δ*ska* and 5448::*ska*$_{ALAB49}$ was significantly reduced ($P < 0.05$; 50% vs. 10% mortality) (Figure 3.7). Conversely, the virulence of 5448::*ska*$_{NZ131,}$ is increased when compared to the wild-type 5448 strain, although these data were not statistically significant ($P > 0.05$; 100% vs. 50%

mortality) (Figure 3.7). Taken together, these data suggest that the unique Plg activation kinetics/properties displayed by the different SK variants affect the pathogenesis of GAS.

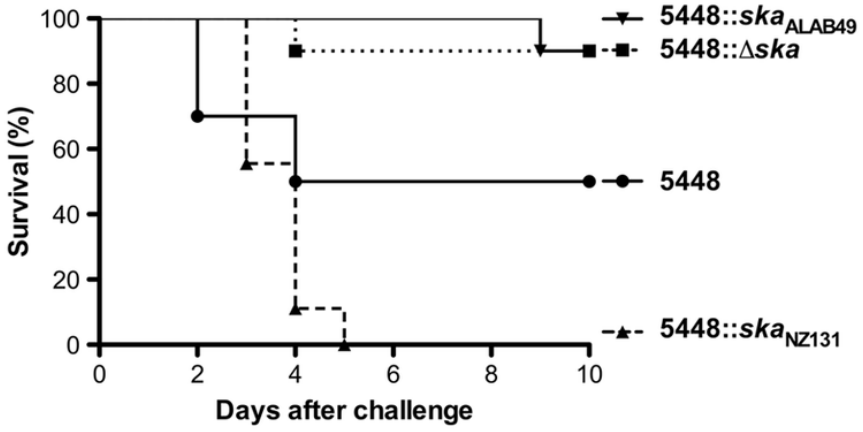

Figure 3.7: Survival curves after subcutaneous infection of humanised Plg transgenic *AlbPLG1* mice with GAS strain 5448 and isogenic mutants. 5448 (3.9 x 10^7 CFU/dose; *n* = 10) (Maamary *et al.*, 2010), 5448::*ska*$_{ALAB49}$ (4.6 x 10^7 CFU/dose; *n* = 10), 5448Δ*ska* (3.7 x 10^7 CFU/dose; *n* = 10) or 5448::*ska*$_{NZ131}$ (3.7 x 10^7 CFU/dose; *n* = 9). Differences in survival of humanised Plg transgenic mice infected with GAS strains 5448, 5448::Δ*ska*, 5448::*ska*$_{ALAB49}$, or 5448::*ska*$_{NZ131}$ were determined by the log-rank test. Differences were considered statistically significant at $P < 0.05$. Virulence of strains 5448Δ*ska* and 5448::*ska*$_{ALAB49}$ were significantly reduced ($P < 0.05$) vs 5548. Conversely, the virulence of 5448::*ska*$_{NZ131}$, was increased when compared to the wild-type 5448 strain, although these data were not statistically significant ($P > 0.05$; 100% vs. 50% mortality) All statistical tests were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

3.5 Discussion

GAS is a versatile human pathogen capable of causing a wide range of human diseases. The broad pathogenicity of GAS is underpinned by the genetic diversity displayed by clinical isolates of the species. There is a large amount of evidence in the literature describing how the absence or presence of virulence genes or changes in the complex regulatory mechanisms controlling the expression of these genes can alter the pathogenicity of a particular GAS isolate (Sumby *et al.*, 2006, Walker *et al.*, 2007, Kreikemeyer *et al.*, 2003, Musser and Shelburne, 2009, Castellino and Ploplis, 2005). Similarly, allelic variation of specific virulence genes may also influence the pathogenicity of GAS isolates however, these changes may be more subtle and therefore more difficult to characterise.

Allelic variation of the *ska* gene has been well characterised (Kalia and Bessen, 2004, Kapur *et al.*, 1995, McArthur *et al.*, 2008). Bioinformatical analyses of predicted SK protein sequences suggested that SK variants maintain similar secondary structure despite differences in the amino acid sequences (Kapur *et al.*, 1995). In this study, the six SK proteins were specifically chosen as representatives of divergent sequence clusters and despite significant amino acid sequence differences, all recombinant SK proteins displayed similar secondary structures as indicated by individual CD spectra. This data further supports the hypothesis that selection pressure may be placing structural constraints on SK molecules (Kapur *et al.*, 1995). However, biochemical analysis of the SK variants presented in this study clearly demonstrates that these

variants do display a number of different phenotypic properties, which alter the ability of these molecules to interact with and activate human Glu-Plg.

GAS SK variants display significant differences in ability to non-proteolytically generate an active site in Glu-Plg. Type-2b SK variants could not induce the formation of an active site in Glu-Plg. Additionally, type-2b SK variants have a 25 fold less affinity for Glu-Plg when compared to other SK variants which all displayed similar high affinities (Table 3.1). Therefore, the inability of type-2b SK variants to produce an active site in Glu-Plg may be the result of the type-2b variants failing to interact with Glu-Plg. However, type-1 SK, group-C SK and type-2a SK molecules which all bind to Glu-Plg with similar, high affinity, also displayed different rates of active site formation in Glu-Plg. These data suggest that while the formation of the nascent SK-Plg complex plays a role in the generation of a conformationally re-arranged active site in Glu-Plg, other protein specific changes may also be affecting this process.

SKc mutants with deletions or site directed amino acid changes within the α domain, also form complexes with Glu-Plg that display delays in the generation of amidolytic activity (Fay and Bokka, 1998, Wang *et al.*, 1999a, Rodríguez *et al.*, 1995, Young *et al.*, 1995). In particular, residues 1-59 of the SK α domain have been shown to be critical for SK mediated Plg activation (Young *et al.*, 1995, Mundada *et al.*, 2003, Reed *et al.*, 1999). SK mutants (such as α domain truncation mutants and numerous amino terminal site directed mutants) also display reduced amidolytic activity, reduced Plg affinity and increased susceptibility to α_2-AP inhibition (Fay and Bokka, 1998, Sazonova *et al.*, 2004, Rodríguez *et al.*, 1995, Boxrud *et al.*, 2000, Mundada *et al.*, 2003). Upon mixing

of SK and Glu-Plg, SK is rapidly cleaved at the Lys_{59}-Ser_{60} peptide bond once bound to Plg (Shi *et al.*, 1994). The N-terminal peptide remains associated (non-covalently) with the SK-Plg complex and is required for non-proteolytic active site induction and stabilisation of the activator complex (Parrado *et al.*, 1996, Young *et al.*, 1995, Wang *et al.*, 1999a, Mundada *et al.*, 2003). For SK to induce an active site in Plg, the SK Ile_1 residue must be positioned within the Plg molecule so that it can form a salt bridge with Asp_{740} of Plg (Wang *et al.*, 1999a). SK mutants lacking this residue cannot induce an active site in Plg through non-proteolytic mechanisms (Wang *et al.*, 1999a, Mundada *et al.*, 2003). While all SK variants examined in this study had an N-terminal Ile residue, cluster type-2b SK proteins contain two specific amino acid changes (Q86R and E134Q) in the α domain regions that come in direct contact with the Plg moiety (Law *et al.*, 2012)(Figure 3.2). These changes (or potentially others within the β and γ domains) may slow the initial interaction of SK with Glu-Plg and also possibly prevent the correct positioning of the N-terminal fragment within the SK-Plg complex thereby preventing (or slowing) non-proteolytic active site formation in Glu-Plg.

The physiological inhibitor of plasmin, α_2-AP, is a member of the serpin family and tightly regulates the activity of plasmin in plasma (Aoki *et al.*, 1993). When plasmin reacts with α_2-AP, the serpin is cleaved resulting in a covalently bound complex of plasmin and α_2-AP that is inactive (Shieh and Travis, 1987). Activator complexes consisting of Plg/plasmin and therapeutic SK (SKc) are known to be resistant to α_2-AP inhibition (Cederholm-Williams, 1979). Similarly, we found complexes of plasmin and type-2a SK (SK_{5448} and SK_{NS696}) variants were also resistant to inhibition by α_2-AP, while complexes of plasmin and type-2b SK variants (SK_{ALAB49} and $SK_{NS88.2}$) or type-1

SK (SK_{NZ131}) were not (Figure 3.5B). The susceptibility of type-2b SK for inhibition by α_2-AP and inability of this variant to non-proteolytically generate in active site in Glu-Plg suggests that GAS isolates expressing a type-2b SK molecule have evolved novel mechanisms to control SK mediated Plg activation. The apparent requirement of plasmin for type-2b SK mediated activation of Plg also suggests that Plg activation may be restricted to areas where there is free plasmin (i.e. at the sites of fibrinolysis). Alternatively, SK variants may require additional cofactors (either host or bacterial in origin) to facilitate successful Plg activation, such as: fibrinogen, SEN, GAPDH or PAM (McArthur *et al.*, 2008, Gaffney *et al.*, 1988, Lahteenmaki *et al.*, 2001).

Subversion of the host Plg activation system is a well-documented pathogenic mechanism used by GAS and other bacterial pathogens to cause disease (Boyle and Lottenberg, 1997, Coleman and Benach, 1999, Castellino and Ploplis, 2005). SK mediated Plg activation has been shown to play a critical role in the invasive pathogenesis of GAS (Sun *et al.*, 2004, Castellino and Ploplis, 2005). M1T1 GAS strains are considered a highly virulent clone capable of causing serve invasive disease of humans. Consequently, the M1T1 GAS strain 5448 used in this study (which contains a type-2a *ska* allele) is highly virulent for the human Plg transgenic mouse strain that was used in these experiments (Cole *et al.*, 2011, Maamary *et al.*, 2010, Walker *et al.*, 2007). In this study, the acquisition of the type-1 *ska* allele increased M1T1 virulence. Similarly, GAS strain NZ131 (type-1 SK) has previously been shown to be virulent in other mouse models of GAS infection. (Kuo *et al.*, 1998, Tsao *et al.*, 2001, Li *et al.*, 2011). When the type-2a *ska* allele of the M1T1 strain 5448 is replaced with a type-2b allele (ska_{ALAB49}), the invasive pathogenesis of this strain is reduced,

similar to a level seen for the isogenic *ska* deletion mutant (5448Δ*ska*) (Figure 3.7). Previous studies conducted in our laboratory have shown that wild-type ALAB49 is avirulent in this mouse model (Maamary *et al.*, 2010). This result indicates that type-2b SK cannot reproduce the *in vivo* function of a type-2a SK in a M1T1 genetic background and suggests the requirement of plasmin by type-2b SK to form an efficient activator complex affects the invasive pathogenesis of GAS. Previous studies have shown a strong association between cluster type-2b alleles and skin tropic *emm* pattern D strains containing the high affinity Plg binding M-protein PAM (Kalia and Bessen, 2004). Taken together, these observations suggest that type-2b SK proteins require bacterial co-factors present only in a subset of strains for efficient plasmin acquisition or that reduced/restricted Plg activation kinetics produced by type-2b SK *in vivo* may be beneficial for successful long term skin infections such as impetigo. Similarly, the Plg activator staphylokinase from the ubiquitous skin coloniser, *Staphylococcus aureus*, also requires plasmin for efficient Plg activation, which suggests this mechanism of Plg activation may be advantageous for skin colonisation (Rampling and Gaffney, 1976). Additionally, Plg acquisition and activation mechanisms have been documented for commensal strains of oral streptococci, which also suggests this process may also be involved in maintaining long term infections (Kinnby *et al.*, 2008).

This study confirms the importance of SK mediated Plg activation in GAS pathogenesis and highlights a mechanism whereby variability in this important virulence factor can influence the pathogenesis of this organism. Characterising GAS SK variants represents a novel approach to elucidate the mechanism of SK-mediated Plg activation. Therefore, future comparative studies that characterise GAS SK variants in more detail will help to

identify critical residues involved in SK function and could assist the rational design of new drugs targeting this specific interaction which may be useful in treating GAS infections.

Chapter 4:

Site Restricted Plasminogen Activation Mediated by Group A streptococcal Streptokinase Variants

Preface

The protease activity harnessed by group A streptococcus is critical for disease progression in humans and influences pathogenic outcomes. Genetic variants of the potent Plg activator protein, SK, which is secreted by group A streptococcal isolates, display differences in the mechanism of Plg activation and have previously been shown to influence invasive pathogenesis. Bacterial and host-derived Plg activation system cofactors have been widely implicated in the subversion of the fibrinolytic system by bacteria. Furthermore, plasmin(ogen) ligands naturally assist in regulating protease activity to areas required in the healthy host. Here we examined the effect of these co-factors on SK variant interaction with the Plg activation system. We show these interactions have a role in restricting protease activity to specific microenvironments for some group A streptococcal isolates and SK variation may contribute to the broad disease spectrum associated with this pathogen.

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4.1 Abstract

Streptokinase (SK) is a secreted Plg activator and virulence factor of group A streptococcus (GAS). Among GAS isolates, SK gene sequences are polymorphic and are grouped into two sequence clusters (cluster type-1 and cluster type-2) with cluster type-2 being further classified into sub-clusters (type-2a and type-2b). Here we examine the role of bacterial and host-derived cofactors in SK mediated Plg activation. All SK variants, apart from type-2b, can form an activator complex with Glu-Plg (Glu-Plg). Specific ligand-binding induced conformational changes in Glu-Plg mediated by fibrinogen, Plg-binding group A streptococcal M protein (PAM), fibrinogen fragment D or fibrin, were required for type-2b SK to form a functional activator complex with Glu-Plg. In contrast with type-1 and type-2a SK, type-2b SK activator complexes were inhibited by α_2-antiplasmin unless bound to fibrin or to the GAS cell surface via PAM in combination with fibrinogen. Taken together, these data suggest that type-2b SK Plg activation may be restricted to specific micro-environments within the host such as fibrin deposits or the bacterial cell surface through the action of α_2-antiplasmin. We conclude that phenotypic SK variation functionally underpins a pathogenic mechanism whereby SK variants differentially focus Plg activation, leading to specific niche adaption within the host.

4.2 Introduction

Streptokinase (SK) is a plasminogen (Plg) activator secreted by *β*-haemolytic streptococcal species. SK from *Streptococcus equisimilis* isolate H46A (SKc) is the most characterised SK protein due to its widespread use as a thrombolytic therapeutic since approval in 1977 (McArthur *et al.*, 2012). Unlike host Plg activators that activate Plg by limited proteolytic cleavage, SKc binds to Plg inducing conformational changes in the protein that result in the formation of an active site and the production of an enzymatically active complex, termed SK-Plg* (known as the 'Conformational Activation Pathway' or 'Pathway I'). The conformationally activated SK-Plg* complex can then sequester substrate Plg and proteolytically convert these molecules to the broad-spectrum serine protease plasmin via cleavage at the Arg_{561}-Val_{562} peptide bond (Boxrud *et al.*, 2000, Boxrud *et al.*, 2004). Plasmin, which has a higher affinity for SK than Plg, rapidly displaces Plg in the SK-Plg* complex to produce an activated SK-plasmin complex that is the main catalyst responsible for the full conversion of Plg to plasmin (known as 'Direct Proteolytic Activation Pathway' or 'Pathway II') (Boxrud *et al.*, 2000, Boxrud *et al.*, 2004).

The process of Plg activation in a healthy individual is strictly controlled. Multiple regulatory mechanisms function in a co-ordinated manner to restrict Plg activation to specific locations within the host where the potent protease activity of plasmin is required. Plg activation is greatly influenced by the conformation of the Glu-Plg molecule. Plg contains several structural domains, consisting of the N-terminal PAp domain (also known as the NTP domain and PAN module domain), followed by five

kringle domains (KI-K5) and the carboxy-terminal serine protease catalytic domain (Ponting *et al.*, 1992, Law *et al.*, 2012, Xue *et al.*, 2012, Brown *et al.*, 2001, Brown *et al.*, 2003). Intramolecular binding between lysine residues and the lysine binding sites of these kringles maintains circulating Glu-Plg in a 'closed' conformation that is highly resistant to activation. Upon binding to specific cell surface receptors or ligands such as fibrin, Glu-Plg adopts an 'open' conformation that becomes more susceptible to activation (Castellino and Ploplis, 2005). While soluble plasmin is readily inhibited by the circulating inhibitor α_2-antiplasmin (α_2-AP), plasmin that remains bound to receptors/ligands may be resistant to inhibition, thereby restricting plasmin activity to specific foci such as the cell surface or fibrin thrombi (Irigoyen *et al.*, 1999).

Plg activation by SKc is not affected by these regulatory mechanisms as the closed conformation of Glu-Plg does not prevent activation and because plasmin activation activity displayed by the SKc-Plg* activator complex is not inhibited by α_2-AP (Xue *et al.*, 2012, Cederholm-Williams, 1979). Thus, SKc-mediated Glu-Plg activation can rapidly generate high levels of soluble plasmin activity as activator complexes sequester and activate substrate Plg while bypassing host regulation mechanisms (Parry *et al.*, 2000). Based on these observations, it is now a widely held view that the human specific pathogen, *Streptococcus pyogenes* (group A streptococcus, GAS) hijacks the host Plg activation system by generating unregulated soluble and cell bound plasmin activity that is used to overcome immune defences and break down tissue barriers to facilitate dissemination (Walker *et al.*, 2005, Sun *et al.*, 2004, Khil *et al.*, 2003). However, recent research that has characterised SK variants from different GAS isolates suggests these variants may play differing roles in the pathogenesis process (Cook *et al.*,

2012, Zhang *et al.*, 2012). SK produced by GAS displays considerable genetic and phenotypic diversity (Kalia and Bessen, 2004, McArthur *et al.*, 2008, Kapur *et al.*, 1995, Zhang *et al.*, 2012, Cook *et al.*, 2012). Phylogenetic studies of *ska* sequences from GAS isolates have revealed two main sequence clusters (cluster type-1 and 2) with cluster type-2 sequences being further subdivided (cluster type-2a and 2b) (Kalia and Bessen, 2004, McArthur *et al.*, 2008). Epidemiological studies have shown the type-2b *ska* linage to be largely restricted to skin tropic, *pam* positive GAS isolates expressing the high affinity plasminogen-binding group A streptococcal M protein PAM (Kalia and Bessen, 2004).

Despite significant sequence variation, SKc, type-1 and type-2a SK variants display several important phenotypic similarities, such as a high affinity for Glu-Plg and an ability to conformationally activate Glu-Plg *via* 'Pathway I'. Conversely, type-2b SK variants have low affinity for Glu-Plg and cannot induce an active site in this protein through conformational rearrangement (Cook *et al.*, 2012, Zhang *et al.*, 2012). In addition, the exchange of *ska* allelic variants in a virulent M1T1 GAS isolate (strain 5448; cluster type-2a) alters pathogenesis in a murine model of invasive infection (Cook *et al.*, 2012). While these observations confirm the importance of Plg activation in GAS pathogenesis, the rationale and mechanisms defining how SK variants influence virulence are yet to be determined.

This study examines the role of bacterial and host-derived cofactors in Glu-Plg activation mediated by different SK variants with a view to determine how these interactions may influence GAS pathogenesis. Our data suggest Glu-Plg activation by

type-2b SK variants is restricted by ligand induced conformational changes in the Glu-Plg molecule and that soluble type 2b SK-Plg* activator complexes are inhibited by α_2-AP. Taken together, we propose the activation of Glu-Plg by type-2b SK variants is restricted to specific microenvironments at the infection foci which may promote long term skin colonisation.

4.3 Materials and Methods

4.3.1 Bacterial strain, culture conditions and reagents

Escherichia coli strain M15 [pREP4] was used as a host for protein expression and was cultured at 37°C in Luria-Bertani broth. GAS isolates ALAB49 (Svensson *et al.*, 2000), ALAB49Δ*pam*, NS88.2 (McKay *et al.*, 2004) and NS88.2*prp* (Sanderson-Smith *et al.*, 2008) were used in this study. All streptococcal strains were routinely cultured at 37°C on horse-blood agar (Biomerieux, Sydney, NSW, Australia) or in static liquid cultures of Todd-Hewitt broth (BD, Sydney, NSW, Australia) supplemented with 1% (w/v) yeast extract (Oxoid, Adelaide, SA, Australia) (THY medium). Where appropriate, antibiotics were used for selection at the following concentrations: kanamycin, 50 μg/mL and ampicillin, 100 μg/mL. Glu-Plg, plasmin, Fg, FgD, FgE and α_2-AP were purchased from Haematologic Technologies, Essex Junction, VT, USA. The chromogenic substrate H-D-Val-Leu-Lys-pNA·2HCl (S-2251) was obtained from Chromogenix, Mölndal, Sweden. Thrombin from human plasma was purchased from Sigma-Aldrich, Sydney, NSW, Australia and 4-methylumbelliferyl p-guanidinobenzoate (MUGB) was from Marker Gene Technologies, Inc., Eugene, OR, USA.

4.3.2 Cloning, expression and purification of recombinant proteins

Recombinant SK variants, plasminogen-binding group A streptococcal M proteins (PAM, Prp), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and streptococcal enolase (SEN) were cloned, expressed in *E. coli*, purified, and characterised as previously described (Sanderson-Smith *et al.*, 2006, Cook *et al.*, 2012, Derbise *et al.*, 2004, Cole *et al.*, 2006b).

4.3.3 Non-proteolytic active site generation in Glu-Plg

Non-proteolytic active site generation in Glu-Plg by SK variants was examined using the fluorescent active site MUGB in a POLARstar Omega fluorescence spectrophotometer (BMG LABTECH, Ortenberg, HE, Germany). Glu-Plg (200 nM) was added to a black 96-well microtitre plate containing 1 μM MUGB in assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) at 37°C. To initiate the reaction, SK was added to a final concentration of 400 nM in a total volume of 100 μL and the development of fluorescence was monitored continuously with excitation at 355 nm and emission at 460 nm. To test the effect of various Plg ligands and co-factors on non-proteolytic active site generation in Glu-Plg by SK variants, each of these molecules were incubated in various combinations for 10 min at 37°C before addition of MUGB, at the following concentrations: SEN (200 nM), GAPDH (200 nM), PAM (200 nM), Fg (200 nM), Fg fragment D (FgD) (200 nM), Fg fragment E (FgE) (200 nM). For active site experiments in the presence of plasma, SK was added to a 1:10 dilution of pooled EDTA-treated human plasma and MUGB (1 μM). Data was normalised by subtracting a control reaction of all protein species without the addition of SK and 1 μM MUGB. This accounted for intrinsic fluorescence associated with buffer and protein species, as well as non-specific hydrolysis of MUGB over the course of reactions. Fluorescence measurements were expressed as the fractional change in the initial fluorescence $(F_{obs}-F_o)/F_o = \Delta F/F_o$. Values for rates of active site generation were calculated using the initial linear portion of the reactions as $(\Delta F.F_o^{-1}).min^{-1}$. This is subsequently referred to in the simplified form as ΔFU/min. To calibrate relative fluorescence units and convert these measurements to [SK-Plg*], standard curves of known concentrations of the fluorescent

product 4-methylumbelliferone were constructed. The fluorescence measurements were made in the assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) at 37°C containing 400 nM SK and 200 nM Glu-Plg, with the same instrument settings as MUGB experiments. Values for rates of active site generation were calculated using the initial linear portion of the reactions as pmol SK-Plg*.min^{-1}. Initial rates of active site generation were compared using a two-tailed unpaired students t-test using GraphPad Prism 5 (GraphPad, San Diego, CA, USA).

4.3.4 Plasminogen activation by variant SK-Plg complexes

The capacity of variant SK-Plg complexes to activate substrate Glu-Plg was studied by the addition of SK (final concentration 5 nM) to assay buffer (10 mM HEPES, 150 mM NaCl, 0.01% Tween-20, pH 7.4) containing a large excess of Glu-Plg (500 nM) and S-2251 (500 μM) in a total volume of 100 μL. The parabolic generation of plasmin was monitored by change in absorbance at 405 nm and measured over 30 min at 37°C using a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). To assess the role of co-factors and ligands, each was pre-incubated with Plg for 10 min at 37°C at the following concentrations: Fg (500 nM), PAM (500 nM), α_2-AP (50 nM). For quantitative comparison of ligand effects, change in $A_{405nm,}$ which is a function of S-2251 substrate cleavage by plasmin generated during the activation of Glu-Plg by SK, was plotted against t^2. The velocities of these reactions were then calculated from the gradient of $A405_{nm}$ *vs* t^2. All data transformation and linear regressions were performed in GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

To examine the role of Plg binding to the GAS cell surface of type-2b expressing strains, whole GAS cells were used as an exogenous source of the cell receptors PAM/Prp to assess the effect on Plg activation, with PAM and Prp knock-out strains used as controls. For these experiments a single colony of each strain (NS88.2, NS88.2*prp*, ALAB49 and ALAB49Δ*pam*) was inoculated in THY medium for growth overnight at 37°C. The overnight culture ($A600_{nm}$ = 1.1–1.3) was diluted (10% v/v) in pre-warmed THY medium and grown to an $A600_{nm}$ = 0.5. The cells were collected by centrifugation (5,000 *g* for 10 min) and washed twice with sterile PBS (3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, and 135mM NaCl, pH 7.4) followed by resuspension of 1 x 10^8/CFU of each GAS strain per replicate in 90 μL assay buffer (10 mM HEPES, 150 mM NaCl, 0.01% Tween-20, pH 7.4) containing Glu-Plg (500 nM) and repeated in the presence of absence of α_2-AP (250 nM) and/or Fg (500 nM). Protein mixtures were incubated for 10 min at 37°C in a 96-well plate to bind Plg to the cell surface. To initiate Plg activation, SK_{ALAB49} or $SK_{NS88.2}$ and S-2251 were added to the wells at 5 nM and 500 μM respectively, in a final volume of 100 μL. The exponential generation of plasmin was monitored by change in absorbance at 405 nm was measured over 30 min at 37°C using a SpectraMax Plus 384 spectrophotometer with shaking between measurements (Molecular Devices, Sunnyvale, CA, USA).

4.3.5 Effect of fibrin on the amidolytic activity of variant SK-plasminogen complexes

To study the amidolytic activity of SK-Plg complexes bound to insoluble fibrin matrices, fibrin-coated micro-titre plate wells (96 wells/plate) were prepared by incubating Fg (3 μM) and thrombin (0.8 NIH U/mL) in 50 μL of sample buffer (0.05 M

Tris, 0.02 M $CaCl_2$, 0.1% Tween-20, pH 7.75) at room temperature for 4 h. Wells were blocked for 1 h with 3% bovine serum albumin and washed five times with washing buffer (0.05 M Tris, 0.1% Tris-20, pH 7.75). Glu-Plg (20 nM) in 100 uL of assay buffer (10 mM HEPES, 150 mM NaCl, 0.01% Tween-20, pH 7.4) was bound to the fibrin clots for 1 h. Wells were washed 5 times with assay buffer. To measure the amidolytic activity of complexes, each SK variant (40 nM) and S-2251 (500 μM) was added to wells, in the presence or absence of α_2-AP (250 nM) and change in absorbance at 405 nm was measured over 30 min at 37°C using a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

4.3.6 Plasma clot lysis assay

Cross-linked plasma clots were prepared using human plasma pooled from healthy donors, which had been collected using K2EDTA BD vacutainers (BD, North Ryde, NSW, Australia). Clotting was initiated by adding human thrombin (0.8 NIH U/mL) and $CaCl_2$ (20 mM) to plasma at room temperature. Immediately after mixing, 50 µL of the polymerising plasma was transferred to a 96-well micro-titre plate. The plasma clots were formed at room temperature for 2 h and washed once with 50 µL of plasma. Clot lysis was performed by adding 50 µL of pooled plasma containing variant SK (50 nM) to the clot at 37°C. The clot lysis process was monitored by change in turbidity at 340 nm using a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). As a control, plasma clots were incubated with 50 µL of plasma without addition of SK to monitor stability during the course of the assay.

