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

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

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Declaration of Authenticity

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

Martina Louise Sanderson-Smith

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

Bertrand Russell 1872-1970

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List of Abbreviations

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

List of Publications and Conference Presentations

Publications [*]

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Summary

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

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

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

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

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

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

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

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

1 Introduction

1.1 Overview

The Gram positive bacterium Group A Streptococcus (*Streptococcus pyogenes*; GAS) is a major etiological agent of skin and mucosal infections in humans. A historical perspective of the nature and classification of GAS and GAS disease is given below. A key feature of certain GAS is the ability to invade from cutaneous and mucosal surfaces to deep tissue sites, resulting in serious invasive infections. Numerous reports have indicated a resurgence in GAS invasive infection, which has yet to be fully explained. One hypothesis involves the interaction of GAS with the host plasminogen activation system, the main enzyme of which has the ability to cleave connective tissue and the extra-cellular matrix (ECM). The plasminogen activation system is subject to tight regulation by a number of plasminogen activators (uPA and tPA) and inhibitors (PAI-1, PAI-2, PN-1, α_2-antiplasmin and α_2-macroglobulin). Subversion of this system by GAS may have grave pathological consequences. The plasminogen-binding group A streptococcal M-like protein (PAM) is a cell surface protein which may facilitate the subversion of the plasminogen activation system by GAS, and as such, is the main focus of this thesis.

1.2 Classification of group A streptococcus

1.2.1 Lancefield classification

The Lancefield system provides a serological basis for the classification of the Genus *Streptococcus*. Grouping of *Streptococcus* is based on the presence of a specific carbohydrate in the cell wall, which is identified via a precipitin test using antisera (Lancefield 1962). This system identifies several groups, designated A through to O (excluding I and J). Group A, B and D streptococci are the most important in terms of

human pathogenesis. Group B streptococci include *Streptococcus agalatiae* and *S. mutans*, while *S. pneumoniae* and *Enterococcus faecalis* are examples of group D streptococci. The Lancefield group A consists solely of *S. pyogenes* (Lancefield 1962). Further differentiation of *S. pyogenes* can be achieved using both serological techniques such as M-typing, and molecular techniques such as *vir*-typing and *emm*-sequence typing which enable distinction of individual strains within the group (Lancefield 1962; Beall *et al.* 1996; Gardiner *et al.* 1997).

1.2.2 M-typing

M proteins (coded for by *emm* genes) are elongated alpha-helical coiled-coil proteins that form a fibrillar layer on the surface of GAS. The extensive antigenic heterogeneity of M protein fibrils provides the basis for a serological typing scheme involving M protein antisera (Fischetti 1989). Using this technique, more than one hundred different M-types have been identified.

While serological typing based on M protein profile is one of the most commonly and widely used modes of classifying GAS, a number of GAS isolates are unable to be classified this way due to a lack of reactivity with reference M-antisera. This is believed to result from the genetic heterogeneity of the *emm* genes encoded by these strains (Cunningham 2000). More extensive and discriminatory modes of classification known as *vir*-typing and *emm*-sequence typing have been developed to overcome this problem.

1.2.3 Vir-typing

Vir-typing is based on long polymerase chain reaction (PCR) and restriction fragment length polymorphism analysis of a 4-7 kb region of the GAS genome. Known as the *vir* or *mga* regulon, this region encodes one or more genes of the *emm* family arranged in

tandem, and other genes encoding putative GAS virulence factors (Gardiner and Sriprakash 1996). *Vir*-typing was developed primarily to enable the classification of M-non-typeable strains of GAS (Gardiner *et al.* 1998). This mode of classification overcomes the limitations of other typing methods, as it is applicable to all GAS isolates, highly discriminatory and reproducible. This has allowed the study of isolates from infected individuals that could not previously be classified, particularly in the Northern Territory of Australia (Gardiner and Sriprakash 1996; Gardiner *et al.* 1997). The heterogeneity demonstrated by *vir*-typing appears to be mediated by variation in the *emm* gene, rather than structural diversity in the *vir* regulon (Gardiner *et al.* 1998).

1.2.4 Emm-sequence typing

The basis of *emm*-sequence typing is nucleotide sequence analysis of the M protein gene (*emm*). Compensatory frameshift mutations, insertion and deletion events and intergenic and intragenic recombination have all been found to contribute to M protein variation. This variation is the most likely reason for cross-reactivity of certain GAS with reference M-antisera, and leads to the finding that numerous strains of GAS are M-non-typeable (Relf *et al.* 1994). Similarities between various *emm* genes in the hypervariable repeat region has led to the classification of strains into families of *emm* sequence types (Relf *et al.* 1992). Sequence types are proposed on the basis of distinct sequence differences in the 5' hypervariable region of *emm* genes. Homology of at least 95% within 160 nucleotides must be shown by isolates in order for them to be considered the same *emm*-sequence type (Beall 2003). Both the *vir*-typing and *emm*-sequence typing modes of classification are highly concordant (Gardiner *et al.* 1998).

1.2.5 Emm-patterning

M-like protein genes are linked and highly homologous to the M protein genes (*emm* genes). Most GAS strains have one of five *emm* patterns (designated A through to E), which are based on the number of *emm* and *emm*-like genes, their sub-family content and their relative arrangements on the chromosome (Figure 1.1). For strains with multiple *emm* or *emm*-like genes, the centrally positioned gene is used to determine the *emm* sequence type of the strain (Beall *et al.* 1996; Svensson *et al.* 1999).

Emm-related genetic markers for throat and skin isolates have also been identified. *Emm* patterns A-C and D are regarded as genetic markers for the throat and skin respectively, as the principle reservoir for GAS in the human host. However, this association has been found to be weaker in tropical regions such as the Northern Territory of Australia (Bessen *et al.* 1996; Bessen *et al.* 2000).

1.3 Group A streptococcal disease and epidemiology

1.3.1 Epidemiology

Group A streptococcal disease may manifest as uncomplicated infections such as impetigo and pharyngitis; invasive infections (in which *S. pyogenes* penetrate normally sterile sites) including necrotising fasciitis, bacteraemia, and streptococcal toxic shock syndrome (STSS); or immunologically mediated post-infection sequelae such as acute rheumatic fever (ARF) and glomerulonephritis. Asymptomatic colonisation of the throat also occurs which may be of importance for GAS persistence and transmission within communities, and act as an important niche for genetic recombination (Fischetti 1997).

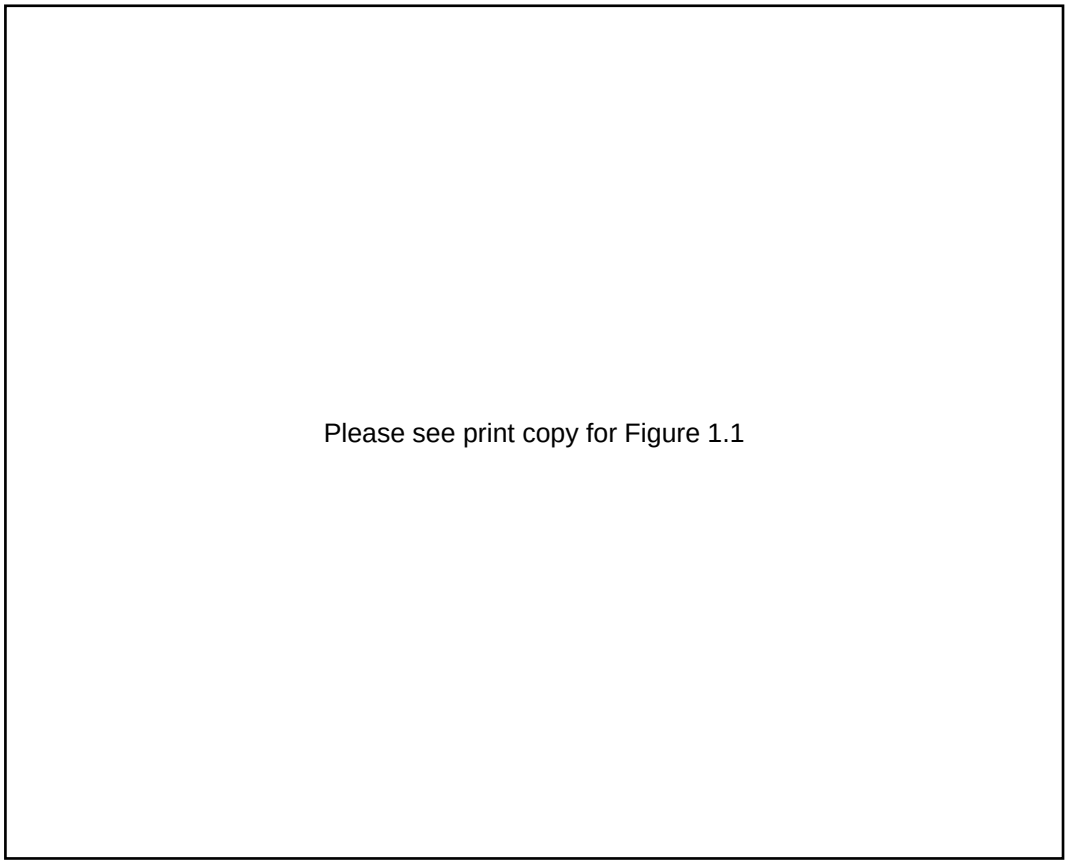


Figure 1.1 Arrangement of *emm* and *emm*-like genes in the GAS chromosome. Most GAS strains have one of five *emm* patterns (A-E), based on the sub family (SF) content of the *emm* gene region. The *emm* and *emm*-like genes are represented by four *emm* gene SF types that are based on nucleotide sequence differences in the 3'-end sequence (modified from Svensson et al., 1999).

The twentieth-century saw a decline in the prevalence of serious infection caused by GAS in the Western World, with the vast majority of GAS infections resulting in only simple, non-invasive diseases (Stevens *et al.* 1989). Proposed reasons for this decline include improved socio-economic conditions and timely treatment of streptococcal infections with antibiotics. A re-emergence of severe invasive GAS infection in industrialised countries in the mid 1980's highlighted the need to further classify and explain the virulence of this organism (Cone *et al.* 1987; Markowitz and Kaplan 1989; Stevens *et al.* 1989; Kaplan 1991).Recent estimates indicate that 18 million people worldwide suffer from serious GAS disease, which results in over half a million deaths annually Currently, approximately 1.78 million new cases of severe streptococcal infection emerge each year (Carapetis *et al.* 2005). Early reports indicated that invasive disease in the industrialised world was caused by a small number of "virulent clones", including serotypes M1, M3 and M18 (Forni *et al.* 1995). However, the high proportion of certain *emm* types in invasive disease often reflects their prevalence in non-invasive disease in the general population (Eriksson *et al.* 1998; Haukness *et al.* 2002; Johnson *et al.* 2002).

In developing nations, the epidemiology of GAS disease is less well-described, and GAS infection remains endemic in many areas (Carapetis *et al.* 1999). A diversity of *emm* types is often found in such regions and a high proportion of isolates are non-typeable by conventional serological methods (Bergner-Rabinowitz and Ferne 1978; Pruksakorn *et al.* 2000). This is also the case in the Northern Territory of Australia, a region with high incidences of impetigo (Gardiner and Sriprakash 1996) and bacteraemia (Carapetis *et al.* 1999) and one of the highest rates of rheumatic fever in the world (Carapetis and Currie 1997). Many aspects of the epidemiology of GAS infection in the Northern Territory are more akin to those of developing nations than industrialised nations. Firstly, the high

incidence of GAS infection in general (Gardiner and Sriprakash 1996; Gardiner *et al.* 1997) and invasive infection specifically (Carapetis *et al.* 1999; Towers 2002), is not correlated with specific M or *vir*-types. Secondly, the diversity and turnover rate of GAS strains reported is exceptionally high (Carapetis *et al.* 1999). Thirdly, streptococcal pharyngitis is rare (Carapetis *et al.* 1997) and invasive GAS infection is secondary to endemic skin infection (Carapetis *et al.* 1999) rather than being secondary to pharyngitis as described in more urbanised populations (Fiorentino *et al.* 1997; Stollerman 2001; Haukness *et al.* 2002). Similarly, ARF occurs in as many as 20 per 1000 residents in the Northern Territory (Carapetis *et al.* 1996) and thus might not be caused solely by throat-tropic isolates as is generally found in industrialised countries (Stollerman 2001).

The reasons behind the resurgence in GAS disease, and the putative global shift in GAS epidemiology have yet to be conclusively established, however possible contributing factors include a lack of host immunity to certain GAS serotypes, and the horizontal gene transfer of virulence factors between serotypes (Stevens, 2000).

1.3.2 Non-invasive diseases

Isolates found in pharyngeal or uncomplicated skin infection sites are considered to be non-invasive. Common non-invasive diseases include the skin disease impetigo and pharyngitis of the throat.

Impetigo lesions usually occur on exposed areas such as the face and extremities, and often precede the formation of suppurative sores. More deeply extending lesions that result in the formation of shallow ulcers in the epidermis are known as ecthyma (Bisno and Stevens 1996). Skin infections such as impetigo are endemic among the Aboriginal population of the Northern Territory, with up to 70% of children afflicted (Currie 1993).

Symptoms of pharyngitis include the sudden onset of sore throat, pain on swallowing, fever, headache, abdominal pain, nausea and vomiting (Bisno 2001). Pharyngitis is transmitted via respiratory droplets during close contact with patients, and as such, spreads rapidly through populations in confined areas. This accounts for the predominant occurrence of streptococcal pharyngitis in school-aged children (Bisno 2001).

1.3.3 Invasive diseases

Many GAS infections are characterised by their ability to cross host tissue barriers. Invasive GAS are defined as those strains isolated from a normally sterile site such as the blood or deep tissue (Eriksson *et al.* 1998). Severe invasive infections caused by GAS include necrotising fasciitis, bacteraemia and streptococcal toxic shock-like syndrome (STSS).

Necrotising fasciitis is a rapidly progressive destructive infection, which often leads to mortality or long-term morbidity. This disease is characterised by such symptoms as necrosis and inflammation of fascia and subcutaneous tissues (Kaul *et al.* 1997). Generally beginning at the site of an apparent trauma, patients with necrotising fasciitis will present with swelling, heat and tenderness within 24 h of the initial lesion. The rapid spread of these symptoms is followed by the formation of blisters and bullae containing clear, yellow fluid and necrosis of the subcutaneous tissue. Necrotising fasciitis also has severe systemic symptoms, including shock and organ failure (Bisno and Stevens 1996; Stevens 2000). Pre-disposing factors for necrotising fasciitis include muscle strain, childbirth, splinters, burns, blunt trauma, cuts and abrasions (Bisno and Stevens 1996).

Invasion of the bloodstream by bacteria, known as bacteraemia, is a severe streptococcal infection that can result in inflammation of the heart and abdominal cavity, as well as meningitis. Bacteraemia facilitates the spread of the pathogen to many other parts of the body and can lead to systemic illness, resulting in high fever, blood coagulation and eventually, organ failure (Bisno and Stevens 1996).

Perhaps the most severe form of GAS infection, patients with STSS suffer from severe acute hypotension and multi-organ failure (Basma *et al.* 1999). Adults between the ages of 20 and 50 years seem to be most susceptible to STSS (Weiss and Laverdiere 1997). Early symptoms of this disease depend on the site of primary infection, but can include malaise, chills, fever, nausea, vomiting and diarrhoea. Left untreated, STSS will result in persistent fever, extreme pain at the site of infection, severe shock and organ failure (Bisno and Stevens 1996; Stevens 2000). Certain host factors have been linked to increased susceptibility to STSS, in particular the disruption of mucosal and cutaneous barriers by viral infections, burns, lacerations and childbirth, which may provide GAS with access to normally sterile sites. However, no known route of entry can be identified for approximately 50% of STSS patients (Stevens 2000).

1.3.4 Post infection sequelae

Seemingly mild streptococcal infections occasionally lead to severe post-infection sequelae (a morbid condition or symptom following GAS disease). ARF and glomerulonephritis, both believed to be immunologically mediated, are sequelae that cause significant morbidity and mortality within the Aboriginal population of Australia (Carapetis *et al.* 1996).

ARF usually follows infection of the throat with certain serotypes of GAS, and expresses its most serious pathology in the heart (Pruksakorn *et al.* 1992). ARF appears to occur when individuals with an inherited immunological abnormality encounter GAS strains with rheumatogenic potential. The potential of certain strains to cause ARF may be related to the presence of antigenic epitopes cross-reactive with human tissue (Dale and Beachey 1985). Anti-streptococcal antibodies cross reactive with the heart, joint and brain have been found in the sera of ARF patients (Carapetis *et al.* 1996). Abnormal expression of self-antigens stimulated by GAS infection may also contribute to this condition. Subsequent destruction of tissues by the host's cellular immune system may be triggered by these events (Carapetis *et al.* 1996). This is evidenced by the finding that certain components associated with the cellular immune response such as T-lymphocytes and macrophage cells have been found to be associated with ARF affected tissues (Carapetis *et al.* 1996). Symptoms of ARF include arthritis (inflammation of the joints), carditis (inflammation of the heart) and chorea (inflammation of the central nervous system) (Denny 1994). Arthritis is the most common symptom, occurring in approximately 75% of patients. ARF associated arthritis involves the large joints, but does not result in permanent damage (Cunningham 2000). Carditis is the most severe manifestation of ARF, often resulting in serious injury to heart valves, and the onset of heart disease (Cunningham 2000). The rate of ARF among the Aboriginal population of Australia has been reported to be as high as 20 cases per 1000 people. The extremely low incidence of group A streptococcal throat infection among the same population contradicts the widely held belief that ARF occurs directly as a result of throat infection with GAS, suggesting that isolates responsible for skin infections may also have rheumatogenic potential (Carapetis *et al.* 1996).

Acute glomerulonephritis appears to follow impetigo infections, and primarily effects children between the ages of 2 and 10 (Hricik *et al.* 1998). This condition is usually characterised by hematuria, oliguria, acute renal failure and fluid retention (Hricik *et al.* 1998). However, occasionally sporadic and epidemic acute glomerulonephritis can occur with few or none of these manifestations (Yoshizawa *et al.* 1996). Deposits of IgG and the complement component C3 in the glomeruli of patients with glomerulonephritis suggest that immune complex formation may be involved in causing damage to renal tissue, but the mechanism involved is as yet unclear. It has been suggested that streptococcal antigens bind to the glomeruli, causing the host immune system to attack its own tissue (Hricik *et al.* 1998). Only certain strains of GAS have been identified as causative agents of glomerulonephritis. Some M-types commonly associated with impetigo have been found to be highly nephritogenic, such as M49 and M55 GAS strains (Bisno and Stevens 1996; Hricik *et al.* 1998). Isolates of the M1 serotype have also been implicated in a glomerulonephritis epidemic in the Northern Territory (Relf *et al.* 1992; Gardiner and Sriprakash 1996). Post-streptococcal glomerulonephritis is a reversible disease, with many patients recovering spontaneously. However, hypertension, recurrent proteinuria and chronic renal insufficiency can develop in some patients, causing long-term morbidity (Hricik *et al.* 1998).

1.3.5 Treatment of GAS disease

The preferred method for treatment of GAS disease is still the rapid administration of antibiotics. Despite regular use, GAS remains highly susceptible to treatment with penicillin, with erythromycin proving useful for patients with penicillin allergies (Weiss and Laverdiere 1997). Antibiotic treatment is most effective for non-invasive infections such as pharyngitis and impetigo, while combination therapy can often be effective in the treatment of more serious infections such as erysipelas and cellulitis (Weiss and

Laverdiere 1997; Stevens 2000). In serious cases of invasive disease such as necrotising fasciitis, surgical debridement and amputation may also be required (Stevens 2000). Antibiotic treatment of conditions such as STSS, ARF and glomerulonephritis is often complemented by treatment of the associated symptoms (Hricik *et al.* 1998; Stevens 2000).

To date, a successful vaccination strategy has not been developed against GAS. While numerous GAS proteins have been studied for their vaccine potential, strain to strain variation and cross reactivity of various epitopes with human tissue has hindered the development of a GAS vaccine (Cunningham 2000). The potential for GAS to cause serious disease and the unexplained resurgence in serious GAS infections indicates the need to develop a comprehensive understanding of those factors that may contribute to streptococcal virulence.

1.4 Group A Streptococcal virulence determinants

The initial stages of GAS pathogenesis involve adherence to, and colonisation of the epithelial cells of the skin and mucosal surfaces. Once an organism has colonised the host, mechanisms are required to adapt or adjust to this new environment, often requiring avoidance of the host immune response. For some isolates, colonisation is followed by tissue invasion, involving penetration of the skin or mucosal barriers. GAS express a multiplicity of both putative and confirmed virulence determinants associated with the various stages of GAS infection. A full discussion of these virulence factors is beyond the scope of this review, however, Table 1.1 outlines some of the characterised GAS virulence determinants and their potential role in pathogenesis. Several recent reviews also discuss in detail virulence factors associated with GAS pathogenesis

(Cunningham 2000; Bisno *et al.* 2003; Hynes 2004). However, the most widely characterised and diverse GAS virulence factor is the M protein. This review will focus on M protein and other virulence factors that interact with the host plasminogen activation system (PAS).

1.4.1 M protein

Coded for by the *emm* gene, M protein is the major surface protein and virulence factor of GAS. The *emm*-family of genes consists of *emm*, *mrp* and *enn* genes. GAS strains can contain one to three of these alleles in the *mga* regulon, which controls the expression of *emm*-family genes (Hollingshead *et al.* 1993). These genes are flanked by *mga* and *scpA* (Kehoe 1994). The *mga* gene encodes a 62 kDa protein that activates transcription via binding to a consensus site found in the promoters of the genes in this regulon, while the *scpA* gene encodes C5a peptidase (O'Connor and Cleary 1986; Perez-Casal *et al.* 1991; McIver *et al.* 1995). The organisation of the *mga* regulon varies between GAS strains, particularly in the number and type of *emm*-family genes present. However, the *mga* regulon is ubiquitous throughout GAS, and is regulated by a variety of environmental factors such as carbon dioxide levels (McIver *et al.* 1995; Navarre and Schneewind 1999). Genes under the control of the *mga* regulon are expressed during the exponential growth phase of GAS (McIver and Scott 1997).

Fibres of M protein on the GAS cell wall have been shown to consist of coiled-coil alpha-helical M protein molecules of between 50-60 nm in length (Fischetti 1989). The N-terminal of the M protein contains a signal sequence, which targets the protein for expression at the cell surface. This is followed by a hypervariable domain, and a series of repeats designated the a and b repeat domains. The highly conserved c and d repeat domains follow these variable repeats, while the C-terminal contains a conserved

region, which covalently links the protein to the GAS cell wall (Fischetti 1989). The number, and type of repeats varies significantly between different M proteins. M proteins are involved in multiple stages of GAS pathogenesis, including adhesion, internalisation, immune evasion and tissue invasion. Reports on the adhesive properties of M protein have been conflicting. While certain M proteins have been found not to mediate adhesion, M1 type GAS, which express the fibronectin binding protein M1, but no other fibronectin binding proteins, were found to attach to human lung epithelial cells. Furthermore, this attachment was deemed dependent on the presence of serum, plasma fibronectin or the ECM protein laminin, and the expression of protein M1 (Cue *et al.* 1998). Recently, inactivation of the *emm* 3 gene resulted in decreased adherence to human epithelial cell by M3 GAS, indicating that protein M3 also functions as an adhesin (Eyal *et al.* 2003). It is also believed that M protein may contribute to the aggregation of GAS at the site of attachment to the appropriate host tissue, conferring a selective advantage in colonisation (Caparon *et al.* 1991). The role of M protein in GAS adhesion to host cells may be dependent on both GAS serotype and the host cell type. This is supported by the findings of Shrager *et al*, who established that M6 protein mediates GAS adherence to skin epithelial cells, but not soft palate epithelial cells, and that M24 protein mediates adhesion to both cell types, while M18 protein does not (Schrager *et al.* 1998).

Table 1.1 Proposed GAS virulence determinants[A]

Virulence Determinant	Proposed Role In Infection	References
M Protein and the *emm* gene superfamily	Various functions including adhesion, immune evasion and multiple binding functions. Discussed in detail in text	(Fischetti 1989; Cunningham 2000; Bisno *et al.* 2003; Hynes 2004)
Plasminogen receptors streptococcal enolase, glyceraldehyde-3-phosphate dehydrogenase and streptokinase	Various functions including plasminogen binding and activation. Discussed in detail in text.	(Coleman and Benach 1999; Lahteenmaki *et al.* 2005; Walker *et al.* 2005)
Streptococcal pyrogenic exotoxins (Spes)	Superantigens. Interact with major histocompatibility complex class II antigens resulting in the concomitant release of cytokines.	(Hackett and Stevens 1992; Norrby-Teglund *et al.* 1994; Norrby-Teglund *et al.* 1994; Hynes 2004)
Cysteine protease (SpeB)	Cysteine protease involved in cleavage of host ECM proteins and activation of host metalloproteases and protease activators	(Kapur *et al.* 1993; Burns *et al.* 1998)
DNAses (A-D)	DNA degradation.	Reviewed in (Bisno *et al.* 2003)
Lipoteichoic Acid	ECM protein receptor. Putative Adhesin.	(Ofek *et al.* 1982)

Protein F/Sfb1	Fibronectin and fibrinogen binding function may promote the adherence to and invasion of host cells by GAS. IgG binding function may facilitate evasion of the host immune system.	(Hanski and Caparon 1992; Hanski *et al.* 1992; Molinari *et al.* 1997; Katerov *et al.* 1998; Medina *et al.* 1999)
FbaA, FbaB, Serum opacity factor (SOF), protein H	Fibronectin receptors. May promote the adherence to and invasion of host cells by GAS. SOF has also been shown to degrade eukaryote serum protein apolipoprotein A1.	(Courtney *et al.* 1994; Kreikemeyer *et al.* 1995; Terao *et al.* 2001; Courtney *et al.* 2002; Terao *et al.* 2002)
PrtF2, Pfbp, FBP54	Putative adhesins based on their interaction with the host ECM proteins fibronectin and fibrinogen.	(Courtney *et al.* 1996; Molinari *et al.* 1997; Rocha and Fischetti 1999)
Streptococcal collagen like surface proteins 1 and 2	Putative adhesins.	(Rasmussen *et al.* 2000; Lukomski *et al.* 2001)
Laminin binding protein	Putative adhesin based on interaction with ECM protein laminin.	(Terao *et al.* 2002)
SibA	Immunoglobulin binding protein. May facilitate immune evasion.	(Fagan *et al.* 2001)
Immunoglobulin G degrading enzymes (Ides, Mac)	Cleavage of surface bound IgG, leading to inhibition of phagocytosis and polymorphonuclear leukocyte activity.	(Lei *et al.* 2001)
Hyaluronic acid capsule	Confers resistance to phagocytosis *in vitro*, and may be involved in the persistence of GAS in throat epithelial cells. Binding to CD44 may facilitate invasion.	(Schrager *et al.* 1996; Moses *et al.* 1997; Ashbaugh *et al.* 2000)

Streptococcal C5a peptidase (ScpA), *S. pyogenes* cell envelope proteinase (SpyCEP)	Cleavage of complement component C5a and IL-8 respectively, facilitating the prevention of phagocytic activity.	(O'Connor and Cleary 1986; Edwards *et al.* 2005)
Streptococcal inhibitor of complement-mediated lysis (SIC)	Clusterin receptor. Activity may facilitate evasion of the host immune system.	(Akesson *et al.* 1996; Hartas and Sriprakash 1999; Hoe *et al.* 2002)
Streptolysins O and S	Lysis of erythrocytes and lung epithelia may facilitate destruction of host-tissue barriers.	(Ferretti *et al.* 1991)
HtrA protease	Promotes thermal stability and resistance to oxidative stress. Exact role in virulence unknown.	(Jones *et al.* 2001)
Heme-binding proteins	Iron sequestration.	(Lei *et al.* 2002)
Streptococcal protective antigen (Spa)	Required for full virulence of GAS isolate M18. Exact role in virulence unknown.	(McLellan *et al.* 2001)
Protein G-related alpha -2 macroglobulin - binding protein	Protease inhibitor. Regulation of protease activity at the GAS cell surface may facilitate survival in host.	(Rasmussen *et al.* 1999)

[A] Also see the following recent reviews: Cunningham, M. W. (2000). "Pathogenesis of group A streptococcal infections." Clin Microbiol Rev **13**(3): 470-511, Bisno, A. L., M. O. Brito, et al. (2003). "Molecular basis of group A streptococcal virulence." Lancet Infect Dis **3**(4): 191-200, Hynes, W. (2004). "Virulence factors of the group a streptococci and genes that regulate their expression." Front Biosci **9**: 3399-433.

Some M proteins have also been found to have fibrinogen and fibronectin binding properties, and are thought to play an important role in fibrinogen-mediated GAS adherence to human cells (Kehoe 1994). The fibronectin dependent binding of human complement factor CD46 by M protein appears to mediate adherence to keratinocytes (Rezcallah *et al.* 2005). Certain M proteins also interact with glycosaminoglycans. Although not well characterised, such an interaction may provide yet another mechanism via which M protein mediates GAS adhesion to host cells (Frick *et al.* 2003).

In addition to a role in GAS adhesion, proteins M1 and M3 have been shown to facilitate the internalisation of GAS by host cells (Hasty and Courtney 1996; Molinari *et al.* 1997; Cue *et al.* 1998; Eyal *et al.* 2003). While the exact role of GAS internalisation by host cells in pathogenesis is unclear, one hypothesis is that internalisation by host cells enables the bacterium to resist antibiotic treatment, thus allowing persistence of the pathogen in the host (Neeman *et al.* 1998).

In order to survive in the host, bacteria must be able to avoid clearance by the immune system. M proteins are highly variable, and provide GAS with a type specific capacity to resist phagocytosis. The primary immune response to infection by GAS involves antibodies that recognise epitopes on M proteins, thus providing protection against further infection. However, this immunity is type specific, therefore protection against one M-serotype does not confer immunity to other serotypes (Lancefield 1962). M proteins can exhibit altered antigenic epitopes due to size variation and recombination between repeat sequences in the N-terminus (Fischetti *et al.* 1985; Hollingshead *et al.* 1986), thus varying an important antigenic target of the immune response. For certain M

types, protective immunity is strain-specific, rather than type-specific. This occurs as a result of genetically distinct subpopulations existing within individual M types (Musser *et al.* 1995; Penney *et al.* 1995).

M proteins have also been shown to provide GAS with resistance to phagocytosis by host immune cells. This ability has been attributed to various binding properties of the M protein. Fibrinogen binding by M protein is believed to mask receptors on the bacteria from the complement component C3b, thereby preventing ploymorphonuclear leukocyte recognition of GAS (Whitnack and Beachey 1985). M protein is also able to bind the complement control factor H, which binds to the c-repeat domain, and a factor H-like protein (FHL-1), which binds to the hypervariable domain of M protein (Johnsson *et al.* 1998). Factor H down-regulates the production of C3b (Kehoe 1994), hence, M proteins block the interaction of the C3b component of the complement system with polymorphonuclear leukocyte receptors, thus conferring antiphagocytic properties on GAS (Cleary *et al.* 1991). However, it should be noted that factor H binding by M protein does not appear to occur under physiologically relevant conditions, and certain types of GAS are able to resist phagocytosis independently of factor H (Perez-Caballero *et al.* 2000; Kotarsky *et al.* 2001).

Other complement factors with which M protein has been associated include the complement regulator C4b-binding protein and complement receptors CD11b and CD18. The C4b-binding protein binds to the hypervariable domain of certain M proteins, including M22, thus impeding complement mediated phagocytosis (Berggard *et al.* 2001; Carlsson *et al.* 2003). Additionally, the association between GAS and

human neutrophils has been shown to be inhibited as a result of interference with the receptors CD11b and CD18 by M5 protein (Weineisen *et al.* 2004).

The binding of immunoglobulins by GAS is thought to be a major method by which the bacteria evade the host defence system. There are numerous immunoglobulin-binding proteins expressed by GAS, comprising both M and M-like proteins and non-M proteins. M and M-like proteins such as Arp4 and Mrp4 bind the Fc region of IgA and IgG respectively (O'Toole *et al.* 1992). The binding of immunoglobulins by GAS prevents the binding of antibodies to phagocytes, and hence phagocytosis of bacterial cells. Antibody binding at the cell surface may also function to prevent recognition of bacterial cells as foreign by the immune system, effectively hiding them from the host defences (Woolcock 1988). Interestingly, it has recently been shown that co-operation between two separate binding sites in M22, for C4b-binding protein and IgA, is responsible for the antiphagocytic properties of the M22 GAS strain (Carlsson *et al.* 2003). This further highlights the importance of M proteins multiple binding properties in GAS virulence.

M proteins have also been implicated in tissue invasion. The role of M protein in invasion may stem from the interaction of some M proteins with plasmin(ogen), which is involved in the lysis of fibrin clots and tissues, and may facilitate tissue destruction and dissemination (Ringdahl and Sjobring 2000).

1.5 The plasminogen activation system

Certain highly virulent strains of GAS have the ability to invade from cutaneous and mucosal surfaces to deep tissue sites (Lottenberg *et al.* 1994). It is thought that one

possible mechanism for tissue invasion involves the generation of plasmin at the GAS cell surface. The classical role of plasmin in humans is degradation of the protein fibrinogen. Due to its broad specificity, plasmin has also been implicated in a host of other functions (Plow *et al.* 1995).

The broad proteolytic activity of plasmin requires tight *in vivo* regulation of the plasminogen system. Uncontrolled activation or subversion of this system can have grave physiological consequences such as tumour metastasis (Plow *et al.* 1995). The subversion of the plasminogen activation system (PAS) by GAS may also be pathogenically relevant. It is therefore important to understand the interactions between *S. pyogenes* and the PAS.

1.5.1 Plasminogen and plasmin

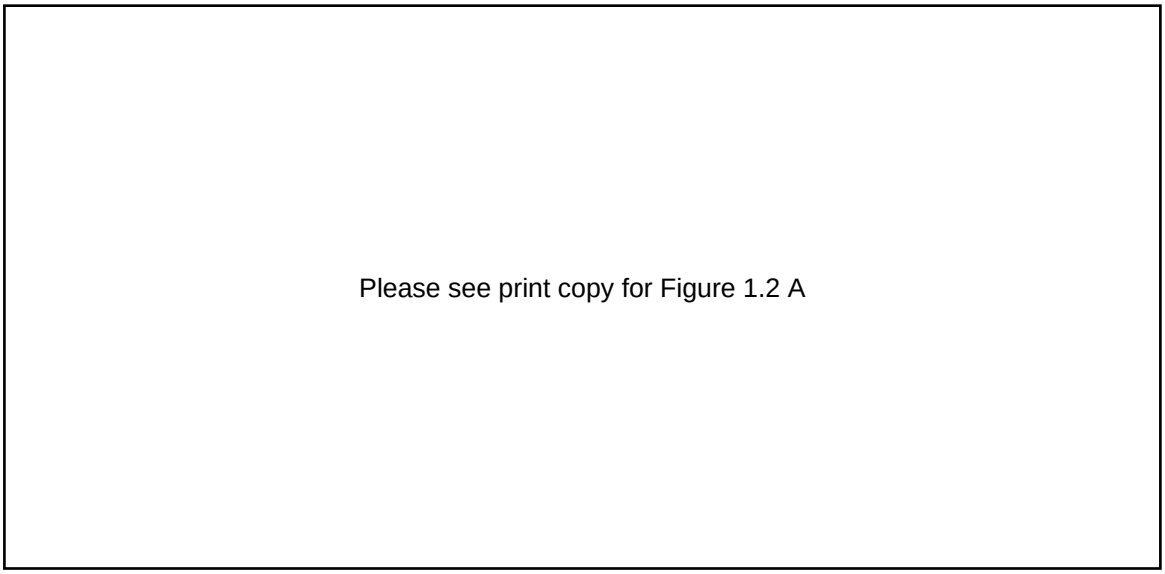
Human plasminogen is a single chain 92 kDa glycoprotein found in the plasma and extracellular fluids (Figure 1.2 A). Produced mainly by the liver, plasminogen is found in the blood at concentrations of approximately 2 μM (Andreasen *et al.* 1997). The circulating form of plasminogen is known as Glu-plasminogen as a consequence of the glutamic acid residue at the N terminus. Cleavage of the Lys^{76} – Lys^{77} peptide bond leaves the plasminogen molecule with a Lys residue at the-N terminus (Lys-plasminogen). Plasminogen is the inactive form of the enzyme plasmin, a serine protease formed by the cleavage of plasminogen at a single site (Arg^{560}-Val^{561}) by specific mammalian plasminogen activators (Dano *et al.* 1985; Ponting *et al.* 1992; Andreasen *et al.* 1997). The resulting two-chain plasmin molecule consists of a light chain containing the serine protease active site in the C-terminal region, and a heavy chain consisting of an N-terminal peptide (NTP), as well as five characteristic kringle domains in the N-terminal region (Ponting *et al.* 1992). The amino acid residues His^{603},

Asp^{646} and Ser^{741} make up the catalytic triad of the serine protease domain. This domain catalyses the hydrolysis of peptide bonds, resulting in peptides with arginine and lysine residues at the C-terminal (Ponting *et al.* 1992) . The kringle domains of plasmin(ogen) mediate interactions with multiple ligands, including fibrinogen and mammalian cellular plasmin(ogen) receptors (Ranson and Andronicos 2003). The kringles contain lysine-binding sites (LBS) comprised of a hydrophobic cleft formed by aromatic residues, that bind C-terminal lysine residues and internal lysine residues of receptors, thus mediating the majority of the interactions involving plasmin(ogen) and its many ligands (Dano *et al.* 1985; Ponting *et al.* 1992).

While the crystal structure of Glu-plasminogen has yet to be elucidated, a number of studies support the hypothesis that this molecule exists in a closed, spiral conformation (Figure 1.2 B). However, the binding of small lysine analogues to Glu-plasminogen, and its interaction with both C-terminal and internal lysine residues induces a conformational change resulting in a more open, U-shaped Glu-plasminogen (Christensen and Molgaard 1992; Weisel *et al.* 1994). Experimental evidence suggests that the LBS motifs of kringles 1, 4 and 5 maintain the closed conformation of Glu-plasminogen via intramolecular interactions with lysine residues in the NTP (McCance and Castellino 1995). Thus, the disruption of these intramolecular interactions by the binding of lysine residues results in a more open conformation, which also stabilises the structure of the protease domain, thus preserving its enzymatic activity (Ueshima *et al.* 1996). Lys-plasminogen lacks the N-terminal peptide and has a more open, U-shaped conformation than Glu-plasminogen. In addition, Lys-plasminogen is more readily activated to plasmin by the plasminogen activators (Claeys and Vermylen 1974; Weisel *et al.* 1994). This is characteristic of the more open conformation, as the conformational

change of Glu-plasminogen from closed to open also renders it more susceptible to activation (Mangel *et al.* 1990).

A



B

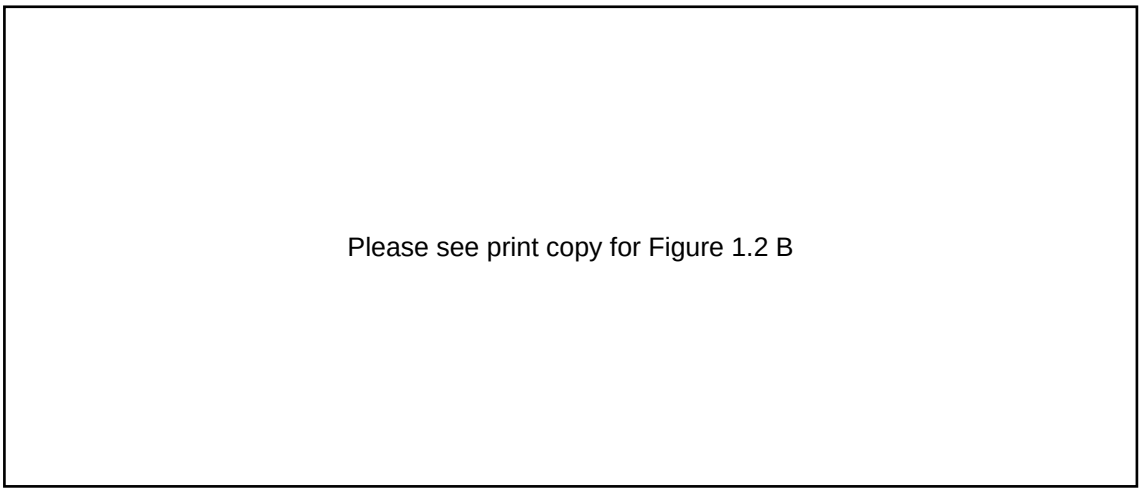


Figure 1.2 Schematic of the protein plasmin(ogen). **A** Cleavage of plasminogen forms plasmin, a two-chain molecule with serine protease activity. The light chain of plasmin contains the serine protease catalytic domain in the C-terminal region. The heavy chain consists of a 77 residue N-terminal peptide (NTP), and five kringle domains near the N-terminus. These kringles contain lysine-binding sites, and mediate the binding interactions of plasmin(ogen) with its many ligands (adapted from Andreasen *et al,* 1997). **B** The binding of glu-plasminogen to cell surface receptors induces a conformational change resulting in a more open, U-shaped Glu-plasminogen. The disruption of intramolecular interactions within plasminogen by the binding of lysine residues results in a more open, readily activatable conformation (adapted from Walker *et al*, 2005).

1.5.2 Physiological roles of the plasminogen activation system

The most well characterised function of plasmin is its role in fibrinolysis. The cleavage of fibrinogen by thrombin mediates its conversion to fibrin monomers. These monomers link spontaneously to form a fibrin network. Such a network makes the blood plasma at the site of formation less fluid, causing the blood to clot (Doolittle 1984). Fibrin formation plays important roles in protecting and repairing tissue and providing temporary connective tissue in hemostatic and inflammatory processes. These events are followed by the selective degradation of fibrin, catalysed by plasmin (Lewis *et al.* 1984). Another essential role of plasmin-mediated proteolysis in normal human physiology is the cleavage of ECM and basement membranes during processes such as wound repair, embryo implantation and inflammation (Dano *et al.* 1985; Saksela and Rifkin 1988; Andreasen *et al.* 1997; Plow 1999). ECM components such as fibronectin, laminin, vitronectin and proteoglycans are plasmin substrates; the activation of latent metalloproteases by plasmin allows cleavage of other components including collagen (Richardson *et al.* 1988; Gordon *et al.* 1993; Werb 1997). Cell surface proteolytic activity of plasmin can assist in the migration and proliferation of host cells, and surface-bound plasmin is protected from its inhibitors (Plow *et al.* 1995; Felez 1998). Plasminogen has also been implicated in cancer metastasis, mediating tumour cell invasion into surrounding normal tissue via plasmin-mediated breakdown of basement membranes and the ECM (Andreasen *et al.* 1997; Ranson and Andronicos 2003).

1.5.3 Regulation of the plasminogen activation system

There are two main host activators of plasminogen; tissue type plasminogen activator (tPA) and urokinase type plasminogen activator (uPA). Due to its high affinity for fibrin, tPA is primarily involved in fibrinolysis; while uPA, bound to its specific high affinity

cell surface receptor uPAR, mediates cell-associated proteolysis for degradation of tissue barriers (Dano *et al.* 1985). The synthesis of the plasminogen activators is tightly regulated by a myriad of effector molecules, including growth factors, peptide and steroid hormones and phorbol esters, each of which is, in turn, subject to its own regulatory system (Saksela and Rifkin 1988; Aguirre Ghiso *et al.* 1999). Thus, the PAS is subject to highly complex levels of control.

In addition to regulation of expression of the various PAS components, the broad proteolytic activity of plasmin necessitates tight *in vivo* regulation of the PAS. The primary inhibitors of the PAS are serine protease inhibitors (serpins) (Kruithof *et al.* 1995). Serpins prevent plasminogen activation by formation of 1:1 complexes with uPA or tPA; *in vivo* the most significant of these are plasminogen activator inhibitor 1 (PAI-1), plasminogen activator inhibitor 2 (PAI-2), and protease nexin 1. The second level of PAS regulation by serpins is plasmin inhibition primarily by α_2-antiplasmin; but also by α_2-macroglobulin if the concentration of the former is compromised either locally or systemically (Travis and Salvesen 1983; Saksela and Rifkin 1988). Binding of α_2-antiplasmin to plasmin is lysine-dependent (Wiman and Collen 1978; Longstaff and Gaffney 1992); thus plasmin bound to cell-surface receptors is resistant to inhibition by α_2-antiplasmin due to occupation of the kringle domains by cell surface lysine residues (Dano *et al.* 1985).

Cell-surface binding events also play a vital role in plasminogen activation. Plasminogen, plasmin, and the plasminogen activator tPA bind to both fibrin and the cell surface via lysine residues. Such a co-assembly brings plasminogen and its activators into close proximity, thus facilitating increased plasminogen activation (Plow *et al.* 1995). The

plasminogen activator uPA is also able to bind to the mammalian cell surface via a specific uPA receptor, uPAR, and remain catalytically active (Vassalli *et al.* 1985). The interaction between uPA and its receptor is believed to localise the activator activity to the cell surface, thus facilitating the migration of various cells through basement membranes and connective tissue (Saksela and Rifkin 1988). Plasmin binding to the mammalian cell surface initiates a variety of amplification loops. For example, once activated at the cell surface, plasmin-mediated proteolysis of membrane proteins may expose C-terminal lysine residues that function as additional plasminogen binding sites (Plow *et al.* 1995). In addition, lysine dependent cell surface binding disrupts the intramolecular plasminogen interactions that maintain a closed, activation resistant conformation of plasminogen. The resultant conformation is open, leaving the activation domain exposed, and thus more easily activated (Andronicos *et al.* 2000). Furthermore, the occupation of the kringle domains by cell surface lysine residues prevents the action of many plasminogen inhibitor molecules (Dano *et al.* 1985).

1.6 Interactions between the plasminogen activation system and GAS

Plasmin(ogen) receptors and activators have been found to be expressed by a large number of bacteria, including Group A, C and G streptococci, *Streptococcus pneumoniae*, *Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella enteritidis*. The potential of these bacteria to cause highly invasive infections, and the finding that such a wide variety of bacterial pathogens interact with the host PAS suggests that acquisition of plasmin(ogen) is a major factor in bacterial virulence (Sun *et al.* 2004; Lahteenmaki *et al.* 2005; Walker *et al.* 2005). Fibrin clots deposited as a result of infection can capture bacteria. Surface-associated plasmin activity can facilitate fibrinolysis, preventing clot

formation, or promote the release of bacteria from a formed clot (Wang *et al.* 1995; Coleman and Benach 1999). Furthermore, plasmin degradation of fibrinogen can initiate the release of products that effect blood vessel permeability and the accumulation of inflammatory cells (Dano *et al.* 1985; Castellino and Ploplis 2005). The direct degradation of ECM and basement membrane proteins, and the activation of matrix metalloproteases by plasmin may enable bacteria to break down host tissue barriers. This is evidenced by the repeated demonstration that plasmin coated bacteria are capable of penetrating ECM or basement membranes (Korhonen *et al.* 1992; Lahteenmaki *et al.* 1995; Eberhard *et al.* 1999). Plasmin may also provide organisms with the capacity to degrade immunoglobulins and complement proteins, thereby inhibiting the host immune response (Dano *et al.* 1985; Coleman and Benach 1999; Lahteenmaki *et al.* 2001). Subversion of the host PAS by pathogens may therefore have wide-ranging physiological consequences. Many strains of GAS are able to interact with the human PAS via both surface-associated and secreted proteins.

1.6.1 Streptokinase

Various prokaryotic plasminogen activators have been identified, including streptokinase from GAS and staphylokinase from *Staphylococcus aureus*. Streptokinase is secreted by group A, C and G streptococci. The activator activity of streptokinase from individual streptococcal isolates is often specific for plasminogen of the normal mammalian host, with inefficient activation of plasminogen from other mammalian species described (Marcum and Kline 1983; Wohl *et al.* 1983; Schroeder *et al.* 1999). The structure of streptokinase may play a role in determining the host range of streptococci. This impacts significantly on the design of animal models of GAS infection as, for example, murine plasminogen is resistant to activation by GAS streptokinase (Yakovlev *et al.* 1995). The streptokinase gene (*ska*) is found in all GAS M-type strains, however, the presence of *ska* in the GAS genome

does not necessarily result in the expression of functional streptokinase by all GAS isolates (Ferretti *et al.* 1991; Tewodros *et al.* 1995).

The streptokinase protein consists of 414 amino acids with three distinct domains designated α, β and γ based on structural and functional characteristics (Wang *et al.* 1998). Two highly variable regions within the β domain of the streptokinase protein have been identified, known as V1 (residues 174-244) and V2 (residues 270-290) (Huang *et al.* 1989; Kapur *et al.* 1995). Classification of variants of *ska* is based on sequence differences within the variable region (Johnston *et al.* 1992; Kalia and Bessen 2004) and over 80 variants have been described (Kapur *et al.* 1995; Kalia and Bessen 2004). Differences in the level of streptokinase production and antigenicity of the protein are found between genotypes. It is yet to be determined if this correlation between genotype and expression extends to the virulence of the isolate.

Unlike other plasminogen activators native to the human plasminogen system, streptokinase is not itself a protease. Rather, it binds plasminogen in a 1:1 stoichiometric ratio, inducing conformational changes to produce a complex possessing both plasminogen activator activity and plasmin activity (Wang *et al.* 1998; Parry *et al.* 2000). The binding of streptokinase to plasminogen exposes the plasminogen active site, allowing it to become functional. The active site of this complex is composed of the same catalytic triad as in the plasmin molecule without the requirement for cleavage of the Arg_{560}-Val_{561} bond of plasminogen (Cederholm-Williams *et al.* 1979; Wiman 1980; Wang *et al.* 1998). This conformational change is believed to involve the γ domain of streptokinase. A second plasminogen molecule then interacts with the binary complex, resulting in conversion of this second molecule to plasmin. This lysine-dependent step is thought to involve the

kringle domains of the plasminogen molecule (Cederholm-Williams *et al.* 1979; Wiman 1980; Young *et al.* 1998; Lin *et al.* 2000). Proteolytic activity of the streptokinase-plasminogen complex is not inhibited by α_2-antiplasmin, the inhibitory action of which requires access to plasmins' lysine binding sites (Cederholm-Williams *et al.* 1979; Wiman 1980). Furthermore, the streptokinase-plasminogen complex shows a high specificity for plasminogen, and is an extremely efficient activator of the zymogen (Parry *et al.* 2000).

Whilst the crystal structure of the catalytic unit of plasmin complexed with streptokinase has been solved (Wang *et al.* 1998), molecular models of activation of GAS receptor-bound plasminogen by streptokinase have not yet been elucidated in detail. It is known that the extended conformation of Glu-plasminogen, such as that induced by lysine-dependent binding to mammalian plasminogen receptors, has a higher affinity for streptokinase (Boxrud and Bock 2000). Thus plasminogen binding to GAS cell-surface receptors may facilitate formation of the initial plasminogen-streptokinase complex. Activation of GAS cell-surface receptor-bound plasminogen by streptokinase has also been demonstrated *in vitro* (Berge and Sjobring 1993; Ringdahl and Sjobring 2000).

1.6.2 Indirect plasminogen binding

Acquisition of plasmin activity by some GAS isolates requires human fibrinogen in addition to plasminogen and streptokinase (Figure 1.3). According to this model, fibrinogen bound to the GAS cell surface provides a target for the lysine-dependent binding of a pre-assembled plasminogen-streptokinase complex; and subsequent molecular rearrangements render the binding interaction lysine-independent (Wang *et al.* 1995). The trimolecular complex possesses plasmin activity, in addition to activating fluid phase plasminogen in the presence of host inhibitors (D'Costa and Boyle 1998). Thus, plasminogen acquisition via the fibrinogen-dependent pathway results in the creation of an

unregulatable surface protease and an immobilised plasminogen activator (Wang *et al.* 1995).

Neither streptokinase, nor plasminogen alone can bind to surface-bound fibrinogen, and streptokinase knockout mutants confirm a key role for streptokinase in the acquisition of plasminogen via the indirect pathway (Christner *et al.* 1997). Increasing concentrations of streptokinase, beyond those required to activate all plasminogen present, augment plasminogen binding to GAS in the presence of fibrinogen. This suggests a role for streptokinase, apart from plasminogen activation, in plasminogen binding by the indirect pathway (Wang *et al.* 1995).

Several GAS cell surface proteins have been shown to bind fibrinogen. These include M protein (Whitnack and Beachey 1985), fibronectin binding protein 54 (Courtney *et al.* 1994), Protein F1/SfbI (Katerov *et al.* 1998), and serum opacity factor (Courtney *et al.* 2002). Fibrinogen-binding capacity is the limiting factor in the ability of many GAS strains to acquire plasmin activity (Wang *et al.* 1995). Deletion of the fibrinogen-binding M protein in GAS isolates significantly reduces plasmin binding by the indirect pathway (Christner *et al.* 1997). The interaction between GAS, fibrinogen, streptokinase and plasminogen confers an exceptionally stable cell-associated enzymatic activity which can lyse fibrin clots despite the presence of the plasmin inhibitor α_2-antiplasmin (Wang *et al.* 1995). It has been demonstrated that GAS isolates associated with invasive infection bind significantly higher levels of plasminogen via the indirect pathway than isolates associated with non-invasive infection. Indirect acquisition of plasminogen by GAS may therefore play a key role in the establishment of invasive disease by this organism (McKay *et al.* 2004).

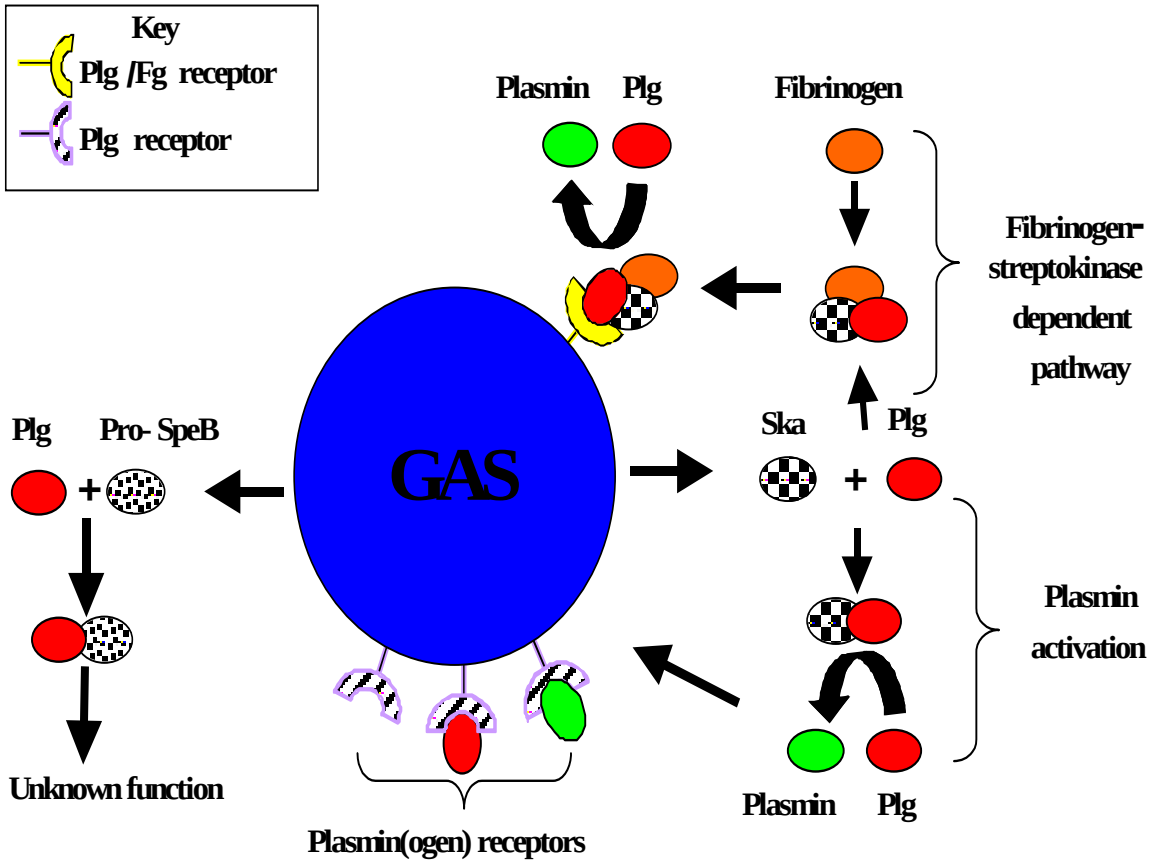


Figure 1.3 Proposed interactions of plasmin(ogen) with GAS plasminogen binding proteins. Abbreviations: Plg, plasminogen; Ska, streptokinase; Pro-SpeB, SpeB precursor. GAS interact with plasminogen both indirectly and directly. The indirect interaction is thought to involve a fibrinogen dependent pathway whereby a pre-assembled plasminogen-streptokinase complex binds to GAS cell surface receptor bound fibrinogen. Via the direct pathway, a GAS cell surface plasminogen receptor directly interacts with circulating plasmin(ogen) (adapted from (Sanderson-Smith *et al.* 2004).

1.6.3 Direct plasminogen binding

As well as the indirect plasmin(ogen)-binding mechanism, GAS can bind plasmin(ogen) directly (Figure 1.3). Plasmin retains its enzymatic activity when bound directly to GAS and may provide localised proteolytic capability (Lottenberg *et al.* 1987). Furthermore, plasminogen bound to GAS can be activated by tPA (Kuusela *et al.* 1992; Berge and Sjobring 1993; Pancholi and Fischetti 1998). Three different plasmin(ogen)-binding proteins expressed at the surface of GAS have been identified: these are streptococcal surface enolase (SEN) (Pancholi and Fischetti 1998), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Broder *et al.* 1991; Pancholi and Fischetti 1992), and the plasminogen–binding group A streptococcal M-like protein (PAM) (Berge and Sjobring 1993). A secreted plasminogen binding protein, the nephritogenic plasminogen-binding protein (NPBP) has also been reported (Poon-King *et al.* 1993).

The 45 kDa SEN is a glycolytic enzyme that functions as a plasmin(ogen) receptor when expressed at the surface of GAS (Pancholi and Fischetti 1998). With a higher affinity for Lys-plasminogen than Glu-plasminogen or plasmin, SEN is found on the surface of most streptococcal serotypes, and possesses more than one site for interaction with plasminogen and plasmin (Pancholi and Fischetti 1998; Derbise *et al.* 2004). These multiple binding sites may contribute to the strong plasmin(ogen)-binding activity of SEN. The high affinity for plasminogen is mediated in part by the presence of two lysine residues (Lys^{434} and Lys^{435}) at the C-terminus (Pancholi and Fischetti 1998) which mediate the interaction of SEN with Lys-plasminogen, and stabilise the conformation of SEN's plasminogen binding site (Derbise *et al.* 2004).

Plasmin and plasminogen bound to SEN are significantly (but not completely) resistant to inactivation by α_2-antiplasmin (Pancholi and Fischetti 1998). Enolase has also been identified as a plasmin(ogen)-binding molecule on the surface of several eukaryotic cell types (Redlitz *et al.* 1995; Ranson and Andronicos 2003). Plasminogen has been shown *in vitro* to act as a linker molecule between enolase at the surface of pharyngeal cells, and SEN at the surface of GAS, thus facilitating the adhesion process (Pancholi *et al.* 2003). When this bridging plasminogen molecule is activated by tPA to plasmin, it can digest intercellular junctions and disrupt cell monolayers in ECM models (Pancholi *et al.* 2003). Antibodies to SEN have been shown to opsonise streptococci and enhance phagocytosis, suggesting an involvement in non-type specific protection against GAS (Pancholi and Fischetti 1998). Anti-SEN antibodies have been found to cross react with human enolase, possibly contributing to the onset of immunologically mediated post-infection sequelae (Fontan *et al.* 2000).

GAPDH is a 41 kDa glycolytic enzyme found in a wide variety of M-types and located at the GAS cell surface (Lottenberg *et al.* 1992; Pancholi and Fischetti 1992). This plasmin receptor has a relatively low affinity for plasmin and Lys-plasminogen, with almost no affinity for the circulating Glu-plasminogen (Lottenberg *et al.* 1987). Plasmin is bound by means of the C-terminal Lys^{334} on GAPDH, and while bound to this receptor, is able to remain enzymatically active in the presence of α2-antiplasmin (Broder *et al.* 1991). In addition to its plasmin binding activity, GAS GAPDH exhibits an affinity for several other mammalian proteins. Interaction with fibronectin, cytoskeletal proteins and lysozyme suggests a potential role for GAPDH in GAS adherence. The interaction between GAPDH and lysozyme may be of particular importance in GAS colonisation of the throat, due to the high concentration of lysozyme in saliva (Pancholi and Fischetti 1992). Cell-surface

GAPDH is functionally and structurally homologous to the cytoplasmic form of GAS GAPDH. The mechanisms via which the glycolytic, cytoplasmic proteins GAPDH and SEN are transported to the GAS cell surface without the presence of a signal peptide are yet to be elucidated. However, there is evidence to suggest that glycolytic enzymes may anchor to the GAS surface via an interaction with M proteins (Winram and Lottenberg 1996; D'Costa *et al.* 2000).

NPBP is a 46 kDa secreted protein that binds to plasmin(ogen) in a lysine dependent manner and is the precursor of the streptococcal pyrogenic exotoxin B (speB), a major GAS virulence factor. The lysine-dependence of the NPBP-plasminogen interaction raises the possibility that, like the cell surface-bound receptors, plasmin bound to NPBP is protected from α_2-antiplasmin (Poon-King *et al.* 1993), which also binds initially via the lysine-binding domains (Lahteenmaki *et al.* 2001).

1.6.4 The plasmin(ogen)-binding group A streptococcal M-like protein (PAM).

GAS associated with certain infections express M proteins that bind plasminogen and plasmin directly and with high affinity. The first of these M proteins to be identified was PAM, isolated initially from M53 serotype GAS (Berge and Sjobring 1993). PAM is a 42 kDa protein of 388 amino acid residues, which binds both plasmin and Glu-plasminogen highly efficiently (Figure 1.4). The major plasmin(ogen)-binding site of PAM is located in a segment of 30 amino acid residues containing two characteristic tandem repeats in the N-terminal variable region. These repeat regions, each approximately 13 amino acids in length, have been designated the a1 and a2 repeats. The a1 and a2 repeat regions are identical in nine out of 13 amino acid residues, with three out of the four non-identical

residues having the same charge and polarity (Berge and Sjobring 1993). Similar binding motifs have been identified in other GAS isolates associated with skin infection (Svensson *et al.* 1999), and are associated with isolates from both invasive and non-invasive disease (McKay *et al.* 2004). The a1 repeat regions are followed by a highly conserved region consisting of the c1, c2 and c3 repeats, each of which is approximately 45 amino acids in length (Berge and Sjobring 1993; Ringdahl and Sjobring 2000). The c repeats are highly homologous with corresponding regions of other members of the M protein family (Berge and Sjobring 1993).

PAM lacks the typical C-terminal lysine residues of many plasminogen receptors; rather, internal lysine residues in the a1/a2 repeat regions of PAM and an internal histidine residue in a1are thought to bind to the lysine-binding kringle 2 of plasmin(ogen) (Wistedt *et al.* 1995; Wistedt *et al.* 1998; Schenone *et al.* 2000; Rios-Steiner *et al.* 2001). It has been suggested that, of the two lysine residues, most of the plasmin(ogen) binding capacity is due to the lysine residue in the first repeat (Wistedt *et al.* 1995).

PAM binds to kringle 2 of plasminogen in such a way that the catalytic domain of the zymogen remains accessible to activation by streptokinase (Ringdahl *et al.* 1998). Mutants of GAS strain AP53 exhibiting a PAM-positive phenotype and a streptokinase-negative phenotype have been shown to be unable to convert bound plasminogen to plasmin, demonstrating the co-operative role of these two proteins in plasmin acquisition by GAS (Ringdahl *et al.* 1998). It has also been suggested that the a-repeat region of PAM binds t-PA; hence the hypothesis that the co-localisation of t-PA and plasminogen by PAM may enhance plasminogen activation at the GAS cell surface (Wistedt *et al.* 1995).

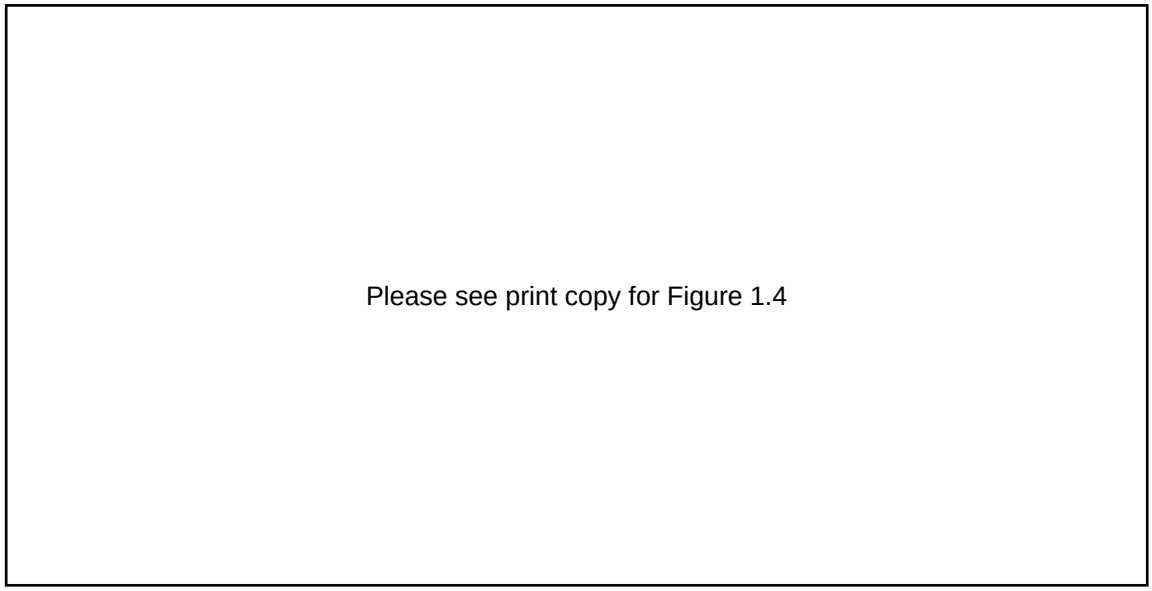


Figure 1.4 Schematic of the PAM protein. The N-terminal domain contains a signal sequence, and a wall-spanning domain can be found in the C-terminal region of the PAM protein. The plasmin(ogen) binding region is found in the N-terminal variable domain, and consists of two repeat regions designated the a1 and a2 repeats. The amino acid sequence of the a1/a2 repeats is shown, and the two lysine residues that are involved in plasmin(ogen) binding by PAM, are shown in red. The sequence from which the oligonucleotide used in this study was designed is underlined in green. The binding domain is followed by a highly conserved region, consisting of three repeat domains designated c1, c2 and c3 (modified from Svensson *et al.*, 1999).

PAM-associated plasmin activity is not inhibited by α_2-antiplasmin (which also binds to kringle 2 of plasmin) and thus is not subject to host regulation (Berge and Sjobring 1993). It has also been shown that PAM-dependent plasminogen binding is unable to be inhibited by the presence of fibrinogen, IgG or IgA, thus indicating that unlike various other M proteins, PAM is unable to interact with these host proteins (Berge and Sjobring 1993). The affinity of plasmin(ogen) for PAM is therefore, highly specific.

The *pam* genotype is largely restricted to *emm* pattern D GAS. Pattern D isolates show high levels of plasminogen binding, with PAM positive pattern D GAS segregating into a group with a strong tendency to cause impetigo (Svensson *et al.* 1999). Population based surveillance of invasive disease in Connecticut found that less than 2% of *emm* pattern D

isolates were associated with invasive infection. It has been hypothesised that PAM-directed plasminogen binding may be an important component of the pathogenesis of localised infection, rather than invasive disease (Svensson *et al.* 1999). Given that the skin appears to be a major portal of entry for invasive GAS infection amongst Northern Territory isolates (Carapetis *et al.* 1999), the *pam*-positive genotype may indeed be associated with skin tropism in these strains.