4.3.7 Plasma fibrinogen depletion by SK variant plasminogen activation

The level of plasma Fg depletion by each SK variant was determined by a modified version of the sodium sulphite precipitation method (Rampling and Gaffney, 1976). Briefly, SK variants (50 nM, final concentration) were added to 100 µL of pooled EDTA-treated human plasma and incubated at 37°C for 30 min. Each aliquot was immediately mixed with aprotinin (20 µM) to inhibit plasmin activity, and precipitated with 900 µL of 10.5 % (w/v) sodium sulphite by incubation in a 37°C water bath for 15 min. Precipitant Fg was cleared from solution by centrifugation at 1,500 x *g* for 15 min. The supernatant was carefully decanted and the cleared Fg washed with 500 µL of 10.5 % (w/v) sodium sulphite, re-centrifuged and the supernatant removed. To dissolve the collected Fg, 1 mL of 4 M urea in 0.1 N NaOH was added to each tube and incubated at 100°C for 15 min. Finally, the A_{280} of each solution was measured using a NanoDrop 2000c spectrophotometer (Thermo Fischer Scientific, Scoresby, VIC, Australia) and remaining Fg concentration determined by comparison to control plasma aliquots prepared without addition of SK. Statistical data analysis was conducted *via* one-way ANOVA with Tukey's multiple-comparison test. Data sets were considered significantly different at $P < 0.05$. All analysis was performed using GraphPad Prism 5 (GraphPad, San Diego, CA, USA).

4.3.8 Accession numbers

The nucleotide sequences of the *ska* genes used in this study are deposited in the GenBank database: H46A: K02986.1, NZ131: CP000829.1, 5448: JQ650489, NS696: JQ650488, ALAB49: AY234134.1 and NS88.2: JQ650490. Details of data submission can be found at: GenBank: www.ncbi.nlm.nih.gov.

4.3.9 Ethics approval:

Permission to collect human blood was obtained from the University of Wollongong Human Ethics Committee (HE08/250). Blood was taken from healthy adult volunteers, who provided informed, written consent.

4.4 Results

4.4.1 Non-proteolytic active site generation in Glu-Plg by variant SK

Active site generation in Glu-Plg by SK variants (SK-Plg*) was examined using the fluorescent active site titrant MUGB. This allowed generation of SK-Plg* (conformational activation, Pathway I) to be measured directly. To calibrate relative fluorescence units and convert these measurements to [SK-Plg*], standard curves of known concentrations of the fluorescent product 4-methylumbelliferone were constructed. The fluorescence measurements were made in the assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) at 37°C containing 400 nM SK and 200 nM Glu-Plg, with the same instrument settings as MUGB experiments. We concluded that 1.0 mol 4-methylumbelliferone/mol SK-Glu-Plg* was formed. It was previously identified that SK_{NZ131} (Type 1) displays the fastest rate (3.2 pmol SK-Plg*.min^{-1}) of conformational activation of Glu-Plg, followed by SKc (1.9 pmol SK-Plg*.min^{-1}). SK_{NS696} and SK_{5448} (type-2a variants) both display very slow rates of active site generation (0.34 and 0.27 pmol SK-Plg*.min^{-1} respectively) while SK_{ALAB49} and $SK_{NS88.2}$ (type-2b variants) fail to induce an active site in Glu-Plg (Figure 4.1A) (Cook *et al.*, 2012). As SK mediated Glu-Plg activation is affected by the conformation of Glu-Plg, experiments were conducted in the presence of Fg and Fg fragments D (FgD) and E (FgE) to the assess effect on conformational rearrangement of Plg by these molecules. In the presence of Fg and Fg fragment D, SK_{NZ131} (type-1) and SKc displayed increased rates of active site generation (Fg = 0.35 and 0.33 pmol SK-Plg*.min^{-1} respectively and FgD = 0.36 and 0.33 pmol SK-Plg*.min^{-1} respectively) (Figure 4.1B and C). The rate of active site generation by type-2a variants (SK_{NS696} and SK_{5448}) was also significantly enhanced (1.9-2.0 and 2.6-3.0 fold respectively), but the rates were less than those observed for SK_{NZ131} and SKc

(Figure 4.1B and C). The type-2b variants (SK_{ALAB49} and $SK_{NS88.2}$) could generate an active site in Glu-Plg when combined with Fg or FgD, however this occurred at a slow rate of 0.12-0.19 pmol SK-Plg*.min^{-1} for both variants with each ligand (Figure 4.1B and C). FgE had minimal effect on the rate of active site generation for the SK variants and did not allow type-2b SK to generate an active site in Glu-Plg (Figure 4.1D). This indicated that binding of Glu-Plg to Fg or FgD, but not FgE, results in a conformational change in the Glu-Plg molecule that readily allows non-proteolytic active site generation (Pathway I) by SK variants.

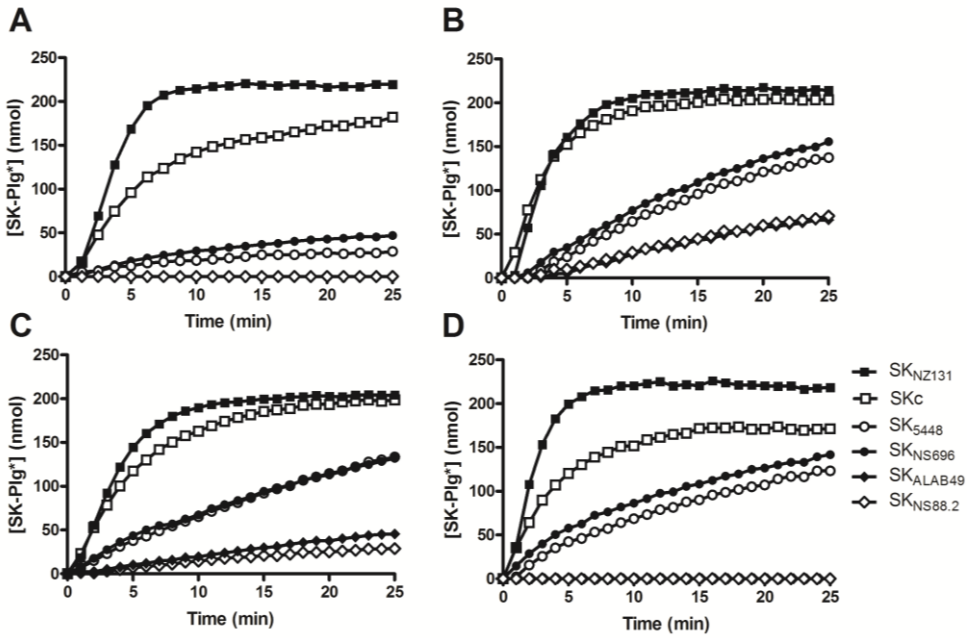


Figure 4.1: Effect of Fg and Fg fragments on non-proteolytic active site generation in Glu-Plg and plasminogen activation by SK variants. Glu-Plg alone (200 nM) (**A**) or in the presence of 200 nM Fg (**B**), FgD (**C**), or FgE (**D**) were added to 1 µM MUGB in assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) at 37 °C to preform for 10 min. To initiate the reaction, SK was added to a final concentration of 400 nM in a total volume of 100 µL and the development of fluorescence was monitored continuously with excitation at 355 nm and emission at 460 nm. Panel (**A**) appears in Cook *et al.* (2012) and is included for comparison. Data shown represent minimum of two independent experiments (n = 3). Error less than 10% are not shown for clarity of presentation.

4.4.2 Effect of GAS cell surface Plg receptors on non-proteolytic active site generation in Glu-Plg by variant SK

The binding of plasmin(ogen) to the bacterial cell surface via cell wall associated proteins such as plasminogen-binding group A streptococcal M proteins (PAM, Prp), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and streptococcal enolase (SEN) has been well documented (Lahteenmaki *et al.*, 2001, Walker *et al.*, 2005, Castellino and Ploplis, 2005). To assess the effect of these bacterial Plg receptors on SK-mediated

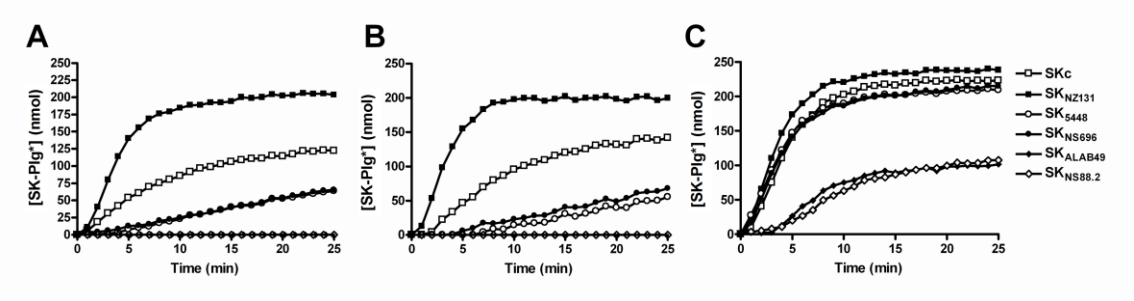


Figure 4.2: Effect of GAS cell surface Plg receptors on non-proteolytic active site generation in Glu-Plg and plasminogen activation by SK variants. Glu-Plg (200 nM) in the presence of 200 nM SEN (**A**), GAPDH (**B**), or PAM (**C**) were added to 1 µM MUGB in assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) at 37 °C to preform for 10 min. To initiate the reaction, SK was added to a final concentration of 400 nM in a total volume of 100 µL and the development of fluorescence was monitored continuously with excitation at 355 nm and emission at 460 nm. Data shown represent minimum of two independent experiments (n = 3). Error less than 10% are not shown for clarity of presentation.

conformational activation of Glu-Plg, active site generation assays were performed in the presence of recombinant GAPDH, SEN and PAM. Pre-binding Glu-Plg with either SEN or GAPDH had no effect on active site generation in this molecule by any of the

SK variants (Figure 4.2A and B). Interestingly, when Glu-Plg was pre-bound with PAM, type-2b variants (SK_{ALAB49} and $SK_{NS88.2}$), were able to non-proteolytically induce an active site in Glu-Plg. Furthermore, the rate of active site generation mediated by SKc, and type-2a variants (SK_{NS696} and SK_{5448}) were all enhanced, producing activation rates similar to type-1 SK (SK_{NZ131}), which was not affected by any of the bacterial co-factors (Figure 4.2C).

4.4.3 Non-proteolytic active site generation in human plasma by variant SK

To assess Glu-Plg activation under conditions that are more physiologically relevant, active site generation experiments were repeated in the presence of EDTA treated human plasma pooled from several donors. When added directly to plasma, SKc displayed the fastest rate of conformational activation of Glu-Plg, followed closely by SK_{NZ131} (Type 1) (0.033 and 0.025 ΔFU/min respectively) (Figure 4.3A). SK_{NS696} and SK_{5448} (type-2a variants) both displayed slow rates of Glu-Plg activation (0.004 and 0.002 ΔFU/min) while SK_{ALAB49} and $SK_{NS88.2}$ (type-2b variants) failed to induce an active site in Glu-Plg (Fig. 3A). This result indicates that type-2b SK variants do not form active complexes with Glu-Plg when circulating in plasma despite the presence of Fg. When this experiment was repeated in the presence of PAM, type-2b variants (SK_{ALAB49} and $SK_{NS88.2}$) were able to induce an active site in Glu-Plg (Figure 4.3B) indicating that the presence of PAM is required for type-2b SK variants to generate plasmin activity through conformational activation of Glu-Plg in plasma. Given that Fg and α_2-AP are present at significant concentrations in plasma, these results suggest that active type-2b SK-Glu-Plg* complexes formed in the presence of plasma Fg are not resistant to inhibition by α_2-AP unless PAM is also available. Additionally, in the

presence of PAM SK_{NS696} and SK_{5448} (type-2a variants) displayed 7 and 9.6 fold increases ($P < 0.01$) in active site generation, while reaction rates produced by SKc and type-1 SK_{NZ131} were not affected (Figure 4.3B). This suggests that interaction of Glu-Plg with PAM via kringle 2 produces a conformation that is favourable for activation by cluster 2 SK variants.

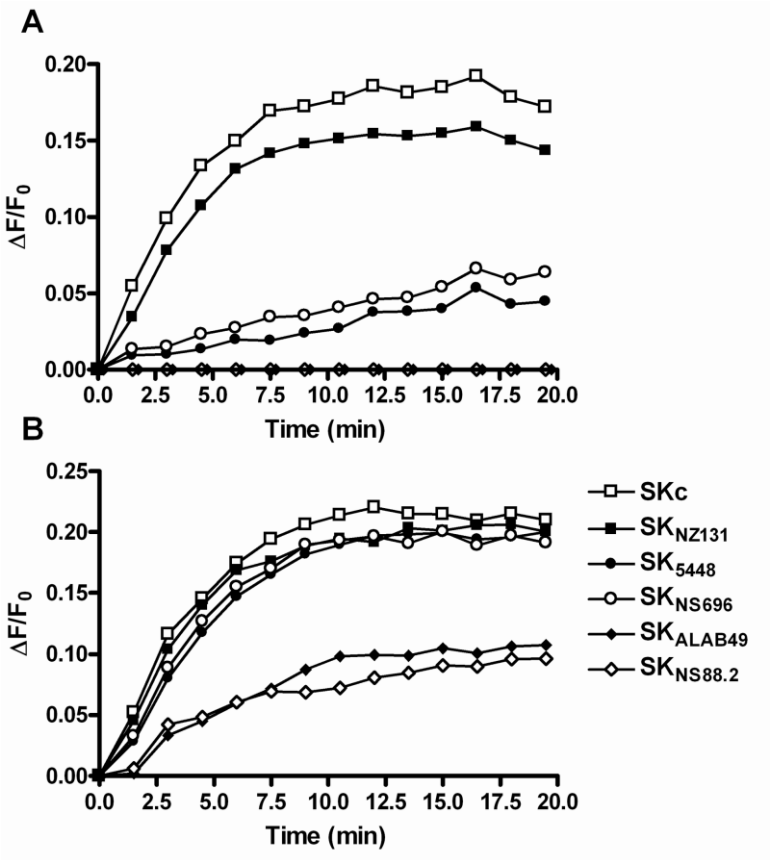


Figure 4.3: Non-proteolytic active site generation in Glu-Plg and plasminogen activation by SK variants in human plasma Pooled human plasma was added (1:10 dilution) to 1 µM MUGB in assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) at 37 °C in the absence (**A**) or presence of 200 nM PAM (**B**). To initiate the reaction, SK was added to a final concentration of 400 nM in a total volume of 100 µL and the development of fluorescence was monitored continuously with excitation at 355 nm and emission at 460 nm. Data shown represent minimum of two independent experiments (n = 3). Error less than 10% are not shown for clarity of presentation.

4.4.4 Regulation of soluble Plg activation activity of type-2b SK-Plg variants by host α_2-AP

To investigate how Plg ligands affect the inhibition of SK-Plg activator complexes by α_2-AP, a catalytic amount of SK (5 nM) was added to a 100 fold excess of substrate Glu-Plg, with various combinations of Fg and PAM in the presence or absence of α_2-AP (Figure 4.4). As expected, SKc could efficiently activate substrate Glu-Plg (v = 6.9 $mAB_{405nm}.min^{-2}$) and the initial velocity for rate of activation was partially reduced by the presence of α_2-AP (2.1 $mAB_{405nm}.min^{-2}$) (Figure 4.4A). As type-2b SK variants cannot generate an active site in Glu-Plg, no Plg activation activity was observed in the reaction with SK_{ALAB49} (Figure 4.4A). In the presence of either Fg or PAM, the initial velocity of SKc mediated Plg activation was enhanced (2.2 and 2.6 fold respectively) and was not inhibited by α_2-AP (Figure 4.4B and C). As type-2b SK variants can non-proteolytically generate an active site in the presence of Fg (Figure 4.1B) and PAM (Figure 4.2C), type-2b SK activator complexes could subsequently activate substrate Glu-Plg in the presence of either of these ligands (v = 1.75 and 0.6 $mAB_{405nm}.min^{-2}$ respectively) (Figure 4.4B and C). However, the activity displayed by these activator complexes was not resistant to inhibition by α_2-AP (Figure 4.4B and C). When SK was added in the presence of both PAM and Fg together, both SKc and type 2b SK (SK_{ALAB49}) could efficiently activate substrate Plg at an initial velocity of 30 $mAB_{405nm}.min^{-2}$ and this activity was not inhibited by α_2-AP (Figure 4.4D).

To determine if similar results would be observed on the bacterial cell surface, whole GAS cells were used as an exogenous source of Plg receptors (PAM/Prp) in Glu-Plg activation assays. In the presence of WT GAS cells (ALAB49 and NS88,2), type-2b SK

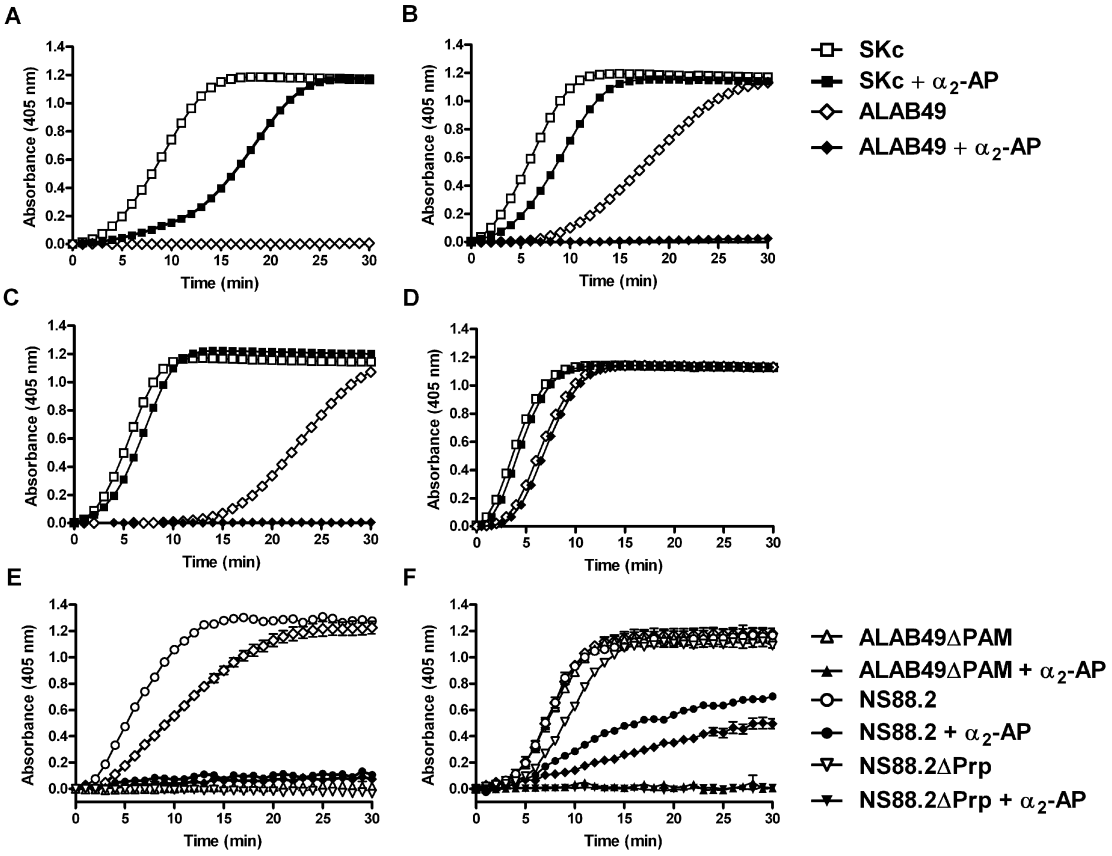


Figure 4.4: Effect of Fg and PAM on inhibition of SK variant mediated Plg activation ability by α_2-AP SK (5 nM final concentration) was added to assay buffer (10 mM HEPES, 150 mM NaCl, 0.01% Tween-20, pH 7.4) containing S-2251 (500 µM) with combinations of (**A**) Glu-Plg (500 nM), (**B**) Glu-Plg (500 nM) and equimolar Fg, (**C**) Glu-Plg (500 nM) and equimolar PAM, or (**D**) Glu-Plg (500 nM) with equimolar Fg and PAM. Panels **A**-**D** were tested for resistance to inhibition with α_2-AP (50 nM). Whole cell GAS experiments were conducted as above with $1x10^8$ cells added as a source of exogenous PAM/Prp with ΔPAM/Prp strains used as controls. To these cells (**E**) Glu-Plg (500 nM) or (**F**) Glu-Plg (500 nM) and equimolar Fg were added. All combinations were tested in the presence or absence of α_2-AP (250 nM). The generation of Plg activation activity was monitored at an absorbance of 405 nm at 37 °C. Data represent minimum of two independent experiments in duplicate. Data represent mean +/- SEM.

variants (SK_{ALAB49} and $SK_{NS88.2}$) could readily initiate substrate Glu-Plg activation. In the presence of isogenic PAM/Prp knock-out strains (ALAB49Δ*pam* and NS88.2*prp*), type-2b SK variants could not initiate substrate Glu-Plg activation confirming the essential role of PAM/Prp in type-2b SK-mediated Glu-Plg activation (Figure 4.4E). α_2-AP was able to inhibit Plg activation at the cell surface in these assays (Figure 4.4E). As expected, in the presence of purified Fg, type-2b SK variants could initiate Glu-Plg activation in the presence of both WT and knockout GAS strains (Figure 4.4F). Additionally, in the presence of Fg and WT GAS cells, cell surface Plg activation activity was not inhibited by α_2-AP, whereas in the presence of Fg and ALAB49Δ*pam* and NS88.2Δ*prp* cells, Glu-Plg activation activity was inhibited (Figure 4.4F). This data demonstrates a critical role for both PAM/Prp and Fg as synergistic cofactors in the generation of unregulated Plg activation activity on the surface of GAS strains expressing type-2b SK.

4.4.5 The effect of fibrin on the activity of type-2b SK variants

Therapeutic SK is considered a fibrin-independent agent because it rapidly generates plasmin in the blood at sites distant from fibrin clots. While type-2b SK variants do not readily activate circulating Glu-Plg, we investigated if this SK variant could activate conformationally modified Glu-Plg when bound to fibrin. Fibrin clots were produced *in vitro* by the addition of thrombin and calcium chloride to purified human Fg. Glu-Plg was then bound to fibrin and activated by the addition of SK variants (Figure 4.5). In the absence or presence of fibrin, SKc could readily activate Glu-Plg, while type-2b variants (SK_{ALAB49} and $SK_{NS88.2}$) could only activate Glu-Plg that was bound to fibrin (Figure 4.5A and B)

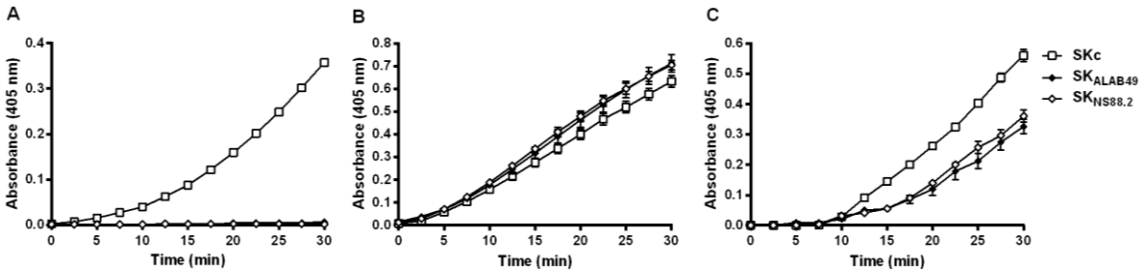


Figure 4.5: Generation of amidolytic activity and resistance to inhibition of variant SK-Plg complexes formed in the absence or presence of a fibrin clot (A) Complex formation was initiated by addition of variant SK (40 nM) to assay buffer (10 mM HEPES, 150 mM NaCl, 0.01% Tween-20, pH 7.4) containing Glu-Plg (20 nM) and chromogenic substrate S-2251 (500 µM) then monitored at $A_{405.}$ **(B)** Fibrin clots were formed for 4 h at RT in a 96-well plate by the addition of thrombin (0.8U/mL) and $CaCl_2$ (20 mM) to recombinant fibrinogen (3 µM). Plg (20 nM) was bound to the fibrin clots for 1 h at 37°C. Bound Plg was washed before complex formation was initiated and monitored at A_{405} by addition of variant SK (40 nM) to assay buffer (10 mM HEPES, 150 mM NaCl, 0.01% Tween-20, pH 7.4) chromogenic substrate S-2251 (500 µM) at 37°C. (**C**) The ability of SK-Plg complexed formed in the presence of fibrin were also assessed for susceptibility to inhibition by α_2-AP (200 nM). Data represent three independent experiments (n = 3). Data represent mean +/- SEM

As type-2b SK variants were able to generate Plg activation activity in the presence of fibrin, we compared the capacity of SKc and type-2b SK variants to lyse plasma clots produced from human plasma. As expected SKc displayed efficient fibrinolytic activity (Figure 4.6A). Both type-2b SK proteins (SK_{ALAB49} and $SK_{NS88.2}$) displayed fibrinolytic activity with potency similar to that of SKc (Figure 4.6A). Additionally, plasma fibrinogen depletion during these activation experiments in plasma was high in SKc mediated lysis reactions (~99 %) but was significantly reduced in both SK_{ALAB49} ($P < 0.05$) and $SK_{NS88.2}$ ($P < 0.01$) mediated lysis reactions (~40% and ~19% respectively)

(Figure 4.6B). Taken together, these data indicate the plasmin activity generated by type-2b SK variants is restricted to the surface of the fibrin clot.

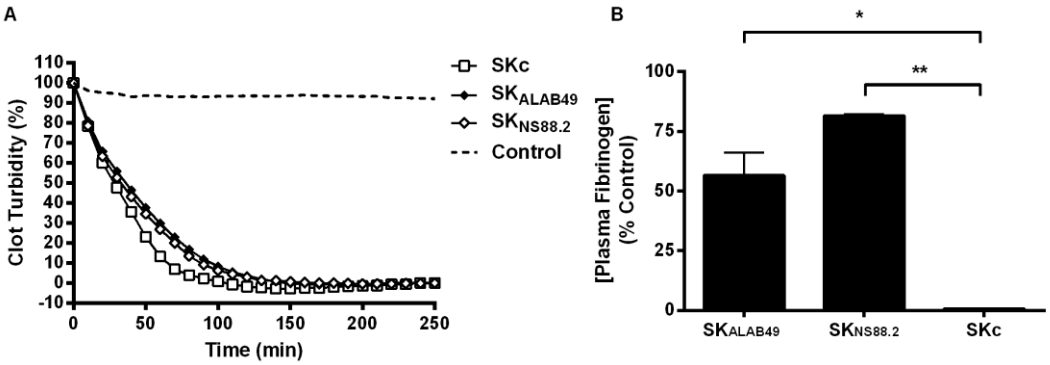


Figure 4.6: Induction of plasma clot lysis by SK variants and capacity to deplete plasma Fg (A) Fibrin clots were formed in pooled EDTA treated human plasma by the addition of thrombin (0.8U/mL) and $CaCl_2$ (20 mM). Variant SK molecules were then suspended in fresh plasma to a final concentration of 50 nM over the plasma clot and lysis was monitored by measuring a reduction in turbidity at 340 nm at 37°C (**B**) Group C SK or type-2b SK variants (each at a concentration of 5 or 50 nM) were added to plasma for 30 min at 37°C. Plasmin activity was inhibited by the addition of aprotinin (20 µM) and plasma fibrinogen concentration was measured by the sodium sulphite precipitation method. Results are standardised to control reactions, which lacked SK. Data represent three independent experiments in duplicate. Data represent mean +/- SEM. Asterisks indicate statistical significance as determined by one-way ANOVA, $P < 0.05$ (*) and $P < 0.01$ (**).

4.5 Discussion

Plasmin is a potent protease with many important physiological functions including fibrin clot dissolution, tissue repair/remodelling and cell migration (Plow *et al.*, 1995a). Regulation of the Plg activation system is essential for the maintenance of homeostatic function and the conversion of Plg into plasmin (the critical first step involved in this process) is a major regulatory target (Castellino and Ploplis, 2005). The conformation of the Glu-Plg molecule greatly influences the regulation of Plg activation. Glu-Plg conformation may be altered upon interaction with ligands, primarily through lysine binding sites located within the kringle domains, and it is now well recognised that these interactions play a central role in modulating key steps in Plg activation by host and bacterial Plg activators (Castellino and Ploplis, 2005, Gladysheva *et al.*, 2007, Rijken and Lijnen, 2009).