While a link between *emm* pattern and niche separation is evident amongst some GAS isolates, there is still a high level of recombination between the housekeeping genes of strains of all *emm* patterns. A recent study suggests that certain streptokinase alleles are co-inherited with *pam* and thus may also be associated with skin tropism (Kalia and Bessen 2004). Amongst GAS isolates that were otherwise genetically distinct, the *pam* gene showed strong linkage disequilibrium with a *ska* allele containing a specific plasminogen-docking domain. No strains containing this *ska* 2b allele were associated with nasopharyngeal infection. The authors suggest that the coinheritance of *pam* and *ska* 2b has arisen as a result of an epistatic interaction between the two genes (Kalia and Bessen 2004). Any selective pressure that has impacted on the inheritance patterns of these genes may be related directly to their interaction with the PAS, and the advantage this confers in the pathogenesis of skin infection. In a murine model of impetigo, inactivation of the *pam* gene produced a GAS mutant attenuated for virulence as determined by tissue pathology and net bacterial growth within the wound. The attenuation of virulence may have resulted from reduced plasminogen binding, or attenuated antiphagocytic M protein activity (Svensson *et al.* 2002). Additionally, recent studies have shown that an isogenic *pam*-negative$^-$ mutant strain of GAS is attenuated for virulence in a human-plasminogen transgenic murine model of infection (Sun *et al.* 2004). This further highlights the importance of focusing

plasminogen on the GAS cell surface in the pathogenesis of certain isolates. Svensson *et al*. have hypothesised that PAM may be involved in interrupting normal wound healing processes at the site of impetigo infection via the sequestering of plasminogen, or that degradation of fibrinogen networks at the site of infection by PAM-associated plasmin prolong the presence of GAS in superficial wounds (Svensson *et al.* 1999). The interaction of PAM with plasminogen may provide a useful model for the analysis of the pathogenic significance of bacterial interactions with the host PAS.

1.7 Aims and objectives

The endemicity of GAS infection and its sequelae in developing nations, together with the resurgence of GAS invasive infections in industrialised countries, necessitates the study of factors governing GAS persistence and invasion. Interactions of GAS with key components of the PAS may carry selective advantage leading to both of these phenomena. A mounting body of clinical, epidemiological and animal work supports the notion of an important role for plasminogen activation in GAS virulence. New models of the PAS interaction with GAS also suggest that some of the factors determining GAS persistence or invasion are adapted for specific host tissue niches. The cell surface protein PAM is a high affinity plasminogen receptor, which has a potential role in the pathogenesis of certain GAS strains. However, the details of PAM associated GAS virulence remain unclear. As such, the overall aims of this thesis were to examine both the interaction of PAM with plasminogen and the role of PAM in GAS pathogenesis. More specifically, the aims of this thesis were to:

- Determine the level of variation in the *pam* gene in a selection of GAS isolates, and identify any links between variation and GAS virulence or epidemiology.
- Assess the impact of naturally occurring variation in PAM on plasminogen binding function, and host immune recognition.

- Investigate potential interactions of PAM with other host factors.
- Further characterise the binding function of the prototype PAM with plasminogen, via the construction of a number of site directed mutants.
- Use site directed mutagenesis to eliminate the plasminogen binding function of a PAM variant isolated from the highly virulent GAS strain NS88.2 for use in future virulence studies involving murine models of infection.

2 Materials and methods

2.1 General materials

All reagents used in this study were of analytical grade, and, unless otherwise stated, were purchased from Crown Scientific. The compositions of all solutions not given below are outlined in Appendix 1.

2.2 General methods

Any variations to the general methodology outlined below are described in the relevant results chapter of this thesis.

2.2.1 Bacterial culture methods

2.2.1.1 Escherichia coli

E. coli containing plasmid DNA used for DNA sequence analysis and recombinant protein expression were grown on Luria-Bertani (LB)-agar plates containing 100 μg/ml ampicillin (Ap_{100}) and/or 50 μg/ml kanamycin (Km_{50}). Plates were incubated at 37^{o}C for 16-18 h. For the growth of liquid cultures, single colonies were selected for each strain and grown in LB-broth containing 100 μg/ml Ap and/or 50 μg/ml Km at 37^{o}C overnight, with shaking at 225rpm.

2.2.1.2 Streptococcus pyogenes

A total of 30 streptococcal strains, obtained from the Menzies School of Health Research (Darwin, Australia), were used in this study. Bacteria were grown on horse blood agar plates (Microdiagnostics, Australia) for 16-18 h at 37°C. Single colonies were used to inoculate liquid cultures, which were grown in Todd Hewitt broth (THB;

Difco Laboratories, USA) containing 1% (w/v) yeast (THBY) at 37°C without shaking for 16-18 h.

2.2.2 Agarose gel electrophoresis

DNA was resolved through a 1% (w/v) TAE agarose gel by electrophoresis for 1 h at 60 V in a Bio-Rad Minisub™ (Bio-Rad, USA) using 1x TAE electrophoresis buffer. DNA was visualised by ethidium bromide staining (Sambrook *et al.* 1989) and recorded using the Novaline Gel Documentation System (Novex, Australia). 1 kb ladder or λ *Hin*dIII molecular weight markers were used to determine the size of DNA bands.

2.2.3 DNA extraction and purification

2.2.3.1 Plasmid extraction from E. coli

Plasmid DNA was extracted using the Wizard® *Plus* SV DNA purification kit (Promega, USA). Bacterial cells were harvested by centrifugation for 5 min at 10,000 x *g*. The supernatant was discarded and 250 µl of Cell Resuspension Solution (Promega, USA) was added to the pellet. Following resuspension of the cells by vortexing, 250 µl of Cell Lysis Solution (Promega, USA) was added, and the solution mixed by inverting the microfuge tube four times. The cell suspension was then incubated until clearing of the lysate was visible. 10 µl of Alkaline Protease Solution (Promega, USA) was added to the cell lysate and mixed by inverting the microfuge tube four times. Following a 5 min incubation at room temperature, 350 µl of Wizard® *Plus* SV Neutralisation Solution (Promega, USA) was added, and the sample immediately mixed by inversion. The bacterial lysate was then centrifuged at 16, 000 x *g* for 10 min.

Following centrifugation, the cleared lysate was transferred to a Wizard Spin Column (Promega, USA), and centrifuged at 16,000 x *g* for 1 min. The supernatant was discarded, and 750 µl of Column Wash Solution (Promega, USA) added. The column was centrifuged as above and the flow-through discarded. A 250 µl volume of column wash solution (Promega, USA) was then added to the column, which was centrifuged at 16,000 x *g* for 2 min. DNA was eluted in dH_2O in a volume of 50 µl by centrifugation at 16,000 x *g* for 1 min. All centrifugation was performed using an Eppendorf 5415C bench centrifuge.

2.2.3.2 Extraction of streptococcal DNA

Streptococcal DNA was extracted and purified using the DNeasy Tissue Kit (Qiagen Inc, USA) according to the following protocol. Streptococcal isolates were grown in THBY (Difco Laboratories, USA) as previously described (2.2.1.2). Cells were harvested in a microcentrifuge tube by centrifugation for 10 min at 5,000 x *g*. The cell pellet was then resuspended in 180 µl of enzymatic lysis buffer and incubated for 30 min at 37°C. Following the addition of 25 µl of proteinase K (Qiagen Inc, USA) and 200 µl of Buffer AL (Qiagen Inc. USA), the sample was mixed by vortexing and incubated at 70°C for 30 min. A 200 µl aliquot of 100% ethanol was then added to the sample, which was again vortexed and added to a DNeasy spin column (Qiagen Inc. USA). The spin column was centrifuged at 6,000 x *g* for 1 min and the flow-through discarded. Following the addition of 500 µl of Buffer AW1 (Qiagen Inc. USA) the column was again centrifuged at 6,000 x *g* for 1 min and the flow-through discarded. 500 µl of Buffer AW2 (Qiagen Inc. USA) was then added to the column, which was centrifuged at 16,000 x *g* for 3 min. The flow-through was discarded and DNA eluted from the column by the addition of 200 µl of buffer AE (Qiagen Inc. USA) and

centrifugation at 6,000 x *g* for 1 min, following a 1 min incubation at room temperature. The elution step was performed twice. All centrifugation was performed using an Eppendorf 5415C bench centrifuge.

Streptococcal DNA was also obtained from the Menzies School of Health Research (Darwin, Australia).

2.2.3.3 DNA extraction from agarose gels

DNA previously electrophoresed on an agarose gel was purified using the Wizard® SV Gel and PCR Clean-up System (Promega, USA). The DNA band of interest was excised from the agarose gel, before being combined in a microfuge tube with 10 µl of Membrane Binding Solution (Promega, USA) per 10 mg of agarose gel slice. Following vortexing, the mixture was incubated at 60°C until the gel slice was fully dissolved. The dissolved gel mixture was then placed on an SV Minicolumn assembly and incubated for 1 min at room temperature. Following centrifugation of the column at 16,000 x *g* for 1 min, the column was washed by the addition of 700 µl of Membrane Wash Solution (Promega, USA). The column was then centrifuged as described previously, and the flow-through discarded. This wash step was then repeated using 500 µl of Membrane Wash Solution (Promega, USA), and centrifugation at 16,000 x *g* for 5 min. The SV Minicolumn was then placed in a microfuge tube, and 50 µl of nuclease free water added to the column and incubated at room temperature for 1 min. DNA was eluted from the column by centrifugation for 1 min at 16,000 x *g,* and stored at –20 °C. All centrifugation was performed using an Eppendorf 5145 C bench centrifuge.

2.2.4 Restriction enzyme digestion of plasmid DNA

Plasmid DNA was routinely subjected to restriction enzyme digestion in order to facilitate ligation with PAM genes, as well as to confirm the presence of insert DNA in recombinant pCR2.1 or pGEX2T constructs. Commonly used restriction enzymes include *Eco*RI and *Bam*H1. Restriction digests were performed for 1 h at 37 °C, and consisted of 400 ng of plasmid DNA and 2 U of restriction enzyme in the appropriate 1 x buffer.

2.3 Molecular characterisation of PAM

2.3.1 Cloning of PAM genes into pCR2.1

On the basis of their hybridisation to an oligonucleotide probe homologous to the a1 and a2 repeat regions of PAM during Southern hybridisation analysis, 15 GAS isolates were identified as PAM positive. The PAM genes from these GAS isolates were cloned into the plasmid pCR®2.1 (Invitrogen, USA; Figure 2.1) by the following method. Chromosomal DNA from GAS isolates was PCR amplified using primers MI, 5'-AGAAAATTAAAAACAGGTACGGCAT-3', and MII, 5'-AGTTGTTTCACCTGTTGATGGTAA-3'. These primers anneal to the 5' and 3' ends of the PAM gene, respectively (Berge and Sjobring 1993). The PCR reaction mixture used for amplification of PAM-like genes consisted of 100 ng of GAS DNA, 1x *Taq* buffer (Qiagen Inc, USA), 0.25 mM dNTPs (Boehringer Mannheim, Germany), 2.5 mM magnesium chloride (Qiagen Inc, USA), 1 U of *Taq* DNA polymerase (Qiagen Inc, USA), and 25 pmol of each primer, made up to a volume of 50 µl with dH_2O. Twenty-five cycles of PCR were performed with a thermocycler (Perkin-Elmer 9000), consisting of a 1 min denaturation step performed at 94°C, a 1 min annealing step at 50°C, and a 1 min extension step at 72°C. A final

extension step of 7 min was also performed. PCR products were visualised by agarose gel electrophoresis as previously described (2.2.2). Bands of approximately 1 kb were then extracted from the gel, and purified to remove excess salts in preparation for ligation. Ligation reactions contained 10 ng of PCR product, 50 ng of vector DNA, 1 µl 10 x ligation buffer, 4 U T4 DNA ligase and sterile dH_2O to a final volume of 10 µl. Following gentle mixing, the ligation reaction was incubated at 14°C overnight. The resulting constructs were then used to transform *E. coli* INV$\alpha F'$ cells (Invitrogen, USA). 2 µl of ligation reaction was added to a single vial of frozen cells, which were then incubated on ice for 30 min. Cells were heat shocked at 42°C for 30 sec and then added to 250 µl of pre-warmed SOC medium. Following shaking at 225 rpm for 30 min at 37°C, the transformation reaction was plated onto LB-agar plates and grown overnight at 37°C. Single colonies were selected for screening and grown in 3 ml of LB-broth containing 100 µg/ml Ap and 50 µg/ml Km at 37°C overnight. Plasmid DNA was extracted from these cells (2.2.3.1) for DNA sequence analysis and Southern hybridisation.

2.3.2 Southern hybridisation analysis

Following the extraction of plasmid DNA from *E. coli* cells, plasmids containing a PAM gene were identified using Southern hybridisation analysis. Prior to electrophoresis, 40 ng of plasmid DNA was digested for 2 h at 37°C with 2 U of *Eco*RI.

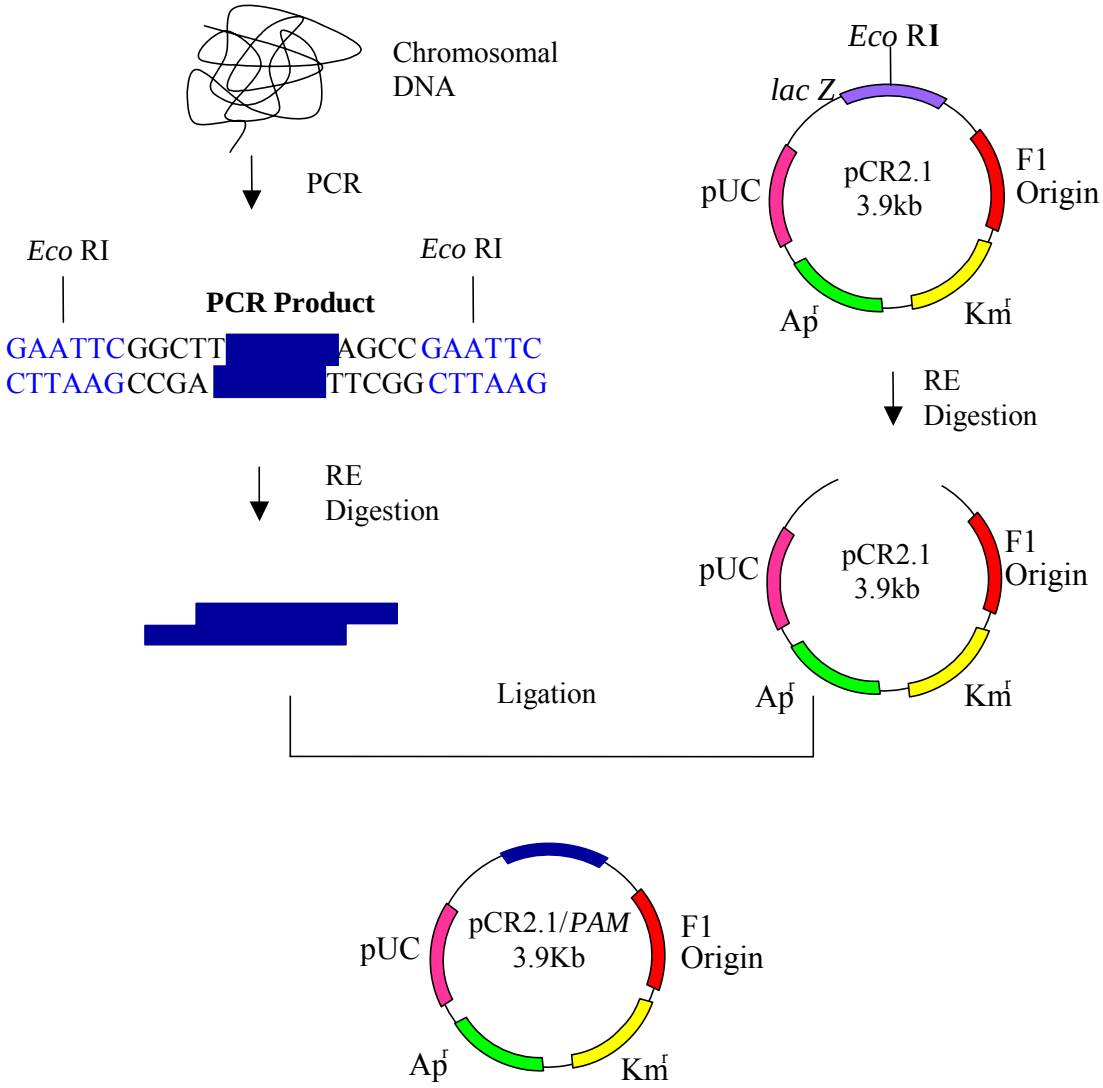


Figure 2.1 Cloning of PAM genes into the vector pCR2.1.PAM genes were amplified from streptococcal DNA using primers MI and MII. Amplicons were subsequently digested using *Eco*RI and ligated with *Eco*RI digested pCR2.1 DNA overnight. These constructs were used to transform TOP10 *E. coli* cells. Positive clones were identified by Southern hybridisation.

2.3.2.1 DNA transfer

Plasmid DNA was subjected to agarose gel electrophoresis as previously described. Digoxigenin-11-ddUTP (DIG) labelled λ-*Hin*dIII markers (Boehringer Manheim, Germany) were also electrophoresed on the gel. Transfer of DNA to a nylon membrane (Boehringer Manheim, Germany) was achieved via capillary transfer overnight, according to the method of Southern (Southern 1975). Plasmid DNA previously identified as containing a PAM gene was then spotted to the membrane to determine the efficiency of the probe, and a sample of unrelated DNA was spotted on the membrane as a negative control. DNA was then cross linked to the membrane using a UV Stratalinker 1800 (Stratagene, USA).

2.3.2.2 3'-end labelling of oligonucleotide probe with digoxigenin-11-ddUTP

The oligonucleotide probe PAM1 (Sigma,USA) 5'–CGACTTAAAA(A/G) CGAGAGACATGA -3' was 3' end labelled using the digoxigenin-11-ddUTP (DIG) oligonucleotide 3'-End labeling kit (Boehringer Manheim, Germany). This probe has high homology with both the a1 and a2 repeat regions of the PAM gene. A 20 µl labeling reaction containing 1x reaction buffer, 5 mM $CoCl_2$ solution, 5 pmol/µl oligonucleotide, 0.05 mM DIG-ddUTP, 2.5 U terminal transferase and dH_2O was incubated at 37°C for 15 min then placed on ice. 1 µl of 200 mM EDTA was then added to terminate the labeling reaction.

2.3.2.3 Detection of PAM genes

Subsequent to DNA transfer, the membrane was pre-hybridised at 42°C for 1 h in DIG-Easy Hyb (Boehringer Manheim, Germany). Hybridisation was performed at 42°C overnight in DIG-labelled PAM-1 probe diluted 1 in 1000 in DIG-Easy Hyb. The membrane was then washed twice in 2 x wash solution for 5 min at room temperature, and twice in 0.5 x wash solution for 15 min at 68°C. A 1 h incubation in blocking solution at 42°C while shaking was followed by incubation of the membrane for 30 min in anti-DIG-AP (Boehringer Manheim, Germany) diluted 1 in 20,000 in blocking solution. The membrane was then washed twice for 15 min in washing buffer and equilibrated in detection buffer for 2 min. CDP-Star™ (Boehringer Manheim) diluted 1 in 100 in detection buffer was then spread over the membrane, which was exposed to medical X-ray film (Fuji, Japan) for 5 min to allow visualisation of plasmids containing a PAM gene.

2.3.3 DNA sequence analysis

A general outline of the DNA sequencing approach used is given below. A more detailed description of primer composition is given where relevant.

2.3.3.1 Calculation of DNA concentration

DNA to be sequenced was electrophoresed on a 1% (v/v) TAE agarose gel as described previously with DNA molecular size markers of known concentration. The DNA concentration of the unknown samples was then estimated visually by comparison of the band intensity with DNA markers.

2.3.3.2 DNA sequencing reactions

DNA sequencing reactions contained 4 µl of terminator ready reaction mix (PE Applied Biosystems, USA), 200-500 ng of plasmid DNA, and 3.2 pmol of primer in a total volume of 10 µl. Oligonucleotide primers (Sigma-Aldrich, USA) were used for DNA sequencing in this study. M13LacZ universal forward and reverse primers (Perkin-Elmer, USA), which anneal to pCR2.1 were used to obtain initial sequence data, along with primers N1 and N2 flanking the a1/a2 repeat region of PAM (Berge and Sjobring 1993). Additional primers listed in Table 2.1 were designed to provide complete sequence data of both DNA strands.

Table 2.1 Oligonucleotide primers designed for DNA sequence analysis of PAM genes.

Primer name	Primer sequence (5'-3')	Gene sequenced
PAMF1	ATAAGCAAGAACATCTTGACGG	All but NS696 M1 protein
PAMR1	CTGTTAATTTCTTGCTTTC	All but NS696 M1 protein
PAMF2	AAAGGGCTTAAGACTGATTTAC	All but NS696 M1 protein
PAMR2	GACCAGCTAATTTGCTGTTTGC	All but NS696 M1 protein
PAMF3	GCAAACAGCAAATTAGCTGCTC	All but NS696 M1 protein
PAMR3	CTTCTCAACATCATCTTTAAGG	All but NS696 M1 protein
NS50.1F1	GCCTTAAAGATGATGCTGAG	NS50.1 PAM
NS265F1	GGCGAACTTAFATTGGAA	NS265/455PAM
NS265R1	GTTTAGTTTTTCAAGGGC	NS265/455PAM
NS1133F1	TCAATGAAAAAGAAGCAGA	NS1133PAM
NS696F1	TCTTGAATTAGCGATAGACAAGCG	NS696 M1 protein
NS696F2	TGCTGAACTTGATAAGGTT	NS696 M1 protein
NS696F3	GGACGCATCACGTGAAGCTAA	NS696 M1 protein
NS696R3	CAGATTCGGCTTGGGTCGAC	NS696 M1 protein
NS696F4	CAGGGTTAGCAAGCCAGACAG	NS696 M1 protein
NS696R4	TTAGCTTCACGTGATGCGTCC	NS696 M1 protein

Cycle sequencing using a Perkin Elmer thermocycler 9000 (Perkin Elmer, USA) consisted of a rapid thermal ramp to 96°C, a constant hold at 96°C for 10 sec, a rapid thermal ramp to 50°C, a constant hold at 50°C for 5 sec, a rapid thermal ramp to 60°C and a constant hold at 60°C for 4 min. This cycle was repeated 25 times. Unincorporated dyes were removed by ethanol precipitation. The sequencing reactions

were added to a 1.5 ml microfuge tube containing 2.0 µl of 3 M sodium acetate and mixed with 50 µl of 95% (v/v) ethanol. These tubes were incubated on ice for 10 min. After centrifugation at 16,000 x *g* for 15 min the ethanol solution was removed and the pellet rinsed with 250 µl of 70% (v/v) ethanol. The tube was then centrifuged again at 16,000 x *g* for 5 min, the ethanol solution removed and the pellet dried in a fume hood for approximately 30 min.

2.3.3.3 DNA sequence gel electrophoresis

DNA sequencing gels were prepared as per the manufacturer's instructions and electrophoresed using a Perkin-Elmer ABI PRISM 377. DNA sequencing polyacrylamide gels were made using a 4% bis-acrylamide solution and buffered with filtered TBE. The gel solution was exposed to amberlite mixed bed ion exchange beads before being filtered using a Nalgene 0.2 µm filter, and degassed. The acrylamide was polymerised by the addition of 400 µl of 10% ammonium persulfate (APS) and 55 µl of N,N,N',N'-tetra-methylethylenediamine (TEMED), and injected into an ABI casting apparatus containing 48 cm glass plates. Once set, sequencing gels were pre-run until the temperature was equilibrated to 51°C. Following the loading of pre-prepared sequencing samples, gels were run continuously for 10 h at a constant 16 mA. Sequence data was analysed using ABI Prism™ DNA Sequencing Analysis Software (Perkin Elmer, USA).

2.3.3.4 Analysis of DNA sequence data

DNA sequences of the PAM genes determined in this study were translated using the etranslate program (http:// www.angis.org.au), and the deduced amino acid sequences aligned using the clustalW program (http:// www.angis.org.au).

In order to characterise the evolutionary relationships between the PAM genes used in the study, the full amino acid sequence of PAM proteins were aligned using ClustalW (Thompson *et al.* 1994). Evolutionary gene trees were then estimated using MrBayes v. 3.1 (Huelsenbeck *et al.* 2001; Ronquist and Huelsenbeck 2003). For the MrBayes analysis, four simultaneous chains were run, with trees sampled every 100 generations for a total of 500,000 generations. Plots of likelihood scores against generation were used to identify when the analysis had reached stationarity. Trees sampled prior to stationarity were discarded (the first 40 trees). The amino acid model was empirically chosen by MrBayes, using the prset aamodelpr=mixed command. This permits jumping between nine alternative amino acid substitution models. The WAG model (Whelan and Goldman 2001) was empirically chosen by MrBayes. The majority rule consensus of all trees generated after stationarity was used to estimate the posterior probabilities of the various nodes in the most likely tree.

2.3.4 Molecular typing and emm pattern analysis

In order to further characterise the GAS strains used in this study, DNA from streptococcal isolates was extracted as described previously (2.2.3.2) and analysed via both *emm* sequence typing (ST) and *emm* pattern PCR. The method used for determining *emm* ST was essentially as described at the *S. pyogenes emm* sequence database (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm). Briefly, PCR amplification of chromosomal DNA was performed using primers emm1 and emm2 (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm), or VUF and SBR (Gardiner *et al.* 1997). PCR cycling consisted of an initial annealing step of 95°C for 30 sec, followed by 30 cycles of 94°C, 15 sec; 60°C 1 min; 68°C, 6 min, annealing, denaturation and extension times respectively. Following purification using agarose gel extraction, amplicons were used as template DNA for DNA sequence analysis

according to the protocol outlined in the general methods section, using primers emm1 and emm2 (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm). The resulting sequences were compared to *emm* sequences in the *S. pyogenes emm* sequence database, and *emm* sequence type assigned on the basis of 95 % identity in the 3' region to known *emm* sequence types.

Emm pattern PCR was performed using the primers outlined in Table 2.2, with *emm* pattern being determine by which primer sets resulted in the formation of extension products (Hollingshead *et al.* 1994; Bessen *et al.* 2000). The PCR reaction mixture used for *emm* pattern PCR contained 2.5 µM dNTP (Boehringer Mannheim, Germany), 25 µM magnesium chloride (Qiagen Inc, USA), 1x *Taq* buffer (Qiagen Inc, USA), 1 U of *Taq* DNA Polymerase (Qiagen Inc, USA), 25 pmol of each primer and 200 ng of GAS chromosomal DNA, made up to a volume of 50 µl with dH_2O. Thirty-five cycles of PCR were performed with a thermocycler (Perkin-Elmer 9000), using a denaturation temperature of 94 °C for 1 min, an annealing temperature of between 60 °C and 64 °C for 1 min, and an extension temperature of 72 °C for 5 min. The extension products were visualised using agarose gel electrophoresis.

2.3.5 Pulsed field gel electrophoresis

To determine if variation within the PAM genes of streptococcal isolates was indicative of wider chromosomal variation, pulsed field gel electrophoresis (PFGE) of the chromosomal DNA of PAM positive GAS isolates was performed. Fifteen PAM negative isolates were also included to enable comparisons of chromosomal variation between PAM positive and PAM negative isolates. Pulsed field samples were prepared

Table 2.2 Primers used to determine the *emm* pattern of GAS strains.

Primer set	Primer sequences (5'-3')	*Emm* patterns yielding products
1	UP-2 (forward) TTCTGGATCCCACTCCCCCCAACAAGTTGC SF1-R (reverse) GTGCTTGACCTTTACCTGGAACAGCTT	A,B,C
2	IG-F (forward) CTGGCCTTTACTCCTTTGATTAACC SF3-R (reverse) GCTGT TTGAGCAGCTCTACC	C, D, E
3	G3-F (forward) CGAGAAGTAGAAAAACGTTATCAAGAAC SF3-R (reverse) GCTGT TTGAGCAGCTCTACC	C, D, E
4	SF4-A (forward) CTCCTAGGTTCAGCTAAGCGTGAGTTG G3-R (reverse) GTTCTTGATAACGTTTTTCTACTTCTCG	D, E
5	SF4-A (forward) CTCCTAGGTTCAGCTAAGCGTGAGTTG SF1-R (reverse) GTGCTTGACCTTTACCTGGAACAGCTT	D
6	G3-F (forward) CGAGAAGTAGAAAAACGTTATCAAGAAC SF2-R (reverse) GTTAGCTTGGGCTACTT	E
7	SF4-A (forward) CTCCTAGGTTCAGCTAAGCGTGAGTTG SF2-R (reverse) GTTAGCTTGGGCTACTT	E

and electrophoresed as previously described (Ramachandran *et al.* 2004). Streptococcal cells were pelleted from overnight culture by centrifugation, and washed twice with TSE. Following resuspension in 200 µl of TE, cells were mixed with an equal volume of 1.5% low melt agarose (Bio-Rad, Richmond CA.). Cell suspensions were then transferred to plug moulds and allowed to set. DNA was released by incubation of the agarose plugs in cell lysis solution at 37°C for 2 h. Plugs were then added to deproteination solution and incubated at 50°C overnight. DNA plugs were subsequently washed with TE, and 3 mm slices cut from the plugs and restriction digested overnight with 20 U of the restriction enzyme *Sma*I (Roche, USA). Plugs were loaded on a 1%

pulsed field certified agarose (Bio-Rad, Richmond CA.) gel with pro λ DNA concatemers (New England Biolabs, USA) and electrophoresed using a CHEF-DRTM electrophoresis cell (Bio-Rad, Sydney) in 0.5 x Tris-borate running buffer according to the following parameters: 6 v/cm, linearly ramped switch times of 2 to 40 sec at 10°C for 24 h. Following electrophoresis, gels were stained in ethidium bromide (1 μg/ml) and visualised under ultraviolet light with a Novex gel documentation system (Novex, Australia). PFGE restriction fragment patterns were analysed using Diversity software (version 2.1, BioRad USA). Genetic similarities were compared by clustering methods (unweighted pair group method with arithmetic means) using the Dice coefficient.

2.4 Characterisation of recombinant PAM variants

2.4.1 Expression and purification of recombinant PAM variants

2.4.1.1 Cloning into pGEX2T

Five naturally occurring variants of the prototype PAM gene were selected for functional studies, including one with 99.7% identity to the prototype PAM amino acid sequence, and 100% identity in the plasminogen-binding domain (PAM_{NS13}). The NS696 M1 protein, from an *emm*1 sequence type (NS696) gene was selected as a negative control for plasminogen binding assays. These genes were amplified from pCR2.1 constructs using *Pfu* polymerase (Stratagene, USA) with the oligonucleotide primers listed in Table 2.3. *Bam*HI and *Eco*RI restriction sites were incorporated into the oligonucleotide primers. The amplification products did not encode a signal peptide at the N-terminus nor the LPXTG motif at the C-terminus for each recombinant protein. Additionally, a C-terminal hexahistidyl tag (6-His) was incorporated into the reverse primer sequence. PCR cycling parameters consisted of thirty cycles of 97 °C, 55 °C and

72 °C for denaturation, annealing and extension reactions respectively. Amplicons were cloned into pGEX-2T (Smith and Johnson 1988; Figure 2.2) resulting in an N-terminal fusion with glutathione-S-transferase, and constructs were transformed into *E.coli* TOP10 (Invitrogen, USA) as described in 2.3.1, using standard procedures (Sambrook *et al.* 1989). The presence of both a 6-His tag and a GST tag on the recombinant protein enabled purification by two methods. DNA sequence analysis was used to confirm the absence of polymerase chain reaction errors in the cloned amplification product.

2.4.1.2 Site-directed mutagenesis

To assess the role of specific binding-site residues in the interaction of PAM with plasminogen, site-specific mutations were introduced into the PAM_{NS13} and $PAM_{NS88.2}$ plasminogen binding sites. The wildtype pGEX2T construct (100 ng) was used as template DNA to create site directed mutants with the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, USA). PCR reactions consisted of 1x *Pfu* reaction buffer (Stratagene, USA), 0.25 mM dNTPs (Boehringer Mannheim, Germany), 2.5 U of *Pfu* Ultra polymerase (Stratagene, USA), and 125 ng of each primer, made up to a volume of 50 µl with dH_2O. Oligonucleotide primers (Sigma-Aldrich) were designed as per the manufacturer's instructions. In general, primers consisted of the desired mutation, flanked on either side by 10-15 bases of correct sequence. The specific primer sequences and details of introduced mutations are outlined in the relevant chapters of this thesis. Following an initial denaturation step (95°C, 30 sec), PCR cycling parameters consisted of sixteen cycles of 95°C for 30 sec, 55°C for 1 min and 68°C for 10 min. An additional, final 7 min extension at 55°C was also performed.

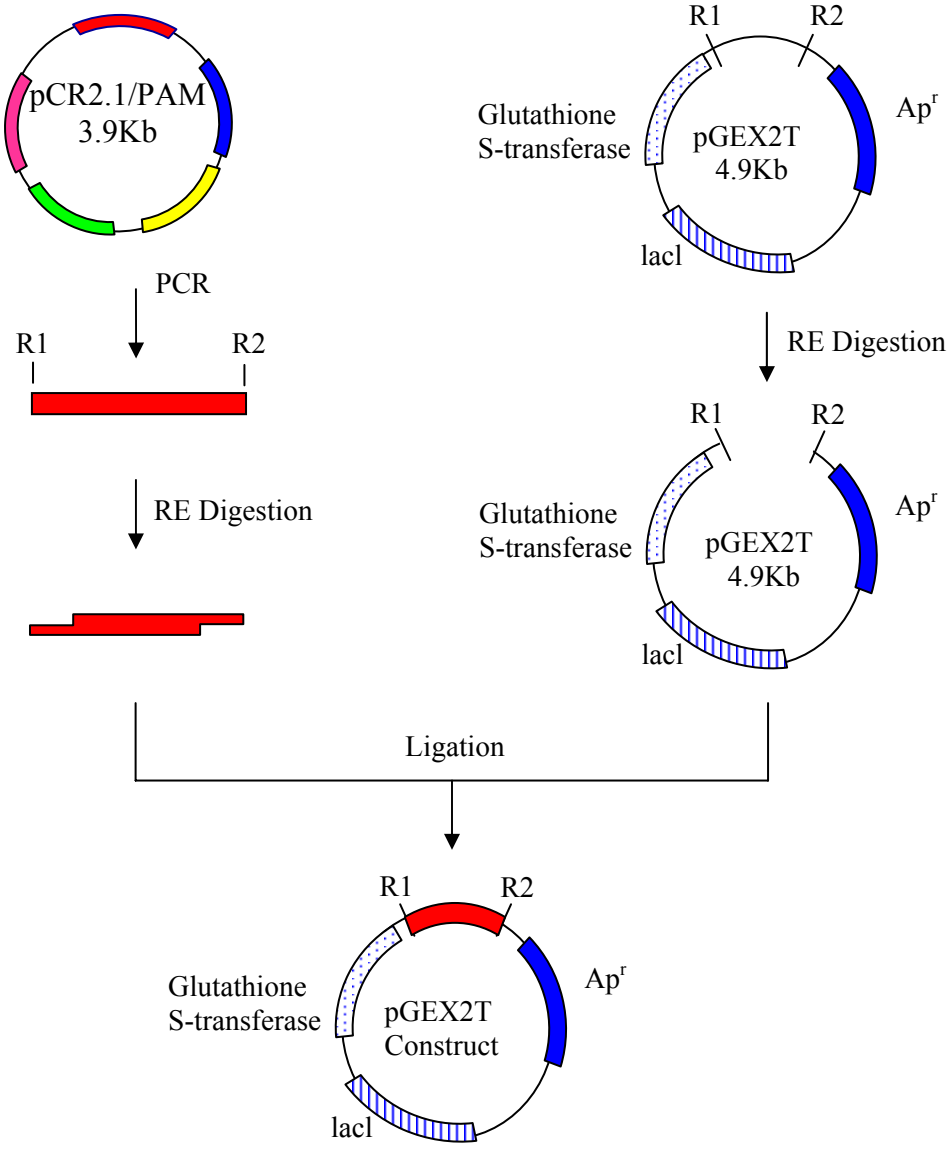


Figure 2.2 Cloning of PAM genes into pGEX2T. PAM genes were amplified from previously constructed pCR2.1 vectors using gene specific primers (Table 2.3). Amplicons were subsequently digested using *Bam*HI and *Eco*RI and ligated with *Bam*H1 and *Eco*RI digested pGEX2T DNA overnight. These constructs were used to transform TOP10 *E.coli* cells. Positive clones were identified by restriction digestion and DNA sequence analysis.

Table 2.3 Primers used for PCR and DNA sequence analysis of PAM/pGEX-2T expression plasmids

Primer	PCR amplification primers
NS13pGEX2TF	5'-GGGGGATCCAATAGAGCAGACGACGCT-3'
NS13pGEX2TR	5'-GGGGAATTCTCAGTGATGGTGATGGTGATGCTGTCTCTTAGTTTCCTT-3'
NS1133pGEX2TF	5'-GGGGGATCCGATCACCCTAGCTATACC-3'
NS53/455pGEX2TF	5'-GGGGGATCCGATCAACCTGTTGATCAC-3'
NS265pGEX2TF	5'-GGGGGATCCGAGAATCACCCTGGATAT-3'
NS696pGEX2TF	5'-GGGGGATCCAACGGTGATGGTAATCCT-3'
	DNA sequence analysis primers
PAMF1	5'-ATAAGCAAGAACATCTTGACGG-3'
PAMR1	5'-CTGTTAATTTCTTGCTTTC-3'
PAMF2	5'-AAAGGGCTTAAGACTGATTTAC-3'
PAMR2	5'-GACCAGCTAATTTGCTGTTTGC-3'
PAMF3	5'-GCAAACAGCAAATTAGCTGCTC-3'
PAMR3	5'-CTTCTCAACATCATCTTTAAGG-3'
NS696F1	5'- TCTTGAATTAGCGATAGACAAGCG-3'
NS696F2	5'- TGCTGAACTTGATAAGGTT-3'
NS696F3	5'- GGACGCATCACGTGAAGCTAA-3'
NS696R3	5'- CAGATTCGGCTTGGGTCGAC-3'
NS696F4	5'- CAGGGTTAGCAAGCCAGACAG-3'
NS696R4	5'- TTAGCTTCACGTGATGCGTCC -3'
NS696pGEX2TF	5'-GGGGGATCCAACGGTGATGGTAATCCT-3'
pGEX2TF	5'-GGGCTGGCAAGCCACGTTTGGTG-3'
pGEX2TR	5'-CCGGGAGCTGCATGTGTCAGAGG-3'

PCR was conducted with a Cooled Palm 96 thermocycler (Corbett Research). Non-amplified template DNA was removed by incubation of reactions for 1 h at 37°C following the addition of *Dpn*I (10 U/ 50 µl reaction). *E. coli* TOP10 cells (Invitrogen, USA) were transformed with 150 ng of *Dpn*I digested PCR product using standard procedures, as described in section 2.3.1 (Sambrook *et al.* 1989). DNA sequence analysis was used to verify the presence of introduced mutations and confirm the absence of random mutations in site-directed mutants.

2.4.1.3 Purification of recombinant PAM variants

Recombinant proteins were expressed and purified essentially as described previously (Smith and Johnson 1988), with the following modifications. 1 L of bacterial culture was incubated at 37°C with shaking at 225 rpm until the culture reached $OD_{600\ nm}$ 0.6, at which point protein expression was induced for 4 h by the addition of 0.1 mM

isopropyl β -D-1 thiogalactopyranoside. Protein was expressed in the presence of 1 mM phenyl-methyl-sulfonyl fluoride (PMSF) to reduce protein degradation. Cells were harvested by centrifugation and resuspended in ice-cold PBS containing 1% Triton-X 100. Cell lysis was achieved by sonication of the cell suspension with a Branson Sonifier 250 (30% duty cycle, microtip output control 2) for 2 min and the addition of a 0.1 x volume of lysis buffer. Following centrifugation, recombinant protein was purified from the cleared lysate using a glutathione agarose (Sigma-Aldrich, USA) column. Prior to elution from the column, the GST tag was removed from the N-terminus of recombinant fusion proteins by the addition of one column volume of thrombin solution (1 U thrombin/µl, PBS pH 8.0). The column was incubated for 5 h at room temperature and the cleaved recombinant protein eluted in PBS pH 8.0. In order to remove thrombin from the protein solution, the C-terminal 6-His tag was utilised to further purify the protein using a Ni-NTA column. Protein solution was added to an equilibrated Ni-NTA agarose (Qiagen, Germany) column and washed with 10 column volumes of PBS. Bound protein was eluted under native conditions with elution buffer and the imidazole removed by dialysis against 10 L of 1 x PBS. Each step of the protein purification process was monitored by 12% SDS-polyacrylamide gel electrophoresis analysis.

2.4.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

In order to assess protein size, composition and purity, protein samples were routinely examined by SDS-PAGE (Laemmli 1970). BioRad Broad Range molecular weight markers were electrophoresed simultaneously to enable protein size estimation. Protein samples were resuspended in 1 x PBS, and then added to an equal volume of either 2 x

reducing SDS-PAGE buffer, or 2 x non-reducing SDS-PAGE buffer. Samples were heated at 100°C for 10 min prior to being loaded on SDS-PAGE gels.

Separation was achieved using 10% or 12% acrylamide gels, with a 4% stacking gel. Gels were electrophoresed for 1 h at 200 v in SDS-PAGE running buffer. The Bio-Rad Mini PROTEAN® 3 Cell system was used for electrophoresis. Following separation, samples were visualised by Coomassie R 250 staining for 1h. Gels were then placed in rapid destain, microwaved for 1 min and incubated for 5 min with gentle agitation. Following this treatment, gels were immersed in final destain until proteins could be properly visualised.

2.4.3 Western and ligand blotting analysis

Western and ligand blotting analysis were routinely used to both identify various protein products, and characterise certain protein-protein interactions.

2.4.3.1 Transfer

Following SDS-PAGE of the protein samples of interest, the acrylamide gel was placed face down on a piece of nitrocellulose membrane that had been pre-soaked in Western transfer buffer. The gel and membrane were then placed between two pieces of pre-soaked filter paper and two pre-soaked fibre pads. The transfer set up was then placed into a Bio-rad Trans-Blot apparatus. Transfer was performed for 1 – 1.5 h at 100 v in Western transfer buffer.

2.4.3.2 Detection

Following protein transfer, the nitrocellulose membrane was blocked by incubation in 10% skim milk/PBS, with gentle agitation. Following 2 x 5 min washes in PBST with gentle agitation, the membrane was transferred to a tray containing either primary antibody, or ligand diluted in 1% Skim milk/PBS. Following this incubation, the membrane was washed as described previously, and then incubated in a solution containing secondary antibody or neutravidin-horseradish peroxidase (HRP; Pierce, USA) with gentle agitation. The membrane was then washed twice for 5 min in PBST, and twice for 5 min in PBS, with gentle agitation, in preparation for detection using diaminobenzidine (DAB). Following a 2 min equilibration in 100 mM Tris-Cl (pH 7.6), the membrane was covered in DAB developing solution (DAB 25 mg, 100 mM Tris-Cl pH 7.6 50 ml, H_2O_2 30 µl) until protein bands were visible. The reaction was stopped by washing the membrane in distilled water.

2.4.4 Determination of protein concentration

The concentration of protein samples was determined using the Bio-Rad D_C Protein Assay (BioRad, USA). BSA standards consisting of BSA in PBS over a range of concentrations were used to construct a protein concentration standard curve. 5 µl of protein sample was placed in a 96-well ELISA plate. 25 µl of reagent A was added to each sample, followed by the addition of 250 µl of reagent B. The reaction was allowed to proceed for 10 min, and the plate read at 750 nm in a Spectramax 250 plate reader (Molecular Devices, USA).

2.4.5 Concentration of protein samples

Where it was necessary to increase the concentration of purified proteins, samples were concentrated using Millipore 30,000 MWCO Centricon concentrators (Millipore, France). The concentrators were pre-washed with sterile MilliQ water, prior to the application of the sample, and were then centrifuged at 2000 x *g* until the desired concentration was reached.

2.4.6 Plasminogen purification

Glu-plasminogen was purified from human plasma using lysine Sepharose-4B affinity chromatography as described previously (Andronicos *et al.* 1997), with the following modifications. Human plasma (300 ml) was diluted with an equal volume of distilled water containing 10 mM EDTA and 2 mM PMSF. Precipitated proteins were allowed to settle and the cleared plasma was batch incubated with 60 ml of lysine-Sepharose 4B (Amersham Biosciences, USA) for 2 h with gentle agitation. Following centrifugation, the resin was poured into a column and washed overnight with PBS (pH 7.4) containing 10 mM EDTA and 2 mM PMSF. The column was then washed with salt wash buffer (0.05 M Na_2HPO_4, 5 M NaCl, pH 7.5), and the plasminogen eluted with PBS (pH 7.4) containing 200 mM ε-amino caproic acid (Sigma-Aldrich, USA). ε-amino caproic acid was removed by dialysis at 4 °C against 10 L of PBS.

2.4.7 Fibrinogen purification

Prior to the use of commercially obtained fibrinogen from human plasma (Sigma-Aldrich, USA) in binding assays, it was necessary to remove contaminating fibronectin. Fibrinogen was solubilised in 0.85% (w/v) NaCl at 37°C for 4 h with gentle agitation. A 10 ml gelatin agarose column (Sigma-Aldrich, USA) was washed with 5 column volumes of 6 M urea in TBS (pH 7.5), followed by equilibration with 5 column volumes

of TBS (pH 7.5). The solubilised fibrinogen was then applied to the column, and the flow-through collected. This was repeated 5 times. Removal of fibronectin was confirmed by SDS-PAGE and Western-blotting techniques as described previously. Western blot analysis was performed using rabbit polyclonal anti-fibronectin primary antibodies, and goat anti-rabbit HRP-conjugated secondary antibodies (BioRad, USA).

2.4.8 Protein labelling

Purified proteins were biotinylated by the addition of 10% (v/v) 1 M $NaHCO_3$ (pH 9), and a 40 molar excess of biotin-X-NHS in dimethyl sulfoxide (Sigma-Aldrich, USA). The reaction was incubated at 4°C overnight with mixing. Free biotin was separated from biotinylated protein by PD-10 gel filtration chromatography (Amersham Biosciences, USA).

2.4.9 Functional characterisation of recombinant proteins

2.4.9.1 Ligand blotting analysis

Ligand blotting analysis of recombinant proteins was conducted using biotinylated glu-plasminogen as described in section 2.4.3. Biotinylated glu-plasminogen was used at a concentration of 2 μg/ml, and neutravidin-HRP diluted 1 in 500, in 1% (w/v) blocking solution.

2.4.9.2 Solid phase microtitre assays

2.4.9.2.1 Plasminogen binding

To further characterise the interaction between recombinant proteins and glu-plasminogen, solid phase microtitre plasminogen binding assays were performed. 96 well microtitre plates (Greiner Bio-one, Germany) were coated with 150 nM recombinant protein (50 μl in 0.1 M $NaHCO_3$) at 4°C overnight. Following three

washes with PiNT, plates were blocked with 50 µl of blocking solution (1% skim milk powder, PiNT) for 1 h at 37°C. Wells were washed as above, and 500 nM biotinylated glu-plasminogen was diluted in a three-fold titration across the plate with blocking solution, in the presence or absence of a 50 fold molar excess of unlabelled glu-plasminogen. Plasminogen was allowed to bind to immobilised proteins for 2 h at room temperature. For competition assays, decreasing concentrations of unlabelled fluid phase PAM_{NS13} or $PAM_{NS88.2}$ (25 µM-0.14 nM) were allowed to compete with immobilised proteins for binding to biotinylated glu-plasminogen. Competitor was titrated three-fold across the microtitre plate prior to the addition of biotinylated glu-plasminogen to all wells, at a final concentration of 500 nM. The assay was incubated for 2 h at room temperature. Following the plasminogen incubation step, microtitre plates were washed three times, and 50 µl of neutravidin-HRP (Progen, Australia) diluted 1:5000 with blocking solution was added to all wells and incubated for 2 h at room temperature. After five washes with PiNT, the reactions were developed by the addition of 50 µl o-phenylenediamine (Sigma-Aldrich, USA) substrate (8 mM Na_2HPO_4 pH5.0, 2.2mM o-phenylenediamine, 3% H_2O_2). Colour development was stopped by the addition of 50 µl of 10 M hydrochloric acid, and the plates were read at 490 nm using a Spectramax 250 plate reader (Molecular Devices, USA).

Data was normalised against the highest and lowest absorbance value for each assay, and non-linear regression analysis performed using GraphPad ® Prism (v4.00, GraphPad software, CA). For the calculation of equilibrium binding dissociation constants (K_d), a one versus two site binding analysis was conducted and the best-fit curve fitted to the data. For competition experiments, a one-site competition curve was

fitted to the data from which the effective concentration of competitor required to inhibit binding by 50% (EC_{50}) was calculated.

2.4.9.2.2 Fibrinogen binding

Solid phase micrtotitre fibrinogen binding assays were performed to analyse the interaction of recombinant PAM variant proteins with human fibrinogen. Assays were performed essentially as for the plasminogen binding analysis, using purified biotinylated fibrinogen at a starting concentration of 325 nM. Due to the insolubility of fibrinogen at concentrations higher than this, only total binding was determined.

2.4.9.2.3 tPA binding

The interaction of immobilised tPA (5 μg/ml) with PAM_{NS13} was analysed using solid-phase microtitre assays as above. Biotinylated fibrinogen was used as a positive control. The concentration of fluid phase- biotinylated PAM_{NS13} and biotinylated fibrinogen used ranged from 2.8 pM– 0.5 μM. tPA was a kind gift from Dr. Rob Medcalf (Department of Medicine, Monash University).

2.4.9.3 Circular dichroism (CD) spectroscopy

To highlight potential variation in protein secondary structure as a result of site-directed mutagenesis, far UV circular dichroism (CD) spectra were obtained for both wildtype and mutant recombinant PAM proteins. CD spectra were acquired using a Jasco J-810 Spectropolarimeter (Jasco, Canada) at room temperature. CD spectra data was recorded from 190 – 250 nm in a 1 cm pathlength cell containing 1.5 ml of protein solution at a concentration of 0.04 mg/ml in 10 mM sodium phosphate buffer (pH 7.4). Recorded

data represents the average of six scans, corrected for buffer baseline. Molar residue ellipticity ([θ]) was calculated using the following formula:

$[\theta] = \theta \times 100 \times$ Molecular weight / concentration (mg/ml) $\times$ distance $\times$ Number of amino acids (Schmid 1989) .

The percentage of α-helix was estimated from the ellipticity at 222 nm using the following formula: % α-helix = $- (\theta_{222\ nm} - 4{,}800)/45{,}400$ (Phillips *et al.* 1981).

2.4.10 Immunological studies

2.4.10.1 Immunisation and challenge of mice

Quackenbush (outbred) mice (n = 20; Animal Resources Centre, Western Australia) were immunised with a commercially synthesised peptide representing the a1 repeat of PAM (NH_2-CDAELQRLKNERHE-COOH) conjugated to keyhole limpet hemocyanin (KLH; Chiron Mimotopes, San Diego, CA), with PepM, or with controls. PepM, a pepsin extract of M protein, was prepared from isolate NS13 as previously described (Beachey *et al.* 1977). PepM was used as a positive control for the immunisation procedure as it has been demonstrated that PepM extracts induce type-specific opsonic antibodies that confer protection upon subsequent challenge with a homologous GAS strain (Beachey *et al.* 1979; Olive *et al.* 2002). Peptide-KLH conjugate, PepM, KLH control or PBS control was administered subcutaneously in a volume of 50 μl at the tail base. Each mouse received a total of 30 μg of immunogen emulsified 1:1 in complete Freunds' adjuvant (Difco Laboratories, Detroit, MI) on day 1 and booster injections at days 21 and 28 with antigen in PBS. Mice were bled on days 20, 27 and 35 post-primary immunisation. All experimental protocols described in this report complied with the Australian Code of Practice for the Care and Use of Animals for Scientific

Purposes (National Health and Medical Research Council, Australia) and institutional ethics requirements.

The NS13 GAS strain was passaged in mice to enhance virulence before challenge. GAS was cultured overnight in THB with 1% (v/v) neopeptone (Difco Laboratories, Detroit, MI; THBN), washed twice in THBN and resuspended in 25% of the original volume. The inoculum dose (CFU/ml) was determined using a previously determined standard curve of OD at 600 nm against CFU/ml. Following overnight incubation at 37^{o}C, colony counts were determined. Groups of immunised (n = 20) and control (n = 20) mice were challenged intraperitoneally with GAS 10 days after the final immunisation. One mouse from each of the PepM and KLH control groups died during the challenge procedure.

2.4.10.2 Detection of murine antibodies and indirect bactericidal assay

Peptide-specific murine serum IgG antibodies and antibody isotypes were determined by ELISA as previously described (Hunter and Greenwood 1962; Hayman *et al.* 1997). Plates were coated with either a1 peptide conjugated to bovine serum albumin (BSA), or BSA alone. The OD of BSA control wells was subtracted from the OD of corresponding a1 peptide-BSA wells to determine the specific anti-a1 peptide response. Titre was defined as the highest dilution that gave an OD of more than three standard deviations above the mean OD of control wells containing non-immunised mouse sera.

Murine sera were assayed for the ability to opsonise GAS *in vitro* as previously described (Brandt *et al.* 1996). Briefly, GAS strains NS13 and NS455 were grown overnight at 37^{o}C in 5 ml THB, followed by serial dilution to 10^{-5} in PBS. For each

murine anti-peptide serum sample, 50 µl of fresh heat-inactivated serum was mixed with 50 µl of the bacterial dilution and incubated for 20 min at room temperature. Following this incubation, 400 µl of non-opsonic heparinised human blood was added and incubated at 37°C with end-over-end mixing. All human blood was tested prior to performing the assay to ensure that it could support the growth of the GAS strain to at least 32 times the CFU of the inoculum in a 3 h incubation at 37°C (Brandt *et al.* 1996). Human blood was obtained according to institutional and National Health and Medical Research Council ethical requirements.

The mixtures were incubated end-over-end at 37°C for 3 h and 50 µl from each tube was plated in duplicate on 2% (v/v) blood THB agar plates. The plates were incubated overnight at 37°C and the number of colonies on each plate was determined. Opsonic activity of the anti-peptide sera (% reduction in mean CFU) was calculated as (1-[CFU in the presence of anti-peptide sera] / [mean CFU in the presence of normal mouse sera]) x 100.

2.4.11 Statistical analysis

For plasminogen binding experiments, a one-way ANOVA was initially used on all data, followed by an unpaired t-test with Welsch's correction to determine if there was any significant difference in the plasminogen binding K_d values for the PAM mutants and PAM_{NS13} or $PAM_{NS88.2}$. For immunisation and challenge experiments, a Kruskal-Wallis test was used to determine if there was any significant variation in the median titres or Opsonisation induced by the four groups of antisera. Dunn's Multiple Comparison test was used for individual comparison of two groups of antisera. Difference in survival curves was determined by logrank test.

3 Characterisation of the plasminogen-binding properties of naturally occurring PAM variants

3.1 Introduction

There is a mounting body of evidence to suggest a role for the plasminogen activation system in streptococcal virulence, and it has recently been hypothesised that PAM–dependent plasminogen binding may confer a selective advantage on GAS during host colonisation and infection (Kalia and Bessen 2004). As such, the distribution of the *pam* gene amongst a subset of Northern Territory GAS isolates, and the potential effect of sequence variation among naturally occurring PAM variants on plasminogen binding function was investigated. Additionally, it was hypothesised that variation within the functional domain of PAM may result from selective pressure by the host immune response. If this is the case, it is expected that the plasminogen binding domain within PAM will be highly immunogenic and this response will be restrictive for propagation of GAS strains in systemic infection. As such, a murine vaccination model was utilised to determine the opsonophagocytic and protective properties of the plasminogen binding domain of PAM.

M protein is considered to be one of the major GAS virulence factors. This is due, in part, to the finding that M protein functions as a receptor for multiple host ligands. One such ligand is the host protein fibrinogen (Cunningham 2000). Studies by McKay *et al* noted that the PAM genotype was positively correlated with plasminogen binding via a fibrinogen dependent pathway (McKay *et al.* 2004). Furthermore, this study found that PAM positive GAS isolates displayed higher levels of fibrinogen binding than PAM negative isolates, raising the possibility that PAM binds fibrinogen directly. Similarly, it has been suggested that the prototype PAM protein interacts directly with the host plasminogen activator tPA (Wistedt *et al.* 1995). The ability of recombinant PAM

variants to bind fibrinogen and tPA was therefore investigated to determine if PAM functions as a receptor for multiple host proteins.

3.2 Results

3.2.1 Cloning of PAM genes

Following PCR amplification of chromosomal DNA with primers MI and MII, PCR products were cloned into the vector pCR2.1 and, the resulting plasmids were used to transform INVαF' *E. coli* cells (Invitrogen, USA). This was done in order to obtain single copies of the PAM genes from GAS isolates NS10, NS50.1, NS88.2, NS265, NS696, NS1133, NS221 and NS253. The PAM genes from six additional streptococcal isolates (NS13, NS59, NS32, NS53, NS223 and NS455) had previously been cloned using the same methodology. Plasmid DNA was extracted from the transformed *E. coli* cells and subjected to Southern hybridisation analysis in order to verify the PAM status of the insert DNA.

Figure 3.1 shows the results of Southern hybridisation analysis for a subset of potential clones containing the PAM_{NS221} (lanes 3-6), PAM_{NS253} (lanes 7-10) and PAM_{NS265} (lanes 11-14) PAM genes. Following restriction enzyme digestion with *Eco*RI, each of the seven plasmids released an insert of approximately 1.1 kb (Figure 3.1 A lanes 4, 6, 8, 10, 12 and 14). Southern hybridisation analysis showed three of these bands to be PAM genes (Figure 7 B lanes 6, 8 and 12). Negative control plasmid DNA (pCR2.1; lane 2) was not detected via Southern hybridisation analysis, while undigested positive control DNA (a previously detected positive clone; lane 1) was detected. Positive clones were identified for all nine PAM genes.

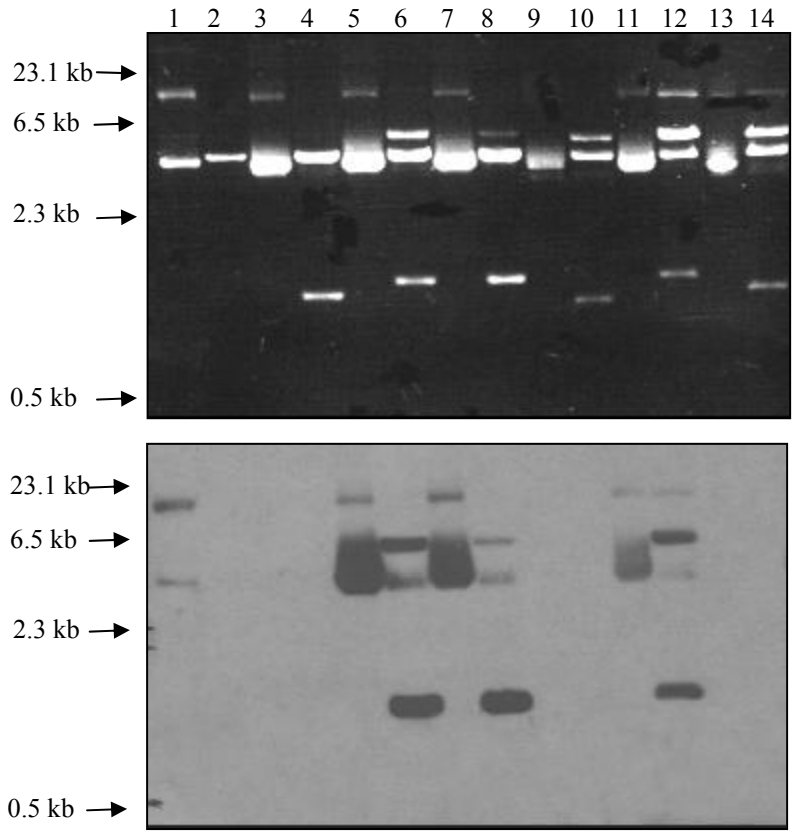


Figure 3.1 Example of Southern hybridisation analysis of clones containing PAM-like genes using DIG-labelled oligonucleotide probe PAM1. **A** Agarose gel electrophoresis. Lanes 3 and 5, 7 and 9, 11 and 13 show uncut pCR®2.1 following cloning with PCR product from GAS isolates NS221, NS253 and NS265 respectively. Lanes 4 and 6, 8 and 10, 12 and 14 show plasmid DNA after restriction enzyme digestion with *Eco*RI. All plasmids released insert DNA of approximately 1.1 kb in size. **B** Southern hybridisation of agarose gel shown in **A**. Three of the plasmids shown contained insert with homology to the a1/a2 repeat region of PAM, indicating the presence of PAM genes. Lanes 1 and 2 show positive (undigested pCR2.1/PAM construct) and negative (undigested pCR2.1) control DNA respectively, while molecular size markers (λ *Hin*d III) are indicated on the left.

3.2.2 DNA sequence analysis

Following successful cloning into pCR2.1, PAM genes were subjected to DNA sequence analysis using oligonucleotide primers to determine the extent of homology to the published sequence of PAM. DNA sequence data was used to deduce the amino acid sequence of PAM proteins using the program Etranslate (www.angis.org.au). Alignment

of deduced amino acid sequences with the prototype PAM sequence was conducted using eclustalW (Thompson *et al.* 1994). The results of the amino acid alignment are shown in Figure 3.2, while Table 3.1 lists the percent identity of each PAM variant gene to the prototype PAM sequence. Each of the PAM proteins appears to consist of a highly conserved signal peptide, followed by a variable domain which corresponds with the plasminogen-binding domain of the published PAM sequence. The a1 and a2 repeat domain is underlined in blue, with the residues involved in the interaction of PAM with plasminogen highlighted in green. This region is followed by a highly conserved domain consisting of two b repeats (147-160), three c repeats (219-278, underlined in purple) and a d repeat region (279-343), in addition to a membrane-spanning domain (344-388, underlined in green). These conserved regions are characteristic of all M and M-like proteins, and tend to vary only in the number, and type of repeats, as evidenced by PAM_{NS455}, which only contains two c repeats as opposed to three.

In addition to constructing a multiple sequence alignment for the full-length protein sequences, the a1/a2 binding motifs from each protein were also aligned (Figure 3.3). The identity of each binding site with the published a1/a2 binding site is given in Table 3.1. This region of the PAM protein was found to be quite divergent, with identities ranging from 52% to 100%. Although the NS696 M protein gave a positive result with the probe PAM1 during Southern hybridisation analysis, the a1/a2 repeat region characteristic of PAM proteins was not present upon sequence analysis. A region with 21% identity to the a1/a2 may be responsible for the positive Southern hybridisation result; however, it does not appear to be a true PAM or PAM-like protein, and subsequent *emm* sequence type analysis identified this gene as an M1 protein gene. The critical residues thought to be involved in the interaction between PAM and the kringle

2 domain of plasminogen (highlighted in green) have been identified as the a1 and a2 lysine residues (Lys^{98} and Lys^{111}), as well as Arg^{101}, His^{102} and Glu^{104} (Berge and Sjobring 1993; Wistedt *et al.* 1995; Schenone *et al.* 2000; Rios-Steiner *et al.* 2001). The a1 lysine residue was conserved in all of the PAM variants studied here, while the a2 lysine residue was conserved in all but PAM_{NS223} and $PAM_{NS88.2}$. Similarly, Arg^{101} and His^{102} were present in all variants, with the exception of $PAM_{NS88.2}$, and Glu^{104} was present in all but PAM $_{NS1133}$, PAM_{NS32}, and $PAM_{NS88.2}$.

It could be hypothesised that variation in the PAM sequence may manifest itself as a preference of some isolates for certain disease states. However, NS10, NS13 and NS59 GAS were isolated from a non-invasive throat infection, an invasive blood infection and a non-invasive wound respectively (Table 3.1), yet the PAM proteins from all three isolates show 100% identity to the prototype a1/a2 sequence and 99% identity to the prototype PAM sequence. Similarly, PAM $_{NS53}$, PAM $_{NS253}$ and PAM $_{NS455}$, all of which have identical a1/a2 repeats, and similar levels of identity throughout the whole PAM sequence, are associated with isolates from non-invasive febrile, non-invasive wound and invasive blood infections respectively. As such, no correlation was found between PAM sequence and the site of infection from which an isolate was obtained.