Fg is known to enhance activation of human Plg by uPA, tPA and SK, however the exact mechanism for this is not defined (Chibber *et al.*, 1985, Cesarman-Maus and Hajjar, 2005). While full length Fg is relatively inert in circulation, fibrin(ogen) fragments that enhance Plg activation can be generated through numerous physiological processes; Fg proteolysis by thrombin during fibrin deposition, fibrinolysis and direct Fg proteolysis by SK-Plg activator complexes at the site of bacterial infection (Yakovlev *et al.*, 2000, Varadi and Patthy, 1983, Chibber *et al.*, 1985, Medved and Nieuwenhuizen, 2003). To differentiate the effect of full length Fg and Fg fragments on Glu-Plg activation by SK variants, the plasmin inhibiting, flurogenic active site titrant (MUGB) was used to measure SK-Plg* activator complex formation. Our results

indicated that full length Fg and FgD accelerated the formation of SK-Plg* for all SK variants and also permitted type-2b SK to generate an active site in Glu-Plg (Figure 4.1B and C and Figure 4.4B). While Glu-Plg and Fg molecules are normally relatively inert in circulation, the interaction between SK, Glu-Plg and Fg must therefore permit a series of conformational modifications that expose binding sites that are not present in native Glu-Plg and Fg molecules. This supports the previously proposed hypothesis that SK, Plg and Fg can interact to form a tri-molecular complex (McArthur *et al.*, 2012, Takada and Takada, 1989, Wang *et al.*, 1995b).

Bacterial Plg receptors interact with plasmin(ogen) to acquire protease activity at the cell surface (Lahteenmaki *et al.*, 2001). However, the influence of bacterial ligands on Glu-Plg conformation and the subsequent effect on activation kinetics by SK variants has not been assessed. PAM binds to Glu-Plg via interaction with the Plg K2 but this bacterial receptor is restricted to skin tropic, *emm* pattern D GAS strains (Svensson *et al.*, 1999). SEN and GAPDH are present in all GAS isolates and display weaker Glu-Plg affinity binding to K1 and K5 through lysine-dependent interactions (Sanderson-Smith *et al.*, 2012). SEN and GAPDH do not affect the generation of the SK-Plg* activator complex for any of the SK variants (Figure 4.2A and B). PAM significantly enhanced active site generation in Glu-Plg by cluster 2 SK variants and more importantly allowed the non-proteolytic generation of an active site in Glu-Plg by type-2b SK (Figure 4.2C and Figure 4.4C). This suggests that differences in the conformational state adopted by Glu-Plg upon interaction with different ligands effects the potential of these ligands to enhance active site generation by SK. Therefore it is possible that some bacterial ligands may play a role in Plg activation while others may only function to bind Plg or plasmin.

Interestingly, Plg/plasmin bound to the bacterial cell surface has also been shown to function as a bridging molecule facilitating bacterial adherence and internalisation of bacteria into keratinocytes/epithelial cells (Siemens *et al.*, 2011, Pancholi *et al.*, 2003). As this process occurs independently of protease activity, Plg receptors with no role in Plg activation may be useful for such purposes.

While the conformation of Glu-Plg plays an important role in the regulation of plasmin generation, the primary regulator of plasmin activity is α_2-AP. The inhibitor α_2-AP binds to plasmin through an initial interaction with the lysine binding sites in the kringle domains which is then followed by a slower irreversible covalent modification of the active centre (Cederholm-Williams, 1979, Gerber *et al.*, 2010). SKc induces an active site in Glu-Plg by the insertion of Ile_1 of SKc into the Glu-Plg amino-terminal binding cleft, which forms a crucial salt linkage with Asp_{740}, in place of Val_{562} of Plg (Wang et al., 1999). SKc-Plg* activator complexes are resistant to α_2-AP inhibition which is thought to be the result of SKc occupying or blocking critical lysine binding sites in Plg and/or through steric shielding of the active site (Cederholm-Williams, 1979). The inability of type-2b SK to induce an active site in Glu-Plg and the fact that type-2b SK-plasmin activator complexes are inhibited by α_2-AP may suggest these variants fail to block α_2-AP binding to specific lysine binding sites in Plg or they are unable to correctly position the N-terminal region of the type-2b SK molecule within Plg (Cook *et al.*, 2012). While type-2b SK-Plg* activator complexes formed in the presence of either Fg or PAM separately can be inhibited by α_2-AP, in the combined presence of PAM and Fg, activator complexes were resistant to α_2-AP inhibition (Figure 4.4D). This complex of multiple ligands may shield the active site by restricting access for α_2-AP and/or may

occupy the lysine binding sites of Plg that are required for initial α_2-AP binding, functions that cannot be imparted by the presence of each ligand alone. As such, type-2b SK variants require additional ligands to assist with the conformational rearrangement of Glu-Plg to induce a proteolytically active site and further additional ligand combinations are required to protect the newly formed active site from inhibition by α_2-AP. This work provides the first comprehensive demonstration of the functional relationship between GAS SK variants, bacterial and host Plg binding ligands and α_2-AP.

It has long been hypothesised that SK-mediated Plg activation, and subsequent recruitment of plasmin activity to the GAS cells surface, acts to prevent plasmin inhibition by α2-AP. By characterising this delicate interplay between bacterial derived SK variants and cell surface receptors such as SEN, GAPDH, M proteins (PAM and Prp) and host proteins such as Fg, fibrin and α_2-AP, we have been able to define a possible mechanism for how these phenotypic variations influence the pathogenesis GAS strains (see Figure 4.7). While type-1 and type-2a SK variants display Plg activation/inhibition mechanisms that are similar to SKc, GAS strains expressing type-2b SK have evolved a distinct and unique system for the interaction with the human Plg activation system that limits soluble plasmin generation. Unlike other SK variants, type-2b SK proteins cannot conformationally induce an active site in Glu-Plg when it has a closed conformation. Type-2b SK can form an activator complex with plasmin and with Plg-PAM or Plg-Fg complexes, however these complexes are readily inhibited by α_2-AP (Figure 4.7A, B, C and D). Type-2b SK will form an activator complex that is

resistant to α_2-AP inhibition when Glu-Plg is bound by a combination of both PAM and Fg or when Glu-Plg is bound to fibrin (Figure 4.7E and F). As a result, Plg activation by

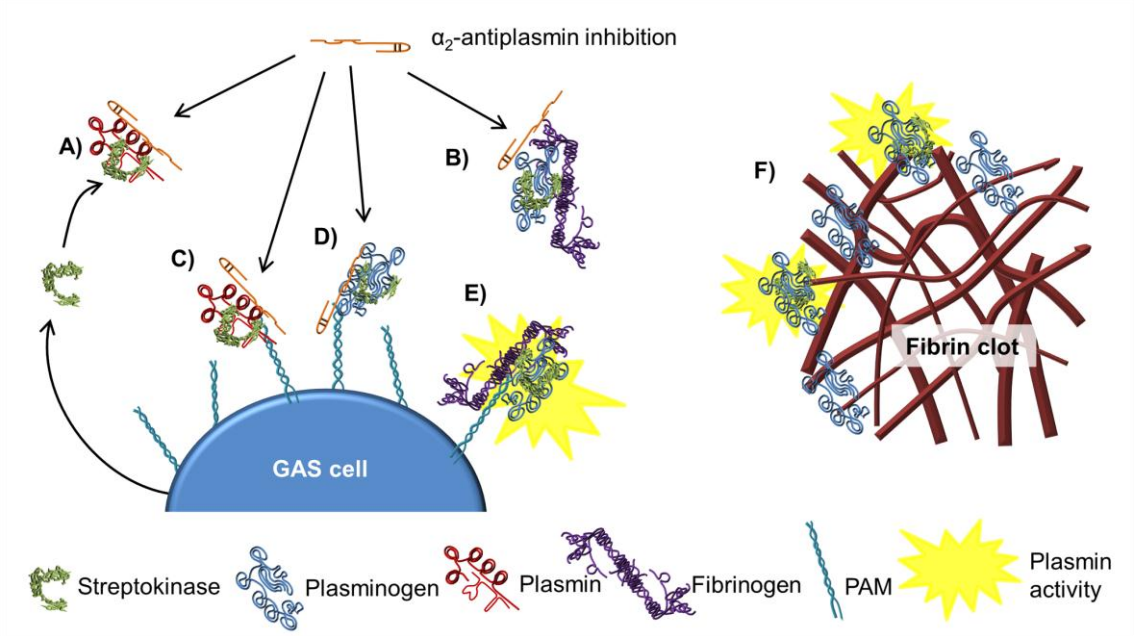


Figure 7. Schematic diagram summarising the site/surface restricted activation of plasminogen by type-2b SK expressing GAS strains. Cluster type-2b SK cannot induce an active site in Glu-Plg. Free complexes of cluster type-2b SK with plasmin (**A**) or Plg/plasmin-Fg (**B**) are inhibited by α_2-antiplasmin. Similarly complexes of cluster type-2b SK with Plg/plasmin with PAM are also inhibited by α_2-antiplasmin (**C** and **D**). Cluster type-2b SK can only generate unregulated plasmin activity when a tri-molecular complex of SK-Plg-fibrinogen is bound to the cell surface PAM (**E**) or Plg is bound to fibrin (**F**). Therefore, plasmin activity generated by cluster type-2b SK in restricted to the bacterial cell surface or to fibrin thrombi.

type-2b SK will be limited by the availability of cofactors and will restrict plasmin activity to specific microenvironments such as the bacterial cell surface and to the fibrin clot surface. This Plg activation mechanism displayed by type-2b SK has similarities to the Plg activation activity of staphylokinase produced by *Staphylococcus aureus*. Both activators fail to generate soluble plasmin activity, as fluid-phase activator complexes

are readily inhibited by α_2-AP. *S. aureus* is a ubiquitous skin coloniser. Similarly, epidemiological studies have shown the type-2b *ska* linage to be largely restricted to *pam* positive, *emm* pattern D GAS strains which have a strong tendency to cause skin infections (Kalia and Bessen, 2004). Therefore, these characteristics could limit systemic bacterial dissemination as there would be low activation of metalloproteases and less degradation of extracellular matrix components (Lu *et al.*, 2011). Plasmin activity that is restricted to the cell surface could promote bacterial persistence in the skin by facilitating evasion of innate immune defences, producing delayed wound healing and allowing transmission to new hosts (Bessen and Tengra, 2010).

Bacterial activators with "fibrin specific" Plg activation activity such as staphylokinase and a truncated form of SKc (SKΔ59) have been proposed as potential thrombolytic therapeutics with improved fibrinolytic activity (Reed *et al.*, 1999, Collen and Lijnen, 1994). The fibrin selectivity displayed by these activators results from 1) a requirement of plasmin to generate a functional Plg activation complex and 2) free activator complexes being inhibited by α_2-AP, thereby restricting plasmin activity to the fibrin clot environment. Similar to these activators, soluble type-2b SK activator complexes are inhibited by α_2-AP. However, unlike staphylokinase and SKΔ59, type-2b SK is not reliant on trace plasmin present in low quantities at the clot surface to produce an activator complex. Type-2b SK activators are capable of activating fibrin bound Glu-Plg which is present in much higher quantities when compared to free plasmin. As the fibrinolytic potency of type-2b SK variants was comparable to SKc (Figure 4.6A) but with significantly reduced Fg depletion (Figure 4.6B), these variants could be useful for thrombolytic therapeutic applications.

Chapter 5:

Elucidating the Molecular Mechanism of Streptokinase Function through the Construction of Chimeric Proteins from *Streptococcus pyogenes* Streptokinase Variants

Preface

Since the discovery of streptococcal fibrinolysis in the 1930s and subsequent therapeutic potential of SK in the mid-1950s, a vast amount of research has focused on elucidating the mechanism of SK activity. However, a definitive description of the molecular mechanism of SK mediated Plg activation is still yet to be determined. This is partially due to the lack of crystal structure data combined with the subtle complexities involved in functionally characterising the activation of Glu-Plg by SK using biochemical techniques. A recent shift in focus to examining the role of SK polymorphism in GAS, has discovered that a myriad of phenotypic differences are displayed by these SK variants and generated new approaches to pinpoint specific amino acid residues involved in the mechanism of SK activity. The purpose of this chapter was to highlight how chimeric versions of GAS SK variants with known phenotype could be used a tool to determine the specific domains regions responsible for the molecular basis of SK phenotypic differences and applied to the SK mechanism in its entirety.

***Publication*: Cook, S. M**., Walker, M. J. and McArthur, J. D., (2014) "Elucidating the molecular mechanism of streptokinase function through the construction chimeric proteins from *Streptococcus pyogenes* streptokinase variants" (*Manuscript prepared*)

5.1 Abstract

Streptokinase (SK) is a bacterial plasminogen activator, secreted by β-hemolytic group A, C and G strains of the genus *Streptococcus*. Among group A streptococcal isolates, SK gene sequences (*ska*) are polymorphic and can be grouped into two distinct sequence clusters (termed cluster type-1 and cluster type-2) with cluster type-2 being further divided into sub-clusters type-2a and type-2b. GAS SK variants display distinct phenotypic differences. Type-1 SK and type-2a SK variants generally display similarities to the elucidated mechanism for SKc, while type-2b SK display a 29-35 fold reduction in affinity for Glu-Plg, cannot activate Glu-Plg via conformational rearrangement, are inhibited by α_2-antiplasmin and require the presence of additional ligands to generate activity. With the discovery of these differences it has now become possible to utilise this information to identify regions of the molecule that are critical for Plg activation function. To determine how specific SK domains contribute to phenotypic differences, hybrid *ska* genes were constructed in which the α, β and γ domain encoding regions of *ska* were exchanged between type-1 (ska_{NZ131}) and type-2b (ska_{ALAB49}) alleles. In this study, far-UV circular dichroism spectroscopy indicated that wild-type SK variants (SK_{ALAB49}, SK_{NZ131} and SKc) and SK chimeras ($SK_{\beta T1}$, $SK_{\beta T2b}$, $SK_{\gamma T1}$ and $SK_{\gamma T2b}$) have similar secondary structures. While, α domain swap chimeras ($SK_{\alpha T1}$ and $SK_{\alpha T2b}$) displayed a significant increase in α-helicity when compared to wild-type SK proteins. Preliminary characterisation of the role of the SK α domain using the chimeric mutant containing the α domain from the active SK_{NZ131} molecule combined with β and γ domains of SK_{ALAB49} was conducted. This chimera ($SK_{\alpha T1}$) was able to generate an active site in Glu-Plg and form an activator complex that could

activate substrate Glu-Plg, albeit at a reduced rate compared to the parental type-1 SK variant (9.4 fold slower rate of active site generation). Taken together these data indicate that amino acid residues within the α domain affect the ability of SK variants to generate an active site in Glu-Plg and also influence the overall structure of the protein. Using variant SK proteins that display known phenotypic differences will allow the identification of regions that are critical for function. This information will produce a greater understanding of the molecular mechanism of SK mediated Plg activation.

5.2 Introduction

SK from the group C streptococcal isolate H46A is the most frequently characterised SK. To date, the majority of expression, purification, structural and functional characterisation studies have been conducted on SK from group C streptococcus due to its extensive use as a thrombolytic therapeutic (McArthur *et al.*, 2012). Group A streptococcus (GAS; *Streptococcus pyogenes*) secrete SK which generates soluble plasmin activity and facilitates the acquisition of protease activity to the bacterial cell surface (McArthur *et al.*, 2012). While SK from group C streptococcal isolates display minimal genetic diversity, SK from human specific GAS isolates display considerable genetic variability (Kalia and Bessen, 2004, McArthur *et al.*, 2008, Kapur *et al.*, 1995, Zhang *et al.*, 2012, Cook *et al.*, 2012).

Genetic variability in GAS confers distinct phenotypic differences between SK variants. Type-1 and type-2a GAS SK variants generally display phenotypic similarity to the therapeutic group C SK variant; these include a high affinity for native Glu-Plg, an ability to form a functional activator complex with Glu-Plg independent of additional cofactor ligands, and resistance to host inhibitory mechanisms such as α_2-antiplasmin . Conversely, type-2b SK variants display a 29-35 fold reduction in affinity for Glu-Plg, cannot activate Glu-Plg via conformational rearrangement, are not resistant to inhibition by α_2-antiplasmin and require the presence of additional ligands such as fibrinogen (Fg) or Plg-binding group A streptococcal M protein (PAM) to overcome host regulation mechanisms and generate proteolytic activity. These phenotypic differences displayed by GAS type-1 and type-2 SK variants have also been shown to affect GAS

pathogenesis (McArthur *et al.*, 2008, Cook *et al.*, 2012, Zhang *et al.*, 2012, Russell and Facklam, 1975).

Nevertheless, further research on the specific structural and biochemical alterations responsible for these phenotypic changes is required to increase our understanding of the molecular basis for SK-mediated Glu-Plg activation. SK variants with known phenotypic differences can be used to elucidate amino acid regions that are important for function through the generation of chimeric SK proteins. In this study, α, β and γ domains were exchanged between type-1 (SK_{NZ131}) and type-2b (SK_{ALAB49}) SK variants producing all six possible chimeric combinations. Structural and biochemical characterisation of these chimeric proteins has revealed a role for inter-domain interactions in co-ordinating native SK structure and highlights the α domain as a critical region involved in the non-proteolytic generation of an active site in Glu-Plg.

5.3 Materials and Methods

5.3.1 Bacterial strain, culture conditions and reagents

GAS isolates NZ131 (The New Zealand Reference Culture Collection, Institute of Environmental Sciences and Research), ALAB49 (Svensson *et al.*, 2000) and *S. dysgalactiae* subsp. *equisimilis* strain H46A (Christensen, 1945) were used in this study. Streptococcal strains were routinely cultured at 37°C on horse-blood agar (Biomerieux, Sydney, NSW, Australia) or in static liquid cultures of Todd-Hewitt broth (BD, Sydney, NSW, Australia) supplemented with 1% (w/v) yeast extract (Oxoid, Adelaide, SA, Australia) (THY medium). *E. coli* strains JM109 and M15[pREP4], were used as hosts for plasmid construction and protein expression respectively and were cultured at 37°C in Luria-Bertani broth. Where appropriate, antibiotics were used for selection at the following concentrations: chloramphenicol, 100 $\mu g.mL^{-1}$; kanamycin, 50 $\mu g.mL^{-1}$; and ampicillin, 100 $\mu g.mL^{-1}$. Glu-Plg, plasmin, and α_2-AP were purchased from Haematologic Technologies, Essex Junction, VT, USA. The chromogenic substrate H-D-Val-Leu-Lys-pNA·2HCl (S-2251) was obtained from Chromogenix, Mölndal, Sweden and 4-methylumbelliferyl p-guanidinobenzoate (MUGB) was from Marker Gene Technologies, Inc., Eugene, OR, USA.

5.3.2 Cloning strategy for construction of chrimeic *ska* allele

To fragment ska_{ALAB49} and ska_{NZ131} and construct *ska* domain swap chimeras, wild-type *ska* alleles were removed from the expression vector pQE-30 by restriction digestion with *Bam*HI and *Pst*I and sub-cloned into pHSGXceIS1 which is a modified pHSG398

generated in our laboratory produced by deleting a 250 bp fragment, which removed the *Nsp*I restriction site from the plasmid backbone (Appendix 7). Once sub-cloned into pHSGXceIS1, the region encoding domain could be removed from full length *ska* via various combinations of restriction enzymes (Figure 5.1). DNA fragments were separated by 0.85% agarose gel electrophoresis and gel purified using Wizard® SV Gel and PCR Clean-up System (Promega, USA) according to manufacturer specifications. Purified domain fragments were cloned into the corresponding purified DNA fragment containing the two residual domains of the alternate gene in pHSGXceIS1 construction vector (Appendix 7). Once each chimeric *ska* had been constructed, the full length chimeric gene was sub-cloned into pQE-30 for DNA sequencing analysis and subsequent protein expression

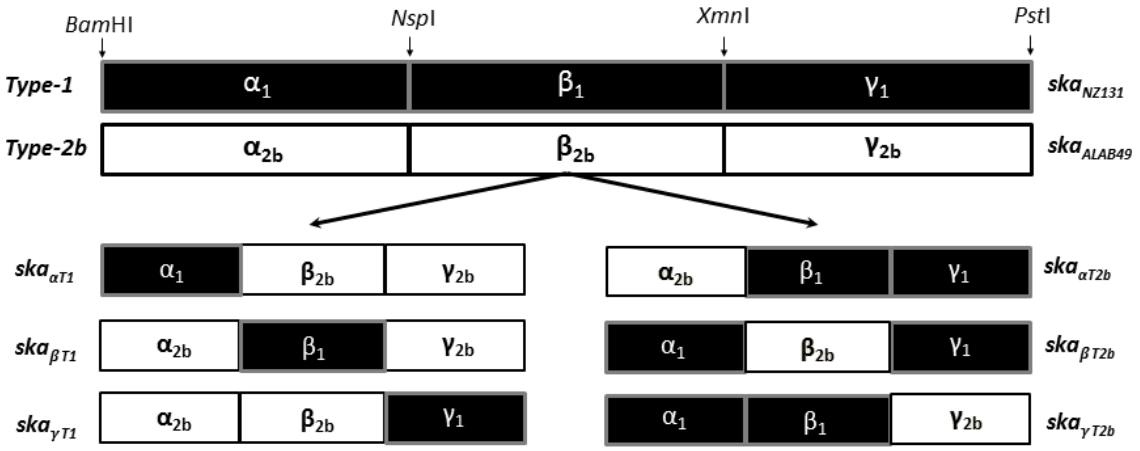


Figure 5.1: Strategy for fragmentation and construction of domain swap chimeras between type-1 and type-2b variants of *ska*. The restriction sites labelled in this schematic were used to release domains separately and clone each into the opposing *ska* allele contained in pHSGXceIS1 for the construction of chimeras. After construction chimeric *ska* alleles were sub-cloned into pQE-30 for recombinant protein expression.

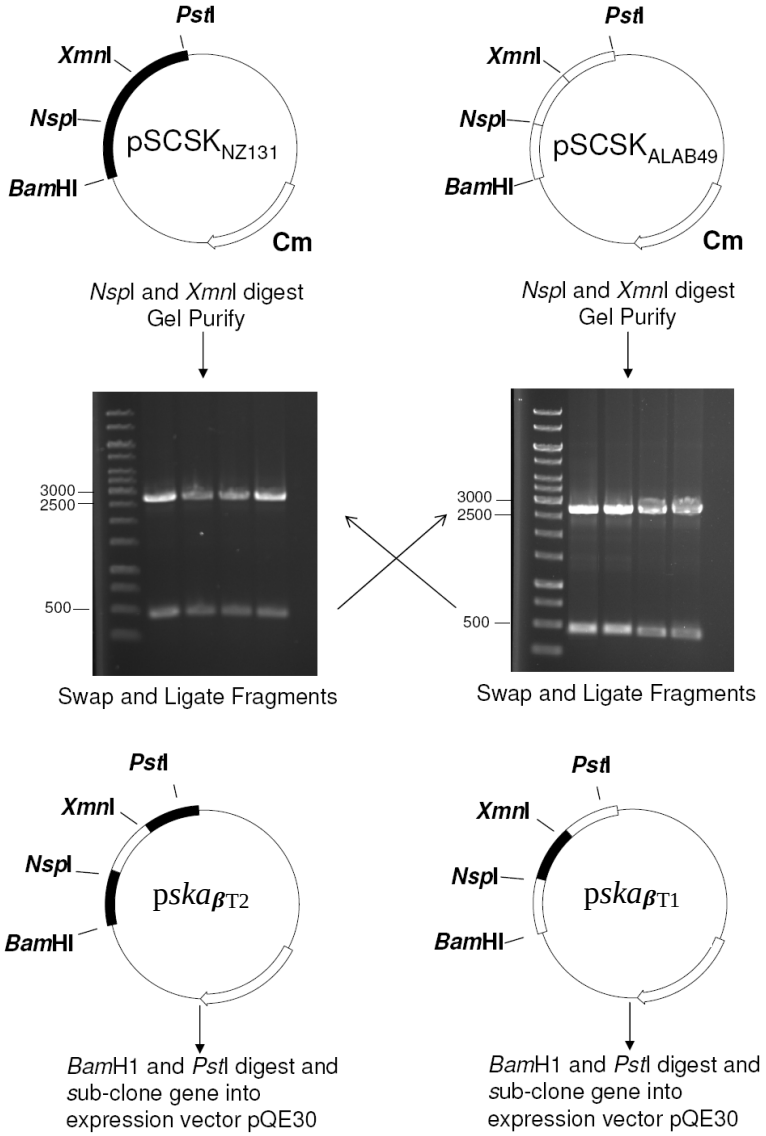


Figure 5.2: Schematic example of the cloning strategy used to create chimeric SK proteins. In this example the *ska* β domain fragment is released from pSC$_{SKNZ131}$ and pSC$_{SKALAB49}$ via restriction digestion with *Nsp*I and *Xmn*I. This digest produces two DNA fragments; an ~2788 bp fragment containing the plasmid vector and the α and γ encoding regions of *ska* and a ~432 bp fragment which encodes the *ska* β domain. As indicated by the arrows, the fragment encoding the β domain is ligated into the plasmid containing the α and γ domains of the other allele. This strategy was employed to construct all six chimeric conformations of type-1 and type-2b *ska*. This schematic outlines the construction of the β domain chimeras. All other chimeras and relating figures are shown in an additional publication (Cook, 2008).

5.3.3 Expression and purification of recombinant SK protein

SK chimeric variants were cloned, expressed and purified as previously described (Cook, 2008, Cook *et al.*, 2012) $SK_{\alpha T1}$, $SK_{\beta T2b}$, $SK_{\gamma T1}$ and $SK_{\gamma T2b}$ were expressed and purified under native conditions while $SK_{\alpha T2b}$ and $SK_{\beta T1}$ were insoluble and thus expressed and purified under denaturing conditions (Appendix 2.4) (Cook, 2008). SK variants further utilised in biochemical and functional analysis underwent secondary purification, as outlined in Chapter 2. See Appendix 2.1 for an example purification of chimera $SK_{\alpha T1.}$

5.3.4 Far-UV circular dichroism (CD) spectroscopy

CD spectroscopy was performed using a J-810 spectropolarimeter (Jasco, Tokyo, Japan) at room temperature. Samples were prepared in 10 mM phosphate buffer (pH 7.4) to a final concentration of 100-300 $\mu g.mL^{-1}$. Spectra representing the average of six scans were collected from 190 nm to 250 nm at 1 nm intervals, with a path length of 1 mm. Molar residue ellipticity (Θ) was calculated using the following formula: $[\Theta] = \Theta$ x 100 x molecular weight (kDa)/concentration ($mg.mL^{-1}$) x path length x number of amino acids.

5.3.5 Non-proteolytic active site generation in Glu-Plg

Non-proteolytic active site generation in Glu-Plg by SK variants was examined using the fluorescent active site MUGB in a POLARstar Omega fluorescence spectrophotometer (BMG LABTECH, Ortenberg, HE, Germany). Glu-Plg (200 nM) was added to a black 96-well microtitre plate containing 1 μM MUGB in assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) at 37°C. To initiate the reaction, SK was

added to a final concentration of 400 nM in a total volume of 100 µL and the development of fluorescence was monitored continuously with excitation at 355 nm and emission at 460 nm. Data was normalised by subtracting a control reaction of all protein species without the addition of SK and 1 µM MUGB. This accounted for intrinsic fluorescence associated with buffer and protein species, as well as non-specific hydrolysis of MUGB over the course of reactions. To calibrate relative fluorescence units and convert these measurements to [SK-Plg*], standard curves of known concentrations of the fluorescent product 4-methylumbelliferone were constructed. The fluorescence measurements were made in the assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) at 37°C containing 400 nM SK and 200 nM Glu-Plg, with the same instrument settings as MUGB experiments.

5.3.6 Plasminogen activation by variant SK-plasmin complexes

The capacity of variant SK-plasmin complexes to activate substrate Plg was assessed. SK was pre-formed with plasmin at 37 °C for 5min. SK-Plasmin complexes (5 nM final concentration) were diluted in assay buffer (10 mM HEPES, 150 mM NaCl, 0.01% Tween-20, pH 7.4) containing a large excess of Glu-Plg (500 nM) and S-2251 (500 µM) in a total volume of 100 µL. The exponential generation of plasmin was monitored by change in absorbance at 405 nm and measured over 35 min at 37°C using a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

5.4 Results

To characterise the phenotypic differences displayed by SK proteins from different GAS isolates, chimeric variants encompassing the six possible domain conformations of a cluster type-1 *ska* (ska_{NZ131}) combined with a cluster type-2b *ska* (ska_{ALAB49}) were constructed and expressed as recombinant proteins. Recombinant chimeric SK proteins

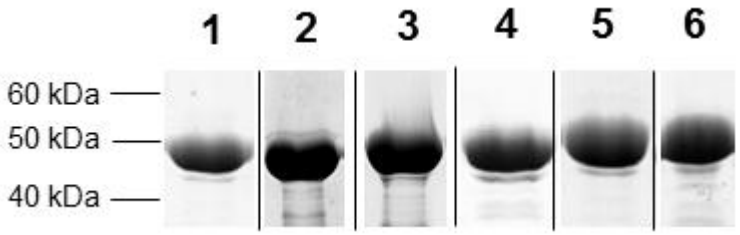


Figure 5.3: SDS-PAGE analysis composite of expressed recombinant chimeric SK proteins used in this study. $SK_{\alpha T1}$, $SK_{\beta T2b}$, $SK_{\gamma T1}$ and $SK_{\gamma T2b}$ were expressed and purified under native conditions while $SK_{\alpha T2b}$ and $SK_{\beta T1}$ were insoluble and thus expressed and purified under denaturing conditions (**A**) Variant $SK_{\alpha T1}$ (lane 1), $SK_{\alpha T2b}$ (lane 2), $SK_{\beta T1}$ (lane 3), $SK_{\beta T2b}$ (lane 4), $SK_{\gamma T1}$ (lane 5) and $SK_{\gamma T2b}$ (lane 6) were separated by 10% SDS-PAGE under reducing conditions. Molecular weight standards in kDa are indicated.

were analysed for size and purity by SDS-PAGE (Figure. 5.3). Proteins ranged in size from 44 to 49 kDa, which is similar to the sizes observed for native SK proteins present in GAS culture supernatants and recombinant wild-type proteins (McArthur *et al.*, 2008, Cook *et al.*, 2012). However, it was observed during the expression and purification of these chimeric proteins under native conditions, that $SK_{\alpha T2b}$ and $SK_{\beta T1}$ were insoluble. Consequently, these proteins were effectively re-purified under denaturing conditions, however all attempts to re-fold these proteins were unsuccessful (data not shown). Due

to time constraints further secondary structural analysis was undertaken on the limited soluble portion of these proteins from these re-folding attempts and further biochemical analyses were limited to the type-1 SK α domain containing chimera $SK_{\alpha T1.}$ For use as positive controls SK from the group C streptococcal isolate H46A (SKc) and wild-type parental GAS variants (SK_{NZ131} and SK_{ALAB49}) were also expressed and purified as shown previously in Chapter 2.

5.4.1 Determination of secondary structure by far-UV circular dichroism (CD) spectroscopy

Far-UV circular dichroism spectroscopy was utilised to compare the secondary structural elements of wild type SK cluster type-1 (SK_{NZ131}), cluster type-2b (SK_{ALAB49}) and group C (SKc), as well as the domain swap chimeras. Despite significant differences in amino acid composition, wild type and chimeric $SK_{\beta T1}$, $SK_{\beta T2b}$, $SK_{\gamma T1}$ and $SK_{\gamma T2b}$ variants had similar molar residue ellipticity spectra across the full range of wavelengths measured (Figure 5.4) (Cook *et al.*, 2012). The output spectra observed was typical of that expected for an amalgamation of α helical (minima at ~ 210 nm maxima at ~190 nm) and anti-parallel β sheet (minima at ~215 nm and maxima at 195 nm) spectra indicative of the known structure of SK (Wang *et al.*, 1998). However, when the type-1 (SK_{NZ131}) α domain was incorporated with the β and γ domains of cluster type-2b (SK_{ALAB49}) or the type-2 (SK_{ALAB49}) α domain was incorporated with the β and γ domains of cluster type-1 (SK_{NZ131}) these SK chimeras ($SK_{\alpha T1}$ and $SK_{\alpha T2b}$) showed an increase in α-helicity when compared to the parental SK protein (Figure 2.5). These data suggest that inter-domain interactions involving the α domain influence the structure of the SK molecule.

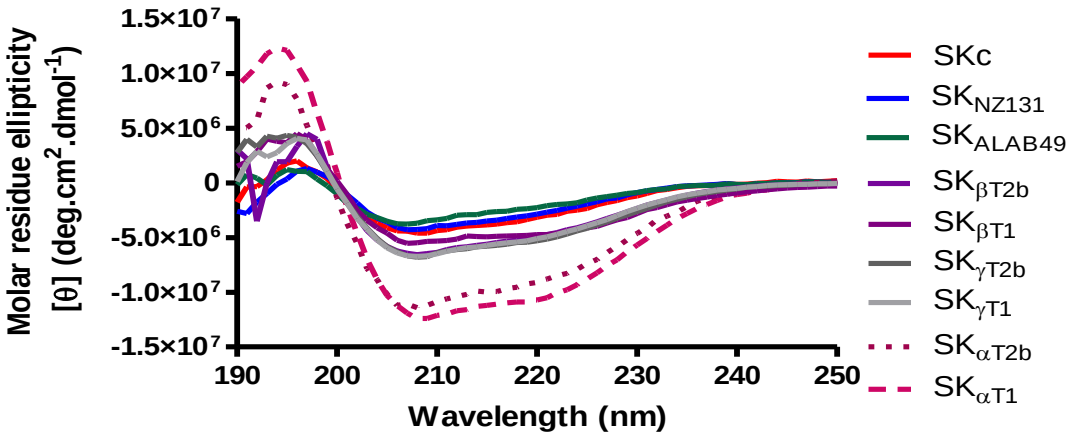


Figure 5.4: Far-UV circular dichroism spectroscopy analysis of recombinant SK proteins used in this study. Wild-type SK variants (SK_{ALAB49}, SK_{NZ131} and SKc) and SK chimeras ($SK_{\beta T1}$, $SK_{\beta T2b}$, $SK_{\gamma T1}$ and $SK_{\gamma T2b}$) have similar molar residue ellipticity values and CD out-put spectrum structure across the full range of wavelengths indicating no differences in secondary structure. While, α domain swap chimera ($SK_{\alpha T1}$ and $SK_{\alpha T2b}$) displayed structural changes with a significant increase in α-helicity when compared to wild-type SK proteins

5.4.2 Non-proteolytic active site generation in Glu-Plg by variant SK

Active site generation (SK-Plg* formation) in Glu-Plg by SK variants and chimeras was examined using the fluorescent active site titrant 4-methylumbelliferyl p-guanidinobenzoate (MUGB). This allowed generation of SK-Plg* to be measured through Conformational activation (Pathway I), independent of direct proteolytic activation encountered in the presence of plasmin (Pathway II) (Figure 1.5 and Figure 1.6)(Boxrud *et al.*, 2004). SK_{NZ131} (type-1 variant) generated the greatest number of active sites at the fastest rate (~5 min) followed by SKc (group C) (Figure 5.4). As expected, SK_{ALAB49} (type-2b variant) could not non-proteolytically generate an active site in Glu-Plg (Figure 5.4) (Cook *et al.*, 2012). These data indicate that type-2b SK

variants rely on the presence of plasmin to generate activity *via* Pathway II (Cook *et al.*, 2012). Interestingly when the α domain from SK_{NZ131} was incorporated with the β and γ domains of SK_{ALAB49} to produce $SK_{\alpha T1}$, the chimera was able to generate an active site in Glu-Plg through non-proteolytic mechanisms (Figure 5.4). This suggests that specific residues within the α domain of SK are required for the generation an active site in Glu-Plg via non-proteolytic mechanisms (Pathway I).

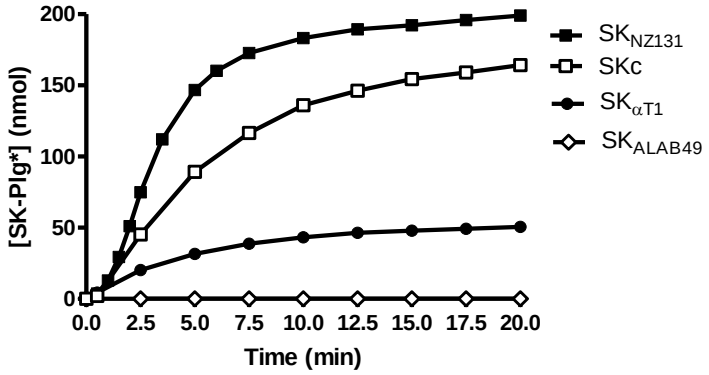


Figure 5.5: Non-proteolytic active site generation in Glu-Plg by SK variants and influence of Glu-Plg conformation on SK-Plg* generation. Glu-Plg (200 nM) was added to 1 µM MUGB in assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) at 37 °C. To initiate the reaction, SK was added to a final concentration of 400 nM in a total volume of 100 µL and the development of fluorescence was monitored continuously with excitation at 355 nm and emission at 460 nm.

5.4.3 Plasminogen activation by variant SK-plasmin-plasmin activator complexes

The ability of variant SK-plasmin complexes to sequester and activate substrate Plg was determined by mixing preformed, stoichiometric SK-plasmin activator complexes with a large excess of substrate Glu-Plg and monitoring the generation of plasmin activity

using the chromogenic substrate, S-2251. All variant SK-plasmin complexes examined in this study were capable of efficient substrate Plg activation (Figure 5.5). The fastest rates of substrate Plg activation activity were observed for group C SK (SKc) and type-1 SK (SK_{NZ131}). Similar rates of Plg activation observed for type-2b SK (SK_{ALAB49}) and the SK chimera ($SK_{\alpha T1}$) indicate that the chimeric protein is able to form an activator complex with plasmin, and that substrate Plg recognition is not influenced by the modified secondary structure of the chimera. Taken together, these data suggest substrate Plg recognition/activation is largely mediated by interactions with the β and γ domains of SK.

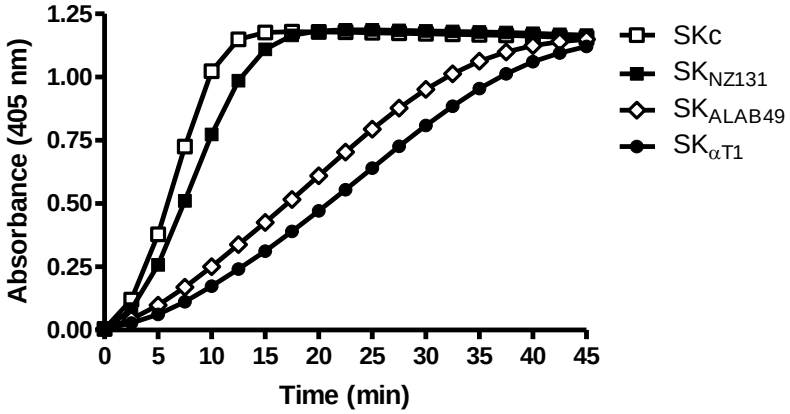


Figure 5.6: Plasminogen activation by variant SK-plasmin activator complexes. SK was pre-formed with plasmin at 37 °C for 5min. SK-Plasmin complexes were diluted to 5 nM final concentration in assay buffer (10 mM HEPES, 150 mM NaCl, 0.01% Tween-20, pH 7.4) containing Glu-Plg (500 nM) and S-2251 (500 μM). The generation of Plg activation activity was monitored at an absorbance of 405 nm at 37 °C.

5.5 Discussion

GAS strains produce SK proteins with distinct genetic and phenotypic variability (Kalia and Bessen, 2004, McArthur *et al.*, 2008, Kapur *et al.*, 1995, Zhang *et al.*, 2012, Cook *et al.*, 2012). While type 1 and type 2a SK variants from GAS possess the hallmark characteristic ability of being able to generate an active site in Glu-Plg through non-proteolytic conformational activation, recent research from our laboratory has shown that type-2b SK variants display distinct phenotypic differences (Cook *et al*., 2012). GAS SK variants with known phenotypic differences but limited amino acid sequence diversity can be used to elucidate protein regions that are important for function through the generation of chimeric SK proteins.

In this study, chimeric SK proteins were produced by exchanging α, β and γ domains between two phenotypic distinct variants, type-1 (SK_{NZ131}) and type-2b (SK_{ALAB49}). When overexpressed as recombinant proteins in *E. coli,* four chimeric variants ($SK_{\alpha T1}$, $SK_{\beta T2b}$, $SK_{\gamma T2b}$ and $SK_{\gamma T1}$) were soluble and were readily purified while $SK_{\alpha T2b}$ and $SK_{\beta T1}$ (both containing the SK_{ALAB49} α domain linked to the β of SK_{NZ131}), were insoluble and could only be purified under denaturing conditions. This suggests the arrangement of a type-2b α domain being followed by a type-1 β domain produces a structural change that affects the solubility of these chimeric proteins. The β domains of type-1 SK (SK_{NZ131}) and type-2b SK (SK_{ALAB49}) share only 63% sequence identity. Therefore it is possible that non-conserved amino acid changes (>25) in the β domain of SK_{NZ131} are preventing interactions with SK_{ALAB49} α domain that are required to

maintain the structural stability of the SK protein. However, no crystal structure data is currently available for SK in an unbound native conformation and therefore the residues involved in stabilising native domain folding, or potential inter-domain interactions cannot be confirmed.

Interestingly, these results are in contrast to those generated by Zhang *et al*., 2013 who produced similar chimeric proteins using a type-1 SK variant (SK_{NS931}) and a type-2b SK variant ($SK_{NS88.2}$). Under the conditions used in their experiments, all recombinant chimeric proteins were soluble and displayed similar secondary structure. It is therefore possible that the amino acid differences between the allelic variants used by Zhang *et al*., (2013) and those used in this study may be residues influencing the native structure of SK. Comparison of amino acid sequences for these variants shows that type-1 SK_{NS931} displays 93.7% amino acid sequence identity to SK_{NZ131} of which 17 of the 21 total amino acid sequence variations are non-conserved. Type-2b SK variants ($SK_{NS88.2}$ and SK_{ALAB49}) display a greater level of homology (97.6% amino acid sequence identity), however non-conserved changes within this amino acid variation can also be observed (8 of 9) (Figure 3.1). While there are numerous non-conserved amino acid variations between these different SK proteins, these changes do not produce detectable functional differences in these proteins (Cook *et al.*, 2012, Zhang *et al.*, 2012). Therefore, these specific residues may have a role in maintaining the structure of free SK. These data further indicate that inter-domain interactions potentially co-ordinate the overall structure of the SK molecule when not bound to Plg. This is an interesting hypothesis as the domains of SKc have been thought to be independently folded within the native molecule and additionally this supports the proposition that selection pressure

may be placing structural constraints on SK (Conejero-Lara *et al.*, 1996, Kapur *et al.*, 1995). More research is required in support of this and such studies could reveal important structural information on the free SK protein.

The α domain of SK has a critical role in SK function and is involved in non-proteolytic generation of the active site in Glu-Plg, substrate Plg activation activity, SK affinity for Plg and susceptibility to α_2-AP inhibition (Fay and Bokka, 1998, Wang *et al.*, 1999a, Rodríguez *et al.*, 1995, Young *et al.*, 1995, Sazonova *et al.*, 2004, Boxrud *et al.*, 2000, Mundada *et al.*, 2003, Sundram *et al.*, 2003, Kim *et al.*, 2002). For SK to induce an active site in Glu-Plg, the SK Ile_1 residue must be positioned within the Glu-Plg molecule so that it can form a salt bridge with Asp_{740} of Glu-Plg (Wang *et al.*, 1999a). Amino acid changes in the 1-59 region (or any other region that elicits structural modifications) may prevent the correct positioning of the N-terminal fragment within the SK-Plg complex, thereby preventing (or slowing) non-proteolytic active site formation in Glu-Plg. Confirming this, site directed mutagenesis studies conducted on these regions in SKc have revealed that mutations in the $^{\alpha}\beta_1$ strand (residues 17–26) of the α domain markedly reduces the formation of an active complex with Glu-Plg and also renders the mutant SK-Plg/plasmin complexes severely defective in activating substrate Plg (Lee *et al.*, 1997, Kim *et al.*, 2000, Mundada *et al.*, 2003, Fay and Bokka, 1998). Therefore, the type-2b specific, non-conserved amino acid differences in this region of $SK_{NS88.2}$ and SK_{ALAB49} (e.g. Ile/Thr_{20}, Asp/Asn_{30}, Lys/Gln_{31}, Lys/Asp_{32}, Phe/Ser_{34} and Asn/Lys_{36}) could be responsible for the inability of these type-2b SK proteins to induce an active site in Glu-Plg (Figure 3.1). Additional non conserved amino acid changes in these proteins (such as Gln/Arg_{45}, Pro/Ser_{71} and Arg/Gln_{86}) may

also influence the type-2b SK phenotype. Site directed mutagenesis studies in SKc have shown that amino acid changes within or near the mobile loop region (residues 45–70) impede the substrate Plg activation activity of the activator complex but do not affect the ability of the protein to form a functional activator complex with Glu-Plg. (Liu *et al.*, 2000, Kim *et al.*, 2000, Wakeham *et al.*, 2002). Furthermore, non-conserved amino acid changes between residues 113-134 (6 in total) have not been examined in SKc and could also potentially effect type-2b SK activity (Figure 3.1).

Here we examined the importance of the α domain for SK function by biochemically examining the α domain swap chimera $SK_{\alpha T1.}$ The α domains of type-1 (SK_{NZ131}) and type-2b (SK_{ALAB49}) SK display 82% sequence identity. SK_{ALAB49} contains 17 non-conserved amino acid changes within α domain when compared to SKc. While, SK_{NZ131} has the ability to non-proteolytically activate Glu-Plg and displays very limited non-conserved amino acid changes in this region (2 in total) (Figure 3.1). Interestingly, $SK_{\alpha T1}$ was able to interact with Glu-Plg and induce an active site in Glu-Plg. This demonstrates that the α domain from type-1 SK contains specific residues that are essential for non-proteolytic activation of Glu-Plg (Figure 5.4). The rate of active site generation displayed by $SK_{\alpha T1}$ was slower than that observed for wild-type SK_{NZ131} (9.4 fold) which suggests active site generation is also influenced by the co-operation of the β and γ domains during conformational rearrangement (Loy *et al.*, 2001). To determine if conformational changes in $SK_{\alpha T1}$ were affecting the ability of this variant to interact with substrate Glu-Plg, $SK_{\alpha T1}$ was complexed with plasmin, bypassing the need to conformationally modify Glu-Plg. The $SK_{\alpha T1}$-plasmin activator complex was able to sequester and activate substrate Glu-Plg at a rate similar to SK_{ALAB49}-plasmin

complexes (Figure 5.6). This showed that substrate Plg activation is not influenced by the modified secondary structure of the chimeric $SK_{\alpha T1}$ molecule and suggests type 2b-specific variation within the β and γ domains of SK may also be influencing substrate Plg acquisition and processing.

Future work completing the purification of all six chimeras used in this study will facilitate the identification of other specific functional regions within the molecule. Additionally, a similar strategy could be applied to the other GAS SK variants including the type-1 and type-2a variants. Now that the biochemical differences of GAS SK variants have been defined, chimeric proteins can also be screened for several different phenotypic variations that can help to assign function to specific domains of the protein. Additionally, the non-conserved amino acid residues within these variant domain sequences represent a finite pool of candidates for specific site directed mutagenic studies to definitively deduce the molecular mechanism of SK mediated Plg activation. The information gained from such studies could also be readily applied to research aimed at the development of improved thrombolytic therapeutics with restricted Plg activation and reduced immunogenicity.

Chapter 6:

Conclusion

As an early mammalian defence mechanism, invading bacterial pathogens are confined to sites of infection through the deposition of fibrin networks, thereby allowing a directed inflammatory response to specifically target the area of capture (Levi *et al.*, 2004). Subversion of the host Plg activation system is a well-documented pathogenic mechanism used by GAS and other bacterial pathogens to generate soluble and cell bound protease activity to circumvent capture by fibrin networks (Boyle and Lottenberg, 1997, Coleman and Benach, 1999, Castellino and Ploplis, 2005). The present study focused on the streptococcal Plg activating protein SK. Preliminary data that lead to the development of this project, demonstrated that allelic variants of SK produced by GAS displayed unique Plg activation properties (McArthur *et al.*, 2008). Given the importance of this virulence factor in GAS pathogenesis, a more comprehensive understanding of how SK variation could influence this process was required. The overall aim of this thesis was to undertake the first extensive phenotypic study of SK variants produced by GAS. Investigating the role of SK polymorphism and effect on GAS virulence has furthered our understanding of Plg activation specific pathogenic mechanisms employed by GAS.

In this study we observed that type-1 and type-2a GAS SK variants display phenotypic similarity to the therapeutic SKc; these include: a high affinity for native Glu-Plg, an ability to form a functional activator complex with Glu-Plg independent of additional cofactor ligands, and resistance of the activator complex to α_2-AP inhibition (Table 6.1). Type-2b SK variants display numerous phenotypic differences when compared to the other SK variants. Interestingly, type-2b SK variants could not induce the formation of an active site in Glu-Plg, a function that until now has been considered archetypal for

SK proteins (Boxrud *et al.*, 2000, Boxrud *et al.*, 2004). Additionally Type-2b SK variants have a 25-30 fold lower affinity for

Table 6.1: Summary of the phenotypic differences observed for the SK variants investigated in this study

Attribute	SKc	Type-1 SK	Type-2a SK	Type-2b SK
Glu-Plg activation (i.e. conformationally induce active site)	✓	✓	✓	×
High Affinity for Glu-Plg	✓	✓	✓	×
High Affinity for plasmin	✓	✓	✓	✓
Forms an activator complex with plasmin	✓	✓	✓	✓
Activator complexes are resistant to inhibition by α_2-antiplasmin	✓	✓	✓	×*
Generates active sites in Glu-Plg in plasma	✓	✓	✓	×
Can initiate fibrin clot lysis	✓	✓	✓	✓

*These complexes are resistant to inhibition when in the presence of a fibrin clot or PAM in combination with fibrinogen.

native Glu-Plg. Unregulated plasmin activity is another characteristic trait usually displayed by SK-Pln activator complexes. However, while type-2b SK variants displayed high affinity for plasmin and could form a functional type-2b SK-plasmin activator complex, this activity was inhibited by α_2-AP. Type-2b SK mediated Glu-Plg activation was influenced by the conformation of Glu-Plg. Type-2b SK variants could activate Glu-Plg when it was bound with fibrinogen, PAM, fibrinogen fragment D or fibrin. Furthermore, in contrast to type-1 and type-2a SK, active type-2b SK-Plg-ligand activator complexes were still inhibited by α_2-AP unless bound to fibrin or to the GAS cell surface via PAM in combination with fibrinogen (Table 6.1). These data suggest

that type-2b SK Plg activation may be restricted to specific micro-environments within the host such as fibrin deposits or the bacterial cell surface through the action of α_2-AP.

This study confirms the importance of SK mediated Plg activation in GAS pathogenesis and highlights a mechanism whereby variability in this important virulence factor can influence the pathogenesis of this organism. Future comparative studies that assess GAS SK sequence variation in relation to the phenotypic differences displayed by the SK variants that have been characterised in this thesis represents a novel approach to further elucidate the mechanism of SK-mediated Plg activation. Such studies will help to identify critical residues involved in SK function and could assist the development of novel therapeutics for treating GAS infections and the development of improved thrombolytics. Preliminary studies here showed that the surface restricted Plg activation displayed by type-2b SK focuses plasmin activity toward fibrin cleavage and prevents plasma fibrinogen degradation which is an unwanted side-effect associated with the current form of therapeutic SK. Additionally, on a broader scale, application of knowledge from this study to a range of other bacterial pathogens (>20 species), which use components of the Plg activation system as a virulence factor may show that indirect regulation of Plg activation may also influence the pathogenesis of other bacterial species.

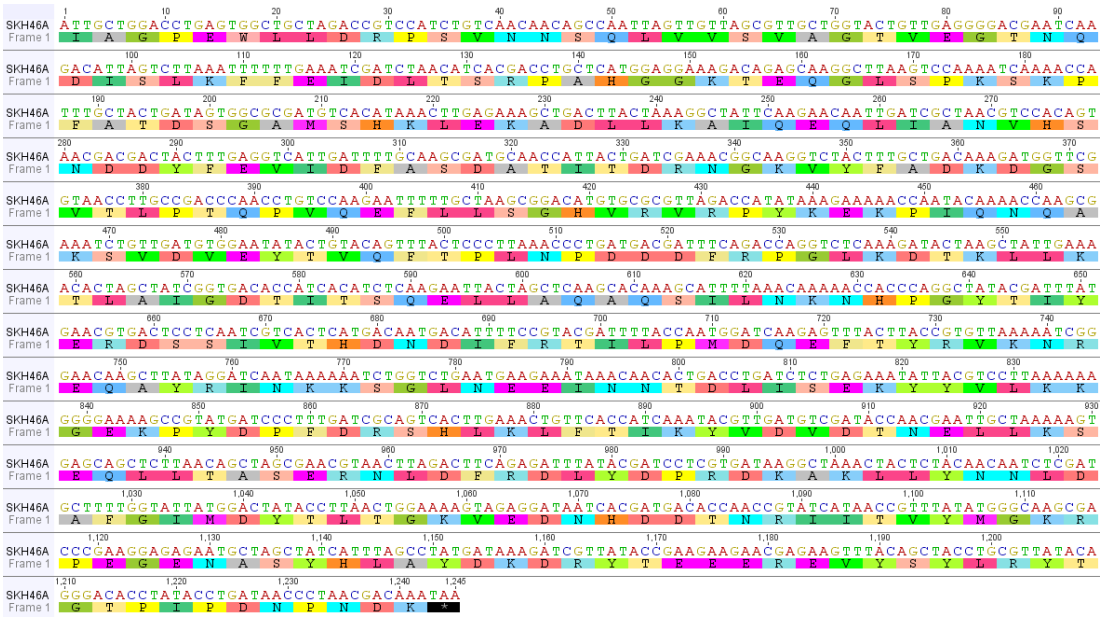
Appendices:

Preface

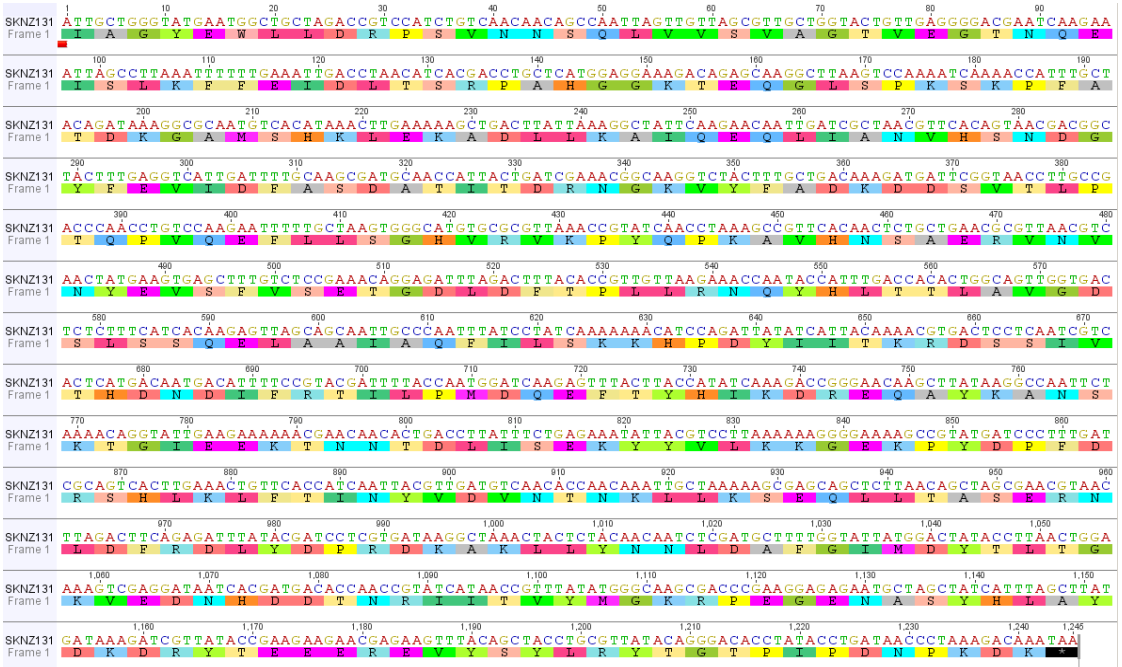
The information presented in the following appendices includes supplementary, preliminary and supporting data to experiments that appear in the research chapters of this thesis (Chapter 2, Chapter 3 and Chapter 4). While these experiments were not included in the publications that came from each body of work, they do provide some background and additional information to the experimental material that appear in the results chapters. Brief methods for these experiments are also included where required.