3.2.3 emm pattern and emm sequence typing

In order to further characterise the strains from which the PAM variants in this study were obtained, GAS isolates were subjected to analysis by *emm* pattern and *emm* sequence typing methods. The results of these analyses are listed in Table 3.1.

```
PAM       RKLKTGTASVAVALTVVGAGLASQTEVKANR------ADDARNEVLRGN----------

PAM  NS10   RKLKTGTASVAVALTVVGAGLASQTEVKANR------ADDARNEVLRGN----------
PAM  NS59   RKLKTGTASVAVALTVVGAGLASQTEVKANR------ADDARNEVLRGN----------
PAM  NS13   RKLKTGTASVAVALTVVGAGLASQTEVKANR------ADDARNEVLRGN----------
PAM  NS53   RKLKTGTASVAVALTVVGAGLASQTEVKADQPVDHHRYTEANNAVLQGR----------
PAM  NS455  RKLKTGTASVAVALTVVGAGLASQTEVKADQPVDHHRYTEANNAVLQGR----------
PAM  NS253  RKLKTGTASVAVALTVVGAGLASQTEVKADQPVDHHRYTEANNAVLQGR----------
PAM  NS265  RKLKTGTASVAVALTVVGAGLASQTEVKAEN---HPGYTAAQNGVLSELPGQA-------
PAM  NS88.2 RKLKTGTASVAVALTVVGAGLASQTEVKADR------YTDAHNAVTQGR----------
PAM  NS221  RKLKTGTASVAVALTVLGAGFANQTEVKAAD---HPSYTAAKDEVLSKF----------
PAM  NS32   RKLKTGTASVAVALTVLGAGFANQTEVKAAD---HPSYTAAKDEVLSKF----------
PAM  NS1133 RKLKTGTASVAVALTVLGAGFANQTEVKAAD---HPSYTAAKDEVLSKF----------
PAM  NS223  RKLKTGTASVAVALTVVGAGFANQTEVKAAD--DHPGAVAARNDVLSGF----------
PAM  NS50.1 RKLKTGTASVAVAVAVLGAGFANQTEVKAKE---HESVTRAREAAIRQMMQQGGRDFAPL



PAM       ---LVRAELWYRQ-----IQENDQLKLENKGLKTDLREKEEELQGLKDDVEKLTADAELQ

PAM  NS10   ---LVRAELWYRQ-----IQENDQLKLENKGLKTDLREKEEELQGLKDDVEKLTADAELQ
PAM  NS59   ---LVRAELWYRQ-----IQENDQLKLENKGLKTDLREKEEELQGLKDDVEKLTADAELQ
PAM  NS13   ---LVRAELWYRQ-----IQENDQLKLENKGLKTDLREKEEELQGLKDDVEKLTADAELQ
PAM  NS53   ---TVSARALLHE-----INKNGQLRSENEELKADLQKKEQELKNLNDDVKKLNDEVALE
PAM  NS455  ---TVSARALLHE-----INKNGQLRSENEELKADLQKKEQELKNLNDDVKKLNDEVALE
PAM  NS253  ---TVSARALLHE-----INKNGQLRSENEELKADLQKKEQELKNLNDDVKKLNDEVALE
PAM  NS265  ---QAFSRAFLHE-----REKNGELRLENEGLKTDLKKKEQELESLKGDVEKLKDEVALE
PAM  NS88.2 ---TVPLRNLLLE-----MDKNSKLRSENEELQAGLQEKKRENEELQAGLQEK--ERELE
PAM  NS221  ---SVPGHVWAHE-----REKNDKLSSENEGLKAGLQEKEQELKNLKADVEKLKDAAELE
PAM  NS32   ---SVPGHVWAHE-----REKNDKLSSENEGLKAGLQEKEQELKNLKADVEKLKDAAELE
PAM  NS1133 ---SVPGHVWAHE-----REKNDKLSSENEGLKAGLQEKEQELKNLKADVEKLKDAAELE
PAM  NS223  ---SVPGNVWYRQ-----HQEIGKLKSEKEELETELQEKEQELKNLKDNVEKLNTEVELE
PAM  NS50.1 LADTIRDNNNLRETLDETKKEIDDLKSENEGLETTLQKKEQE-------LKGLKDDAELQ

PAM       RLKNERH---EEAELERLKSERHDHDKKEAERKALEDKLADKQEHLNGALRYINEKEAEA

PAM  NS10   RLKNERH---EEAELERLKSERHDHDKKEAERKALEDKLADKQEHLNGALRYINEKEAER
PAM  NS59   RLKNERH---EEAELERLKSERHDHDKKEAERKALEDKLADKQEHLNGALRYINEKEAER
PAM  NS13   RLKNERH---EEAELERLKSERHDHDKKEAERKALEDKLADKQEHLNGALRYINEKEAER
PAM  NS53   RLKNERHVHDEEVELERLKNERHDHDKKEAERKALEDKLADKQEHLDGALRYINEKEAER
PAM  NS455  RLKNERHVHDEEVELERLKNERHDHDKKEAERKALEDKLADKQEHLDGALRYINEKEAER
PAM  NS253  RLKNERHVHDEEVELERLKNERHDHDKKEAERKALEDKLADKQEHLDGALRYINEKEAER
PAM  NS265  RLKNERHVHDEEAELERLKNERYEHDKKEAERKALEDKLADKQEHLNGALRYINEKEAER
PAM  NS88.2 DLKD--------AELKRLNEERHDHDKREAERKALEDKLADKQEHLDGALRYINEKEAER
PAM  NS221  RLKNERHDHD---ELERLKNERHDHDKREAERKALEDKLADRQEHLDGALRYINEKEAER
PAM  NS32   RLKNERHDHD---ELERLKNEGHDHDKREAERKALEDKLADKQEHLDGALRYINEKEAER
PAM  NS1133 RLKNERHDHD---ELERLKNERHDHDKREAERKALEDKLADKQEHLDGALRYINEKEAER
PAM  NS223  RLKNERHDHDEEAELNRLREERHDHDKKEAERKALEDKLADKQEHLDGALRYINEKEAER
PAM  NS50.1 RLKNERH---EEAELERLKSERHEHDKKEAERKALEDKLADKQEHLDGALRYINEKEAER
```

```
PAM        KEKEAEQKKLKEEKQISDASRQGLRRDLDASREAKKQVEKDLANLTAELDKVKEEKQISD

PAM  NS10  KEKEAEQKKLKEEKQISDASRQGLRRDLDASREAKKQVEKDLANLTAELDKVKEEKQISD
PAM  NS59  KEKEAEQKKLKEEKQISDASRQGLRRDLDASREAKKQVEKDLANLTAELDKVKEEKQISD
PAM  NS13  KEKEAEQKKLKEEKQISDASRQGLRRDLDASREAKKQVEKDLANLTAELDKVKEEKQISD
PAM  NS53  KEKEAEQKKLKEEKQISDASRQGLRRDLDASREAKKQVEKDLANLTAELDKAKEEKQISD
PAM  NS455 KEKEAEQKKLKEEKQISDASRQ-----------------------------------
PAM  NS253 KEKEAEQKKLKEEKQISDASRQGLRRDLDASREAKKQVEKDLANLTAELDKVKEEKQISD
PAM  NS265 KEKEAEQKKLKEEKQISDASRQGLRRDLDASREAKKQVEKDLANLTAELDKVKEEKQVSD
PAM  NS88.2 KEKEAEQKKLKEEKQISDASRQGLRRDLDASREAKKQVEKDLANLTAELDKVKEEKQISD
PAM  NS221 KEKEAEQKKLKEEKQVSDASRQGLRRDLDASREAKKQVEKDLANLTAELDKVKEEKQISD
PAM  NS32  KEKEAEQKKLKEEKQISDASRQGLRRDLDASREAKKQVEKDLANLTAELDKVKEEKQISD
PAM  NS1133 KEKEAEQKKLKEEKQISDASRQGLRRDLDASREAKKQVEKDLANLTAELDKVKEEKQISD
PAM  NS223 KEKEAEQKKLKEEKQISDASRQGLRRDLDASREAKKQVEKDLANSTAEFDKVKEEKQISD
PAM  NS50.1 REKEAEQKKLKEEKQISDASRQGLRRDLDASREAKKQVEKDLANLTAEFDKVKEEKQISD



PAM        ASRQGLRRDLDASREAKKQVEKGLANLTAELDKVKEEKQISDASRQGLRRDLDASREAKK

PAM  NS10  ASRQGLRRDLDASREAKKQVEKGLANLTAELDKVKEEKQISDASRQGLRRDLDASREAKK
PAM  NS59  ASRQGLRRDLDASREAKKQVEKGLANLTAELDKVKEEKQISDASRQGLRRDLDASREAKK
PAM  NS13  ASRQGLRRDLDASREAKKQVEKGLANLTAELDKVKEEKQISDASRQGLRRDLDASREAKK
PAM  NS53  ASRQGLRRDLDASREAKKQVEKDLANLTAELDKVKEEKQISDASRQGLRRDLDASREAKK
PAM  NS455 ----------------------------------------GLRRDLDASREAKK
PAM  NS253 ASRQGLRRDLDASREAKKQVEKDLANLTAELDKVKEEKQISDASRQGLRRDLDASREAKK
PAM  NS265 ASRQGLRRDLDASREAKKKVEADLAALTAEHQKLKEEKQISDASRQGLSRDLEASREAKK
PAM  NS88.2 ASRQGLRRDLDASREAKKQVEKGLANLTAELDKVKEEKQISDASRQGLRRDLDASREAKK
PAM  NS221 ASRQGLRRDLDASREAKKQVEKDLANLTAELDKVKEEKQISDASRQGPRRDLDASREAKK
PAM  NS32  ASRQGLRRDLDASREAKKQVEKDLANLTAELDKVKEEKQISDASRQGLRRDLDASREAKK
PAM  NS1133 ASRQGLRRDLDASREAKKQVEKDLADLTAELDKVKEEKQISDASRQGLRRDLDASREAKK
PAM  NS223 ASRQGLRRDLDASREAKKQVEKDLANLTAEFDKVKEEKQISDASRQGLRRDLDASREAKK
PAM  NS50.1 ASRQGLRRDLDASREAKKQVEKDLANLTAEFDKVKEEKQISDASRQGLRRDLDASREAKK

PAM        QVEKALEEANSKLAALEKLNKELEESKKLTEKEKAELQAKLEAEAKALKEQLAKQAEELA

PAM  NS10  QVEKALEEANSKLAALEKLNKELEESKKLTEKEKAELQAKLEAEAKALKEQLAKQAEELA
PAM  NS59  QVEKALEEANSKLAALEKLNKELEESKKLTEKEKAELQAKLEAEAKALKEQLAKQAEELA
PAM  NS13  QVEKALEEANSKLAALEKLNKELEESKKLTEKEKAELQAKLEAEAKALKEQLAKQAEELA
PAM  NS53  QVEKALEEANSKLAALEKLNKELEESKKLTEKEKAELQAKLEAEAKALKEKLAKQAEELA
PAM  NS455 QVEKALEEANSKLAALEKLNKELEESKKLTEKEKAELQAKLEAEAKALKEKLAKQAEELA
PAM  NS253 QVEKALEEANSKLAALEKLNKELEESKKLTEKEKAELQAKLEAEAKALKEKLAKQAEELA
PAM  NS265 KVEADLAEANSKLQALEKLNKELEEGKKLSEKEKAELQARLEAEAKALKEQLAKQAEELA
PAM  NS88.2 QVEKALEEANSKLAALEKLNKELEESKKLTEKEKAELQAKLEAEAKALKEQLAKQAEELA
PAM  NS221 QVEKALEEANSKLAALEKLNKELEESKKLTEKEKAELQAKLEAEAKALKEQLAKQAEELA
PAM  NS32  QAEKALEEANSKLAALEKLNKELEESKKLTEKEKAELQAKLEAEAKALKEQLAKQAEELA
PAM  NS1133 QVEKALEEANSKLAALEKLNKELEESKKLTEKEKAELQAKLEAEAKALKEQLAKQAEELA
PAM  NS223 QVEKALEEANSKLAALEKLNKELEESKKLTEKEKAELQAKLEAEAKALKEQLAKQAEELA
PAM  NS50.1 QVEKALEEANSKLAALEKLNKELEESKKLTEKEKAELQAKLEAEAKALREQLAKQAEELA
```

Figure 3.2 ClustalW alignment of deduced PAM amino acid sequences. Black font indicates residues identical to those of the prototype PAM sequence, while red font indicates residues different to the prototype PAM sequence. Important domains are underlined as follows; a repeats, blue; c repeats, purple and membrane spanning domain green. Critical residues involved in the interaction of the a1 repeat with kringle 2 of plasminogen are highlighted in green.

	a1	a2
PAM	DAELQRLKNERH---E	EAELERLKSERHD
PAM $_{NS10}$	**********	**********
PAM $_{NS13}$	**********	**********
PAM $_{NS59}$	**********	**********
PAM $_{NS50.1}$	**********	**********E
PAM $_{NS221}$	A***E*******D	HD*****N****
PAM $_{NS1133}$	A***E*******D	HD*****N****
PAM $_{NS32}$	A***E*******D	HD*****N*G**
PAM $_{NS53}$	EVA*E******VHD*	*V*****N****
PAM $_{NS253}$	EVA*E******VHD*	*V*****N****
PAM $_{NS455}$	EVA*E******VHD*	*V*****N****
PAM $_{NS223}$	EV**E******DHD*	***N**RE****
PAM $_{NS265}$	EVA*E******VHD*	*******N**YE
PAM $_{NS88.2}$	ER**ED**----***-	D***K**NE****
NS696 M1	WDRQRLE*ELEE**KK	**LAIDQA*RD*Y

Figure 3.3 Alignment of the deduced amino acid sequences of PAM variants corresponding to the a1 and a2 repeat regions of the prototype PAM sequence. Identical residues are indicated by *, while – indicates gap introduced by the alignment program ClustalW. Residues involved in a1/a2 domains interaction with kringle 2 of plasminogen are highlighted in green.

Table 3.1 Molecular characteristics of PAM positive GAS strains.

Isolate	Clinical origin	*vir* type	*emm* ST[a]	*emm* pattern	Identity of PAM protein to prototype PAM	Identity of PAM protein to prototype a1/a2 PAM domain
NS10	Throat, uncomplicated	24	53	D	99.7%	100%
NS59	Wound, uncomplicated	24	53	D	99.7%	100%
NS13	Blood, invasive	24	53	D	99.5%	100%
NS253	Wound, uncomplicated	29.1	52	D	88.9%	69%
NS455	Blood, invasive	29.1	52	D	69%	69%
NS53	Febrile and unwell patient, uncomplicated	29.1	71	D	88.7%	69%
NS32	Wound, uncomplicated	29.2	101	D	87.4%	76%
NS1133	Blood, invasive	17.1	101	D	87.6%	79%
NS221	Throat, uncomplicated	17.1	101	D	87.1%	79%
NS223	Infected central venous catheter (leg), blood, invasive	4	4	D	88.9%	69%
NS88.2	Blood, invasive	17.4	98.1	D	85.8%	52%
NS265	Wound, uncomplicated	11	56	D	84.5%	65%
NS50.1	Wound, uncomplicated	12.1	108	D	84.7%	96.5%
NS696	Throat swab, pharyngitis, uncomplicated	78	1	ABC	71.9%	21%

[a] Sequence Type (ST)

Of the GAS isolates that tested positive for the PAM gene via Southern hybridisation, all were found to have an *emm* pattern D *mga* regulon. NS696 was found to have a pattern ABC chromosomal arrangement.

Results of *emm* ST indicate that isolates with the same, or highly similar PAM genes have the same *emm* ST. PAM $_{NS10}$, PAM $_{NS59}$, and PAM $_{NS13}$, all of which have the same a1/a2 region were all found to be from *emm*53 isolates, while NS221, NS1133 and

NS32 were all found to be *emm*101 isolates, and possess PAM genes which are identical except for one residue in PAM_{NS32}. The GAS isolate NS53 was an exception to this finding. This isolate was found to type *emm*71, however, NS455 and NS253, both of which have PAM genes which are highly homologous to the PAM_{NS53}, were found to be type *emm52* strains. As with sequence homology, no association was seen between *emm* ST and GAS site of isolation. As has been found previously, *emm* ST was highly concordant with *vir* type (Gardiner *et al.* 1998).

3.2.4 Pulsed field gel electrophoresis and phylogenetic analysis

To determine if variation within the PAM genes of streptococcal isolates was indicative of wider, chromosomal variation, PFGE of the chromosomal DNA of thirteen PAM-positive GAS isolates was performed. Fifteen PAM-negative isolates were also included to enable comparisons of chromosomal variation between PAM-positive and PAM-negative isolates.

The subset of 28 GAS strains used in this study consisted of 22 different *vir* types and 22 different *emm* sequence types (McKay *et al.* 2004). PFGE analysis (Figure 3.4 A) revealed 23 different fingerprint patterns. The thirteen PAM positive isolates displayed 8 different fingerprint patterns, suggesting that this group represents a genetically diverse subset of isolates. In general, PFGE clustering was concordant with both *vir* and *emm* type. No congruence was seen between the chromosomal patterns for the *emm* locus (*emm* pattern) and PFGE fingerprint, with isolates of all *emm* patterns found to be distributed throughout the dendogram. This was anticipated, as there are only 5 possible *emm* patterns. PAM positive isolates were found to cluster into three groups. Cluster 1 contained PAM positive isolate NS265, cluster 2 contained the PAM positive isolates NS223, NS50.1, NS59, NS13 and NS10, and cluster 3 contained the PAM positive

isolates NS221, NS88.2, NS455, NS253, NS32 and NS1133. Isolates NS10, NS13 and NS59 (cluster 2), and NS455 and NS253 (cluster 3), appear to be clonal as they display identical PFGE patterns, *vir* types and *emm* sequence types. A small number of changes in the PAM sequences of these isolates do not appear to be mirrored by changes to *emm* sequence type or *vir* type. Similarly, isolates NS1133 and NS32 (cluster 3), display identical PFGE patterns and *emm* sequence types, and as such, are likely to be clonal. Phylogenetic analysis of the amino acid sequences of the PAM genes from the PAM positive isolates showed only minor congruence with PFGE analysis (Figure 3.4 B). The PAM proteins were found to cluster in four groups, however, this clustering was not concordant with that seen in the PFGE analysis, suggesting that horizontal gene transfer of PAM is occurring in GAS isolates. This is most evident in the case of GAS isolate NS265. PAM_{NS265} lies close to PAM sequences from cluster 3 isolates (PAM_{53}, PAM_{253} and PAM_{455}), however, GAS strain NS265 is genetically distinct from the other PAM positive isolates subjected to PFGE. The positioning of PAM_{NS223} from cluster 2, in close proximity to PAM proteins from cluster 3 GAS isolates, also suggests horizontal transfer of the PAM gene among GAS strains belonging to distinct PFGE clusters. Additionally, whilst GAS strain NS88.2 falls into the PFGE cluster group 3, $PAM_{NS88.2}$ extends from a separate node of the phylogeny dendogram to all other variants. These data suggest that $PAM_{NS88.2}$ is a phylogenetically distinct gene.

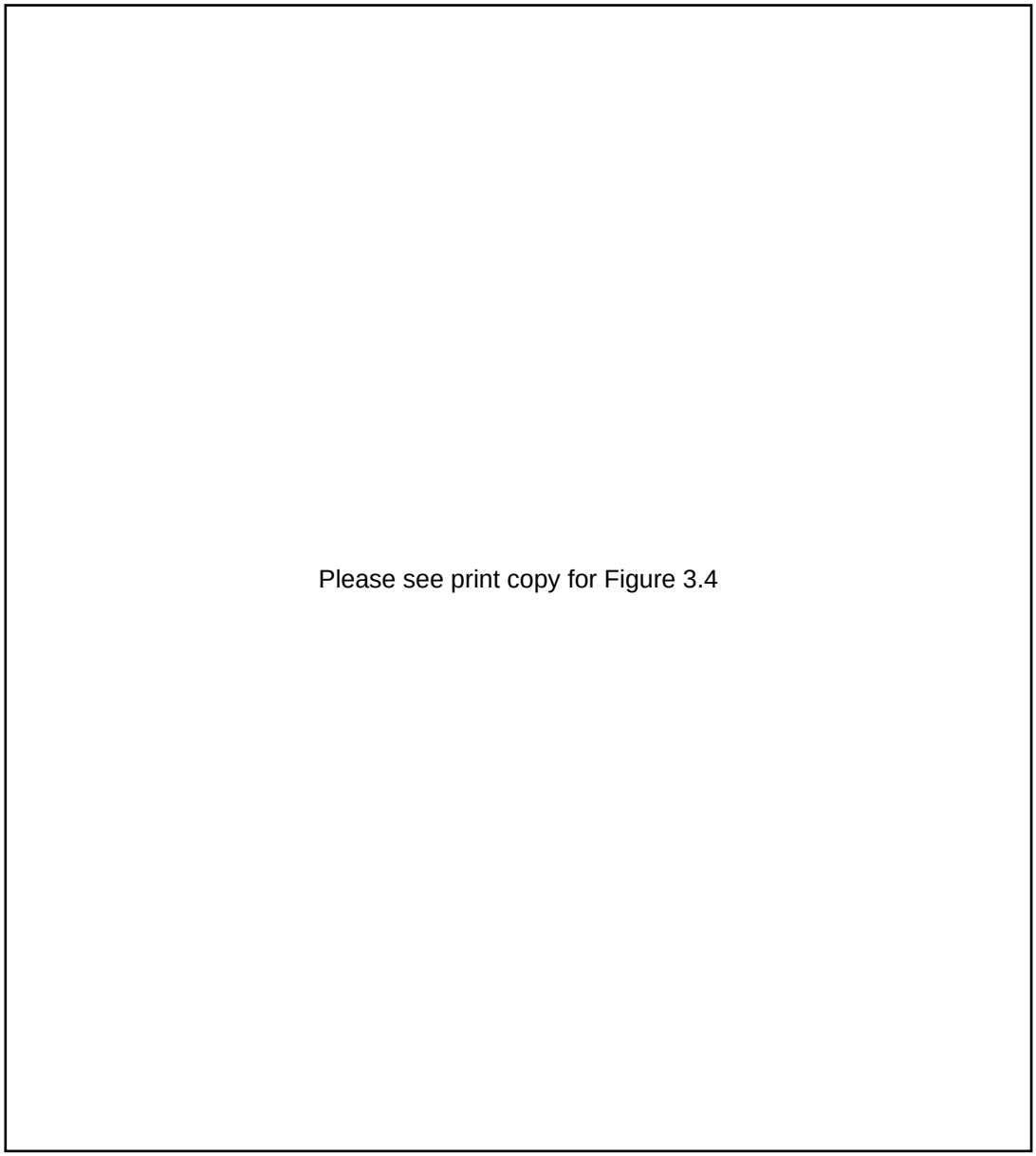


Figure 3.4 Evolutionary analysis of PAM positive GAS isolates**. A** Dendogram generated by Diversity software showing the genetic relationships between 13 PAM positive GAS isolates and 15 PAM negative GAS isolates. The dendogram was constructed by cluster analysis of the PFGE patterns obtained after macrorestrictrion with *Sma*I enzyme, using the unweighted pair group method with arithmetic means. PFGE fingerprint patterns are shown next to the corresponding branches of the dendogram. The strain number is followed by *vir* type, *emm* sequence type, *emm* pattern and PAM status (McKay *et al.* 2004). Patterned branches represent the three different clusters of GAS isolates found in the dendogram (hatched, cluster 1; shaded cluster 2; cross-hatched cluster 3). **B** Phylogeny tree generated by MrBayes analysis of the amino acid sequences of 14 PAM genes (PAM_{NS13} GenBank™ AY351851; PAM_{NS455} GenBank™ AY351857; PAM_{NS265} GenBank™ AY351855; PAM_{NS223} GenBank™ AY351854; PAM_{NS253} GenBank™ AY351853; PAM_{NS53} GenBank™ AY351852; PAM_{NS32} GenBank™ AY351850; $PAM_{NS50.1}$ GenBank™ AY351849; PAM_{NS59} GenBank™ AY351848; PAM_{NS1133}

GenBank™ AY351847; PAM_{NS10} GenBank™ AY351846; PAM_{NS221} GenBank™ DQ136319; $PAM_{NS88.2}$ GenBank™ AY351856; NS696 M1 protein GenBank™ AY351858). Patterned lines correspond to the cluster groups as described above. MLC36 (GenBank™Z32677) and MLC72 (GenBank™Z32678) are plasminogen-binding M protein sequences from group C and group G streptococci respectively (Ben Nasr *et al.* 1994). The M1 protein sequence (Harbaugh *et al.* 1993) was included as an outgroup and for comparison with the NS696 M1 protein.

3.2.5 Cloning and expression of recombinant PAM variants

In order to analyse the plasminogen binding characteristics of naturally occurring PAM variants, the PAM genes from six GAS strains, which had previously been cloned into pCR2.1 were sub-cloned into the expression vector pGEX-2T. Genes were selected for expression based on PFGE cluster group (Figure 3.4), variation both within the plasminogen-binding a1/a2 repeat domain (Figure 3.3, Table 3.1) and other regions within the protein (Figure 3.2, Table 3.1). The gene encoding the NS696 M1 protein, with no significant identity to the prototype PAM a1/a2 repeat region, was selected as a negative control. The presence of insert DNA in the resulting expression constructs was confirmed by restriction digestion of construct DNA with *Eco*RI and *Bam*HI, followed by agarose gel electrophoresis (Figure 3.5). All construct were found to contain inserts of approximately 1.1 kb, with the exception of the PAM_{NS455} expression vector (0.8 kb) and the NS696 M1 protein expression vector (1.2 kb). DNA sequence analysis was used to confirm the lack of polymerase chain reaction errors in the cloned amplification product, and the presence of the correct insert in each vector.

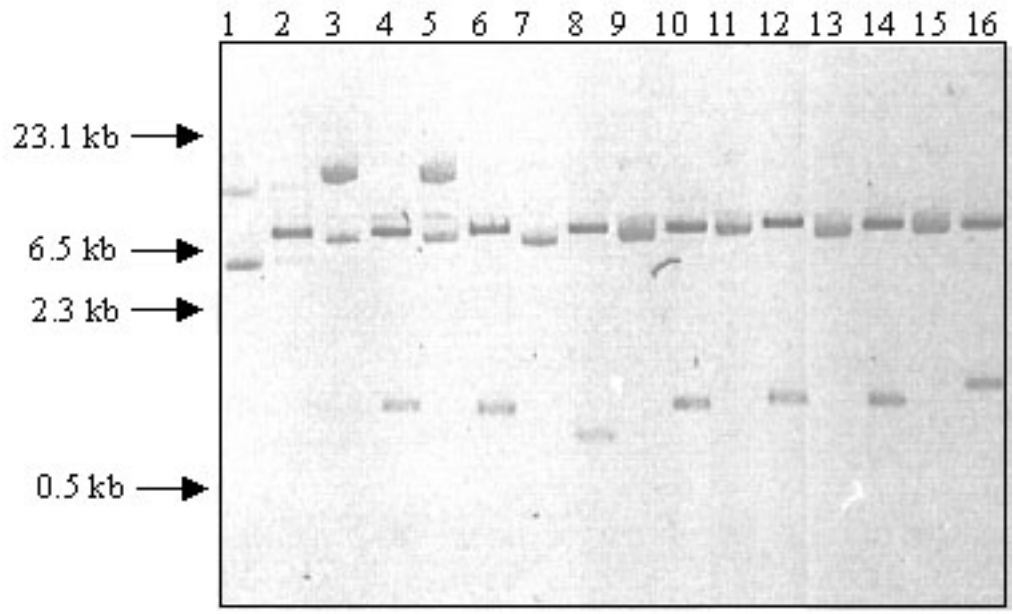


Figure 3.5 Agarose gel electrophoresis and *Eco*RI/*Bam*H1 restriction digestion analysis of pGEX2T/PAM expression constructs. Lanes 1 and 2 show digested and undigested pGEX2T DNA respectively. Lanes 3, 5, 7, 9, 11, 13 and 15 show undigested pGEX2T expression constructs for PAM_{NS13}, $PAM_{NS88.2}$, PAM_{NS455}, PAM_{NS1133}, PAM_{NS53}, PAM_{NS265} and NS696 M protein respectively. Lanes 4, 6, 8, 10, 12, 14 and 16 show *Eco*RI and *Bam*H1 restriction enzyme digested pGEX2T expression constructs for PAM_{NS13}, $PAM_{NS88.2}$, PAM_{NS455}, PAM_{NS1133}, PAM_{NS53}, PAM_{NS265} and NS696 M1 protein respectively. Following digestion, all expression vectors released inserts of approximately 1.1 kb in size, except for the PAM_{NS455} expression construct, which released and insert of 0.8 kb in size, and the NS696 M1 protein expression construct, which released an insert of 1.2 kb in size.

Following expression in *E. coli*, recombinant proteins were purified using glutathione-agarose and Ni-NTA agarose affinity chromatography (Figure 3.6 A). SDS-PAGE analysis (Figure 3.6 B) indicated the proteins were within the expected size range (PAM_{NS13} 42 kDa; PAM_{NS53}, 42 kDa; PAM_{NS1133}, 43 kDa; PAM_{NS455}, 32 kDa; PAM_{NS265}, 45 kDa; $PAM_{NS88.2}$, 40 kDa; NS696 M1 protein, 47 kDa). Proteins appear as doublet bands following SDS-PAGE, which is characteristic of some M proteins (Cunningham 2000).

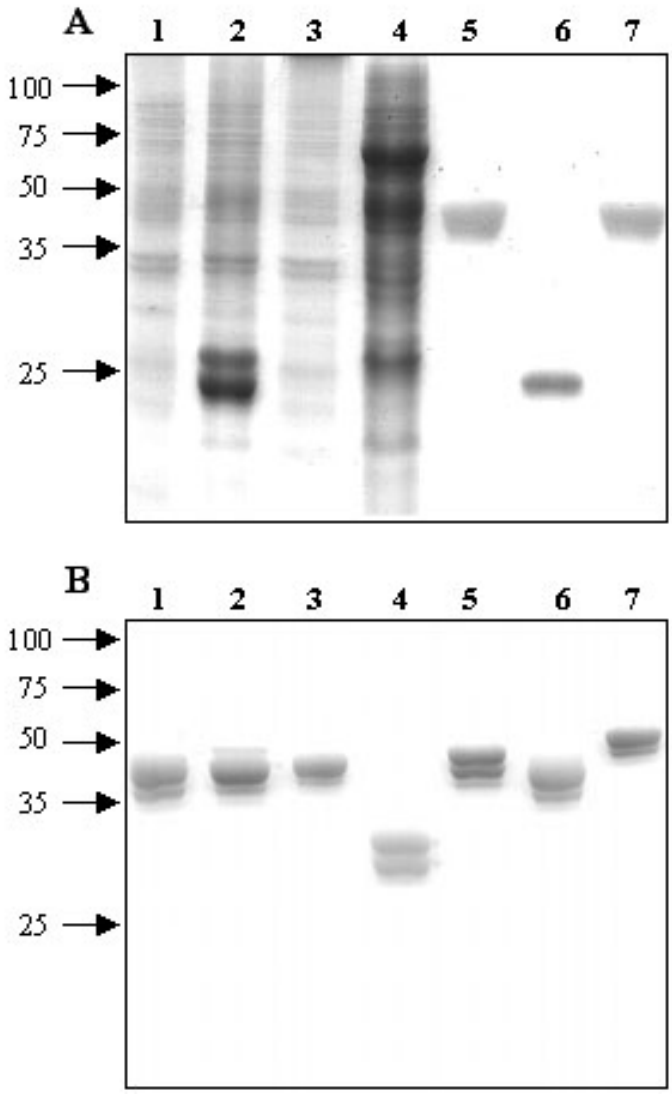


Figure 3.6 SDS-PAGE analysis of recombinant PAM variants. **A** Fractions from each stage of protein purification were analysed by 12% SDS-PAGE. Lane 1, whole-cell lysate of non-induced *E. coli* TOP10 [pGEX-2T]; lane 2, whole-cell lysate of *E. coli* TOP10 [pGEX-2T] 4 h post-induction with 0.1mM IPTG; lane 3, whole-cell lysate of non-induced *E. coli* TOP10 [pGEX-2T/PAM $_{NS13}$]; lane 4, whole-cell lysate of *E. coli* TOP10 [pGEX-2T/PAM $_{NS13}$] 4 h post induction with 0.1mM IPTG; lane 5, PBS elution containing recombinant protein following thrombin cleavage; lane 6, glutathione elution buffer elute containing GST only; lane 7, Ni-NTA native elution buffer elute containing PAM_{NS13} following Ni-NTA purification. **B** 12% SDS-PAGE gel showing purified recombinant PAM variant proteins. Lanes 1 to 7 show variants PAM_{NS13}, PAM_{NS53}, PAM $_{NS1133}$, PAM $_{NS455}$, PAM $_{NS265}$, PAM$_{, NS88.2}$ and NS696 M1 protein respectively. Molecular weight markers are given in kilo Daltons (kDa).

3.2.6 Characterisation of the binding properties of recombinant PAM variants

3.2.6.1 Plasminogen purification and labelling

Glu-plasminogen was purified from human plasma using lysine Sepharose-4B affinity chromatography (Figure 3.7 A). Elution fractions were monitored at 750 nm using the Lowry protein detection assay. Following elution with 200 mM ε-amino caproic acid, purified plasminogen was analysed by 12% SDS-PAGE (Figure 3.7 B). Purified plasminogen ran at close to the expected size (92 kDa) under both reducing and non-reducing conditions, no low molecular weight protein bands indicative of the presence of plasmin were detected (Ponting *et al.* 1992). Western blotting analysis using rabbit polyclonal anti-human plasminogen antibodies at a dilution of 1 in 2000 confirmed the identity of the purified protein (Figure 3.7 C).

For use in plasminogen binding experiments, purified glu-plasminogen was conjugated to biotin-X-NHS. Removal of unconjugated biotin using PD-10 filtration was monitored at 750 nm via Lowry protein detection assay (Figure 3.8 A). Biotinylated plasminogen was typically eluted in fractions 10, 11 and 12, which were then pooled. Western blotting analysis, using neutravidin conjugated horseradish peroxidase confirmed biotinylation (Figure 3.8 B).

3.2.6.2 Plasminogen binding analysis

DNA sequence analysis of a subset of PAM genes identified significant variation within the plasminogen binding domain of this protein. It was hypothesised that this diversity may manifest as a reduction in the plasminogen binding ability of certain variants. As such, the plasminogen binding function of 6 naturally occurring PAM variants, and the NS696 M1 protein was investigated using ligand blotting techniques.

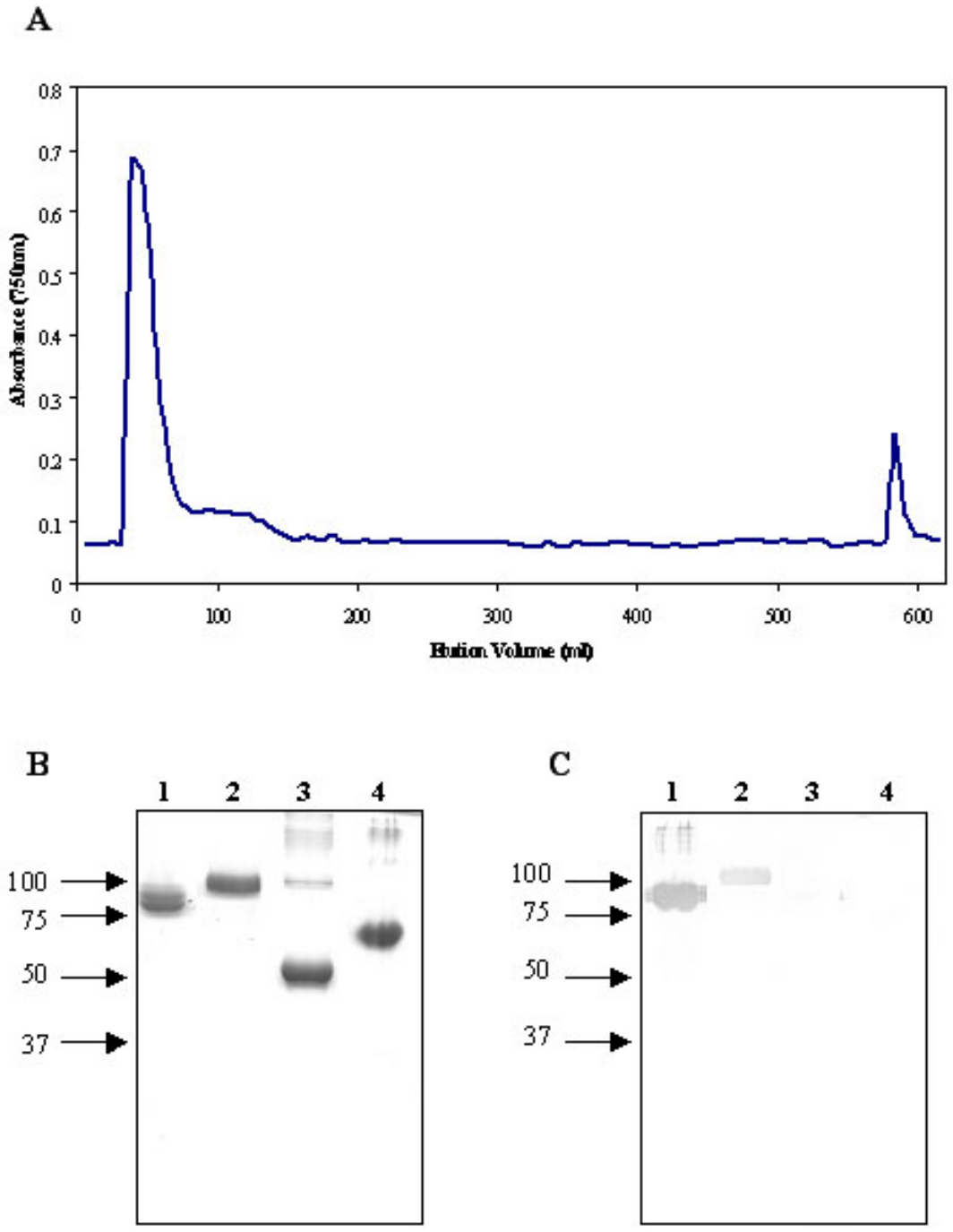


Figure 3.7 Purification of plasminogen from human plasma. **A** Elution profile of the purification of glu-plasminogen from human plasma. **B** 12% SDS-PAGE analysis of purified glu-plasminogen. Plasminogen was electrophoresed under both non-reducing (Lane 1) and reducing (Lane 2) conditions. BSA was also electrophoresed under both non-reducing (Lane 3) and reducing (Lane 4) conditions as a negative control for Western blotting analysis. Molecular weight markers are given in kDa. **C** Western blotting analysis of purified glu-plasminogen using rabbit polyclonal anti-human plasminogen antibodies diluted 1 in 2000. Plasminogen was electrophoresed under both non-reducing (Lane 1) and reducing (Lane 2) conditions. BSA was also electrophoresed under both non-reducing (Lane 3) and reducing (Lane 4) conditions as a negative control. Molecular weight markers are given in kilo Daltons (kDa).