Appendix 1: DNA sequences of variant ska inserts in pQE-30::ska constructs

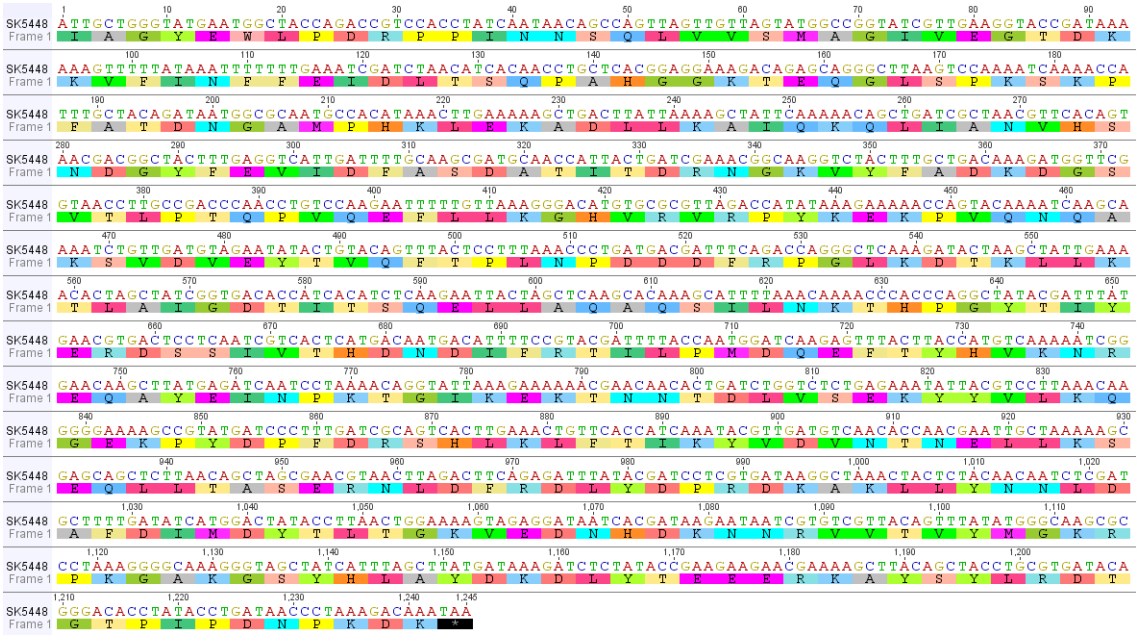
A



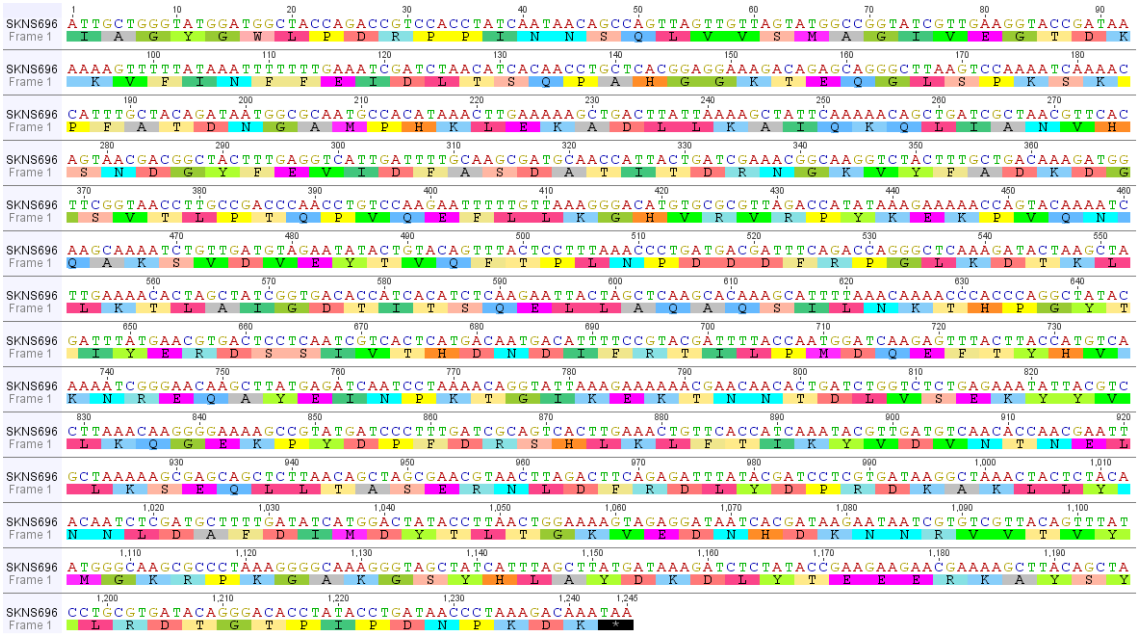
B



C



D



E

F

Figure A1.1 DNA sequence of mature streptokinase data from pQE-30::*ska* variant constructs. A) SKc **B)** SK_{NZ131}; **C)** SK_{5448}; **D)** SK_{NS696}; and **E)** $SK_{NS88.2}$**.** One letter designation of the predicted amino acid sequence is shown below the DNA sequence data. Accession numbers for these sequences are given in the material and methods of Chapter 3 (section 3.3.10)

Appendix 2: Expression and Purification of Recombinant SK Variants

A.2.1 Expression and purification of SK using Ni^{2+}-NTA affinity chromatography

The figures presented here are supplementary to the purification process outlined in Chapter 2.3.4 and Chapter 3.3.2 for expression of the SK variants used in this thesis.

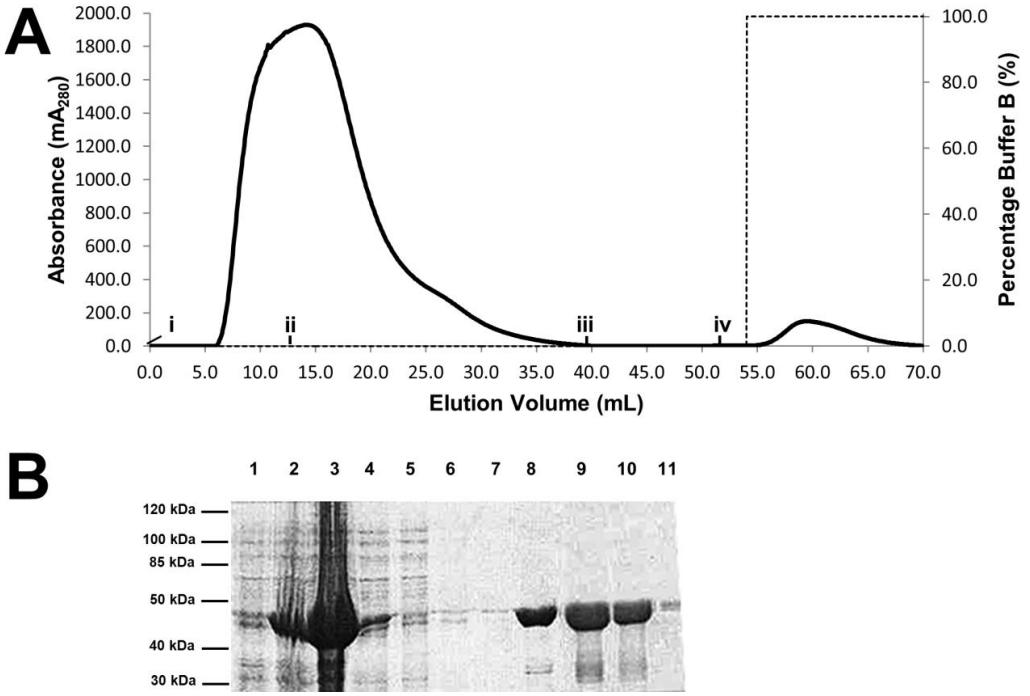


Figure A2.1.1: Expression and purification of native recombinant poly-histidine tagged fusion protein SK_{NZ131} via FPLC Ni^{2+}-NTA affinity chromatography. (**A**) Elution profile from ÄKTAprime plus FPLC showing A_{280} trace (solid line) and percentage elution buffer (dashed line) for $SK_{NZ131.}$ Point *i* represents injection of cleared lysate. Point *ii* represents injection of binding buffer. Point *iii* represents injection of wash buffer and point *iv* represents injection of elution buffer. (**B**) Coomassie stained 10% SDS-PAGE of SK_{NZ131} purification process. Un-induced *E. coli* whole cell lysate (lane 1), 2 h post induction *E. coli* whole cell lysate (lane 2) native lysis pellet containing insoluble protein (lane 3), lysate supernatant containing soluble protein (lane 4) Ni^{2+}-NTA column flow through (lane 5), wash buffer Ni^{2+}-NTA column flow through (lane 6) and Ni^{2+}-NTA column elution fractions (lane 7-11).

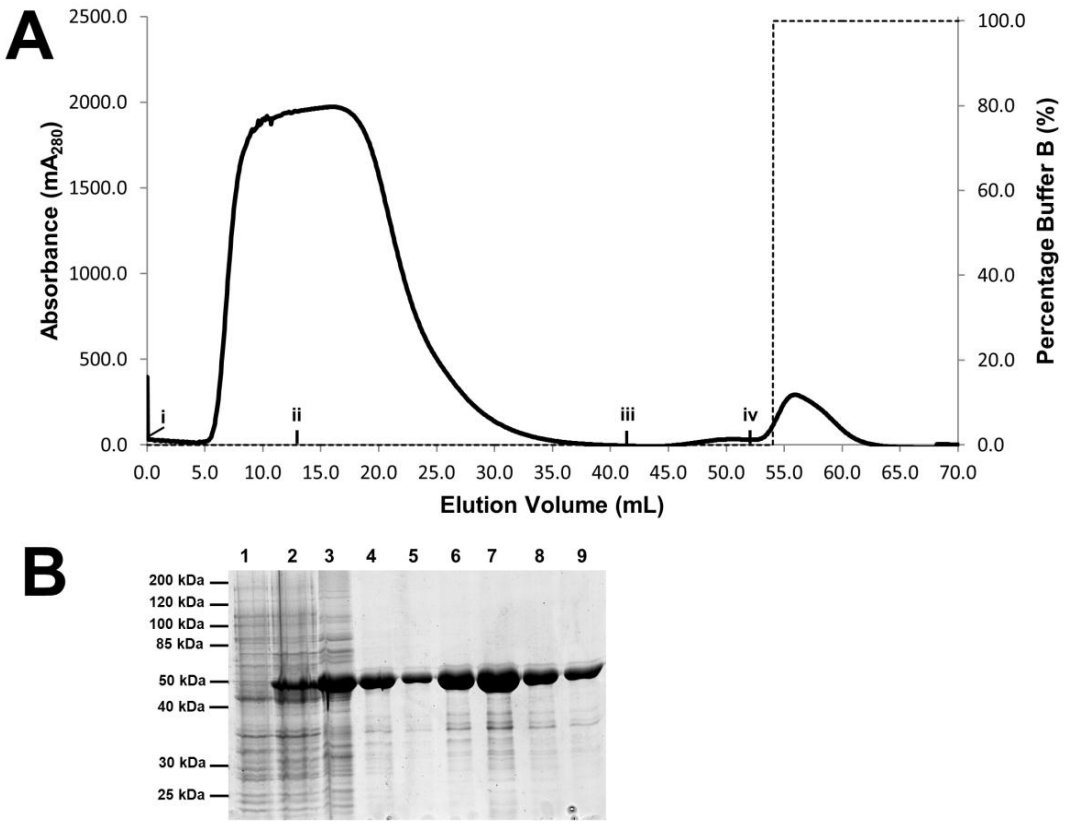


Figure A2.1.2: Expression and purification of native recombinant poly-histidine tagged fusion protein $SK_{\alpha T1}$ via FPLC Ni^{2+}-NTA affinity chromatography. (**A**) Elution profile from ÄKTAprime plus FPLC showing A_{280} trace (solid line) and percentage elution buffer (dashed line) for $SK_{\alpha T1.}$ Point *i* represents injection of cleared lysate. Point *ii* represents injection of binding buffer. Point *iii* represents injection of wash buffer and point *iv* represents injection of elution buffer. (**B**) Coomassie stained 10% SDS-PAGE of $SK_{\alpha T1}$ purification process. Un-induced *E. coli* whole cell lysate (lane 1), 2 h post induction *E. coli* whole cell lysate (lane 2) native lysis pellet containing insoluble protein (lane 3), lysate supernatant containing soluble protein (lane 4) Ni^{2+}-NTA column flow through (lane 5), wash buffer Ni^{2+}-NTA column flow through (lane 6) and Ni^{2+}-NTA column elution fractions (lane 7-11).

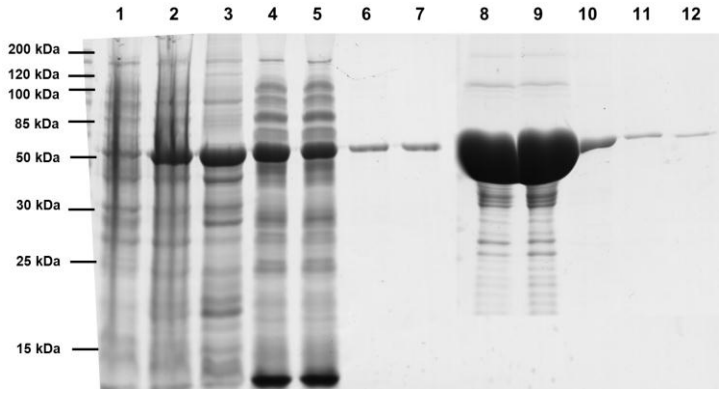


Figure A2.1.3: Expression and purification of native recombinant poly-histidine tagged fusion protein SKc via bench top column Ni^{2+}-NTA affinity chromatography. Coomassie stained 10% SDS-PAGE of SKc purification process. Un-induced *E. coli* whole cell lysate (lane 1), 2 h post induction *E. coli* whole cell lysate (lane 2), lysate supernatant containing soluble protein (lane 3) Ni^{2+}-NTA column flow through (lane 4), wash buffer Ni^{2+}-NTA column flow through (lane 5) and Ni^{2+}-NTA column elution fractions (lane 7-12).

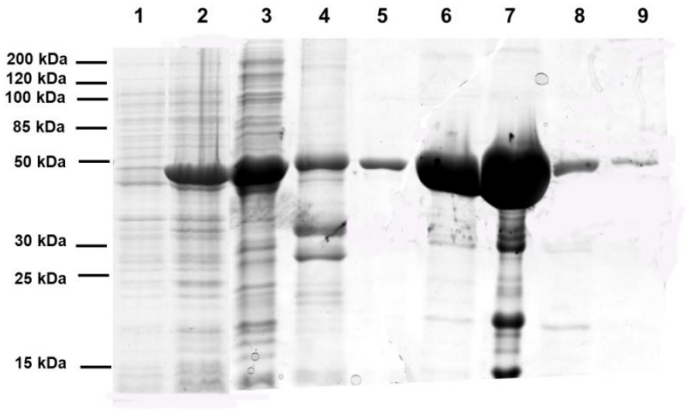


Figure A2.1.4: Expression and purification of native recombinant poly-histidine tagged fusion protein SK_{5448} via bench top column Ni^{2+}-NTA affinity chromatography. Coomassie stained 10% SDS-PAGE of SK_{5448} purification process. Un-induced *E. coli* whole cell lysate (lane 1), 2 h post induction *E. coli* whole cell lysate (lane 2), lysate supernatant containing soluble protein (lane 3) Ni^{2+}-NTA column flow through (lane 4), wash buffer Ni^{2+}-NTA column flow through (lane 5) and Ni^{2+}-NTA column elution fractions (lane 7-12).

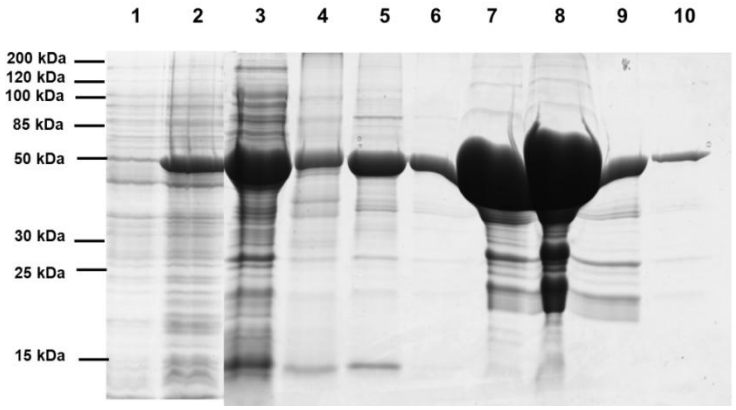


Figure A2.1.5: Expression and purification of native recombinant poly-histidine tagged fusion protein SK_{NS696} via bench top column Ni^{2+}-NTA affinity chromatography. Coomassie stained 10% SDS-PAGE of SK_{NS696} purification process. Un-induced *E. coli* whole cell lysate (lane 1), 2 h post induction *E. coli* whole cell lysate (lane 2), lysate supernatant containing soluble protein (lane 3) Ni^{2+}-NTA column flow through (lane 4), wash buffer Ni^{2+}-NTA column flow through (lane 5) and Ni^{2+}-NTA column elution fractions (lane 6-10).

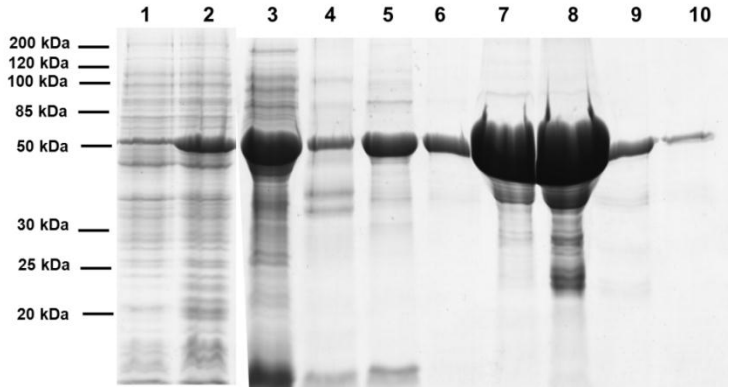
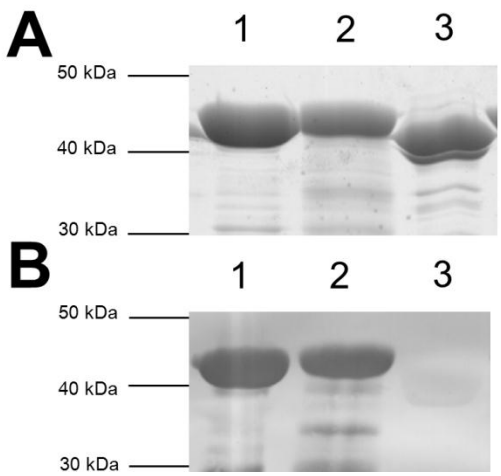


Figure A2.1.6: Expression and purification of native recombinant poly-histidine tagged fusion protein $SK_{NS88.2}$ via bench top column Ni^{2+}-NTA affinity chromatography. Coomassie stained 10% SDS-PAGE of $SK_{NS88.2}$ purification process. Un-induced *E. coli* whole cell lysate (lane 1), 2 h post induction *E. coli* whole cell lysate (lane 2), lysate supernatant containing soluble protein (lane 3) Ni^{2+}-NTA column flow through (lane 4), wash buffer Ni^{2+}-NTA column flow through (lane 5) and Ni^{2+}-NTA column elution fractions (lane 6-10).

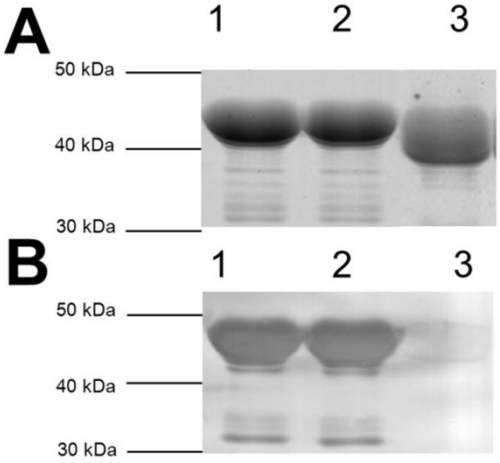
A2.2 Factor Xa cleavage of SK variants

The figures presented here are supplementary to the purification process outlined in Chapter 2.3.4 for Factor Xa cleavage of the remaining SK variants used in this thesis.

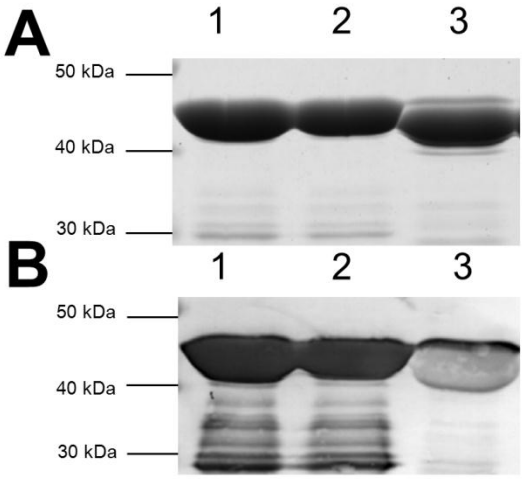
(i)



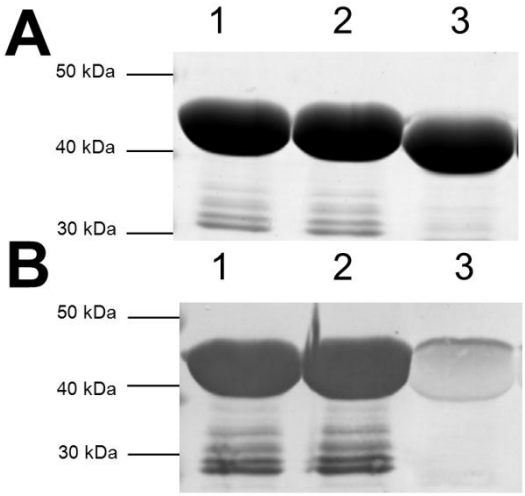
(ii)



(iii)



(iv)



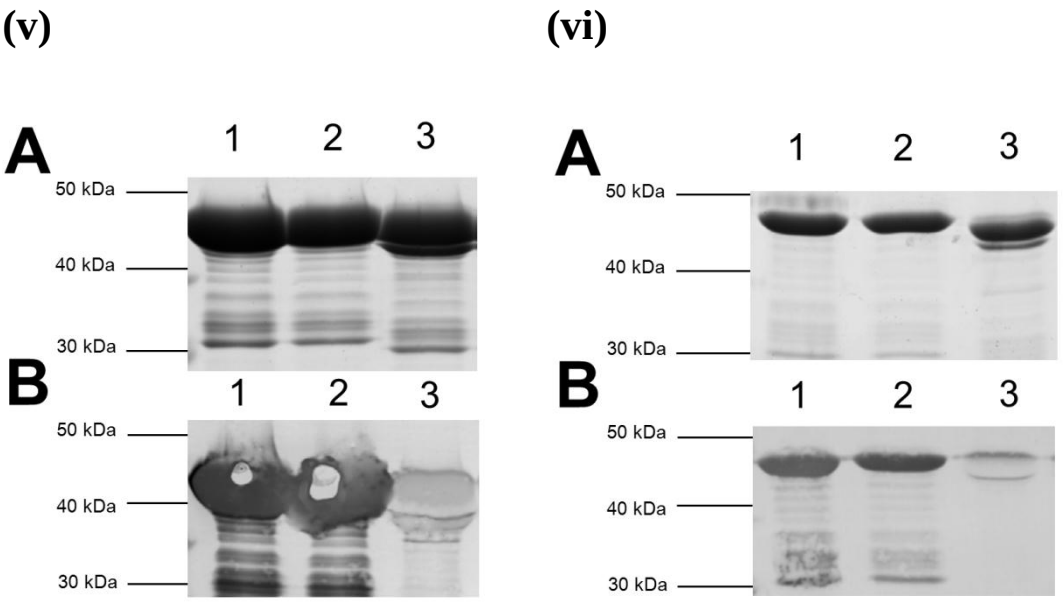
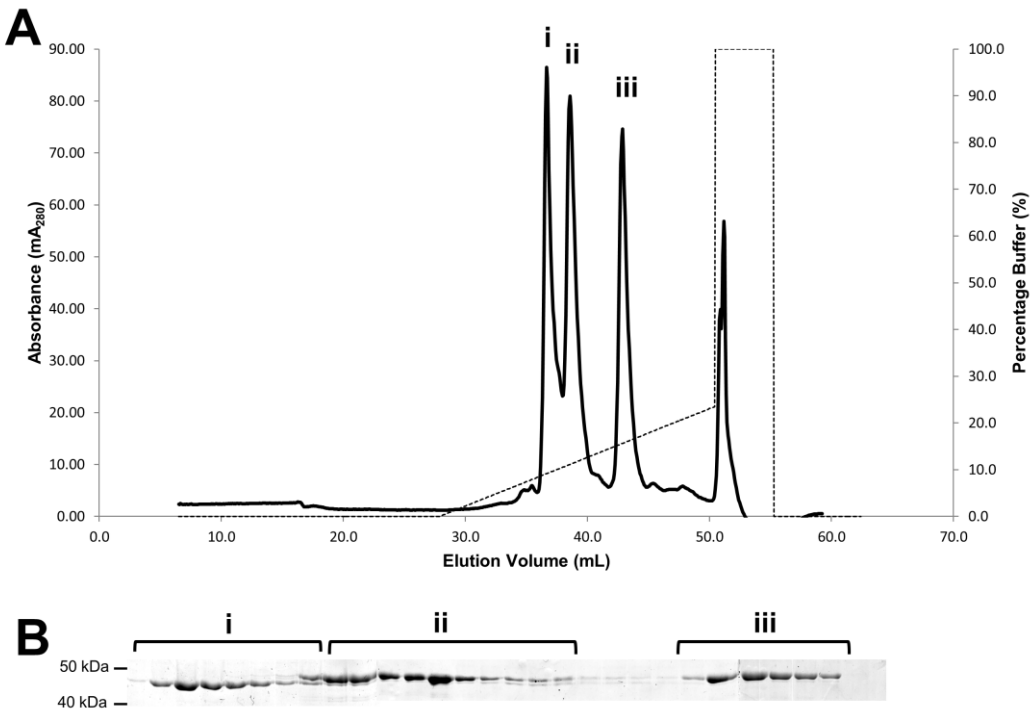


Figure A2.2.1: Factor Xa cleavage of recombinant poly-histidine tagged SK variant fusion proteins. (i) SK_{NZ131}; **(ii)** SKc; **(iii)** SK_{5448}; **(iv)** SK_{NS696}; **(v)** $SK_{NS88.2}$ **(vi)** $SK_{\alpha T1.}$ **(A)** Coomassie stained 10% SDS-PAGE of SK_{ALAB49} cleavage. Lane 1, SK T = 0 h; Lane 2, SK T = 36 h without Factor Xa; Lane 3, SK T = 36 h Factor Xa added. **(B)** Western blot of the duplicate samples using Ni^{2+}-NTA HRP conjugate to detect polyhistidine tags.