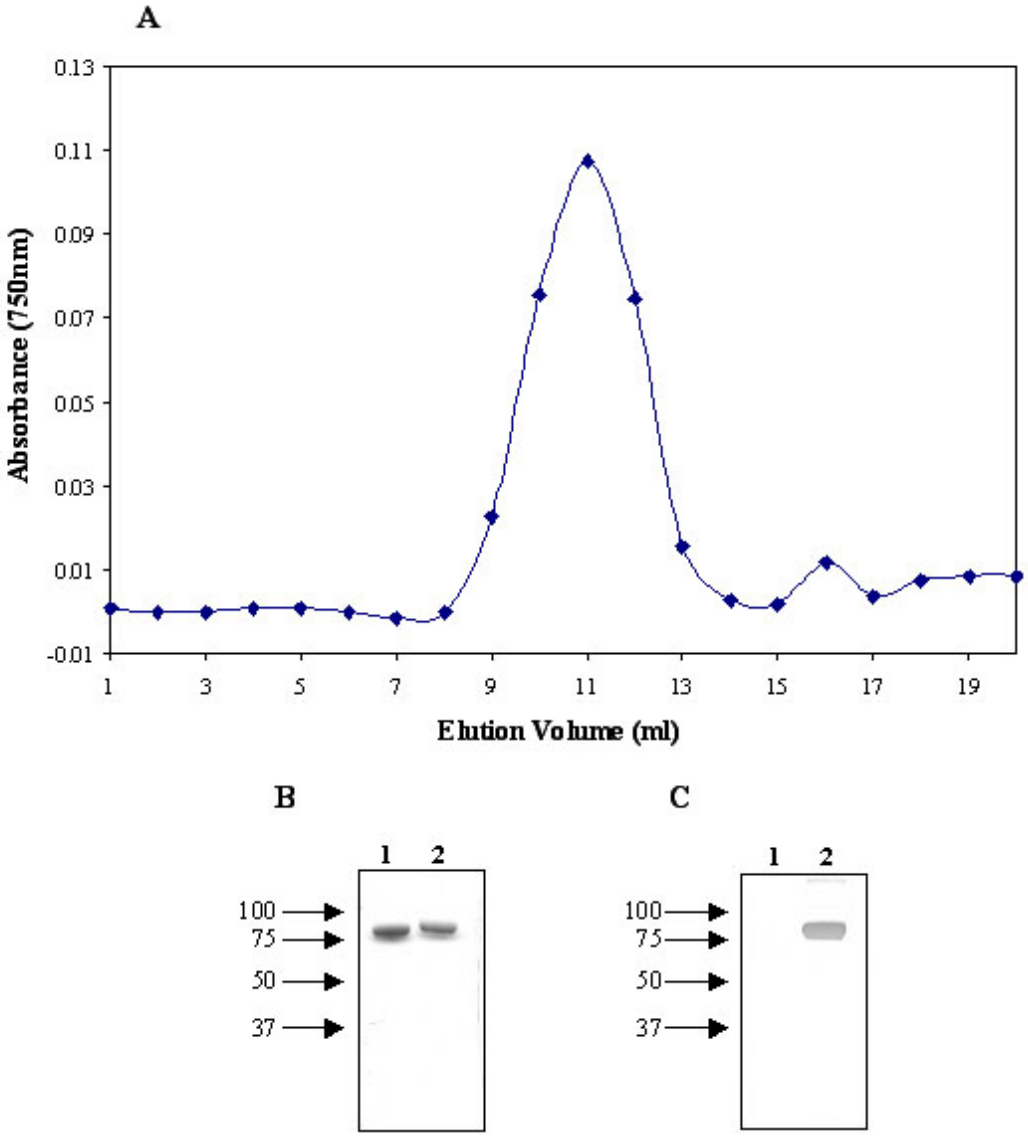


Figure 3.8 Biotinylation of glu-plasminogen. **A** Elution of biotinylated glu-plasminogen from a PD-10 gel filtration column. Plasminogen typically eluted in elution volumes 10, 11 and 12. **B** 12% SDS-PAGE analysis of non-biotinylated (lane 1) and biotinylated (lane 2) glu-plasminogen. Molecular size markers are given in kDa. **C** Western blot analysis utilising neutravidin-HRP of non-biotinylated (lane1) and biotinylated (lane 2) glu-plasminogen. Molecular weight markers are given in kilo Daltons (kDa).

All recombinant proteins reacted with biotinylated glu-plasminogen in a ligand blot analysis, except for the NS696 M1 protein (Figure 3.9).

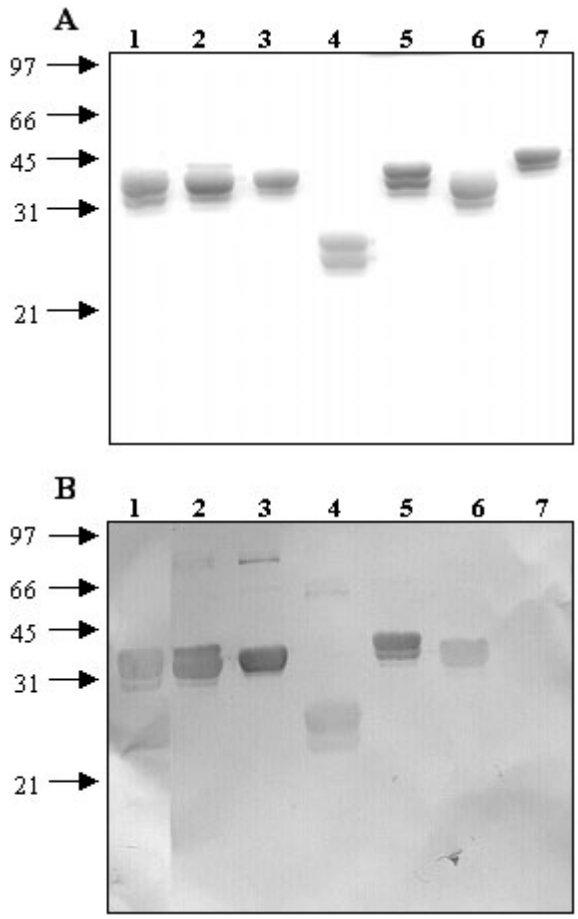


Figure 3.9 Ligand blot analysis of purified recombinant PAM variants. **A** 12% SDS-PAGE gel of recombinant PAM variants used for ligand blotting analysis. Lane 1, PAM_{NS13}; lane 2, PAM_{NS53}; lane 3, $PAM_{PAMNS1133}$; lane 4, PAM_{NS455}; lane 5, PAM_{NS265}; lane 6, $PAM_{NS88.2}$; lane 7, NS696 M1 protein. **B** Ligand blot of purified variant PAM proteins employing biotinylated glu-plasminogen and neutravidin-HRP. Lane 1, PAM_{NS13}; lane 2, PAM_{NS53} lane 3, PAM_{NS1133}; lane 4, PAM_{NS455}; lane 5, PAM_{NS265}; lane 6, $PAM_{NS88.2}$; lane 7, . NS696 M1 protein. Molecular weight markers are given in kilo Daltons (kDa).

The interaction between recombinant PAM variants and glu-plasminogen was further characterised using saturation plasminogen binding assays. Recombinant proteins immobilised to 96-well plates were incubated with increasing concentrations of biotinylated glu-plasminogen, in the presence or absence of a 50 fold molar excess of

unlabelled glu-plasminogen. The recombinant PAM proteins bound plasminogen in a dose-dependent fashion, and saturable binding was achieved with 500 nM plasminogen for 5 of the 6 recombinant proteins after 2 h (Figure 3.10). Only non-specific binding was found for NS696 M1 protein. Non-linear regression analysis was used to determine the affinity of each recombinant protein for glu-plasminogen (Table 3.2). Equilibrium dissociation constants (K_d) were calculated using a best-fit non-linear regression curve. K_d values were found to be consistent with those found for the prototype PAM protein and plasminogen binding M proteins of group C and G streptococci (Berge and Sjobring 1993; Ben Nasr *et al.* 1994; Wistedt *et al.* 1995; Wistedt *et al.* 1998) and ranged from 1.58 nM to 7.58 nM, indicating that each of the PAM variants maintained the ability to bind plasminogen with high affinity. While the range in affinity was small, PAM_{NS13}, which has 100% identity to the prototype PAM in the a1/a2 region was found to have significantly higher affinity for plasminogen than the other PAM variants (PAM_{NS53} $p = 0.001$; $PAM_{N88.2}$; $p = 0.022$; PAM_{NS265} $p = 0.002$; PAM_{NS455} $p = 0.008$; PAM_{NS1133} $p = 0.005$).

Plasminogen binding experiments indicated that sequence variation within the a1 and a2 repeats reduces but does not abolish plasminogen binding affinity by PAM variants. To further explore the PAM variants relative affinity for plasminogen, competition binding experiments were performed. Recombinant PAM variants were immobilised then incubated with biotinylated glu-plasminogen at a saturating concentration (500 nM). Unlabelled fluid phase competitor (PAM_{NS13}) was added at varying concentrations to the immobilised protein prior to the addition of labeled plasminogen.

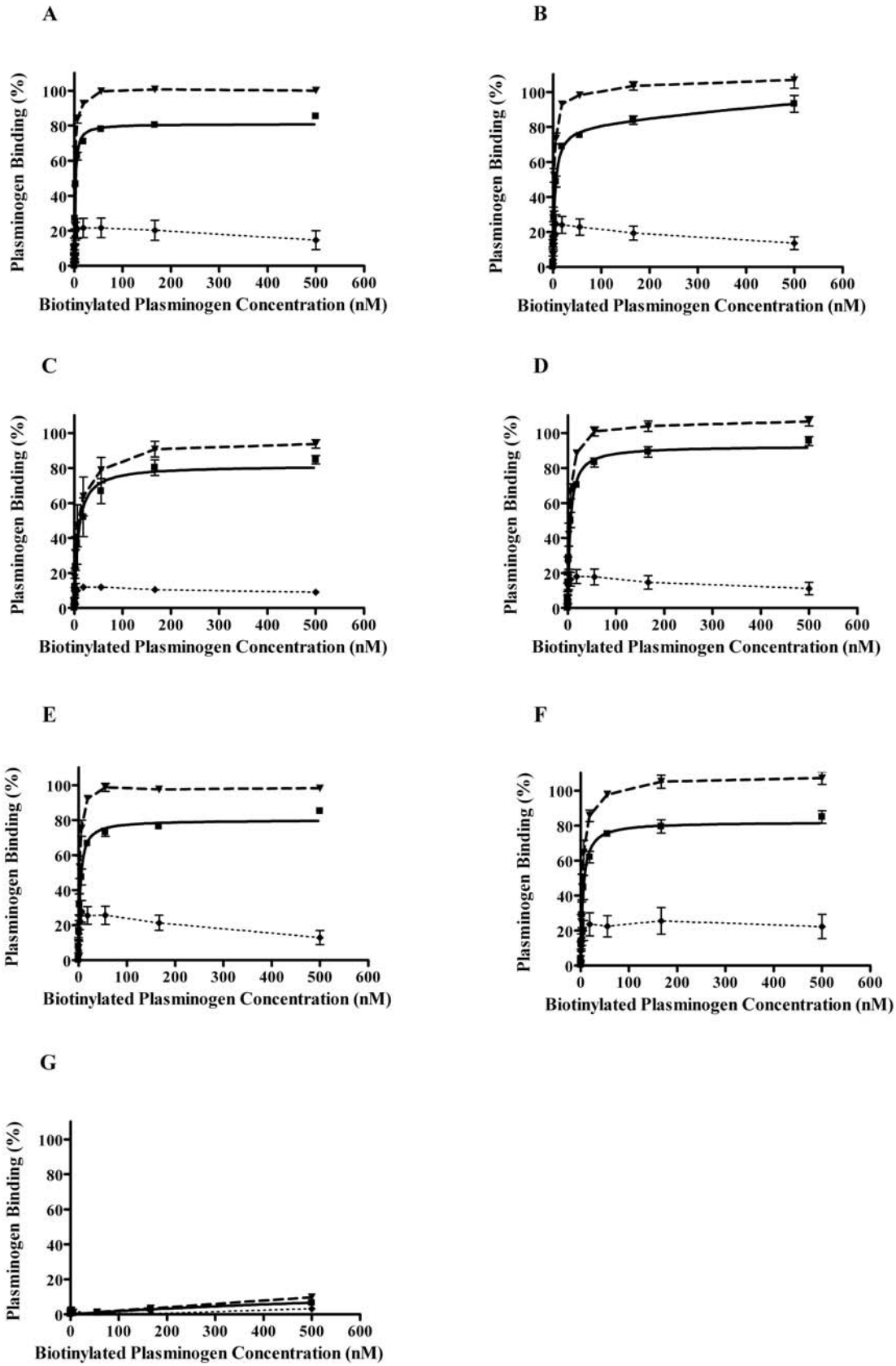


Figure 3.10 Saturation binding analysis of biotinylated glu-plasminogen to immobilised recombinant PAM variant proteins. Biotinylated glu-plasminogen binding to immobilised recombinant protein (**A**, PAM_{NS13}; **B,** PAM_{NS53}; **C,** $PAM_{NS88.2}$; **D**, PAM_{NS265}; **E**, PAM_{NS455}; **F**, PAM_{NS1133}; **G**, $_{NS696}$ M1 protein) was measured in the absence (▼, total binding) and presence

(●, non-specific binding) of a 50 fold molar excess of unlabelled glu-plasminogen. Specific binding (■) was determined by subtracting non-specific binding from total binding at each concentration. A one-site hyperbolic binding function was fitted to the data ($p < 0.05$), from which the binding dissociation constants were determined. Error bars represent the standard error of the mean (SEM; n = 6).

PAM_{NS13} was selected as the competitor as it was found to have the highest affinity for plasminogen and it has a plasminogen binding site which shows 100% identity to the prototype PAM a1 and a2 repeats (Berge and Sjobring 1993; Ben Nasr *et al.* 1994; Wistedt *et al.* 1995; Wistedt *et al.* 1998). The effective concentration of competitor required to inhibit plasminogen binding by 50% (EC_{50}) was determined by fitting a one-site competition curve (Figure 3.11). EC_{50} values ranged from 0.34 μM to 22.06 μM (Table 3.2). These data also suggest that variation within the plasminogen-binding region of these PAM variants decreased the comparative affinity for plasminogen. As expected, there was generally an inverse correlation between K_d and EC_{50} (Table 3.2). Additionally, when immobilised, PAM_{NS13} plasminogen-binding out-competed binding to biotinylated glu-plasminogen by fluid phase PAM variants (PAM_{NS53}, $PAM_{NS88.2}$, PAM_{NS455}, PAM_{NS1133} and PAM_{NS265}). No competition of PAM_{NS13} plasminogen binding was found in the presence of a 50 fold molar excess of any of the PAM variants examined (data not shown), further indicating that the prototype PAM sequence (PAM_{NS13}) provides a higher affinity binding site for plasminogen.

3.2.6.3 Fibrinogen purification and labelling

Contaminating fibronectin was removed from commercially obtained human fibrinogen by gelatin-agarose purification. No contaminating fibronectin was visible following 10% SDS-PAGE analysis of both pre- and post- purification fibrinogen (Figure 3.12 A). The α, β and γ chains of fibrinogen were visible as protein bands of approximately 63, 56 and 47 kDa respectively (Doolittle 1984).

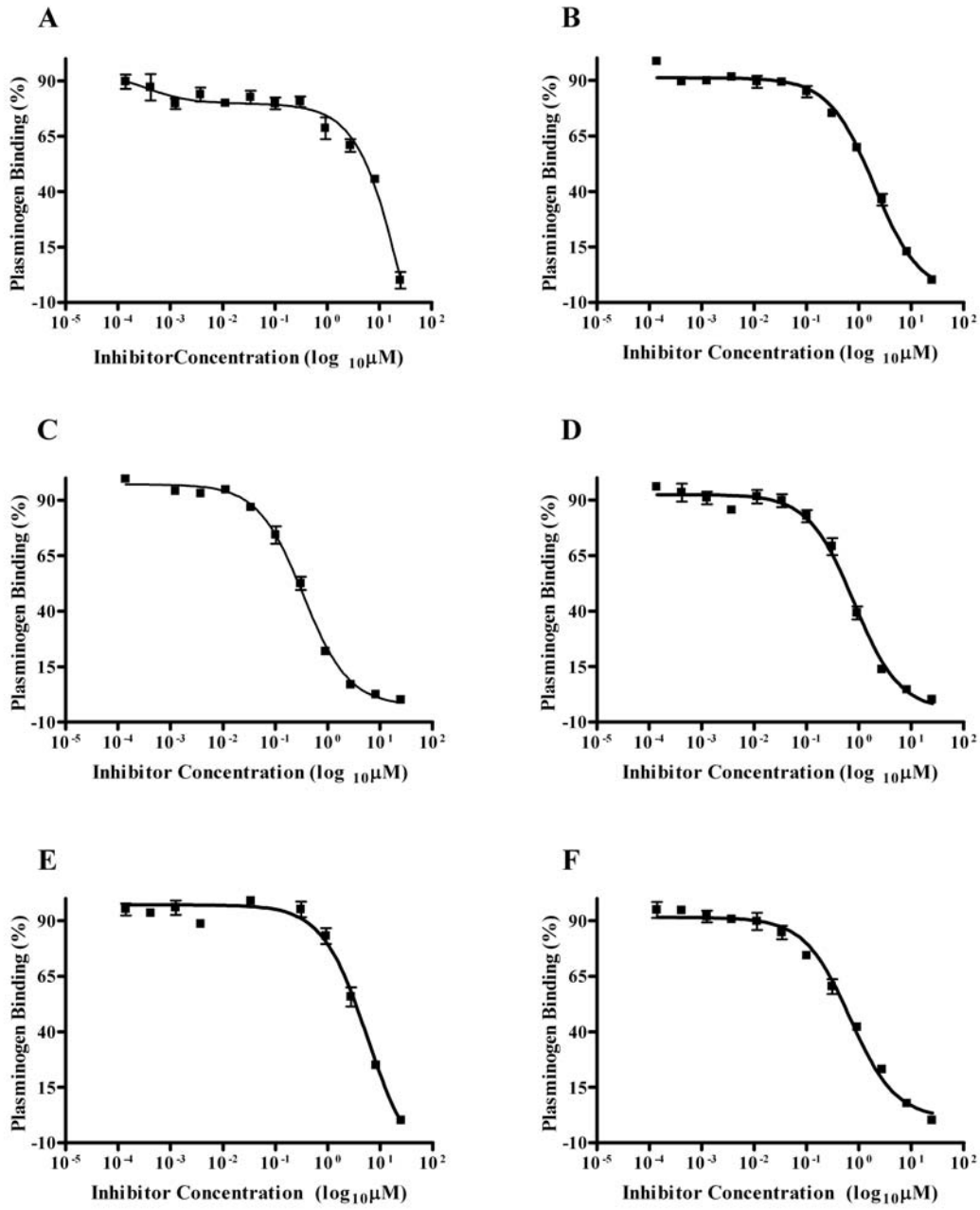


Figure 3.11 Competition of glu-plasminogen binding to immobilised recombinant PAM variants with fluid phase PAM_{NS13}. Binding of biotinylated glu-plasminogen to immobilised **A** PAM_{NS13}, **B** PAM_{NS53}, **C** $PAM_{NS88.2}$, **D** $PAM_{NS265,}$ **E** PAM_{NS455} and **F** PAM_{NS1133} was measured in the presence of varying concentrations of unlabelled fluid phase PAM_{NS13}. Data points are the mean values of triplicate readings, with error bars indicating SEM. One-site competition analysis was performed on data for all recombinant proteins; this analysis was used to determine the concentration of PAM_{NS13} required to inhibit binding of biotinylated glu-plasminogen by 50% (EC_{50}).

Table 3.2 Plasminogen binding properties of recombinant PAM variants.

Recombinant protein	K_d (nM)	EC_{50} (μM)
PAM_{NS13}	1.58	22.06
PAM_{NS455}	3.47	5.77
PAM_{NS1133}	4.68	0.68
PAM_{NS53}	4.86	1.96
PAM_{NS265}	4.99	0.81
$PAM_{NS88.2}$	7.58	0.34
NS696 M1 protein	Non-specific binding only	Not determined

Western blot analysis of purification fractions using rabbit polyclonal anti-fibronectin antibodies revealed decreasing levels of fibronectin in the various purification fractions (Figure 3.12 B).

Purified fibrinogen was conjugated to biotin-X-NHS prior to use in solid-phase fibrinogen binding assays. Removal of unconjugated biotin using PD-10 filtration was monitored at 750 nm via Lowry protein detection assay (Figure 3.13 A). Biotinylated fibrinogen was typically eluted in elution volumes 4 and 5, which were then pooled. Western blotting analysis, using neutravidin-HRP confirmed biotinylation (Figure 3.13 B).

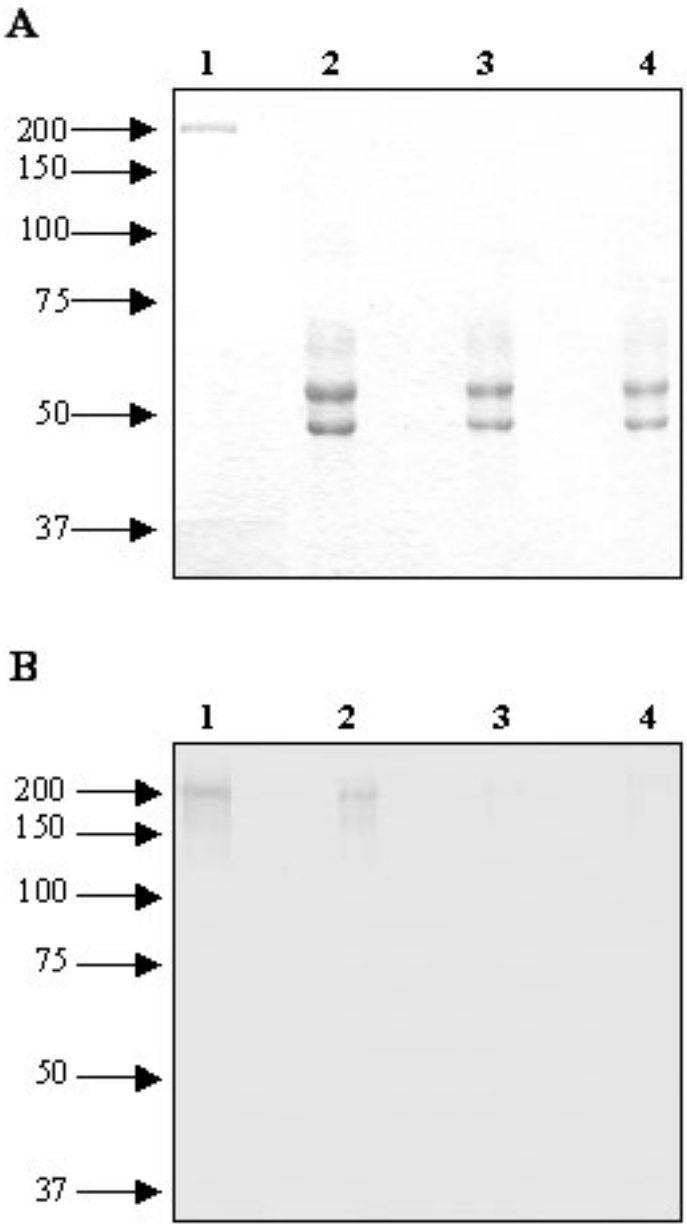


Figure 3.12 Western blot analysis of purified fibrinogen. **A** 10% SDS-PAGE gel showing fibronectin (lane 1), un-purified human fibrinogen (lane 2), gelatin-agarose purification fraction 1 (lane 3), and gelatin agarose purified human fibrinogen (lane 4). The three polypeptides that make up fibrinogen (α, β andγ) appear as protein bands of 63, 56 and 47 kDa respectively. Molecular weight markers are given in kDa. **B** Western bot analysis of gelatin-agarose fibrinogen purification utilising rabbit polyclonal anti-fibronectin antibodies. Lane 1, fibronectin; lane 2, un-purified human fibrinogen; lane 3, elution fraction 1; lane 4, gelatin-agarose purified human fibrinogen. Molecular weight markers are given in kilo Daltons (kDa).

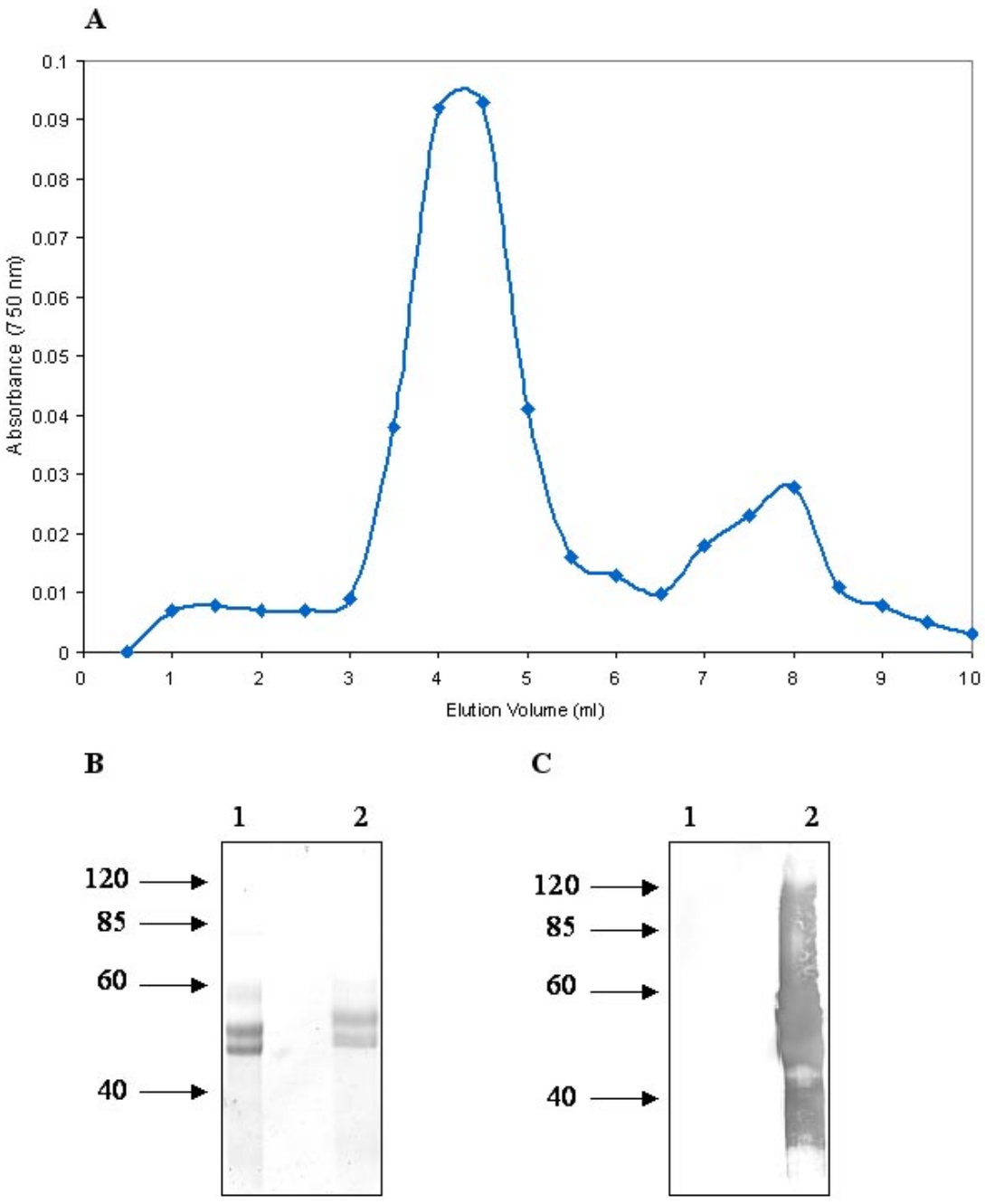


Figure 3.13 Biotinylation of purified fibrinogen**. A** Elution of biotinylated fibrinogen from a PD-10 gel filtration column. Biotinylated fibrinogen typically eluted in elution volumes 4 and 5. **B** 12% SDS-PAGE analysis of non-biotinylated (lane 1) and biotinylated (lane 2) fibrinogen. **C** Western blot analysis of non-biotinylated (lane 1) and biotinylated (lane 2) fibrinogen, utilising neutravidin-HRP. Smearing is the result of degraded protein. Molecular weight markers are given in kilo Daltons (kDa).

3.2.6.4 Fibrinogen binding analysis

The interaction between recombinant PAM variants and fibrinogen was characterised using saturation fibrinogen binding assays. Recombinant proteins immobilised to 96-well plates were incubated with increasing concentrations of biotinylated fibrinogen. All PAM variants bound fibrinogen non-specifically, with the exception of PAM_{NS265}, which approached, but did not reach saturable binding of 300 nM fibrinogen after 1 h. The NS696 M1 protein bound fibrinogen in a dose-dependent fashion, and saturable binding was achieved with 300 nM fibrinogen after 1 h (Figure 3.14). PAM_{NS265} does not appear to contain a fibrinogen binding domain on the basis of database search results and sequence alignment with the NS696 M1 protein.

3.2.6.5 tPA binding analysis

The interaction between PAM_{NS13} and tPA was analysed using solid phase binding assays. PAM_{NS13} was selected for analysis of this interaction as it is 99.5% identical to the prototype PAM protein for which tPA binding ability was originally suggested. Immobilised tPA, glu-plasminogen and BSA were assessed for the ability to interact with increasing concentrations of biotinylated PAM_{NS13} or fibrinogen. Fibrinogen is known to interact with tPA (Dano *et al.* 1985), and as such was used as a positive control, together with glu-plasminogen, which is known to interact with PAM_{NS13}. Neither tPA nor BSA were found to interact with PAM at levels higher than background (Figure 3.15). However, immobilised tPA bound to biotinylated fibrinogen in a specific and saturable fashion as expected. A weak interaction between PAM and tPA was initially reported on the basis of the interaction of a radiolabelled peptide homologous to the a1 repeat of PAM with tPA following transfer to PVDF (Wistedt *et al.* 1995).

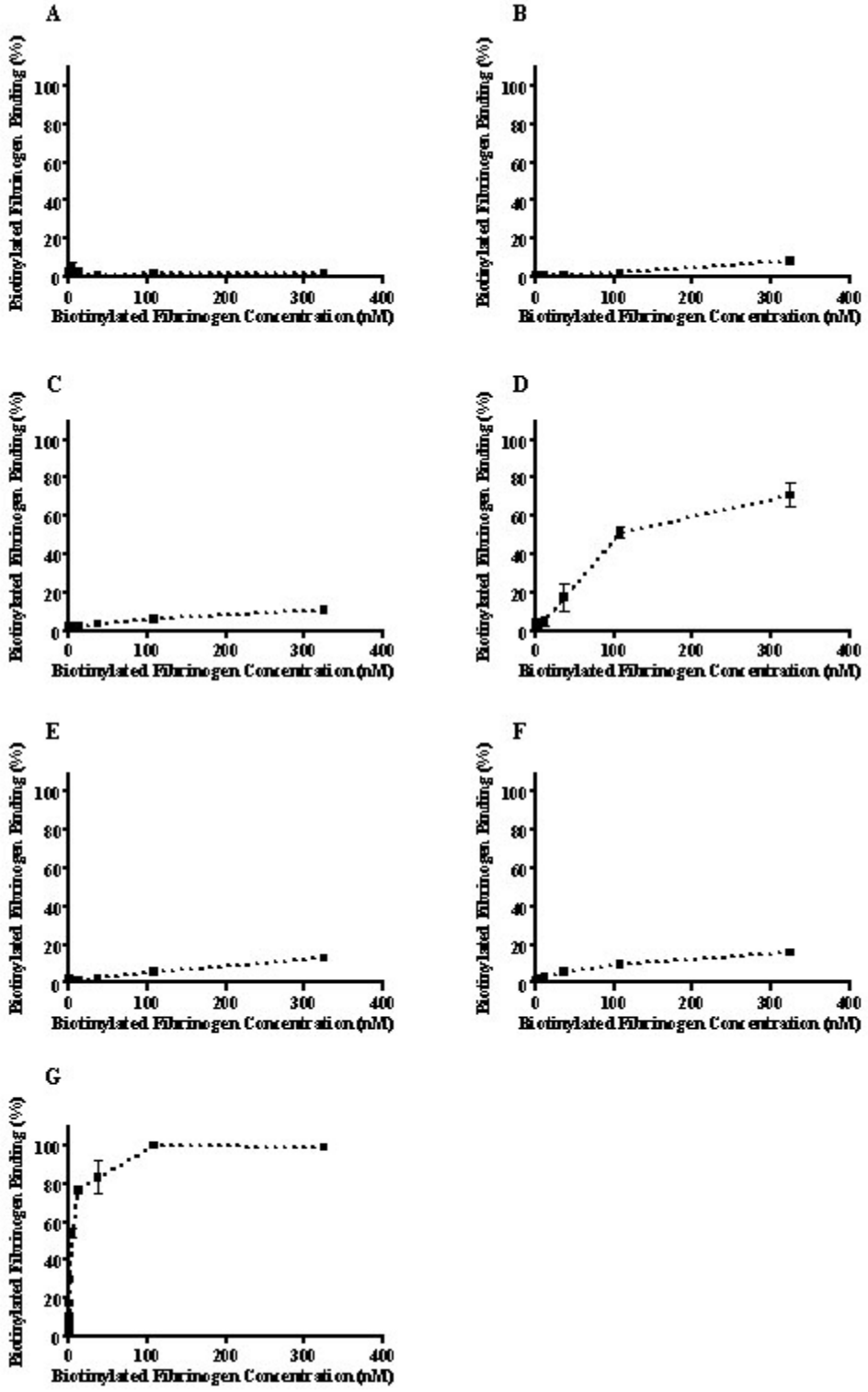


Figure 3.14 Saturation binding analysis of biotinylated fibrinogen to immobilised recombinant PAM variant proteins. **A**, PAM_{NS13}; **B,** PAM_{NS53}; **C,** $PAM_{NS88.2}$; **D**, PAM_{NS265}; **E**, PAM_{NS455}; **F**, PAM_{NS1133}; **G**, $_{NS696}$ M1 protein. Error bars represent the standard error of the mean (SEM; n = 3).