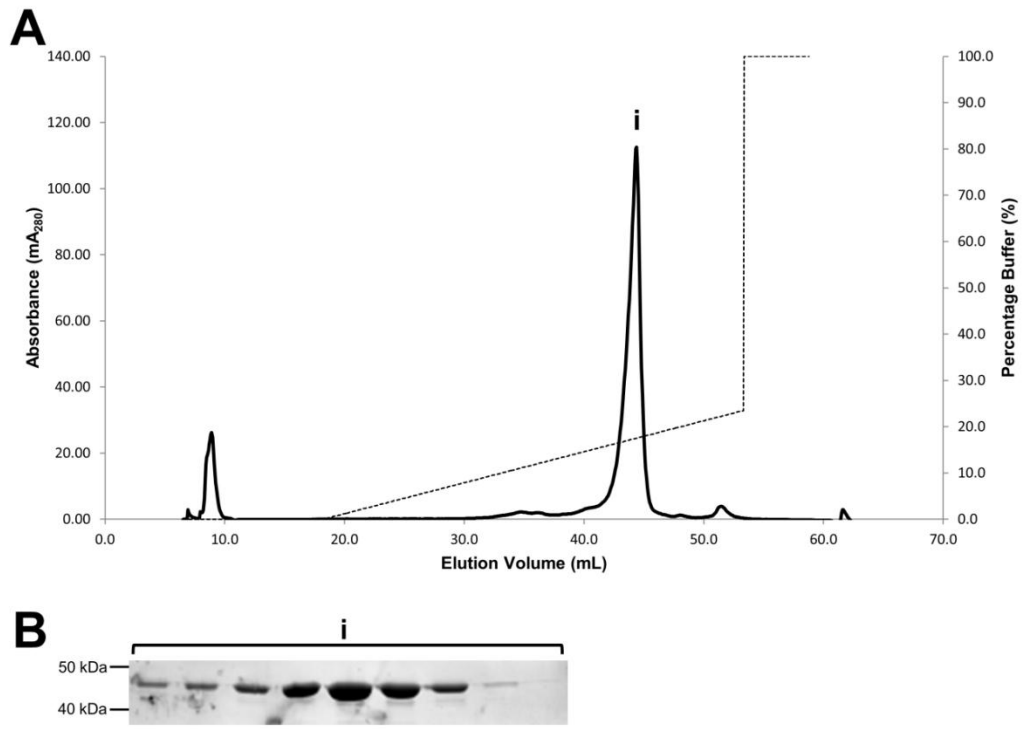
A2.3 Additional anion exchange chromatography

The figures presented here are supplementary to the purification process outlined in Chapter 2.3.6 for Anion-exchange FPLC of the remaining SK variants used in this thesis.

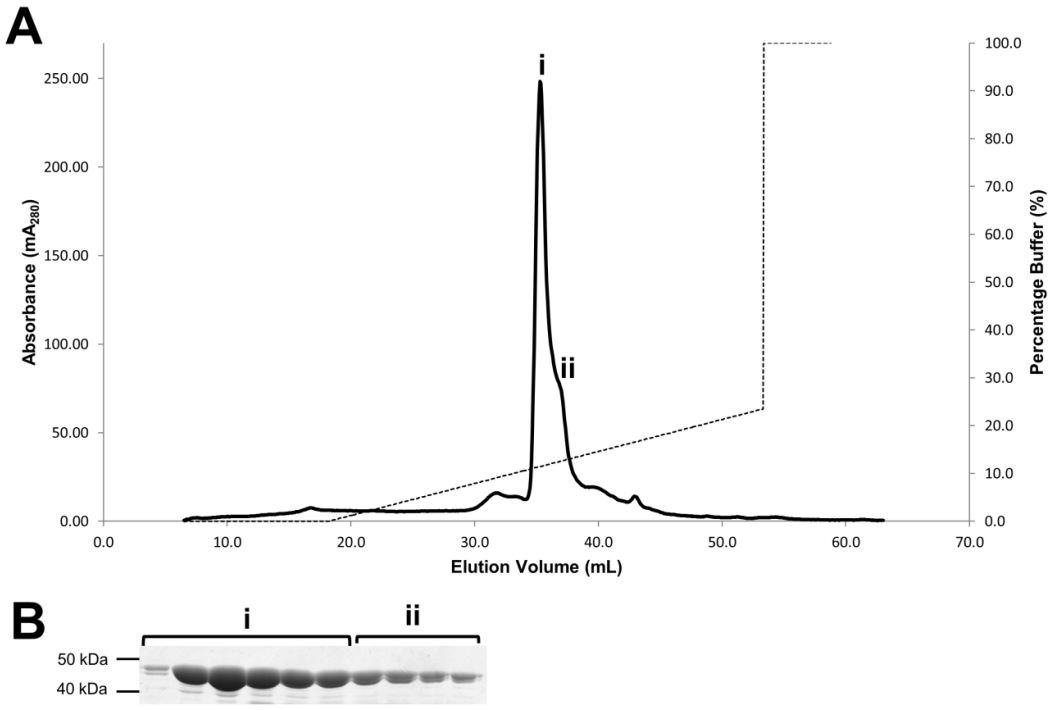
(i)



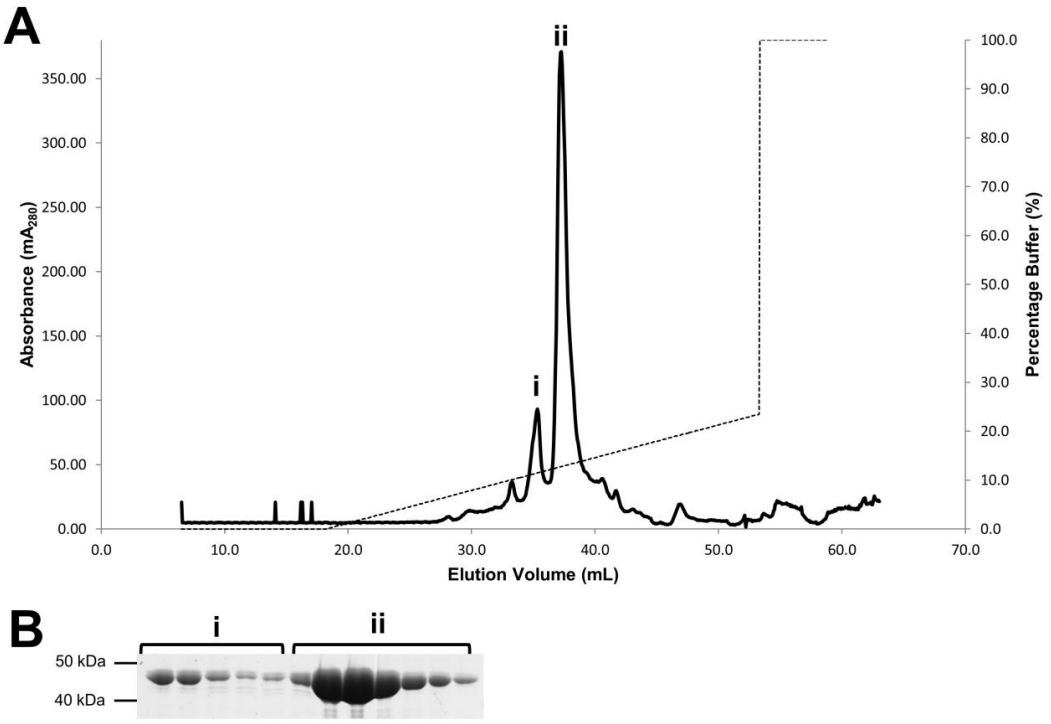
(ii)


A
Absorbance (mA280)
Percentage Buffer (%)
Elution Volume (mL)
i
B
i
50 kDa
40 kDa

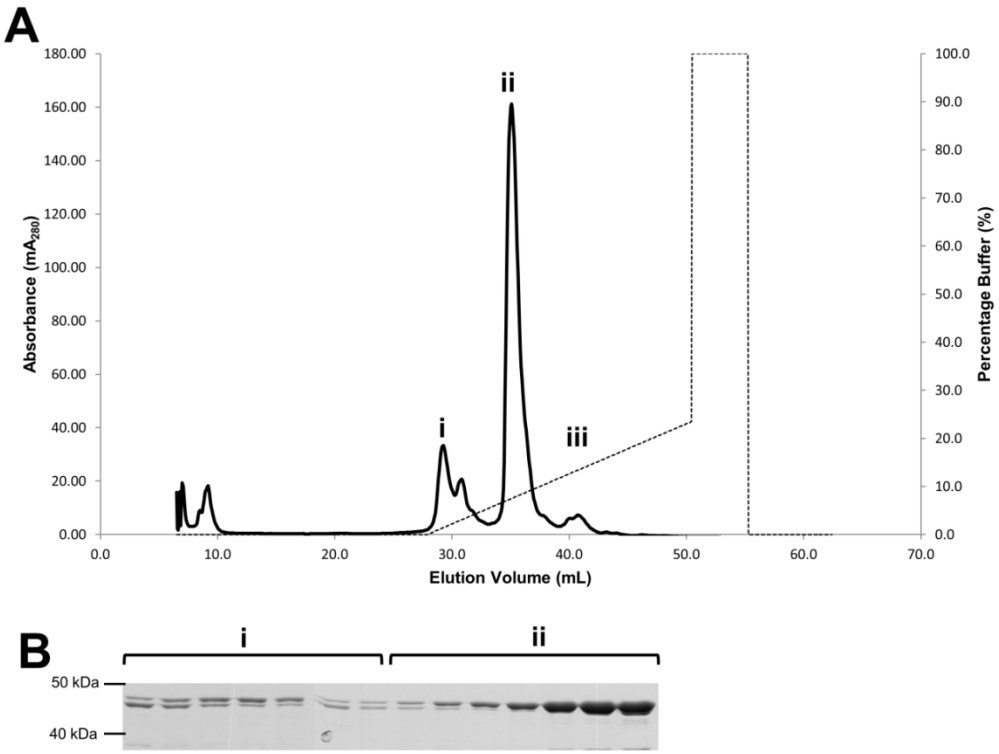
(iii)


A
Absorbance (mA280)
Percentage Buffer (%)
Elution Volume (mL)
i
ii
B
i
ii
50 kDa
40 kDa

(iv)


A
ii
i
Absorbance (mA280)
Percentage Buffer (%)
Elution Volume (mL)
B
i
ii
50 kDa
40 kDa

(v)


A
ii
i
iii
Absorbance (mA280)
Percentage Buffer (%)
Elution Volume (mL)
B
i
ii
50 kDa
40 kDa

(v)

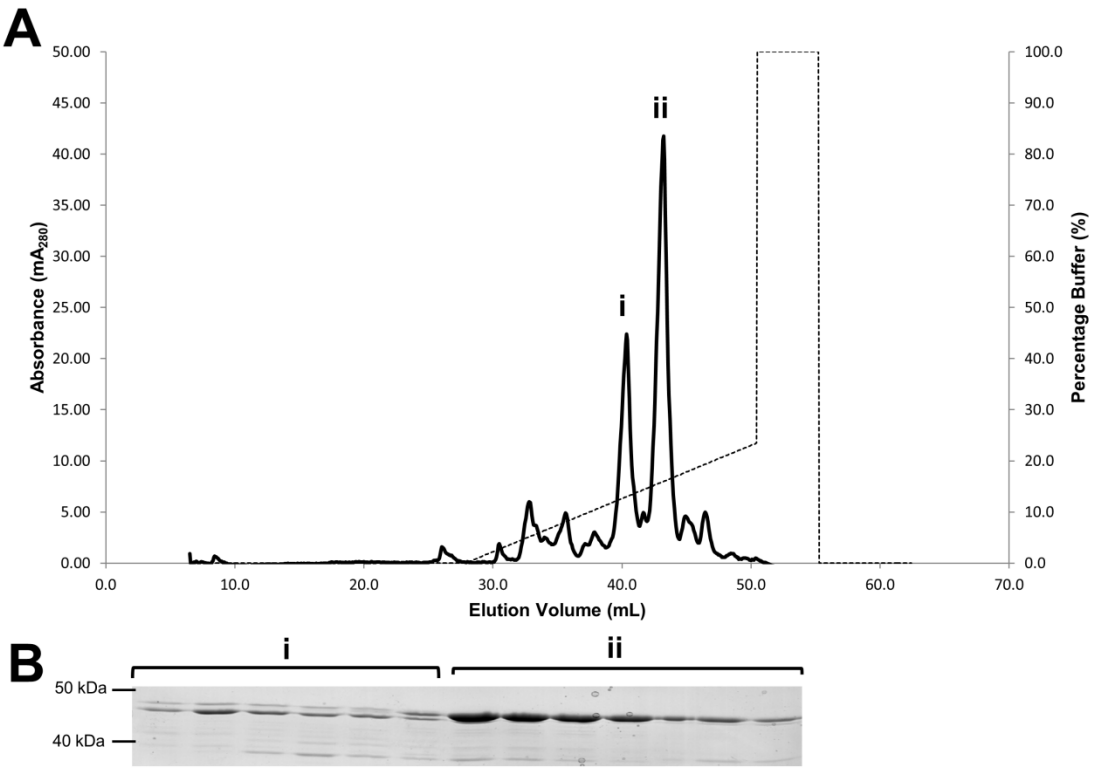


Figure A2.3.1: Anion-exchange fast protein liquid chromatography of SK variants. (i) SK_{NZ131}; **(ii)** SKc; **(iii)** SK_{5448}; **(iv)** SK_{NS696}; **(v)** $SK_{NS88.2}$ **(vi)** $SK_{\alpha T1.}$ **(A)** Elution profile from ÄKTA explorer FPLC showing A_{280} trace (solid line) and percentage of Buffer B (0 -1 M NaCl) (dashed line). Generally three peaks were resolved in the A_{280} trace correlating to truncated SK (peak i), full length 47 kDa SK (peak ii) and a co-elution of both species (peak iii). **(B)** Coomassie stained 10% SDS-PAGE of anion exchange elution peaks. Elution peaks generally correlated to truncated SK (i), full length ~47 kDa SK (ii) and co-elution of both species (iii). Variation in the peak resolution and composition can be clearly identified by comparison of panels A and B of the figure.

A2.4 Denaturing purification protocol for recombinant SK proteins

Recombinant SK protein expression in transformed M15[pREP4] was induced by addition of isopropyl-1-thio-b-D-galactopyranoside to log-phase cells in 1L culture volume at a final concentration of 1 mM. Three hours later, cells were harvested by centrifugation, resuspended in 5 mL of denaturing lysis buffer (Urea 8 M, NaH_2P0_4 100 mM, Tris.Cl 100 mM,pH 8.0) and agitated at room temperature for 1 h. A further 5 mL of denaturing lysis buffer was added to the solution and agitated for a further 15 min at room temperature. The solution was incubated on ice for a further 15 min and subjected to sonication using a Branson Sonifier 250® (Branson, USA) equipped with a micro tip (output limit of 7) to lyse remaining intact cells. Cells were sonicated with a 50% duty cycle for 30 sec then chilled on ice for 30 sec for a total of 2 min sonication. The suspension was centrifuged at 10,000 x *g* for 30 min to clear the lysate of cellular debris and the supernatant removed for Ni^{2+}-NTA affinity chromatography using denaturing purification buffers. Purification of protein from cleared lysate was performed using an ÄKTAprime plus fast protein liquid chromatography (FPLC) system (GE Healthcare Life Sciences, Piscataway,NJ, USA) with two 1 mL HisTrap HP columns in series (QE Scientific, USA). The resin was equilibrated with 10 column volumes of binding buffer (50 mM NaH_2P0_4, 300 mM NaCl, 10 mM Imidazole, pH 8) and once equilibrated, 50 mL of 0.22 µm syringe filtered lysate was loaded onto the column. The column was washed with binding buffer until the A_{280} trace returned to a constant pre-sample injection baseline. The column was then washed with 10 column volumes of washing buffer (Urea 8 M, NaH_2P0_4 100 mM, Tris.Cl 100 mM, pH 6.3) to remove non-specifically bound contaminants. Column bound recombinant SK was eluted from the column by flowing

20 column volumes of elution buffer (Urea 8 M, NaH_2P0_4 100 mM, Tris.Cl 100 mM, pH 4.5) over the column resin and collected in 1mL elution fractions.

Appendix 3: Enzyme Kinetic Analysis of SK variants

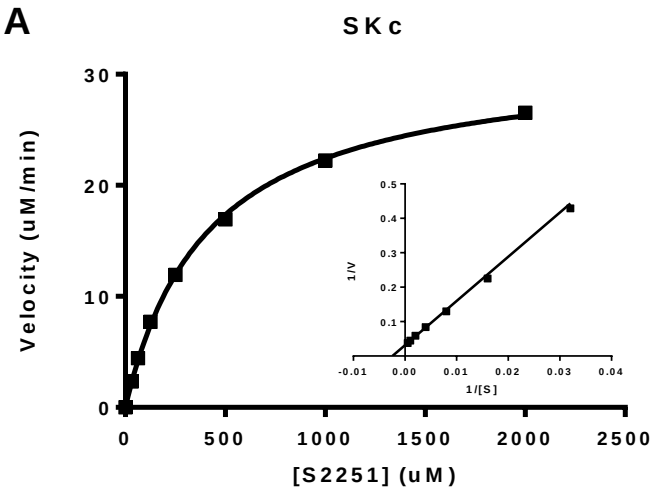
The biochemical experiments shown in this appendix were initially completed as a method to assess differences in the amidolytic (Figure A3.1) and Plg activation activities (Figure A3.2) of the SK variants used in this study. However, after these experiments were completed plasmin was determined to bind with high affinity to SK (several orders of magnitude tighter than Glu-Plg) resulting in preferential formation of the SK-plasmin complex for all SK variants and generation of amidolytic activity via 'Pathway II'. Thus, these considerations affected the kinetic parameters listed in Table A3.1, where the SK variants and Plg were pre-incubated before measurement of the rates of plasmin specific chromogenic substrate, S-2251, hydrolysis. As such, these parameters likely reflect the SK-plasmin complex and not SK-Glu-Plg*, but do show distinct differences between each variant and are valuable data to be considered for future biochemical analyses.

The kinetic parameters listed in Table A3.2 for activation of Glu-Plg were achieved by the addition of SK variants to excess Glu-Plg and S-2251 substrate. After completion of these experiments it was noted that the chromogenic substrate, S-2251, used in these experimentm inhibits the rate of both SK-Plg* and SK-plasmin activation of free Glu-Plg, as S-2251 binds to the active site of theses complexes and interferes with proteolytic activity. While these reactions were appropriately analysed as parabolic progress curves to obtain the initial rate of plasmin formation and subsequently fit by a hyperbola to determine the kinetic parameters, the rates could not be corrected for inhibition due to the presence of S-2251. As Table A3.1 suggests, the kinetic constants

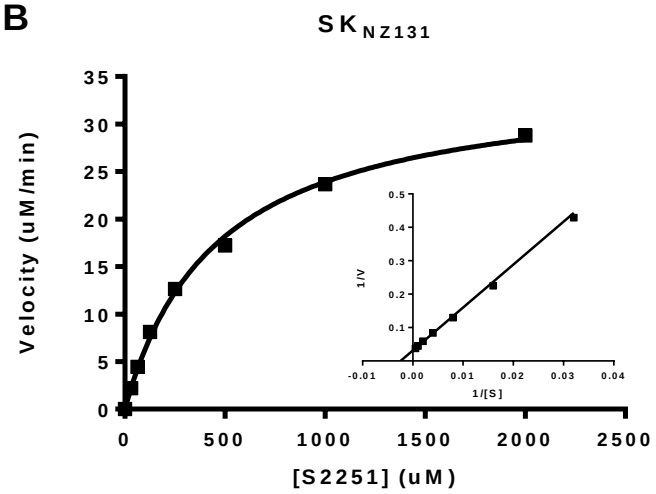
for S-2251 hydrolysis are different for the SK variants, and this may also suggest that the corrections for inhibition by S-2251 will be different for SKc Type-1, 2a, and 2b SK variants. Despite attempting to calculate these inhibition constants, these data could not be successfully generated and as such these data were not considered suitable for publication and were superseded by the S-2251 hydrolysis absorbance versus time curves used in Chapters 2, 3 and 4.

A3.1 Steady state amidolytic kinetics analysis of SK-Plg variants

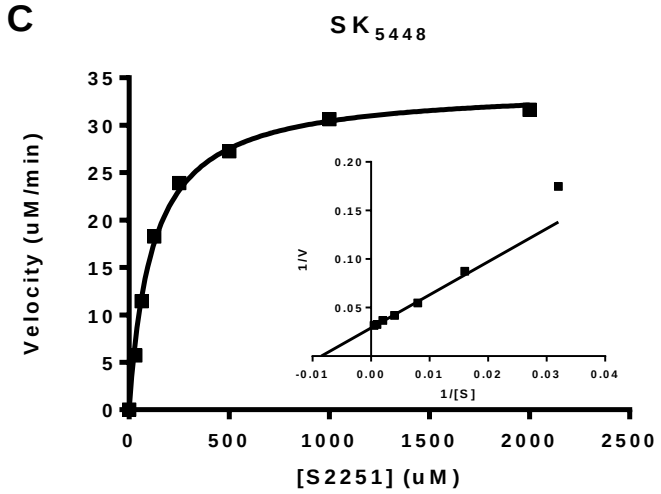
The amidase kinetic parameters of variant SK molecules were studied by the addition of preformed SK-Plg complex (5 – 25 min, final concentration 20 nM) to assay buffer containing S-2251 (30 µM – 2000 µM) in a total volume of 100 µL. The change in absorbance was measured at 405 nm at 37°C. The data were plotted as velocity (µM min^{-1}) versus substrate concentration (µM) and analysed by hyperbolic curve fitting using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). A molar *extinction coefficient* of 10000 M^{-1} cm^{-1} was used for calculation *p*-nitroanilide concentration.



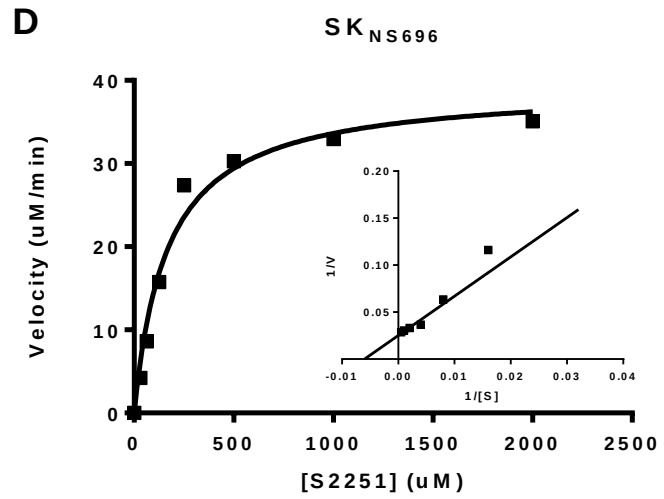
B


SK NZ131
Velocity (uM/min)
[S2251] (uM)
1/V
1/[S]

C


SK 5448
Velocity (uM/min)
[S2251] (uM)
1/V
1/[S]

D


SK NS696
Velocity (uM/min)
[S2251] (uM)
1/V
1/[S]

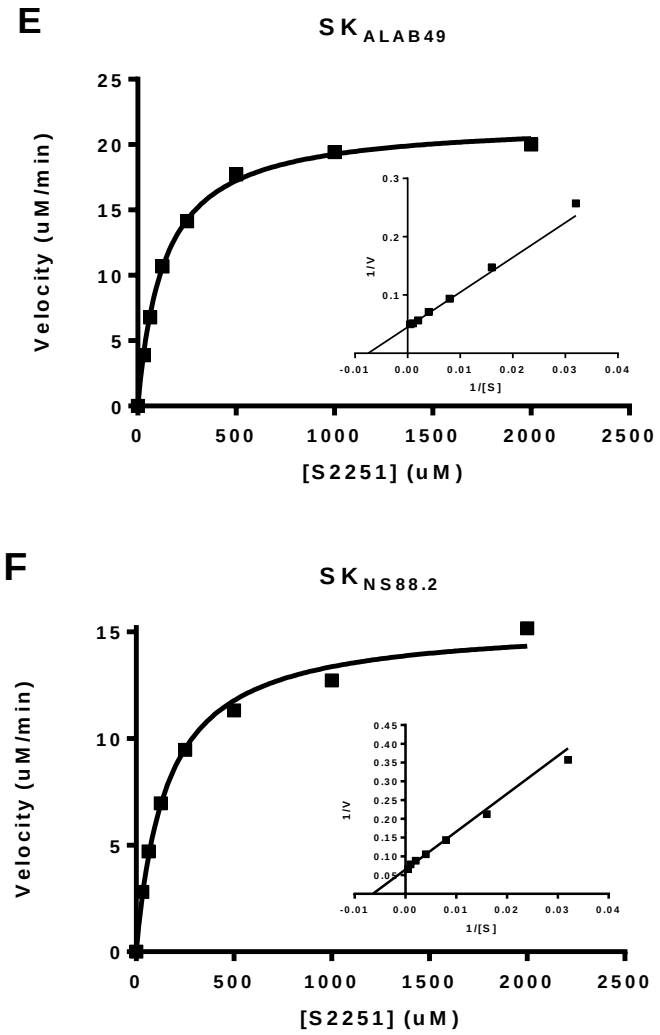


Figure A3.1: Mechaelis-Menten kinetic analysis of the amidase properties of SK variants complexed with Glu-Plg. (**A**) SKc; (**B**) SK_{NZ131}; (**C**) SK_{NS696}; (**D**) SK_{5448}; (**E**) SK_{ALAB49} and (**F**) $SK_{NS88.2}$. All panels: representative Michaelis-Menten curve fits of velocity verses substrate concentration to determine Km and Vmax. The insert of each figure displays the Linewearver-Burk plots from these data and used for kinetic constant determination (shown in Table A3.1). The data presented here is representative of at least three experiments.

Table A3.1. Kinetic constants for amidolysis by variant SK-Plg complexes

	Amidolytic Parameters		
SK Variant	K_m **(μM)**	k_{cat} **(s^{-1})**	k_{cat}/K_m **($\mu M^{-1}s^{-1}$)**
SKc	414 ± 16	26.4 ± 0.4	0.064
SK_{NZ131}	460 ± 33	29.1 ± 0.8	0.063
SK_{5448}	118 ± 9	28.4 ± 0.6	0.241
SK_{NS696}	167 ± 31	32.7 ± 1.7	0.196
SK_{ALAB49}	133 ± 6	18.2 ± 0.2	0.137
$SK_{NS88.2}$	156 ± 17	12.9 ± 0.4	0.083

Values represent the mean ± SEM.

A3.2 Steady state Plg activation assays

The kinetics of Plg activation were studied by the addition of variant SK (final concentration 5-10 nM) to assay buffer containing Plg (300 nM – 1600 nM) and S-2251 (500 μM) in a total volume of 100 μL at 37°C. Initial reaction rates were obtained from the first 300 s by plotting A_{405}/min^2 and apparent Michaelis and catalytic rate constants were calculated by fitting the data to a hyperbolic curve as described previously by Wohl *et al.* (1980) using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Where the overall reaction consists of two separate reactions:

$$\mathbf{SK + Plg} \underset{}{\overset{K_{Plg}}{\rightleftharpoons}} \mathbf{SK.Plg} \xrightarrow{k_{Plg}} \mathbf{SK + Pln} \qquad \mathbf{(1)}$$

$$\mathbf{Pln + S\text{-}2251} \overset{K_s}{\rightleftharpoons} \mathbf{Pln.S\text{-}2251} \xrightarrow{k_s} \mathbf{Pln + pNA + H\text{-}D\text{-}Val\text{-}Leu\text{-}Lys} \qquad \mathbf{(2)}$$

These reactions occur independently of each other and pNA is the p-nitroanilide group released from a chromogenic substrate S-2251 that is detected at an absorbance of 405 nm. K_{plg} and K_s are the dissociation constants for the activation of Plg by SK and for the

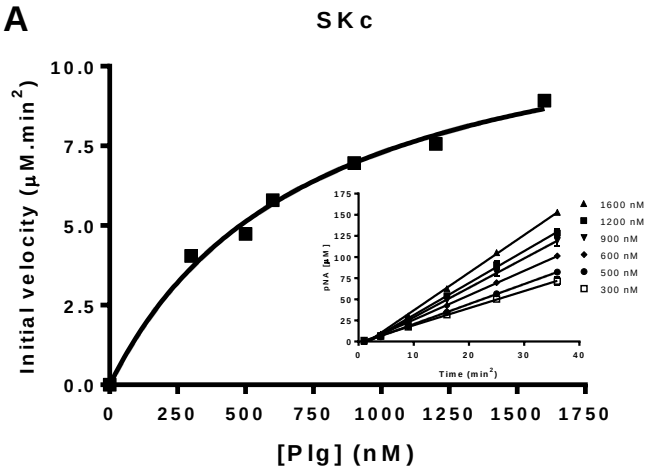
hydrolysis of S-2251 by plasmin (Pln), respectively. *k*plg and *k*s are the catalytic rate constants of the Plg activation and of the amidolysis, respectively. Below, *k* is the apparent Plg activation rate (µM pNA/min^2), k_1 is the Plg activation rate (µM

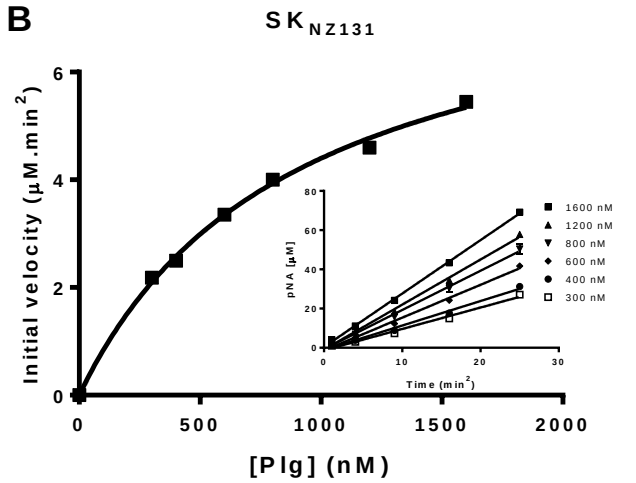
$$[\mathbf{pNA}]_t = \frac{1}{2} \cdot \frac{k_{plg}[SK]_0[Plg]_0}{K_{plg} + [Plg]_0} \cdot \frac{k_s}{\frac{K_s}{[S2251]_0} + 1} \cdot t^2$$

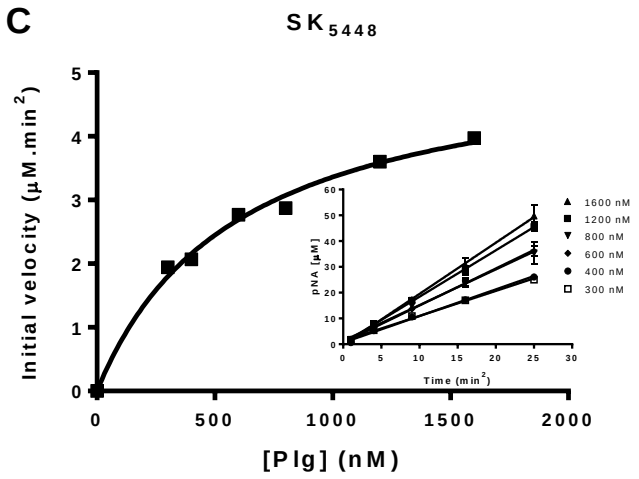
$$[\mathbf{pNA}]_t = \frac{1}{2} \cdot k_1 \cdot k_2 \cdot t^2 = k \cdot t^2 \qquad \textbf{(3)}$$

where, $k_1 = \frac{k_{plg}[SK]_0[Plg]_0}{K_{plg} + [Plg]_0}$, $k_2 = \frac{k_s}{\frac{K_s}{[S2251]_0} + 1}$

Pln/min), and k_2 represents the plasmin activity toward S-2251 (µM pNA/min.µM Pln/min). $[SK]_0$, $[Plg]_0$ and $[S\text{-}2251]_0$ are the initial concentrations of SK, Plg, and S-2251, respectively. $[pNA]_t$ is the concentration of p-nitroaniline at time t. The apparent Plg activation rate *k* was determined by plotting the concentration of pNA released vs. reaction time squared. The k_2 value was calculated from equation 3 using the kinetic parameters on the amidolytic activity of plasmin toward S-2251 as described in section A3.3. The k_1 value was calculated from the values of *k* and k_2 for final determination of kinetic constants of SK mediated Plg activation.


A
SKc
Initial velocity (µM.min²)
10.0
7.5
5.0
2.5
0.0
0
250
500
750
1000
1250
1500
1750
[Plg] (nM)
pNA [µM]
Time (min²)
1600 nM
1200 nM
900 nM
600 nM
500 nM
300 nM


B
SK NZ131
Initial velocity (µM.min²)
6
4
2
0
0
500
1000
1500
2000
[Plg] (nM)
pNA [µM]
Time (min²)
1600 nM
1200 nM
800 nM
600 nM
400 nM
300 nM


C
SK 5448
Initial velocity (µM.min²)
5
4
3
2
1
0
0
500
1000
1500
2000
[Plg] (nM)
pNA [µM]
Time (min²)
1600 nM
1200 nM
800 nM
600 nM
400 nM
300 nM

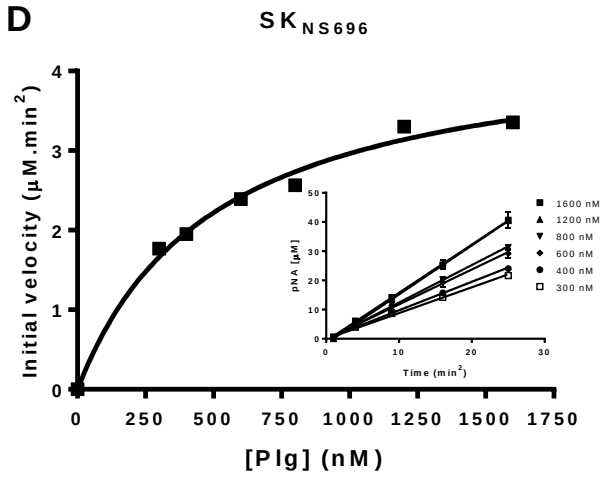


Figure A3.2. Mechaelis-Menten kinetic analysis of the Plg activation activity of SK variants (**A**) SKc; (**B**) SK_{NZ131}; (**C**) SK_{NS696} and (**D**) SK_{5448}. All panels: representative Michaelis-Menten curve fits of velocity verses substrate Plg concentration to determine Km and Vmax. The insert of each figure displays the plots of initial velocity of Plg activation for each substrate concentration, from which the gradients of the lines were used to fit to a modified Michalis-Menten equation that has been derived to determine the kinetic constants of Plg activation specifically (shown in Table A3.2). The data presented here is representative of at least three experiments.

Table A3.2. Kinetic constants for activation of Plg by variant SK-Plg complexes

	Activation Parameters		
Activator	K_m **(nM)**	k_{cat} **(min^{-1})**	k_{cat}/K_m **(uM^{-1} min^{-1})**
SKc	333 ± 60	1.7 ± 0.1	5.1
SK_{NZ131}	432 ± 42	2.3 ± 0.1	8.3
SK_{5448}	112 ± 15	1.5 ± 0.1	13.4
SK_{NS696}	127 ± 22	1.2 ± 0.1	9.5
SK_{ALAB49}	Activates*	Activates*	N/A
$SK_{NS88.2}$	Activates*	Activates*	N/A

Activation experiments were conducted at 37°C as described above. Procedures'. Values represent the mean ± SEM. N/A = Not Applicable. * Delayed Plg activation (> 30 min lag phase due to plasmin contamination) before significant generation of activation resulting in indeterminable kinetic constants.

A3.3 Calculation of plasmin amidase activity

The amidase kinetic parameters of plasmin used to determine the k_2 value for equation 3 (section 3.2) were determined by the addition of plasmin (final concentration 20 nM) to assay buffer containing S-2251 (30 µM – 3000 µM) in a total volume of 100 µL. The change in absorbance was measured at 405 nm at 37°C. The data were plotted as velocity (µM/min) versus substrate concentration (µM) and analysed by hyperbolic curve fitting using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). A molar *extinction coefficient* of 10000 M^{-1} cm^{-1} was used for calculation *p*-nitroanilide concentration. From this experiment the Km and Vmax of plasmin hydrolysis of S-2251 were determined to be 319.5 µM and 27.7 µM/min, respectively.

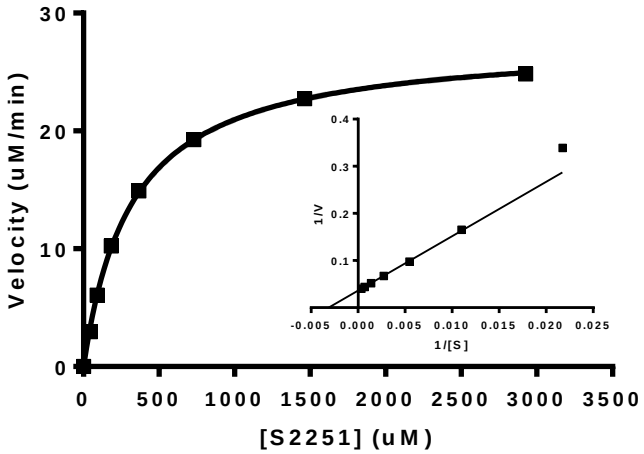


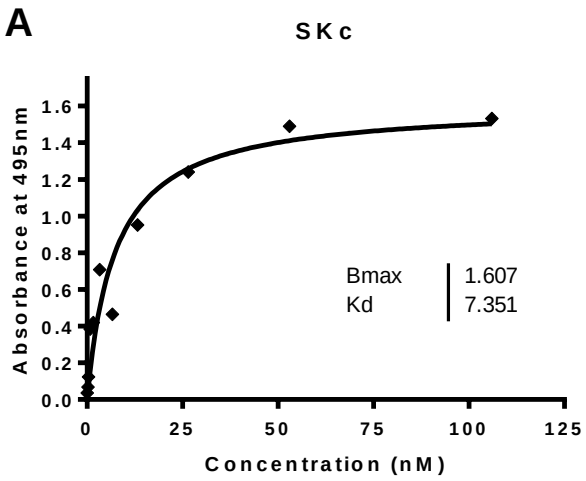
Figure A3.3. Mechaelis-Menten kinetic analysis of the amidase activity of plasmin Representative Michaelis-Menten hyperbolic curve fit of velocity of pNA generation verses substrate concentration to determine Km and Vmax. The insert displays the Linewearver-Burk plot from these data, which was used for kinetic constant determination. The data presented here is representative of at least three experiments.

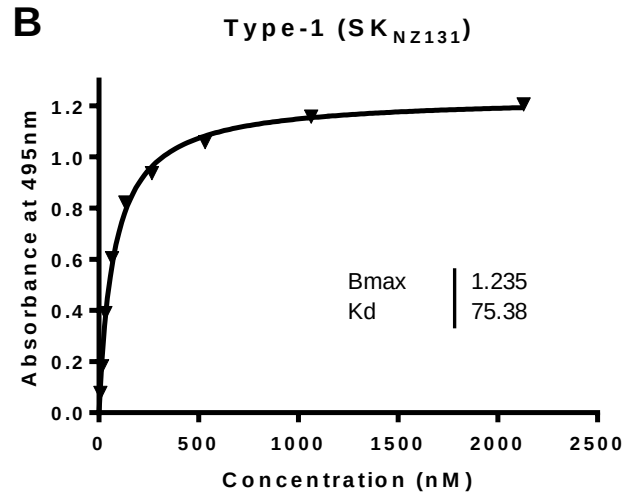
Appendix 4: Binding Interaction Analysis of SK Variants to Glu-Plg and plasmin

A4.1 Solid state binding assays of SK variants to Plg

A preliminary characterisation of the interaction between recombiant SK variant proteins and Glu-Plg was achieved by solid phase microtitre Plg binding assay. 96 well microtitre plates (Greiner Bio-one, Germany) were coated with 5 µg/mL Glu-Plg (50 uL in 20 mM HEPES buffer, 150 mM NaCl, pH 7.4) at 4°C overnight. Following five washes with washing buffer (20 mM HEPES buffer, 150 mM NaCl, 0.05% Tween-20, pH 7.4), plates were blocked with 100 µL of blocking solution (20 mM HEPES buffer, 150 mM NaCl, 5% skim milk, pH 7.4) for 1 h at 37°C. Wells were washed as above and SK (SKc = 105 nM, type-1 SK = 2150 nM, type-2a SK = 150 nM and type-2b SK = 2150 nM) was diluted in a two-fold titration across the plate in binding buffer (20 mM HEPES buffer, 150 mM NaCl, 0.5% skim milk, 0.05% Tween-20, pH 7.4) and incubated at 37°C for 1.5 h. Following SK binding, microtitre plate wells were washed five times and 50 µL of α-SK rabbit polyclonal sera (Jason Mcarthur, Univerity of Wollongong, NSW, Australia) diluted 1:5000 in binding solution was added to all wells and incubated for 1.5 h at 37°C. After five washes with washing buffer, 50 µL secondary α-rabbit HRP conjugate antibody (Pierce, USA) was added to all wells of the microtitre plate at a 1:4000 dilution in binding buffer and incubated at 37°C for 1 h. After five washes with washing buffer the reactions were developed by the addition of 50 µL of o-phenylenediamine (Sigma-Aldrich, Sydney, NSW, Australia) substrate (8 mM Na_2HPO_4, 2.2 mM o-phenylenediamine, 3% H_20_2, pH 5.5). Colour development

was stopped by addition of 50 µL of 10 M HCl and the plates were read at 490 nm using a Spectramax 250 spectophotometer plate reader (Molecular Devices, USA). For the calculation of equilibrium binding dissociation constants (K_D), a non-linear regression, one site binding (hyperbola) was fit to the data using GraphPad Prism v5 (GraphPad software, CA, USA).





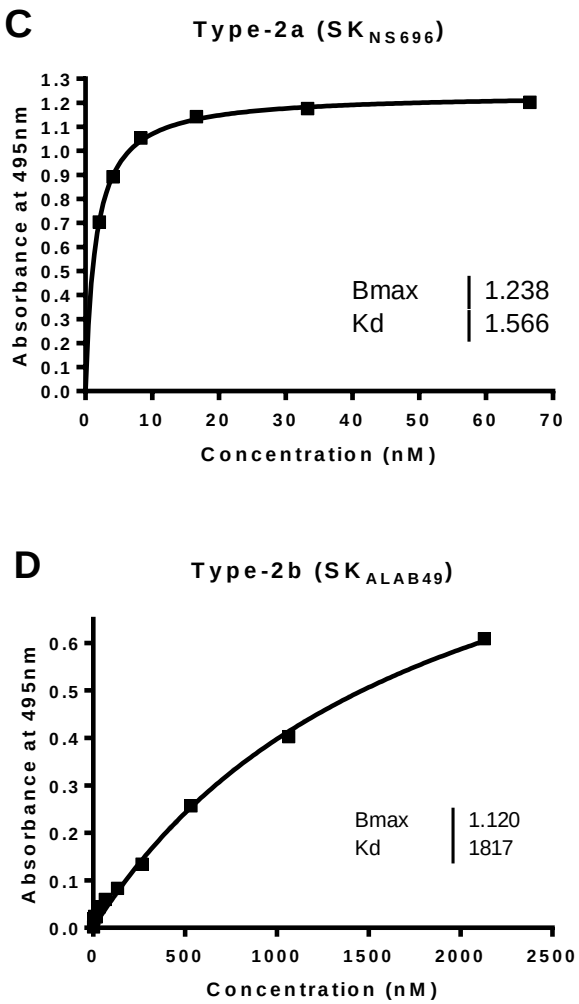
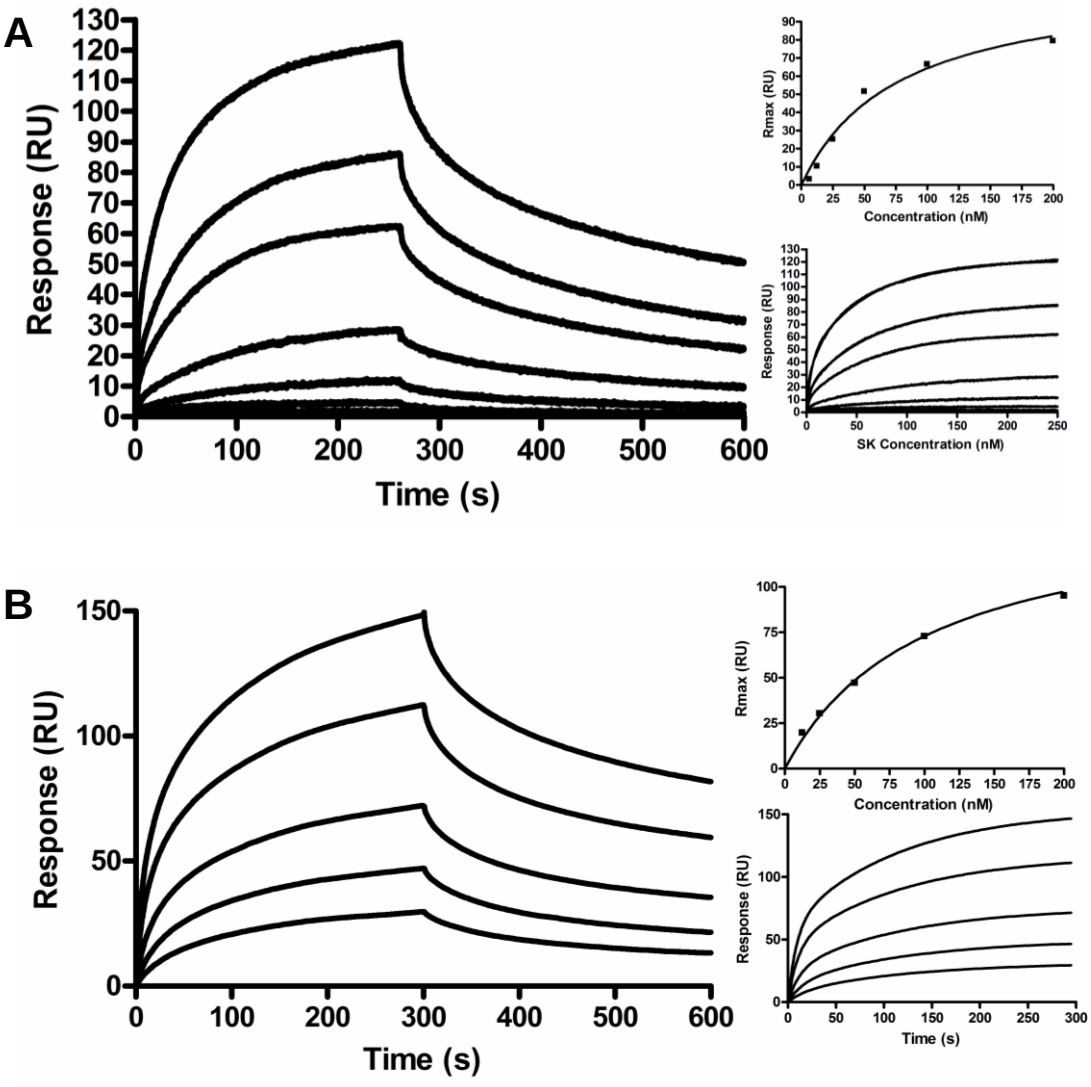
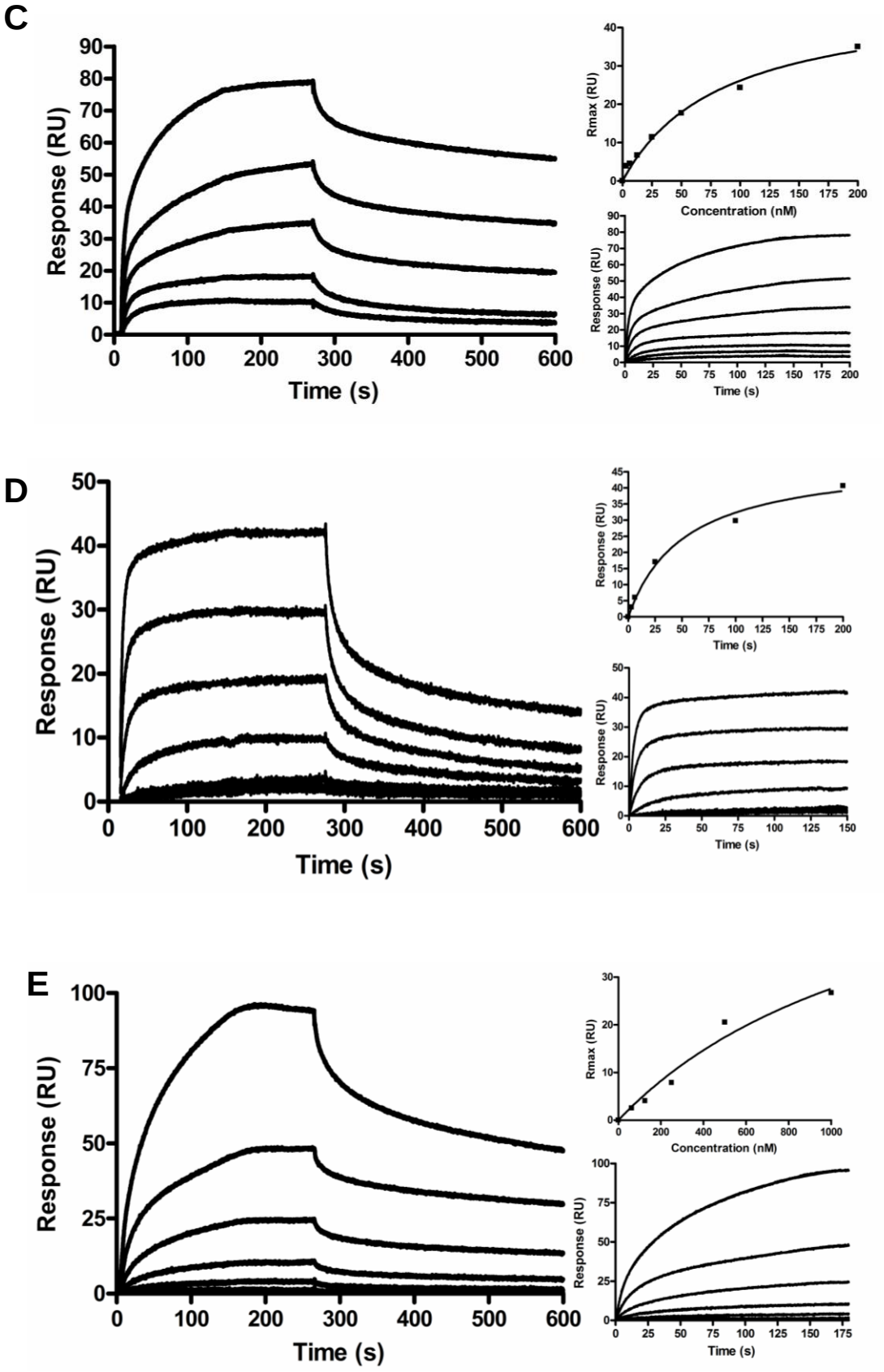


Figure A4.1.1 Solid state binding analysis of SK variants to immobilised recombinant Glu-Plg. SK binding to immobilised recombinant Glu-Plg (**A**) Group C SK (SKc); (**B**) Type-1 SK (SK_{NZ131}); (**C**) Type-2b SK (SK_{NS696}) and (**D**) Type-2b SK (SK_{ALAB49}). Equilibrium binding dissociation constants (K_D) were calculates using a non-linear regression, one site binding (hyperbola) analysis and was fit to the data using GraphPad Prism v5 (GraphPad software, CA, USA).

A4.2 Surface plasmon resonance studies to determine affinity constants for variant SK interaction with Glu-Plg and plasmin

The figures presented here are supplementary to the generation of binding affinity constants given in Table 3.3.6 as outlined in Chapter 3.3.6 for surface plasmin resonance experiments.




C
Response (RU)
Time (s)
Rmax (RU)
Concentration (nM)
Response (RU)
Time (s)
D
Response (RU)
Time (s)
Response (RU)
Time (s)
Response (RU)
Time (s)
E
Response (RU)
Time (s)
Rmax (RU)
Concentration (nM)
Response (RU)
Time (s)

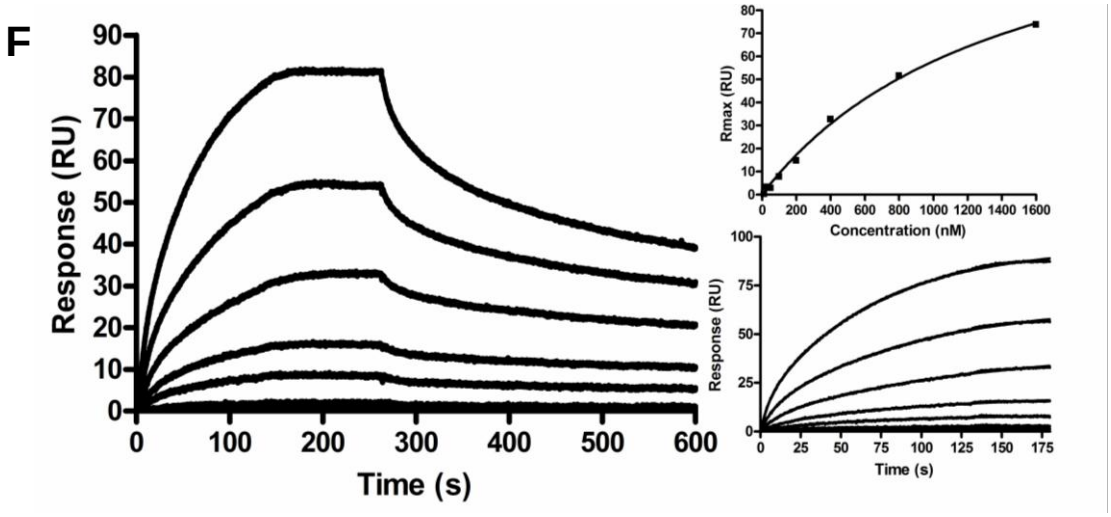
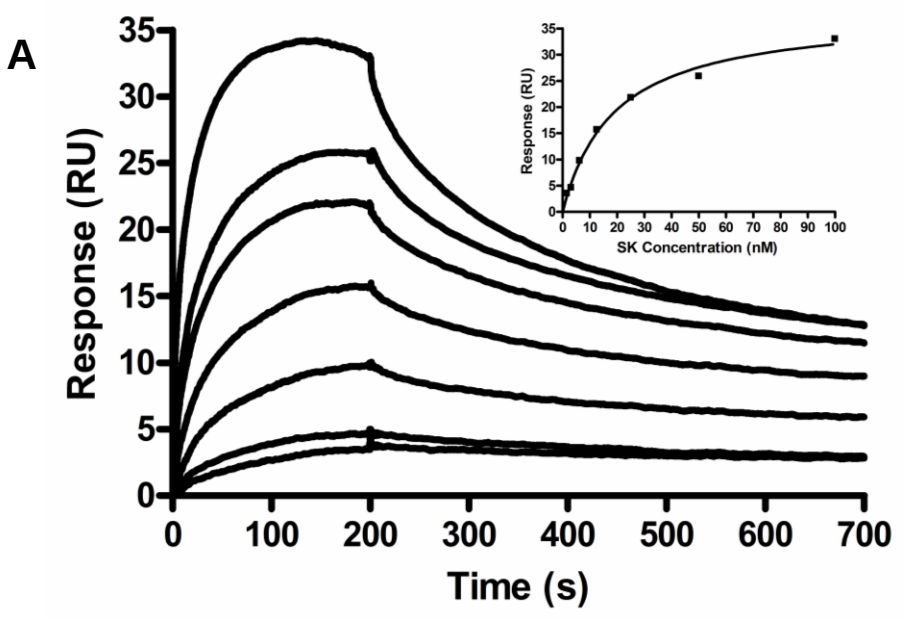
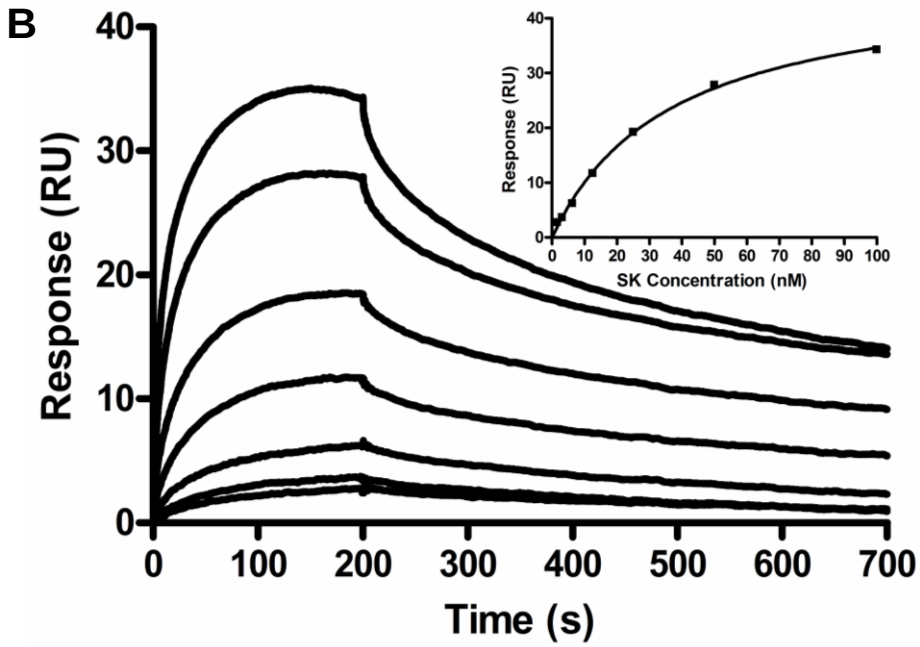
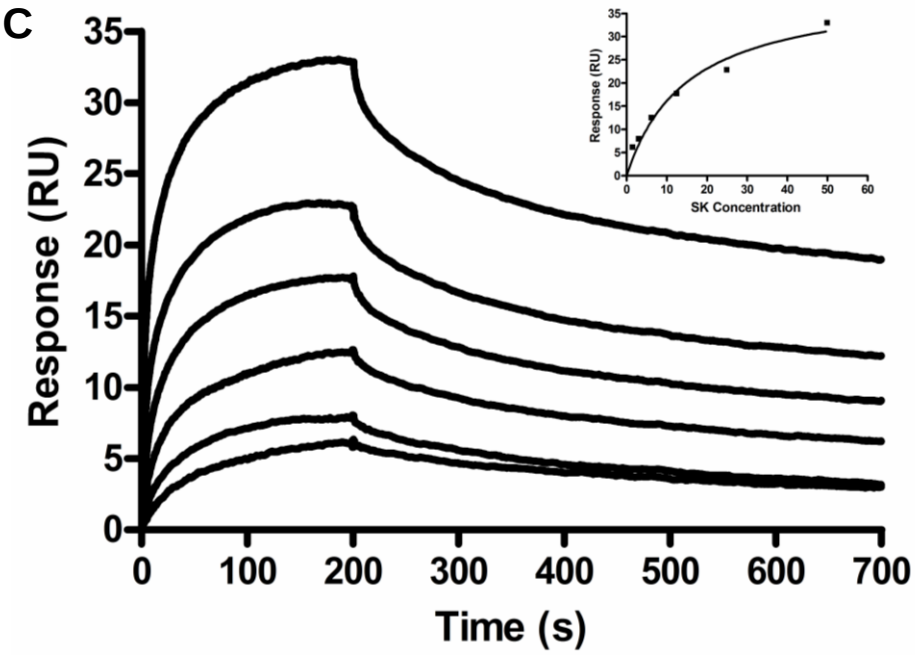
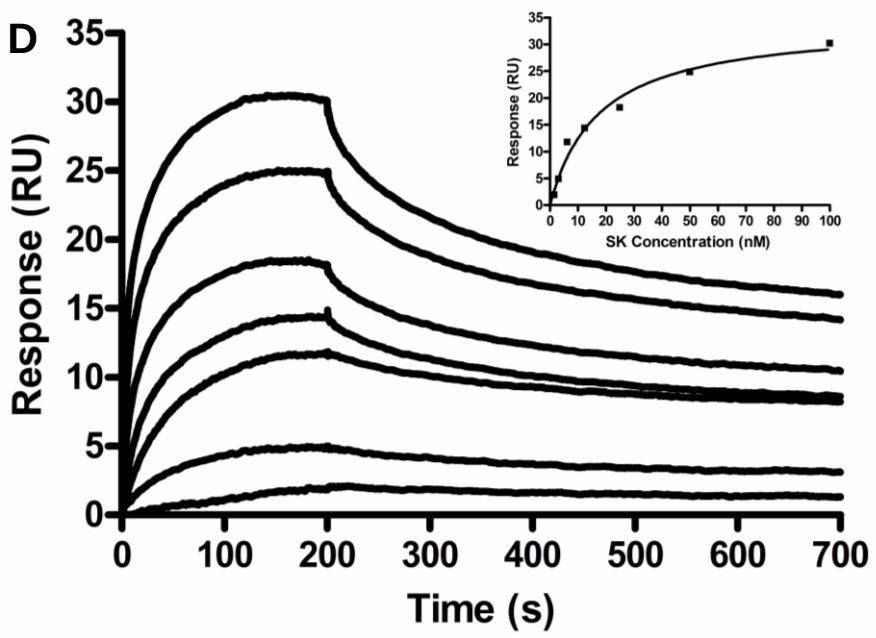