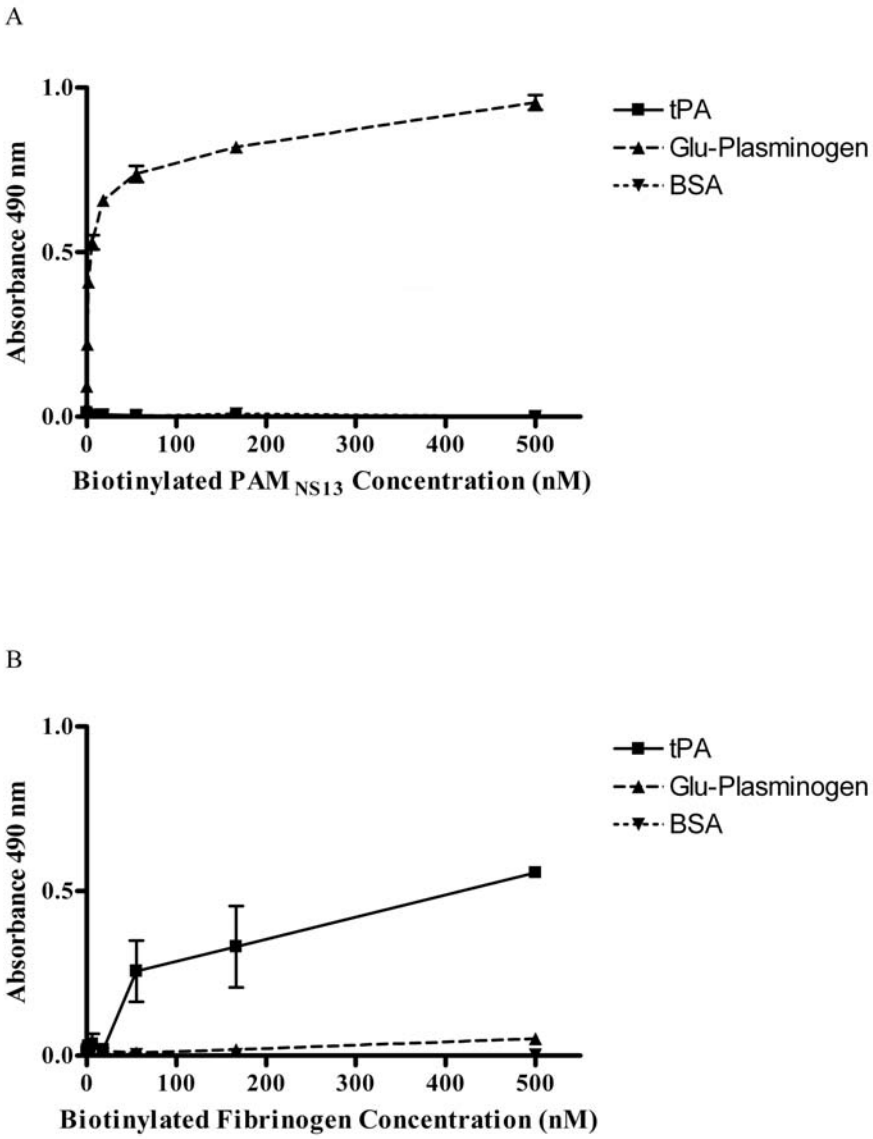


Figure 3.15 Analysis of PAM_{NS13} binding to tPA. Solid phase binding analysis of biotinylated PAM_{NS13} **A** and biotinylated fibrinogen **B** to human tPA, glu-plasminogen and BSA. PAM_{NS13} does not appear to bind human tPA.

However, this data may represent a non-specific interaction with denatured tPA, as results given here do not show any specific interaction between PAM_{NS13} and tPA.

3.2.6.6 Analysis of the impact of PAM sequence variation on immune recognition of GAS

Naturally occurring variation in the a1 region of PAM does not abrogate plasminogen binding function. Whilst there are several potential explanations for substantial sequence divergence in the major functional domain of a protein, the plasminogen-binding region of PAM may be an important target for immune recognition by the host. New variants may be selected for by host immune pressure. Thus, the potential for the a1 region of PAM to elicit protective immunity in a mouse model for GAS infection was investigated. Immunisation of mice with a KLH-conjugated peptide encompassing the a1 region of PAM_{NS13} elicited a significantly higher titre of a1 peptide specific IgG than KLH alone ($p < 0.001$) or PBS ($p < 0.001$) controls (Figure 3.16 A). The anti-a1 peptide-KLH sera also showed significant opsonising activity towards GAS in an *in vitro* bactericidal assay in human blood when compared with the KLH ($p < 0.01$) and PBS ($p < 0.001$) control antisera (Figure 3.16 B). Additionally, anti-a1 peptide-KLH sera showed no opsonising activity against GAS expressing variant PAM_{NS455} when compared to KLH antisera ($p > 0.05$), indicating that variation in the a1 region of PAM can result in a change in immune recognition (Figure 3.16 C).

Mice immunised with the a1 peptide-KLH showed increased survival when challenged with GAS strain NS13 compared to both the PBS ($p = 0.0071$), and KLH ($p = 0.0055$) immunised control mice (Figure 3.16 D), indicating that the a1 repeat of PAM may represent a protective epitope against group A streptococcal infection.

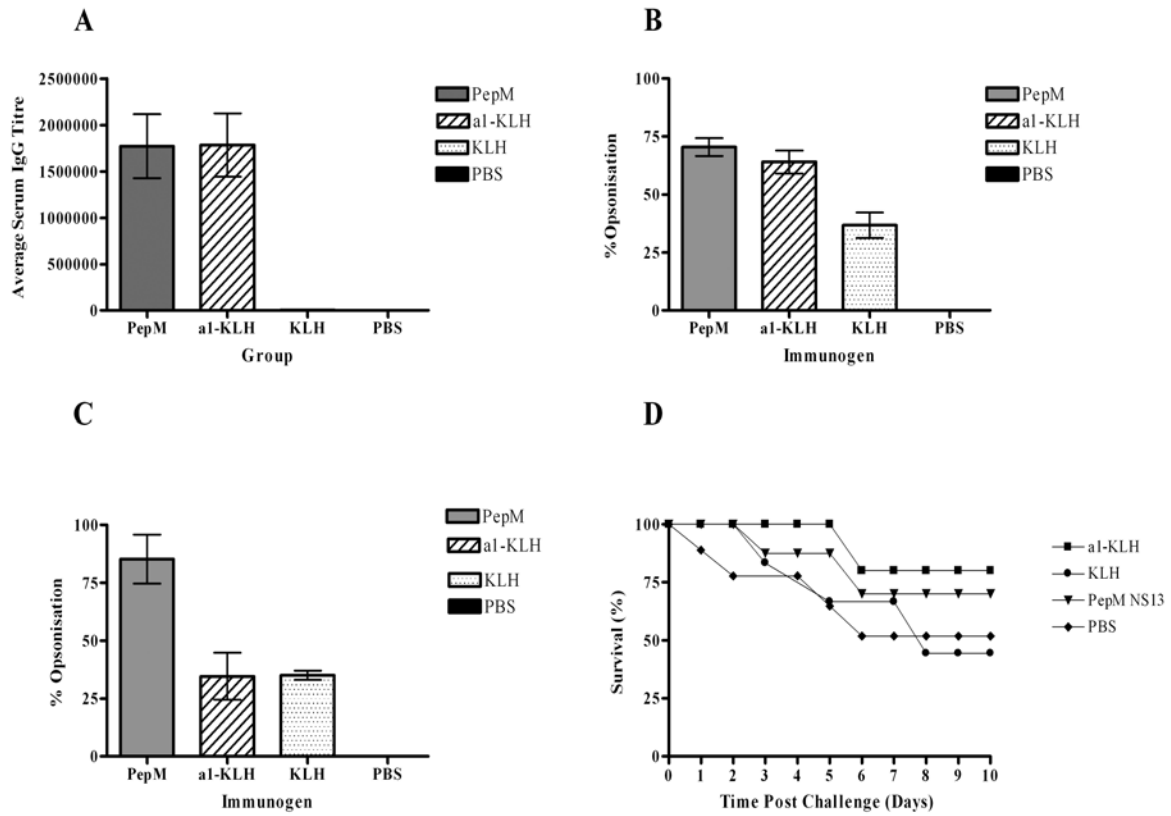


Figure 3.16 Immunisation of mice with a KLH-conjugated peptide representing the a1 region of PAM_{NS13} and subsequent challenge with GAS strain NS13**.** Mice were immunised with either a KLH-conjugated peptide representing the a1 region of PAM_{NS13} (a1 peptide-KLH), a pepsin extract of the cell surface of GAS strain NS13 (PepM positive control), KLH or PBS controls. **A** Titre of antisera as determined by ELISA against the a1 peptide. All estimates were determined in triplicate and data are presented as mean/median $\pm$ SEM of two separate immunisation experiments. **B** Opsonisation (%) of GAS strain NS13 by mouse antisera 7 days after the final immunisation as determined by indirect bactericidal assay. All estimates were determined in triplicate and data are presented as mean $\pm$ SEM of two separate challenge experiments. **C** Opsonisation (%) of GAS strain NS455 by mouse antisera. All estimates were determined in triplicate using pooled mouse sera and data are presented as mean $\pm$ SEM. **D** Survival (%) of immunised mice following challenge with NS13 10 days after the final immunisation. Data represents the combined survival from two separate challenge experiments.

3.3 Discussion

In order to further examine the role of PAM in GAS virulence in a region where GAS infection is endemic, the distribution of PAM genes in a subset of Northern Territory GAS isolates was investigated. Additionally, the interaction of human plasminogen with six naturally occurring PAM variants was investigated. This study indicates that variation in the plasminogen binding region of PAM may reflect the targeting of this region by the host immune response.

No correlation was seen between PAM genotype and site of GAS islolation, with PAM positive GAS strains being isolated from a variety of infection sites. Phylogenetic analysis of 28 GAS isolates by PFGE indicated that the PFGE profile was concordant with *vir* type and *emm* sequence type. However, there was little association between *emm* pattern and PFGE profile. Previous studies of the allelic profiles of neutral housekeeping genes have found a lack of concordance between *emm* pattern and the genetic relatedness of strains (Kalia *et al.* 2002), and as such, this finding is not unexpected. All the PAM positive isolates used in this study were found to be *emm* pattern D. To date, the PAM gene has been found to be primarily associated with *emm* pattern D isolates, with this chromosomal arrangement thought to be a marker for skin tropic GAS isolates (Bessen *et al.* 1996; Svensson *et al.* 1999). Comparison of the PFGE dendogram with a phylogenetic analysis of 13 PAM protein sequences provided evidence to suggest that there has been horizontal gene transfer of the PAM gene during GAS evolution. Horizontal gene transfer is considered to be a major source of genetic variation within *emm* and *emm*-like genes, and appears to have been a common event during GAS evolution (Whatmore *et al.* 1994). Furthermore, phylogenetic analysis of

$PAM_{NS88.2}$ indicated that this protein is phylogenetically distinct from the other PAM variants studied here.

Solid phase plasminogen binding assays indicated that despite variation in the plasminogen binding domain of PAM, all naturally occurring variants maintained the ability to bind plasminogen with high affinity. While the binding characteristics of fragments representing different a1/a2 repeat sequences have previously been investigated (Schenone *et al.* 2000; Rios-Steiner *et al.* 2001), the functional characteristics of M proteins, including fibrinogen and plasminogen binding, may depend largely on their overall structure (Cedervall *et al.* 1997; Wistedt *et al.* 1998), highlighting the importance of using full length protein in functional studies. Binding dissociation constants for the interaction of PAM variants and biotinylated plasminogen were within the same range as reported previously for PAM and plasminogen binding M proteins of groups C and G streptococci (Berge and Sjobring 1993; Ben Nasr *et al.* 1994; Wistedt *et al.* 1995). The degree of sequence similarity in the plasminogen binding regions of the variants used in this study to that of the prototype PAM ranged from 52% to 100%. Overall, this variation has not abolished affinity for plasminogen. The circulating concentration of glu-plasminogen is 2 μM (Dano *et al.* 1985), and as such, the K_d values reported in this work ranging from 1.58 nM to 7.58 nM are within the physiological range of plasminogen and thus are of functional significance.

The maintenance of plasminogen binding function in the presence of sequence divergence may relate to the conservation of key binding site residues. Studies involving the interaction of a polypeptide sequence encompassing the a1 and a2 region of PAM in addition to six residues preceding the a1 repeat (designated VEK-30) with kringle 2 of plasminogen highlighted the importance of Lys^{98}, Arg^{101}, His^{102}, Glu^{104} and

Lys^{111} in the interaction of PAM with plasminogen (Schenone *et al.* 2000; Rios-Steiner *et al.* 2001). In the PAM variants used in this study, Lys^{98}, Lys^{111}, Arg^{101} and His^{102} were conserved in all sequences. Glu^{104} was present in all proteins except PAM_{NS1133}. It is likely that the conservation of these key residues provides the necessary binding site integrity for the interaction with plasminogen despite other sequence variation within this region of PAM. Nonetheless, while PAM_{NS53} and PAM_{NS455} contain identical a1 and a2 repeats, the K_d and EC_{50} values of these proteins vary, suggesting that the overall M protein structure subtly contributes to the capacity to bind plasminogen. PAM_{NS13} displays the highest affinity for plasminogen, contains a binding site identical to that of the prototype PAM sequence, and was able to compete plasminogen binding to other PAM variants at low concentrations, suggesting that this binding site sequence represents the ideal motif for the interaction of plasminogen with PAM.

It has been hypothesised that PAM may function as a receptor for multiple host proteins. However, data presented here indicates that PAM does not interact directly with either fibrinogen or tPA as had been previously suggested (Wistedt *et al.* 1995; McKay *et al.* 2004). The interaction between PAM_{NS265} and fibrinogen appears of a lower affinity than that of the NS696 M1 protein. However, this interaction could not be fully characterised due to the insolubility of fibrinogen at high concentrations, and as such, further investigation of the potential interaction of PAM_{NS265} with fibrinogen requires the use of more sensitive techniques such as BIA-CORE analysis. No direct evidence exists in the literature for the interaction of PAM with the plasma protein fibrinogen. However, a study of GAS isolates from the Northern Territory found that PAM positive GAS bound more fibrinogen then PAM negative GAS, and showed a direct correlation between PAM genotype and the ability to acquire cell surface

plasminogen via a fibrinogen dependent pathway (McKay *et al.* 2004). Many GAS strains express more than one M or M like protein, many of which act as receptors for fibrinogen (Fischetti 1989). Additionally, there are several other fibrinogen binding proteins which have been identified on the cell surface of GAS (Cunningham 2000). Therefore, it is likely that the high fibrinogen binding reported for PAM positive GAS isolates is due to the concomitant expression of fibrinogen binding proteins with PAM, rather then fibrinogen binding to PAM itself. A 1995 study by Wistedt *et al* provided evidence to suggest that PAM may interact directly with tPA. Specifically, ligand blot analysis indicated that the a1 and a2 repeat domain of PAM had a weak affinity for tPA (Wistedt *et al.* 1995). If this is the case, the co-localisation of both plasminogen and a plasminogen activator at the GAS cell surface could result in the generation of large amounts of plasmin. However, no direct interaction between PAM and tPA was detected in this study. The denaturing of proteins in ligand blot studies means that any interaction detected in this fashion is not sufficient evidence of a significant interaction between two proteins (Soutar 1997). To date then, it appears that PAM is a high affinity receptor for the host protein plasminogen, possessing no dual receptor activity.

M protein is one of the major virulence factors of GAS, and consists of a highly variable N-terminal domain, as well as a and b repeat blocks that generate type-specific immunity in the host. Variation within the repeat sequences of M proteins as a result of both point mutations and duplication/deletion events may function as a means of developing antigenic variation (Jones *et al.* 1988; Harbaugh *et al.* 1993; Relf *et al.* 1994). Furthermore, it has been found that a difference of only 4 amino acids in the N-terminus of M3 variants was enough to alter the antigenic profile of the protein, resulting in altered immune recognition (Beres *et al.* 2004). A potential role for the variable a1 region of PAM in eliciting protective immunity in the host was investigated

using a mouse model for GAS infection. The plasminogen binding a1 repeat region of PAM is an orthologue of the a repeat region in other M proteins. The immunogenic properties of the a1 domain have not previously been reported. Type specific anti-M antibodies bind to exposed epitopes within the N–terminus of M protein, thus inhibiting the anti-phagocytic role of this protein, and initiating opsonisation (Jones *et al.* 1988). If the binding of plasminogen by PAM promotes deep tissue dissemination of GAS strains, it may be argued that the antibody response against the plasminogen binding domain of PAM could promote clearance of the infecting GAS in order for it to offer selective pressure for novel variants. The results presented here clearly show that in a murine model, the a1 domain is highly immunogenic and the antibodies against this domain are opsonic, resulting in phagocytic clearance. It has also been demonstrated that anti-sera raised against the prototype a1 sequence does not opsonise GAS expressing the variant protein PAM_{NS455}. It is possible then, that divergence in the a1 and a2 repeats of PAM may have resulted as a response to selective pressure from the host immune system.

4 Characterisation of the plasminogen-binding site of PAM

4.1 Introduction

The plasminogen binding domain of PAM has been shown to be highly variable, however, this diversity does not abrogate plasminogen binding by PAM variants. This finding may, in part, be due to the conservation of key amino acid residues within the plasminogen binding domain. Plasmin(ogen) interacts with its ligands via lysine binding-sites located in the kringle domains of the N-terminus of plasminogen (Ponting *et al.* 1992). PAM lacks the typical C-terminal lysine residues of many plasminogen receptors. Rather, it is thought that internal lysine residues in the a1 and a2 repeat regions of PAM (Lys^{98} and Lys^{111}) mediate binding to kringle 2 of plasminogen (Wistedt *et al.* 1995). Interestingly, whilst kringle 2 has a relatively weak affinity for lysine and arginine derivatives when compared to kringles 1, 4 and 5, it displays a similar affinity for analogues of internal lysine residues (Marti *et al.* 1997). Early reports suggested that Lys^{98} of the a1 repeat contributed to the majority of the plasminogen binding ability of PAM (Wistedt *et al.* 1995), however, studies involving peptides homologous to this region of PAM highlighted a potential role for internal residues His^{102}, Arg^{101} and Glu^{104} in this interaction (Wistedt *et al.* 1995; Ringdahl *et al.* 1998; Schenone *et al.* 2000; Rios-Steiner *et al.* 2001). The relative contributions to binding by these residues in the full length PAM protein have not been fully characterised.

This study aims to further elucidate the interaction of PAM with plasminogen, by assessing the contribution of key residues in both the a1 and a2 repeat domains, to the plasminogen binding function and structure of PAM. To this end, a series of site-directed mutants were constructed as outlined in section 2.4.1.2, in which selected amino acids were converted to alanine residues. Alanine residues were selected as

small, less bulky amino acids facilitate the preservation of protein structure when used in site directed mutagenesis (Argos 1989). The impact of mutagenesis on protein function and structure was determined using ligand blotting, solid-phase binding assays, and CD spectroscopy.

4.2 Results

4.2.1 Site-directed mutagenesis

In order to assess the role of specific binding site residues in the interaction of PAM_{NS13} with plasminogen, a number of site-directed mutants were constructed using the Quickchange™ Site-Directed mutagensis kit. The forward primers used to introduce the desired mutations are given in Table 4.1.

Table 4.1 Forward primer sequences used to construct PAM_{NS13} site-directed mutants. For site-directed mutants containing two or more mutations not encoded in a single primer, mutations were sequentially introduced using previously mutated DNA as a template.

Introduced mutation	Forward mutagenesis primer
K^{98}/A	5'-GATGCTGAGTTGCAACGACTTGCAAACGAGAGACATGAAGAAGCA-3'
K^{111}/A	5'-GAAGCAGAGTTGGAGCGACTTGCAAGCGAGAGACATGATCATGAC-3'
$R^{101}H^{102}E^{104}/A$	5'-CGACTTGCAAACGAGGCAGCAGAAGCAGCAGAGTTGGAGCGA-3'
$R^{101}H^{102}/A$	5'-CGACTTGCAAACGAGGCAGCAGAAGCAGCAGAGTTGGAGCGA-3'
$R^{114}H^{115}/A$	5'-CGACTTGCAAGCGAGGCAGCAGATCATGACAAAAAAGAAGC-3'

The gene encoding wildtype PAM_{NS13} shares 100% homology with the prototype PAM sequence in the a1 and a2 repeat domain responsible for the interaction with plasminogen. The binding site-sequences of the five constructed mutants are shown in Figure 4.1 A. The presence of the introduced mutations were verified by DNA sequence analysis. Following expression in *E. coli*, recombinant proteins of approximately 40 kDa in size were purified using glutathione-agarose and Ni-NTA agarose affinity chromatography (Figure 4.1 B).

A

Recombinant Protein	Plasminogen Binding Site a1	a2
PAM_{NS13}	DAELQRLKNERHE	EAELERLKSERHD
$PAM_{NS13}[K^{98}\ K^{111}]$	DAELQRL**A**NERHE	EAELERL**A**SERHD
$PAM_{NS13}[K^{98}R^{101}H^{102}E^{104}K^{111}]$	DAELQRL**A**NE**AA**E	**A**AELERL**A**SERHD
$PAM_{NS13}[K^{98}R^{101}H^{102}E^{104}K^{111}R^{114}H^{115}]$	DAELQRL**A**NE**AA**E	**A**AELERL**A**SE**AA**D
$PAM_{NS13}[R^{101}H^{102}]$	DAELQRLKNE**AA**E	EAELERLKSERHD
$PAM_{NS13}[R^{101}H^{102}R^{114}H^{115}]$	DAELQRLKNE**AA**E	EAELERLKSE**AA**D

B

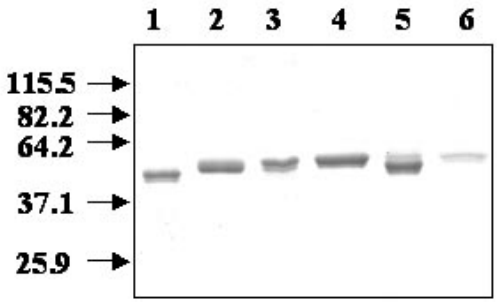


Figure 4.1 SDS-PAGE analysis of PAM_{NS13} site-directed mutants. **A** Alignment of the plasminogen binding domain of wild type PAM_{NS13} with the 5 site-directed mutants constructed in this study. Mutated residues are indicated in bold. **B** 12% SDS-PAGE gel showing the 6 purified recombinant proteins used in this study. Lane 1, PAM_{NS13}; lane 2, $PAM_{NS13}[K^{98}\ K^{111}]$; lane 3 $PAM_{NS13}[K^{98}\ R^{101}\ H^{102}\ E^{104}\ K^{111}]$; lane 4, $PAM_{NS13}[K^{98}\ R^{101}\ H^{102}\ E^{104}\ K^{111}\ R^{114}\ H^{115}]$; lane 5, $PAM_{NS13}[R^{101}\ H^{102}]$; lane 6, $PAM_{NS13}[R^{101}\ H^{102}\ R^{114}\ H^{115}]$. Molecular weight markers are given in kilo Daltons (kDa).

4.2.2 Structural characterisation of mutant proteins

The impact of mutations in the a1 and a2 repeat domains of PAM_{NS13} on protein structure were analysed using far UV CD spectroscopy. CD studies are advantageous for structural studies of proteins as spectra are largely derived from the spatial asymmetry of amino-acids in the protein backbone (Freifelder 1982). As a result, proteins with α-helical and β-sheet structures give distinct CD spectra (Freifelder 1982). Far UV spectra, resulting from measurements in the amide region of 170 nm-250 nm are particularly useful for deriving information on protein secondary structure (Schmid

1989). Although exact protein structure can not be deduced, CD can be applied to mutagensis based studies as it is extremely sensitive to conformational changes (Freifelder 1982).

All proteins used in this study were found to have a CD emission spectrum characteristic of α-helical coiled proteins (Freifelder 1982), displaying two characteristic minima at approximately 210 nm and 220 nm, and a maximum peak at 190 nm (Figure 4.2). This is similar to the CD spectra of other streptococcal M proteins, which are coiled-coil α-helical proteins (Phillips *et al.* 1981). Thus, even after mutagenesis these proteins appear to maintain an α-helical secondary structure. Additionally, for all proteins the two minima are of a similar magnitude, which is indicative of coiled-coil proteins (Graddis *et al.* 1993). Percent α-helicity ranged from 27% to 44% (Table 4.2). Analysis of secondary structure using CD can have up to 10% error, and as such, the variation seen between mutant and wild type proteins in this study does not represent a significant difference (Berengian *et al.* 1997). Analysis of other streptococcal M proteins has found them to contain between 23% and 70% α-helix (Phillips *et al.* 1981; Khandke *et al.* 1988).

4.2.3 Plasminogen binding analysis

PAM_{NS13} has previously been shown to interact with glu-plasminogen with high affinity (K_d = 1.58 nM). The interaction of site-directed mutants with glu-plasminogen was investigated using ligand blotting techniques. All site-directed mutants were found to interact with biotinylated glu-plasminogen in a ligand blot analysis except for $PAM_{NS13}[K^{98}R^{101}H^{102}E^{104}K^{111}R^{114}H^{115}]$ and $PAM_{NS13}[R^{101}H^{102}R^{114}H^{115}]$ (Figure 4.3).

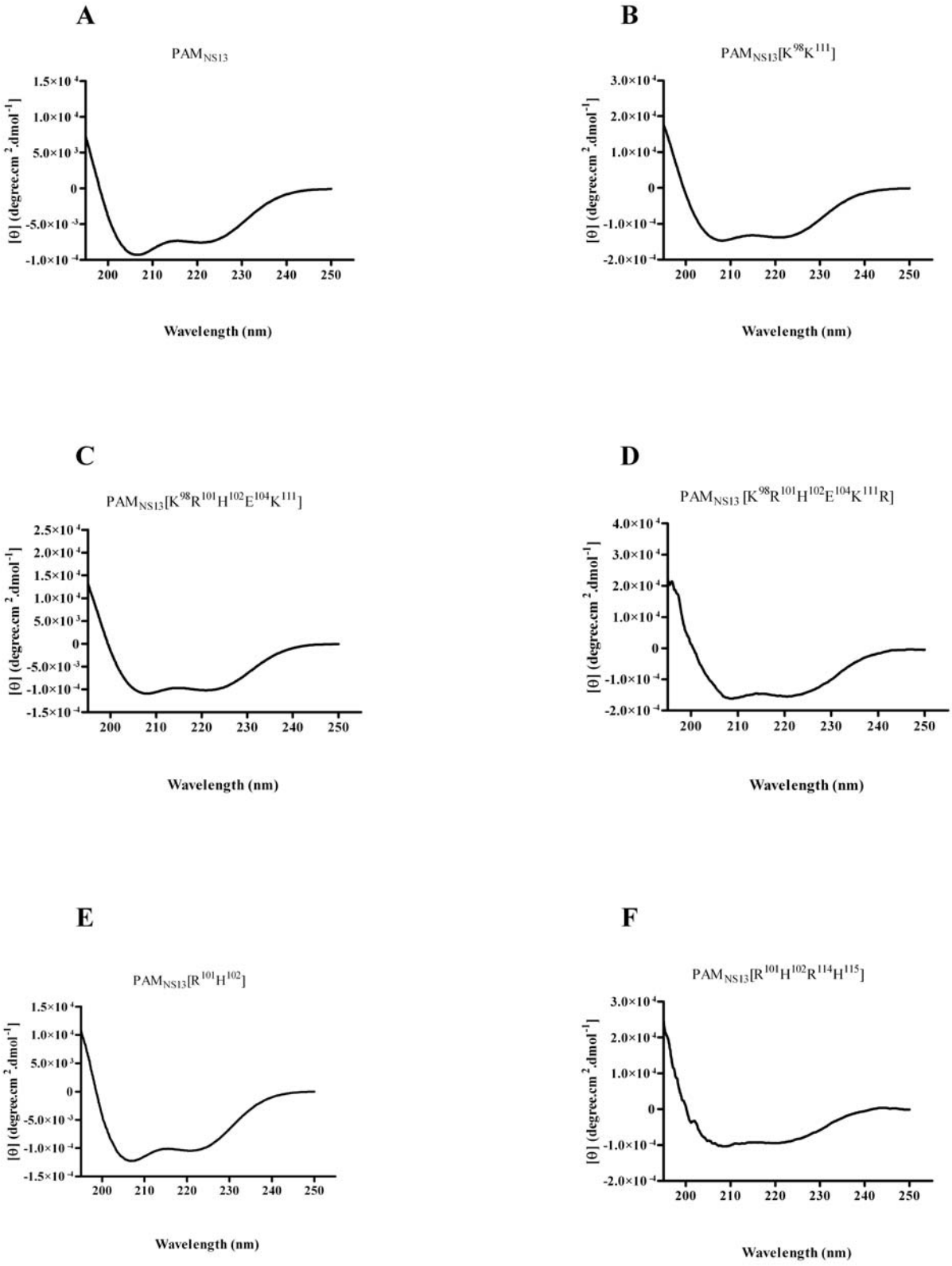


Figure 4.2 Circular dichroism spectra of recombinant PAM_{NS13} mutants. A PAM_{NS13}; B $PAM_{NS13}[K^{98}\ K^{111}]$; C $PAM_{NS13}[K^{98}\ R^{101}\ H^{102}\ E^{104}\ K^{111}]$; D $PAM_{NS13}[K^{98}\ R^{101}\ H^{102}\ E^{104}\ K^{111}\ R^{114}\ H^{115}]$; E $PAM_{NS13}[R^{101}\ H^{102}]$; F $PAM_{NS13}[R^{101}\ H^{102}\ R^{114}\ H^{115}]$ All proteins exhibit CD emission spectrum characteristic of α-helical coiled proteins (Freifelder 1982), displaying two characteristic minima at approximately 210 nm and 220 nm, and a maximum peak at 190 nm.

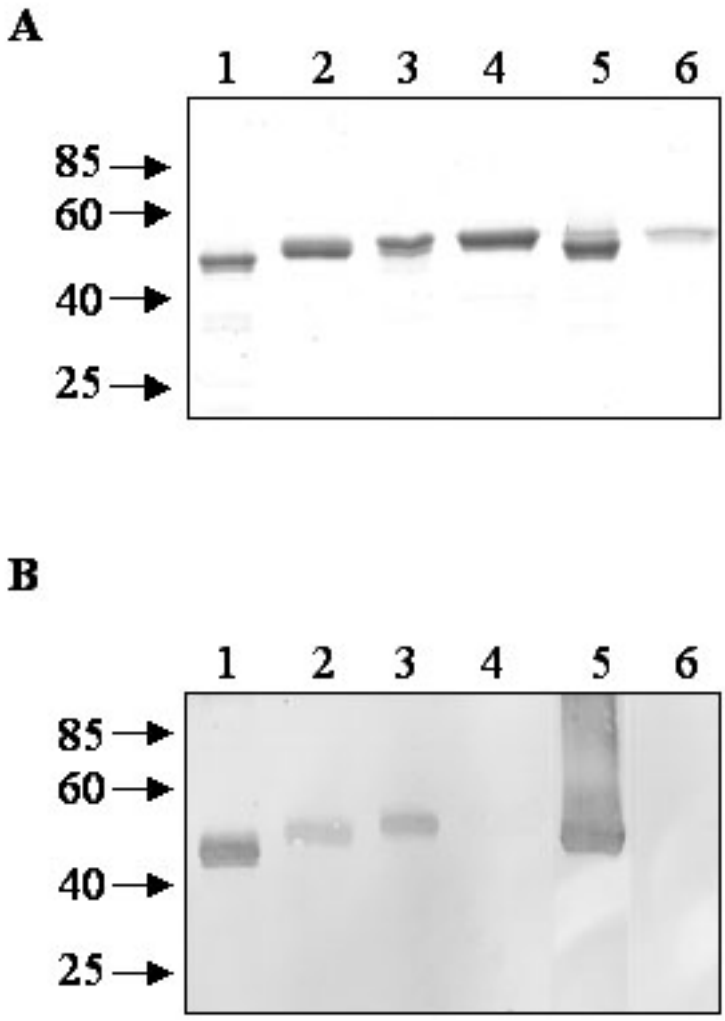


Figure 4.3 Ligand blot analysis of PAM_{NS13} site-directed mutants. **A** 12% SDS-PAGE gel of recombinant PAM_{NS13} mutants used for ligand blotting analysis. Lane 1, PAM_{NS13}; lane 2, $PAM_{NS13}[K^{98}K^{111}]$; lane 3 $PAM_{NS13}[K^{98}R^{101}H^{102}E^{104}K^{111}]$; lane 4, $PAM_{NS13}[K^{98}R^{101}H^{102}E^{104}K^{111}R^{114}H^{115}]$; lane 5, $PAM_{NS13}[R^{101}H^{102}]$; lane 6, $PAM_{NS13}[R^{101}H^{102}R^{114}H^{115}]$. **B** Ligand blot of purified variant PAM proteins. employing biotinylated glu- plasminogen. Lane 1, PAM_{NS13}; lane 2, $PAM_{NS13}[K^{98}K^{111}]$; lane 3 $PAM_{NS13}[K^{98}R^{101}H^{102}E^{104}K^{111}]$; lane 4, $PAM_{NS13}[K^{98}R^{101}H^{102}E^{104}K^{111}R^{114}H^{115}]$; lane 5, $PAM_{NS13}[R^{101}H^{102}]$; lane 6, $PAM_{NS13}[R^{101}H^{102}R^{114}H^{115}]$. Molecular weight markers are given in kilo Daltons (kDa).