Figure 4.2.1 Surface plasmon resonance analysis of the interaction between SK variants and immobilised Glu-Plg. (**A**) Biosensorgram data for the interaction of 200 – 6.25 nM SKc;(**B**) Biosensorgram data for the interaction of 400 – 25 nM SK_{NZ131}; (**C**) Biosensorgram data for the interaction of 200 – 12.5 nM SK_{NS696}; (**D**) Biosensorgram data for the interaction of 200 – 12.5 nM SK_{5448}; (**E**) Biosensorgram data for the interaction of 1600 – 100 nM SK_{ALAB49} and (**F**) Interaction of 1600 – 100 nM $SK_{NS88.2}$. All panels: (top inset) [SK] dose dependent response of specific binding used for kinetic constant determination and (bottom inset) representative fits of the two-component heterogeneous surface model to the association portion of the biosensorgram data. The data presented here is representative of at least three experiments.


A
Response (RU)
Time (s)
SK Concentration (nM)


B
Response (RU)
Time (s)
SK Concentration (nM)


C
Response (RU)
Time (s)
SK Concentration


D
Response (RU)
Time (s)
SK Concentration (nM)

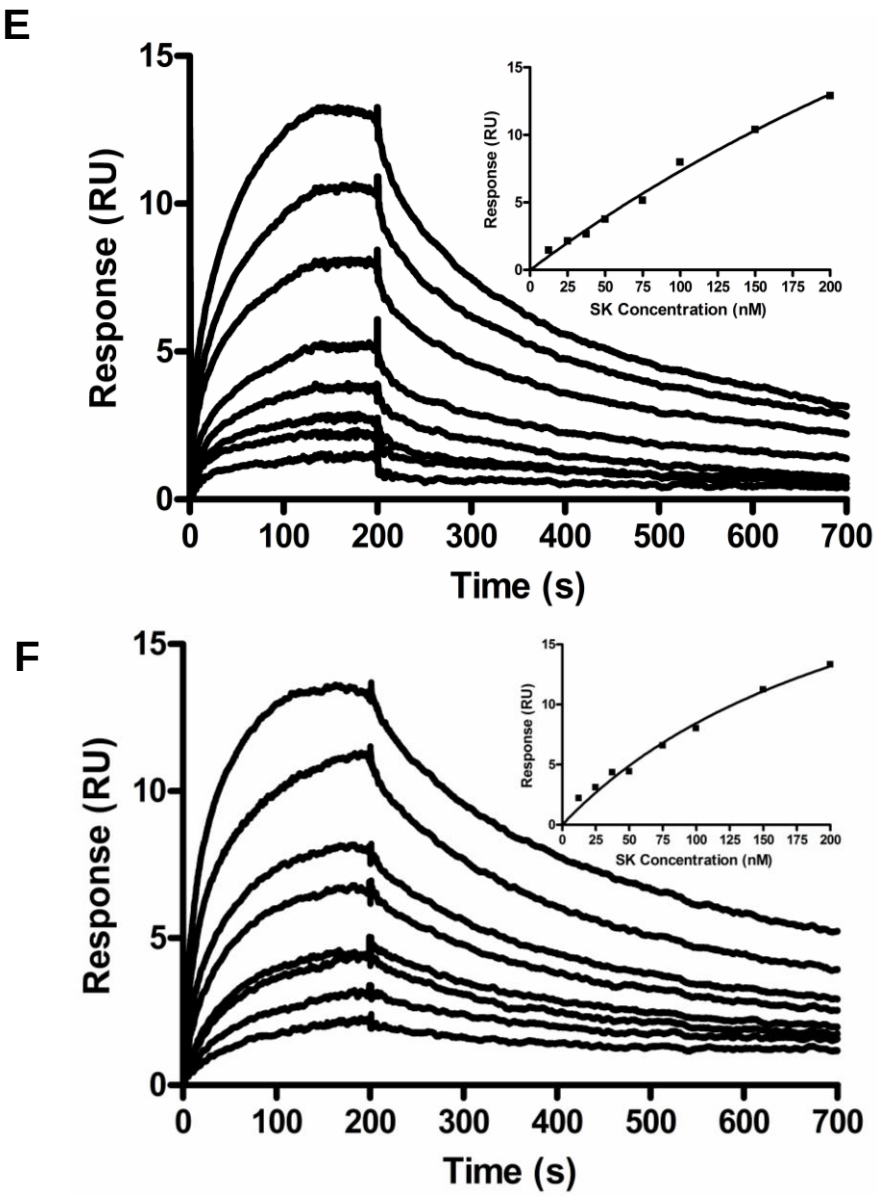


Figure. A4.2.2 Surface plasmon resonance analysis of the interaction between SK variants and immobilised plasmin. (**A**) Biosensorgram data for the interaction of 200 – 1.25 nM SKc; (**B**) Biosensorgram data for the interaction of 200 – 1.25 SK_{NZ131}; (**C**) Biosensorgram data for the interaction of 200 – 1.25 nM SK_{NS696}; (**D**) Biosensorgram data for the interaction of 200 – 1.25 nM SK_{5448} (**E**) Biosensorgram data for the interaction of 200 – 12.5 nM SK_{ALAB49}; (**F**) Interaction of 200 – 12.5 nM $SK_{NS88.2}$. All panels: (top inset) [SK] dose dependent response. The data presented here is representative of at least three experiments.

Appendix 5: Effect of Fibrin on SK Amidolytic Activity and Clot Lysis Capacity

A.5.1 Effect of fibrin on SK variant amidolytic activity

The figure presented here is supplemental to that in Chapter 4.2.5 for additional SK variants not included in that section of the publication.

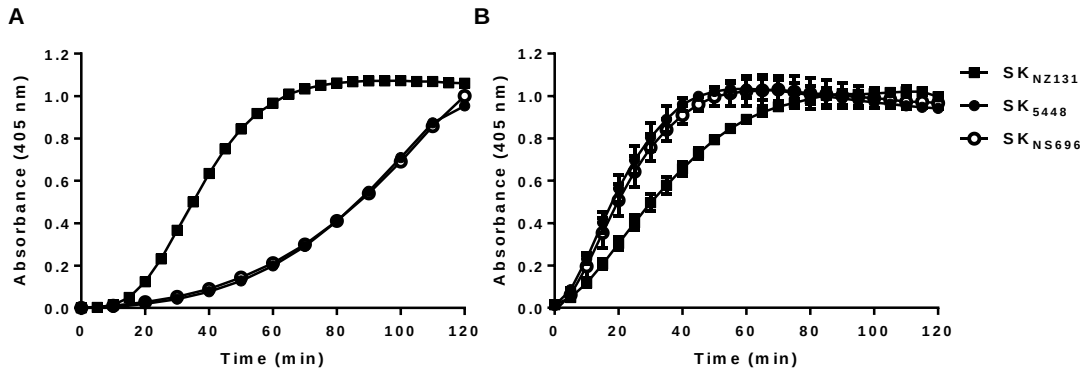


Figure A5.1 Generation of amidolytic activity and the effect of variant SK-Plg complexes formed in the presence of a fibrin clot (A) Complex formation was initiated by addition of variant SK (40 nM) to assay buffer (10 mM HEPES, 150 mM NaCl, 0.01% Tween-20, pH 7.4) containing Glu-Plg (20 nM) and chromogenic substrate S-2251 (500 µM) then monitored at $A_{405.}$ **(B)** Fibrin clots were formed for 4 h at RT in a 96-well plate by the addition of thrombin (0.8U/mL) and $CaCl_2$ (20 mM) to recombinant fibrinogen (3 µM). Plg (20 nM) was bound to the fibrin clots for 1 h at 37°C. Bound Plg was washed before complex formation was initiated and monitored at A_{405} by addition of variant SK (40 nM) to assay buffer (10 mM HEPES, 150 mM NaCl, 0.01% Tween-20, pH 7.4) chromogenic substrate S-2251 (500 µM) at 37°C.

A.5.2 Plasma clot lysis capacity of SK variants

The figure presented here is supplemental to that in Chapter 4.2.6 for additional SK variants not included in that section of the publication.

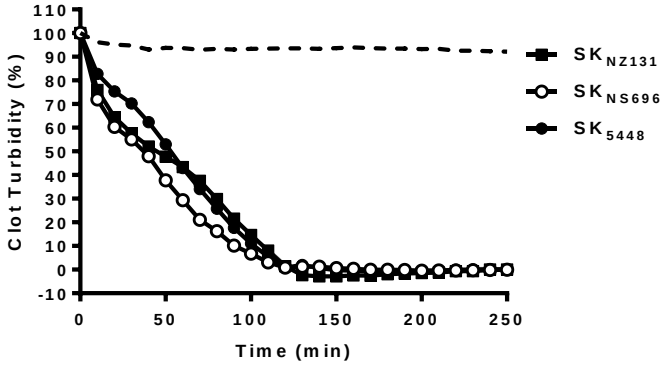


Figure A5.2. Induction of plasma clot lysis by SK variants. Fibrin clots were formed in pooled EDTA treated human plasma by the addition of thrombin (0.8U/mL) and $CaCl_2$ (20 mM). Variant SK molecules were then suspended in fresh plasma to a final concentration of 50 nM over the plasma clot and lysis was monitored by measuring a reduction in turbidity at 340 nm at 37°C

Appendix 6: Buffers, Media and Solutions

Growth and Nutrient Media

Luria Bertani (LB) Broth

Bactotryptone	10 g/L
Yeast Extract	5 g/L
NaCl	10 g/L

LB Agar

Bactotryptone	10 g/L
Yeast Extract	5 g/L
NaCl	10 g/L
Agar	15 g/L

Todd-Hewitt Broth (THY)

Todd-Hewitt Broth powder	30 g/L
Yeast Extract	10 g/L

THY Agar

THY	1L
Agar	15 g/L

***E.coli* Glycerolisation Solution**

Casamino acids	1% (w/v)
Glycerol	10% (v/v)

Agarose Gel Electrophoresis

1X TAE Buffer

Tris-base	40 mM
Glacial Acetic acid	20 mM
EDTA (pH 8.0)	1 mM

0.85% Agarose Gel

Agarose	0.85% (w/v)
Tris-base	40 mM
EDTA	1 mM
Glacial Acetic acid	5.71 %(v/v)

Agarose Loading Buffer

Bromophenol blue	0.005% (w/v)
Glycerol	75% (v/v)

TE Buffer	25% (v/v)

Ethidium Bromide Solution

Ethidium bromide	1 μg/mL
dH_20	

SDS-PAGE Gel Electrophoresis

2 X Cracking Buffer

Tris.HCl	90 mM
Bromophenol blue	0.02% (w/v)
Glycerol	20% (v/v)
SDS	2% (w/v)
β-mercaptoethanol	1% (v/v)

10% Resolving Gel

Bis-acrylamide (37.5:1)	10% (w/v)
Tris.HCl 1.5 M (pH 8.8)	25% (v/v)
SDS	0.1% (w/v)
TEMED (add last)	0.05% (v/v)
Ammonium Persulphate (add last)	0.015% (w/v)

4% Stacking Gel

Bis-acrylamide (37.5:1) 4%w/v)	
Tris-HCl 0.5 M (pH 6.8)	25% (v/v)
SDS	0.1% (w/v)
TEMED (add last)	0.5% (v/v)
Ammonium Persulfate (add last) (w/v)	0.015%

1X SDS-PAGE running buffer

Tris-base	50 mM
Glycine	196 mM
SDS	0.1% (w/v)
pH 8.3	

Coomassie Blue Stain

Coomassie Brilliant Blue R-250	0.2% (w/v)
Methanol	40% (v/v)
Glacial Acetic acid	10% (v/v)

Rapid Destain

Methanol	40% (v/v)
Glacial Acetic acid	10% (v/v)

Wizard® *Plus* SV Minipreps DNA Purification

Cell resuspension solution

Tris.HCl	50 mM
EDTA	10 mM
RNase A	100 μg/mL
pH 7.5	

Cell lysis solution

NaOH	200 mM
SDS	1% (w/v)

Neutralisation solution

Guanidine Hydrochloride	4.09 M
Potassium Acetate	0.759 M
Glacial Acetic acid	2.12 M

Column wash solution

Aotassium Acetate	60 mM
Tris-HCl (pH 7.5)	8.3 mM
EDTA (pH 8.0)	0.04 mM
Ethanol	60% (v/v)

Alkaline Lysis DNA Purification

Solution 1

Glucose	50 mM
Tris-HCl	25 mM
EDTA	10 mM
RNase A	0.1 mg/mL

Solution 2

NaOH	0.2 M
SDS	1% (v/v)

Solution 3

Potassium Acetate	3M
Glacial Acetic acid	11.5% (v/v)

Qiagen-100 Tip Column Buffers

Wash Buffer

NaCl	1.0 M

Isopropanol	15% (v/v)
pH 7	

Elution Buffer

NaCl	1.25 M
Tris.HCl (pH 8.5)	50 mM
Isopropanol	15% (v/v)

Native Ni^{2+}-NTA Purification

Native Lysis Buffer

Lysozyme	1 mg/mL
PMSF	0.1 mM
$MgCl_2$	10 mM
DNase I	5 μg/mL
Triton X-100	0.1% (v/v)
Imidazole	10 mM
NaH_2P0_4	50 mM
NaCl	300 mM
pH 7.4	

Binding Buffer

NaH_2P0_4	50 mM
NaCl	300 mM
Imidazole	10 mM
pH 8	

Wash Buffer

NaH_2P0_4	50 mM
NaCl	300 mM
Imidazole	20 mM
pH 8	

Elution Buffer

NaH_2PO_4	50 mM
NaCl	300 mM
Imidazole	250 mM
pH 8.0	

Western Blot Analysis

Western Transfer Buffer

Methanol	20% (v/v)
Tris-base	23 mM
Glycine	192 mM

SDS	0.001% (v/v)

PBST

NaCl	137 mM
Na_2HPO_4	4.3 mM
KCl	2.7 mM
KH_2PO_4	1.4 mM
Tween-20	0.05% (v/v)
pH 7.4	

TBST

Tris.Cl	20 mM
NaCl	500 mM
Tween-20	0.05% (v/v)

Blocking Buffer

Skim milk powder	5% (w/v)
Made up in PBST	

Dilution Buffer

Skim milk powder	0.5% (w/v)
Made up in PBST	

DAB developing solution

Diaminobenzidine	0.5 mg/mL
Tris.Cl	100 mM
H_2O_2	0.05% (v/v)
pH 7.4	

Factor Xa Cleavage

Dialysis Buffer

Tris.Cl	25 mM
NaCl	500 mM
pH 8.0	

QB Buffer

Tris.HCl	50 mM
NaCl	50 mM
$CaCl_2$	1 mM
pH 6.5	

Anion Exchange Chromatography

Buffer A

Bis-Tris	20 mM
pH 6.6	

Buffer B

Bis-Tris	20 mM
NaCl	1M
pH 6.6	

ESI-MS

ESI-MS Sample Buffer

Ammonium Acetate	10 mM
Formic acid	0.1%
pH 6.8	

Biochemical Assay Buffers

MUGB Assay Buffer

Tris-HCl	50 mM
NaCl	100 mM
pH 7.4	

S-2251 Assay Buffer

HEPES	10 mM
NaCl	150 mM
Tween-20	0.01%
pH 7.4	

BIAcore Assays

Activation Solution

N-ethyl-*N*'-(3-dimethylaminopropyl)carbodiimide	100 mM
N-hydroxysuccimide	25 mM

Running Buffer

HEPES	10 mM
NaCl	150 mM
P-20	0.01%
pH 7.4	

Regeneration Buffer

HEPES	10 mM
NaCl	150 mM

P-20	0.01%
Urea	6 M

Hyaluronic Acid Capsule Assays

Stains-All Solution

Stains-All	20 mg
Galacial Acetic acid	60 µL
50% Formamide	100 mL

Plasma Fibrinogen Depletion Assay

Precipitation Solution

Sodium Sulphite	10.5% (w/v)

Resuspension Solution

Urea	4 M
NaOH	0.1 N

General Buffers

PBS

NaCl	137 mM
Na_2HPO_4	4.3 mM
KCl	2.7 mM
KH_2PO_4	1.4 mM
pH 7.4	

TBS

Tris.HCl	10 mM
NaCl	350 mM
pH 7.4	

TE Buffer

Tris-HCl	10 mM
EDTA (pH 8.0)	1 mM

Phosphate buffer (10 mM)

Na_2HPO_4	0.336 g/L
NaH_2PO_4	1.02 g/L

Appendix 7: Plasmids Used in this Study

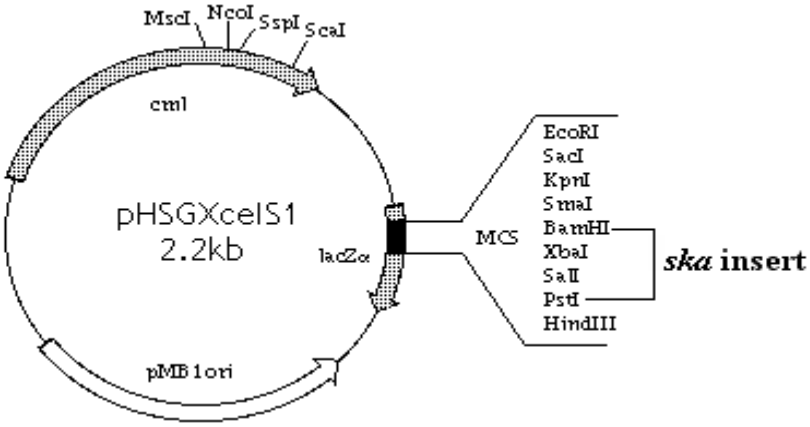


Figure A7.1. Plasmid map of pHSGXceIS1, **an augmented form of pHSG398 that has had *Nsp*I restriction sites removed.** *Nsp*I site were removed from the plasmid backbone by restriction digest with *NspI* (Fermentas, Pittsburgh, PA, USA) followed by treating the DNA preparation with S1 nuclease (Thermo Fischer Scientific, Scoresby, VIC, Australia) to remove single strand over-hangs. The plasmid was then re-ligated with T4 ligase (Fermentas, Pittsburgh, PA, USA) resulting in deletion of *Nsp*I sites. This vector was used for construction of the chimeric *ska* gene in Chapter 5.

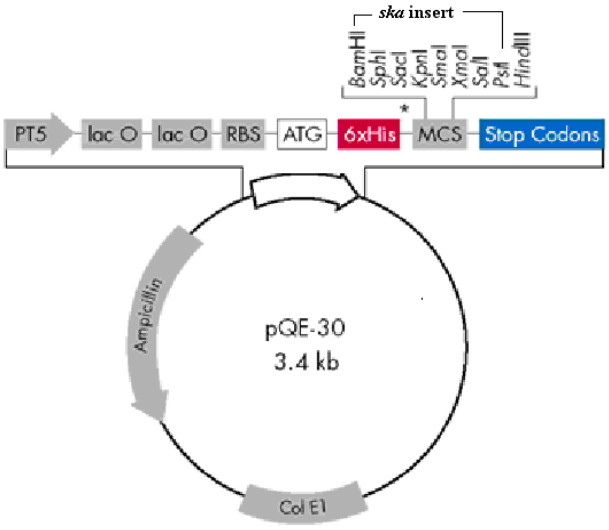


Figure A7.2: Plasmid map of pQE-30 expression vector. PCR primers were designed to incorporate *Bam*HI and *Pst*I restriction sites at the 5' and 3' ends of the amplified *ska* genes used in this study. This allowed cloning into pQE-30 (Qiagen, Valencia, CA, USA) for expression of recombinant SK as a poly-histidine tagged fusion protein.

Appendix 8: Construction of DNA Standard Curve

Using molecular weight markers, standard curves were constructed to allow for the estimation of the size of DNA bands observed on agarose gels. The standard curve constructed based on the markers in Figure A.1.1.1 is shown below (Table A8.1 and Figure A6.1).

Table A8.1: Mobility of DNA markers GeneRuler™ DNA Ladder (Fermentas, Pittsburgh, PA, USA) used for construction of a standard curve (Figure A8.1). The data here is based on the markers present on the gel in Figure A1.1.1

Molecular Weight (kb)	$\log_{10}$ Molecular Weight	Mobility (mm)
10	1	3
8	0.903	4.5
6	0.778	11
5	0.699	14.5
4	0.602	18
3.5	0.544	20.5
3	0.477	23
2.5	0.398	26.5
2	0.301	31
1.5	0.176	36
1	0	43
0.75	-0.125	47
0.5	-0.301	52
0.25	-0.602	58

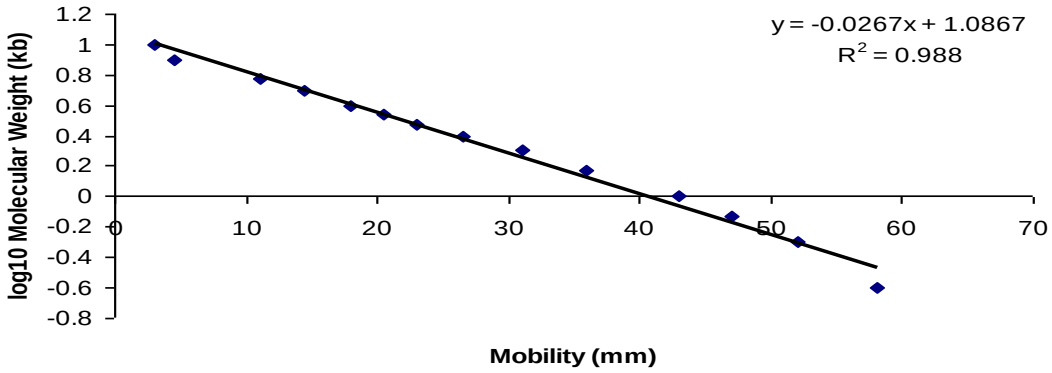


Figure A8.1: Standard curve constructed using GeneRuler™ DNA Ladder (Fermentas, Pittsburgh, PA, USA) from the gel in Figure A.1.1.1

Appendix 9: Construction of Protein Standard Curve

Using molecular weight markers, standard curves were constructed to allow for the estimation of the size of proteins observed on SDS-PAGE gels. The standard curve constructed using the molecular weight markers in Figure 2.4.1B is shown below.

Table A9.1: Mobility of PageRuler™ Unstained Protein Ladder used for construction of a standard curve (Figure A9.1). The data here is based on the markers present on the gel in Figure 2.4.1B

Molecular Weight (kDa)	$\log_{10}$ Molecular Weight	Mobility (mm)
250	2.3979	10
130	2.1139	14
100	2	21
70	1.8451	27
55	1.7403	38
35	1.5440	47
27	1.4314	58

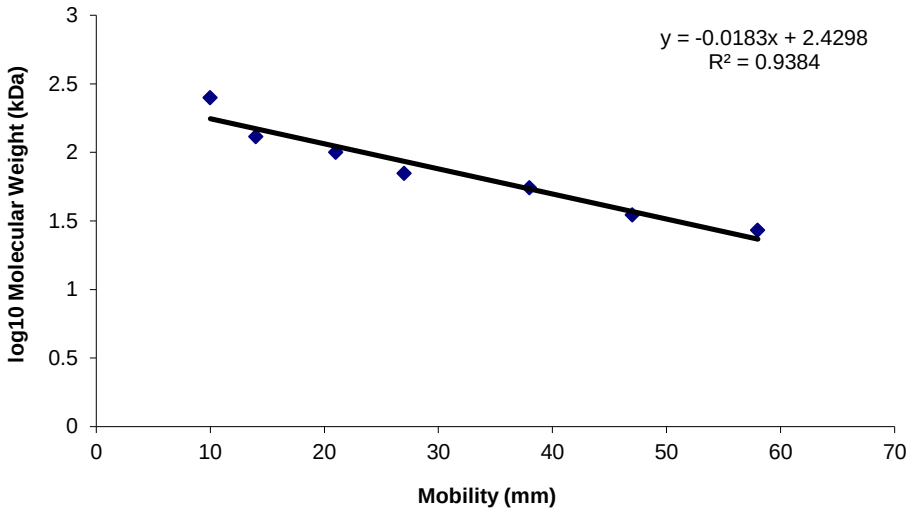


Figure A9.1: Standard curve constructed of PageRuler™ Unstained Protein Ladder (Fermentas, Pittsburgh, PA, USA) from the gel in Figure 2.4.1B

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