To further characterise the interaction between PAM_{NS13} site-directed mutants and plasminogen, solid-phase plasminogen binding assays were performed. The recombinant PAM mutants bound plasminogen in a dose-dependent fashion, and saturable binding was achieved with 500 nM plasminogen for 3 of the 5 mutant proteins after 2 h (Figure 4.3). Non-linear regression analysis was used to determine the affinity of each recombinant protein for glu-plasminogen (Table 4.2). Equilibrium dissociation constants (K_d) were calculated using a best-fit non-linear regression curve. The following site-directed mutants where residues were replaced with alanine, $PAM_{NS13}[K^{98}\ K^{111}]$ and $PAM_{NS13}[K^{98}\ R^{101}\ H^{102}\ E^{104}\ K^{111}]$, bound plasminogen with K_d values of 10.34 nM and 50.24 nM respectively. Whilst this represents a significant decrease in affinity for glu-plasminogen when compared to wild type PAM_{NS13} (K_d = 1.58 nM; $p < 0.05$), binding by these mutants was still specific and saturable. $PAM_{NS13}[R^{101}\ H^{102}]$ bound plasminogen with a K_d value of 1.69 nM, which is not significantly different from that of wild type PAM_{NS13} ($p > 0.05$). Only non-specific plasminogen binding was seen for mutants $PAM_{NS13}[K^{98}\ R^{101}\ H^{102}\ E^{104}\ K^{111}\ R^{114}\ H^{115}]$ and $PAM_{NS13}[R^{101}\ H^{102}\ R^{114}\ H^{115}]$, indicating that the arginine and histidine residues in both repeat domains are critical residues in the interaction of PAM with plasminogen.

To further explore the contribution of these residues to the interaction with plasminogen, competition binding experiments were performed. The effective concentration of competitor required to inhibit plasminogen binding by 50% (EC_{50}) was determined by fitting a one-site competition curve (Figure 4.4). EC_{50} values ranged from 0.25 μM to 22.06 μM. As expected, there was an inverse correlation between K_d and EC_{50} (Table 4.2). This represents a greater than 90% decrease in EC_{50} values for mutant proteins $PAM_{NS13}[K^{98}\ K^{111}]$, $PAM_{NS13}[K^{98}\ R^{101}\ H^{102}\ E^{104}\ K^{111}]$ and $PAM_{NS13}[R^{101}\ H^{102}]$,

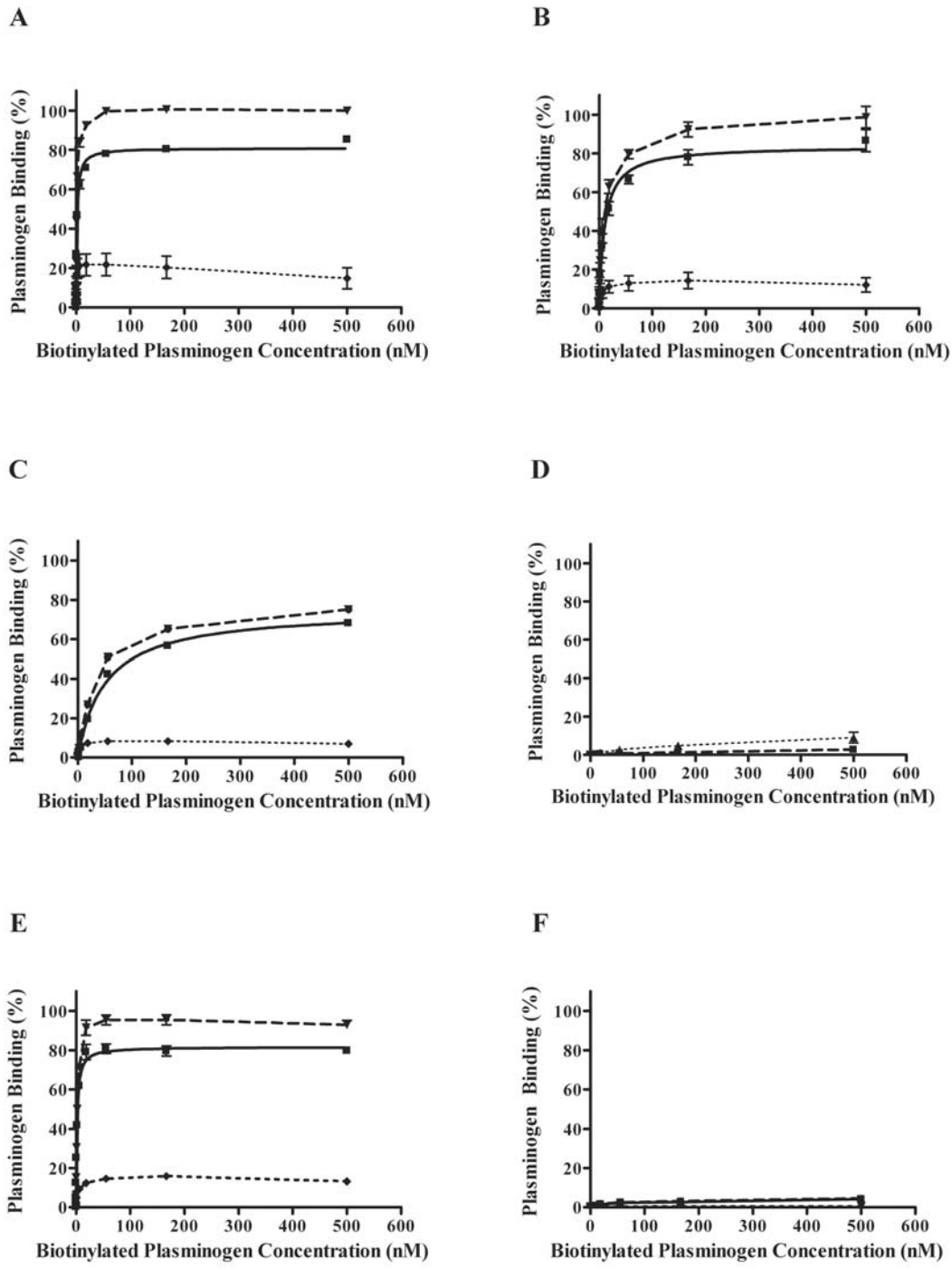


Figure 4.4 Saturation binding analysis of biotinylated glu-plasminogen to immobilised recombinant PAM_{NS13} mutant proteins. Biotinylated glu-plasminogen binding to immobilised recombinant protein (**A** PAM_{NS13}; **B** $PAM_{NS13}[K^{98}\ K^{111}]$; **C** $PAM_{NS13}[K^{98}\ R^{101}\ H^{102}\ E^{104}\ K^{111}]$; **D** $PAM_{NS13}[K^{98}\ R^{101}\ H^{102}\ E^{104}\ K^{111}\ R^{114}\ H^{115}]$; **E** $PAM_{NS13}[R^{101}\ H^{102}]$; **F** $PAM_{NS13}[R^{101}\ H^{102}\ R^{114}\ H^{115}]$) was measured in the absence (▼, total binding) and presence (●, non-specific binding) of a 50 fold molar excess of unlabelled glu-plasminogen. Specific binding (■) was determined by subtracting non-specific binding from total binding at each concentration. A one-site hyperbolic binding function was fitted to the data ($p < 0.05$), from which the binding dissociation constants were determined. Error bars represent the standard error of the mean (SEM; n = 3).

when compared to wild type PAM_{NS13}. These data indicate that whilst mutation of residues Lys^{98}, Arg^{101}, His^{102}, Glu^{104} and Lys^{111} within the plasminogen binding repeats of PAM_{NS13} decreases the avidity of the interaction with plasminogen, the simultaneous mutation of residues Arg^{114} and His^{115} is required to fully abolish binding.

Table 4.2 Functional and structural characteristics of PAM_{NS13} site–directed mutants.

Protein	K_d (nM)	EC_{50} (μM)	% α - helix
PAM_{NS13}	1.58	22.06	27
$PAM_{NS13}[K^{98}K^{111}]$	10.34	0.57	41
$PAM_{NS13}[K^{98}R^{101}H^{102}E^{104}K^{111}]$	50.24	0.25	33
$PAM_{NS13}[K^{98}R^{101}H^{102}E^{104}K^{111}R^{114}H^{115}]$	Non-specific binding only	Not determined	44
$PAM_{NS13}[R^{101}H^{102}]$	1.69	4.87	33
$PAM_{NS13}[R^{101}H^{102}R^{114}H^{115}]$	Non-specific binding only	Not determined	29

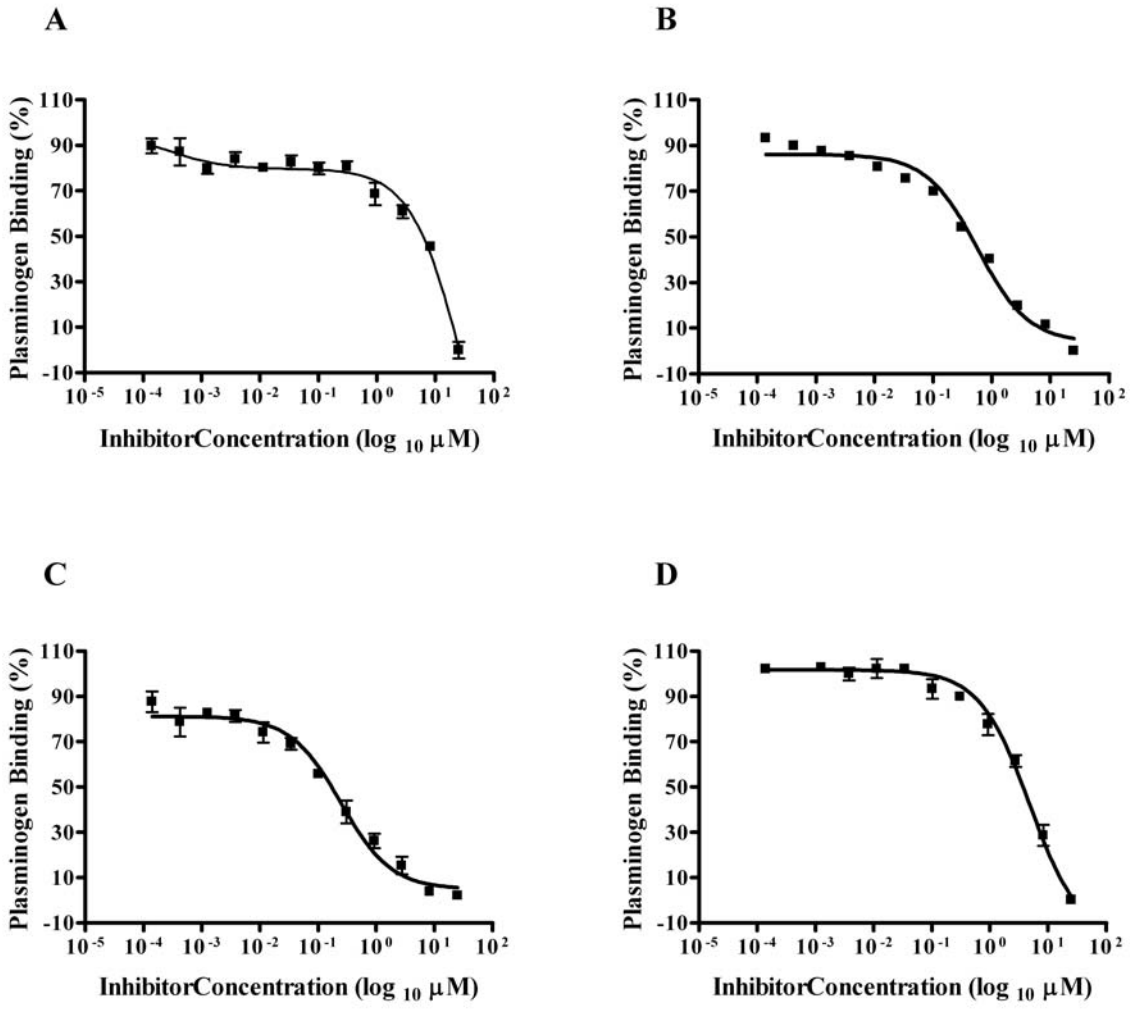


Figure 4.5 Competition of glu-plasminogen binding to immobilised recombinant PAM variants with fluid phase PAM_{NS13}. Binding of biotinylated glu-plasminogen to immobilised **A** PAM_{NS13}, **B** $PAM_{NS13}[K^{98}\ K^{111}]$; **C** $PAM_{NS13}[K^{98}\ R^{101}\ H^{102}\ E^{104}\ K^{111}]$, and **D** $PAM_{NS13}[R^{101}\ H^{102}]$ was measured in the presence of varying concentrations of unlabelled fluid phase PAM_{NS13}. Data points are the mean values of triplicate readings, with error bars indicating the standard error of the mean (SEM; n=3). One-site competition analysis was performed on data for all recombinant proteins; this analysis was used to determine the concentration of PAM_{NS13} required to inhibit binding of biotinylated glu-plasminogen by 50% (EC_{50}).

4.3 Discussion

PAM is a cell surface exposed, high affinity plasminogen receptor expressed by GAS associated with a variety of disease states, and appears to play an integral role in the plasminogen-dependent virulence of PAM positive GAS. In a recent study, it was shown that elimination of PAM dependent plasminogen binding by GAS significantly reduced mortality in mice expressing the human plasminogen transgene (Sun *et al.* 2004). Furthermore, whilst the plasminogen binding domain of PAM is highly variable, this variation does not appear to significantly impact on the high affinity interaction of PAM with plasminogen. The conservation of binding function in spite of sequence diversity is indicative of the physiological significance of this interaction, and the ability of GAS to subvert the host plasminogen activation system (Sanderson-Smith *et al.* 2006).

The a1 and a2 repeat domains in the N-terminus of PAM mediate binding to kringle 2 of plasminogen, with the a1 repeat residue Lys^{98} generally considered to make the greatest contribution to this interaction (Berge and Sjobring 1993; Wistedt *et al.* 1995; Wistedt *et al.* 1998). Using site-directed mutagenesis, this study indicates that residues Arg^{101} and His^{102} within the a1 repeat, together with residues Arg^{114} and His^{115} within the a2 repeat, are critical for the interaction of full length PAM with plasminogen. The interaction with plasminogen of site-directed mutant $PAM_{NS13}[K^{98}\ K^{111}]$ in which residues Lys^{98} and Lys^{111} were mutated to alanines was dose-dependent and specific. Additionally, the K_d value for this interaction was within a physiologically relevant concentration of plasminogen, which circulates in the bloodstream at a concentration of approximately 2 μM (Dano *et al.* 1985). Furthermore, mutation of binding site residues Arg^{101}, His^{102}, Arg^{114} and His^{115} abolished plasminogen binding by PAM_{NS13} despite the

presence of residues Lys^{98} and Lys^{111}. This appears to be the first demonstration of a non-lysine dependent, high affinity interaction between plasminogen and a full-length naturally occurring receptor.

Previous studies involving the interaction of a polypeptide sequence designated VEK-30 with kringle 2 of plasminogen highlighted the importance of PAM a1/a2 residues Lys^{98}, Arg^{101}, His^{102}, Glu^{104} and Lys^{111} in the peptide/plasminogen interaction (Schenone *et al.* 2000; Rios-Steiner *et al.* 2001). The peptide VEK-30 is comprised of the six residues preceding the a1 repeat, the full a1 repeat of PAM, together with the first ten residues of the a2 repeat. Notably, VEK-30 lacks the final three residues of the a2 repeat (Arg^{114}, His^{115}, Glu^{116}). Mutation of residues Lys^{98}, Arg^{101}, His^{102}, Glu^{104} and Lys^{111} in the full-length protein PAM_{NS13} in this study did not eliminate plasminogen binding. However, the avidity of this interaction was significantly reduced, as evidenced by a greater than 90% decrease in EC_{50} values recorded for mutant proteins when compared to wild type PAM_{NS13}. This supports the previous finding that these residues are important in the interaction of PAM with plasminogen. However, the finding that mutation of arginine (Arg^{101} and Arg^{114}) and histidine (His^{102} and His^{115}) residues in both the a1 and a2 repeat was required to fully abrogate plasminogen binding suggests that both repeats are able to mediate high affinity interactions with plasminogen. In order to fully assess the role of residues Arg^{114} and His^{115} in the interaction of PAM with plasminogen, mutagensis of these residues independently of Arg^{101} and His^{102} is required. Residues Arg^{101} and His^{102} of the a1 repeat of PAM have been shown to make numerous saltbridge and hydrophobic electrostatic interactions with recombinant kringle 2, forming a pseudo-ligand similar to the lysine analogue ε- amino caproic acid (Rios-

Steiner *et al.* 2001). It is thus likely that the corresponding residues Arg^{114} and His^{115} in the a2 repeat interact with plasminogen in a similar fashion.

The decrease in plasminogen binding by site-directed mutant $PAM_{NS13}[R^{101}H^{102}R^{114}H^{115}]$ reported here does not appear to be due to loss of secondary structure, as all site-directed mutants displayed CD spectra characteristic of coiled-coil alpha-helical proteins, similar to that of the wild type PAM_{NS13}. Additionally, this mutant displays 29% α-helicity, which is not significantly different to the wild type protein. CD analysis of other streptococcal M proteins has found them to contain between 23% and 70% α-helix (Phillips *et al.* 1981; Khandke *et al.* 1988). However, the spectra for all α-helical proteins are not identical due to the small effect of non-aromatic side chains on the rotary strength of the peptide bond, and occasional helix distortions (Freifelder 1982). It has also been shown that the α-helical structure of the peptide VEK-30, representative of the a1 repeat of PAM, increases from 25% to 75% upon binding to kringle 2 of plasminogen (Rios-Steiner *et al.* 2001). It is possible that a similar structural shift could occur in the full length PAM protein. It should be noted that, for all other mutant proteins reported here, the percent α-helicity was between 33% and 44%. It has been shown that CD spectra can vary by as much as 10% for two different preparations of the same protein, and as such, the variation between proteins seen here does not represent a significant difference (Berengian *et al.* 1997). No correlation was seen between percent α-helicity and plasminogen binding function, suggesting that any secondary structure changes have had only a minor influence on the differences in plasminogen binding described here.

In the previous chapter of this thesis, it was shown that whilst the plasminogen binding domain of PAM is highly variable, this diversity does not abrogate plasminogen binding by naturally occurring PAM variants. In these naturally occurring PAM variants, the Arg and His residues in both a1 and a2 repeat domains are highly conserved (McKay *et al.* 2004). Interestingly, in this study, a loss of plasminogen binding was only observed following simultaneous mutation of both the Arg^{101} and His^{102} residues in the a1 repeat, and the Arg^{114} and His^{115} residues in the a2 repeat. Therefore, conservation of arginine and histidine residues in either repeat domain may compensate for variation elsewhere in the binding repeats, and explain the maintenance of high affinity plasminogen binding by naturally occurring variants of PAM. This study highlights for the first time the importance of highly conserved arginine (Arg^{101} and Arg^{114}) and histidine (His^{102} and His^{115}) residues within the binding site of the plasminogen binding protein PAM, in mediating the interaction of this molecule with plasminogen. Such a finding may have implications for the identification of novel plasminogen binding proteins in the future.

5 Characterisation of the phylogenetically distinct PAM variant $PAM_{NS88.2}$

5.1 Introduction

Increasingly, sequestration of plasminogen is being implicated in the pathogenesis of GAS, and the ability of some GAS isolates to cause severe invasive infection (Sun *et al.* 2004; Walker *et al.* 2005). For a subset of GAS isolates, the ability of PAM to focus plasminogen at the GAS cell surface appears to be critical for virulence (Sun *et al.* 2004). In the Northern Territory of Australia, where streptococcal skin infection is endemic, high rates of invasive infection such as bacteraemia have been reported (Carapetis *et al.* 1999). GAS strain NS88.2 is a Northern Territory isolate associated with invasive blood infection. The PAM gene from this isolate shows 85.8% identity to the prototype sequence of PAM, but only 52% identity to the classical a1/a2 repeat domain of PAM. The major site of variation between $PAM_{NS88.2}$ and the prototype PAM is between residues 29-100 in the N-terminus of the protein. Type specificity of M protein comes from variation within the proteins N-terminus, and, $PAM_{NS88.2}$ appears to be phylogenetically distinct from the other PAM variants characterised in this study. In spite of this, $PAM_{NS88.2}$ is still a high affinity receptor for plasminogen. It is not known whether $PAM_{NS88.2}$ and other PAM proteins interact with plasminogen via similar binding site residues.

Previous studies investigating the role of PAM in GAS disease have focused on isolates associated with uncomplicated infection (Svensson *et al.* 2002; Sun *et al.* 2004). Studies using the hu-skin-SCID mouse model for streptococcal impetigo found that a PAM negative isogenic mutant of GAS strain ALAB49 displayed limited attenuation for virulence when compared to the wildtype ALAB49 (Svensson *et al.* 2002). However, a recently published animal study of GAS infection showed that in mice expressing the human plasminogen transgene, inoculation with the PAM positive GAS strain AP53

resulted in 80% mortality. A PAM negative isogenic mutant of AP53 exhibited only minimal virulence in this model (Sun *et al.* 2004). The creation of isogenic mutants in these studies was achieved using allelic replacement of the PAM gene with the ΩKm-2 interposon. Whilst these authors report a limited impact on factors such as capsule formation due to allelic replacement, results indicate that the PAM negative isogenic mutant Δ*pam*ALAB49 showed reduced resistance to phagocytosis (Svensson *et al.* 2002). The ability of GAS to resist phagocytosis has been largely attributed to the antiphagocytic properties of M protein (Fischetti 1989), thus attenuation in virulence may, in part, be attributed to reduced antiphagocytic properties of mutant strains. Additionally, deletion of genes from the chromosome has the potential to cause polar effects downstream. As such, an ideal mutant for infection studies would involve replacement of a functional PAM gene with one that expresses a full length, conformationally unchanged protein with no plasminogen binding ability.

In studies performed in our laboratory, GAS strain NS88.2 was found to be highly virulent in the human plasminogen transgenic mouse model of infection. All mice infected with this strain died within four days of challenge. However, no mortality was seen in mice following inoculation with the PAM positive strain ALAB49 (McKay 2005). The highly virulent nature of GAS strain NS88.2, together with the finding that $PAM_{NS88.2}$ is phylogenetically distinct from other PAM proteins, makes this strain an interesting candidate for future studies, particularly in murine models of streptococcal infection. This chapter outlines that characterisation of the $PAM_{NS88.2}$ plasminogen binding site, and the subsequent abrogation of binding by recombinant $PAM_{NS88.2}$, for possible future use in the creation of an NS88.2 PAM mutant for use in animal studies.

5.2 Results

5.2.1 Site directed mutagenesis

$PAM_{NS88.2}$ displays only limited identity to the prototype PAM binding site. In order to identify the key residues involved in the interaction of $PAM_{NS88.2}$ with plasminogen, a number of site-directed mutants were created using the Quickchange™ site-directed mutagenesis kit. Residues were selected for mutation based on similarity to key binding site residues in the PAM_{NS13} protein. The forward primers used to introduce the desired mutations are given in Table 5.1.

Table 5.1 Forward primer sequences used to construct PAM_{NS13} site-directed mutants.

Introduced mutation	Forward mutagenesis primer
K^{96}/A	5'-CGAGAGTTAGAAGACCTTGCAGATGCTGAGTTGAAGC-3'
K^{101}/A	5'-GCAGATGCTGAGTTGGCACGACTTAATGAAGAG -3'
$R^{107}H^{108}$/A	5'-CGACTTAATGAAGAGGCAGCAGATCATGACAAAAGAGAAGC -3'

The presence of the desired mutations, together with the absence of other PCR induced mutations was confirmed using DNA sequence analysis. The binding site sequence of wildtype and mutant $PAM_{NS88.2}$ proteins is shown in Figure 5.1A. Following the expression of recombinant proteins in *E. coli*, proteins of approximately 40 kDa were purified as described previously (Figure 5.1B).

5.2.2 Structural characterisation of $PAM_{NS88.2}$ site directed mutants

In order to assess the potential impact of introduced mutations on $PAM_{NS88.2}$ structure, far UV CD spectroscopy was conducted on both wildtype and mutant proteins. As discussed previously, this is a useful technique for structural characterisation of

A

Recombinant Protein	Plasminogen Binding Site
$PAM_{NS88.2}$	ERELEDLKDAELKRLNEERHD
$PAM_{NS88.2}[K^{96}\ K^{101}]$	ERELEDL**A**DAEL**A**RLNEERHD
$PAM_{NS88.2}[K^{96}K^{101}R^{107}\ H^{108}]$	ERELEDL**A**DAEL**A**RLNEE**AA**D
$PAM_{NS88.2}[R^{107}H^{108}]$	ERELEDLKDAELKRLNEE**AA**D

B

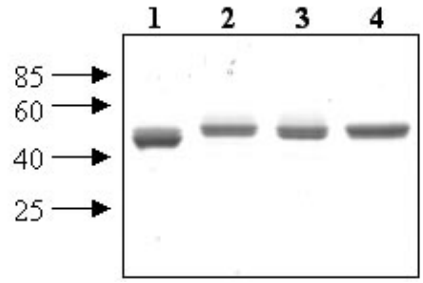


Figure 5.1 SDS-PAGE analysis of recombinant $PAM_{NS88.2}$ site-directed mutants. **A** Alignment of the plasminogen binding domain of wildtype $PAM_{NS88.2}$ with the 3 site-directed mutants constructed in this study. Mutated residues are indicated in bold. **B** 12% SDS-PAGE gel showing the 4 purified recombinant proteins used in this study. Lane 1, $PAM_{NS88.2}$; lane 2, $PAM_{NS88.2}[K^{96}K^{101}]$; lane 3 $PAM_{NS88.2}[K^{96}K^{101}R^{107}H^{108}]$; lane 4, $PAM_{NS88.2}[R^{107}H^{108}]$ Molecular weight markers are given in kilo Daltons (kDa).

mutants. This is particularly important in the case of $PAM_{NS88.2}$ as it is a potential candidate for construction of an NS88.2 GAS mutant for use in animal virulence studies.

All the site-directed mutants constructed displayed similar CD spectra to the wildtype $PAM_{NS88.2}$ (Figure 5.2). Each protein displays two minima at approximately 210 nm and 220 nm, in addition to a maximum peak at approximately 190 nm. As stated previously, this is characteristic of α-helical proteins, and in particular, streptococcal M proteins (Phillips *et al.* 1981). Additionally, as with PAM_{NS13}, the two minima for each protein are of a similar magnitude, a characteristic indicative of coiled-coil α-helical proteins

(Graddis *et al.* 1993). The precent helicity of each protein was found to be as follows: $PAM_{NS88.2}$,, 42.9%; $PAM_{NS88.2}$ [$K^{96}K^{101}$], 37.8%; $PAM_{NS88.2}$[$R^{107}H^{108}$], 40.7%; and $PAM_{NS88.2}$[$K^{96}K^{101}R^{107}H^{108}$], 37.4%. Thus, it appears that each of these proteins are all structurally similar, and site directed mutagenesis has resulted in limited structural changes.

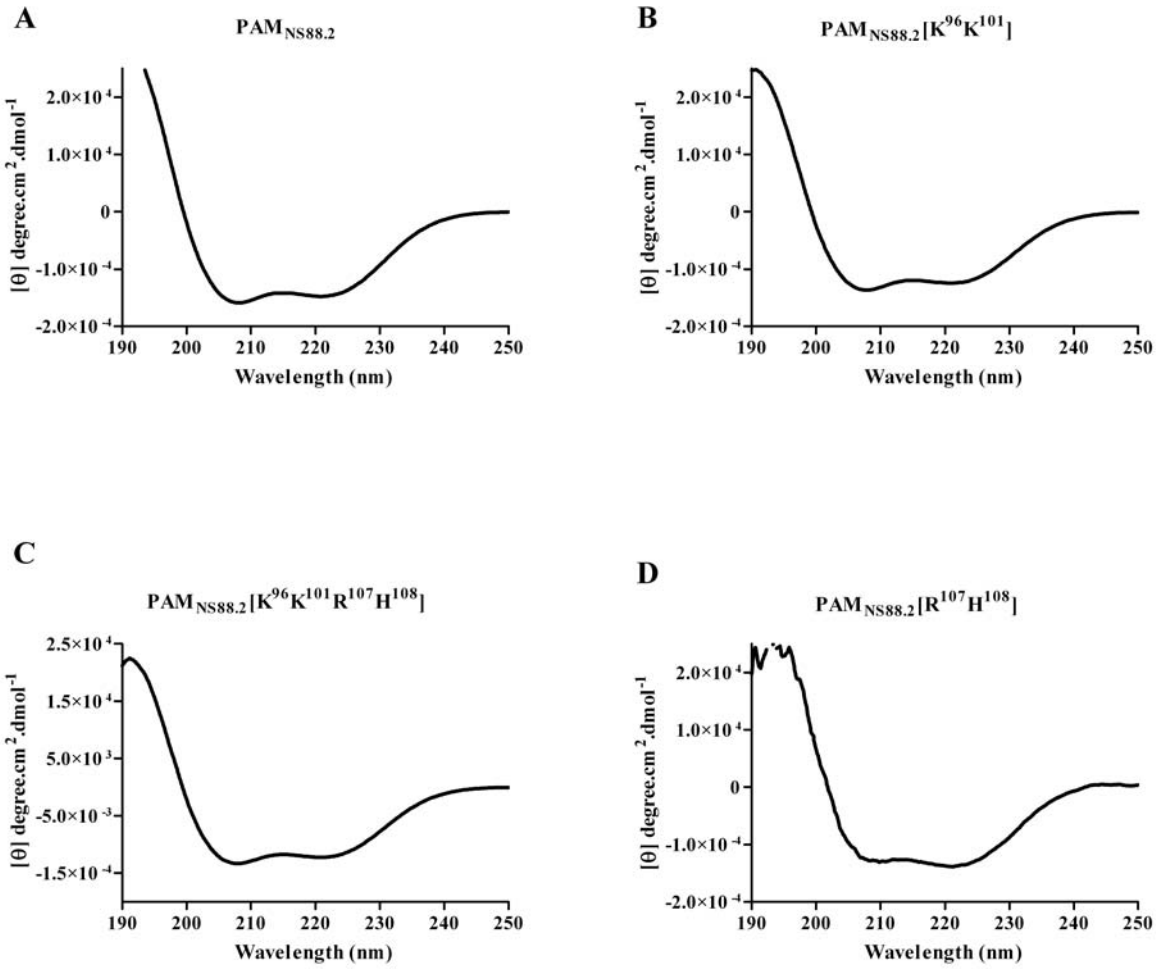


Figure 5.2 Circular dichroism spectra of recombinant $PAM_{NS88.2}$ site-directed mutants. All proteins exhibit CD emission spectra characteristics of α-helical coiled-coil proteins (Freifelder 1982), displaying two characteristic minima at approximately 210 nm and 220 nm, and a maximum peak at approximately 190 nm.

5.2.3 Plasminogen binding analysis

$PAM_{NS88.2}$ has previously been shown to interact with glu-plasminogen with a K_d of 7.58 nM. This represents a physiologically relevant, high affinity interaction. In order to assess the impact of site-directed mutagenesis on the affinity of $PAM_{NS88.2}$, and to identify which residues are required for this interaction, ligand blotting and solid-phase saturation plasminogen binding assays were performed. Wildtype $PAM_{NS88.2}$ and $PAM_{NS88.2}[K^{96}\ K^{101}]$ both interacted with biotinylated glu-plasminogen in a ligand blot analysis. No interaction was seen for $PAM_{NS88.2}[K^{96}K^{101}R^{107}H^{1081}]$ nor, $PAM_{NS88.2}[R^{107}\ H^{108}]$ (Figure 5.3).

To further characterise the interaction of $PAM_{NS88.2}$ site-directed mutants with glu-plasminogen, recombinant proteins immobilised to 96-well plates were incubated with increasing concentrations of biotinylated glu-plasminogen, in the presence or absence of a 50 fold molar excess of unlabelled glu-plasminogen. None of the site-directed mutants interacted with plasminogen in a specific, saturable fashion with 500 nM plasminogen (Figure 5.4 B-D). $PAM_{NS88.2}\ [K^{96}K^{101}]$ approached, but did not reach saturation (Figure 5.4 B). This suggests that whilst residues Lys^{96} and Lys^{101} are not solely responsible for the interaction of $PAM_{NS88.2}$ with plasminogen, they do contribute to this interaction. Only non-specific binding was found for site directed mutants $PAM_{NS88.2}[K^{96}K^{101}R^{107}H^{108}]$ and $PAM_{NS88.2}[R^{107}H^{108}]$. This data indicates that residues Arg^{107} and His^{108} make the greatest contribution to the interaction of $PAM_{NS88.2}$ with plasminogen.

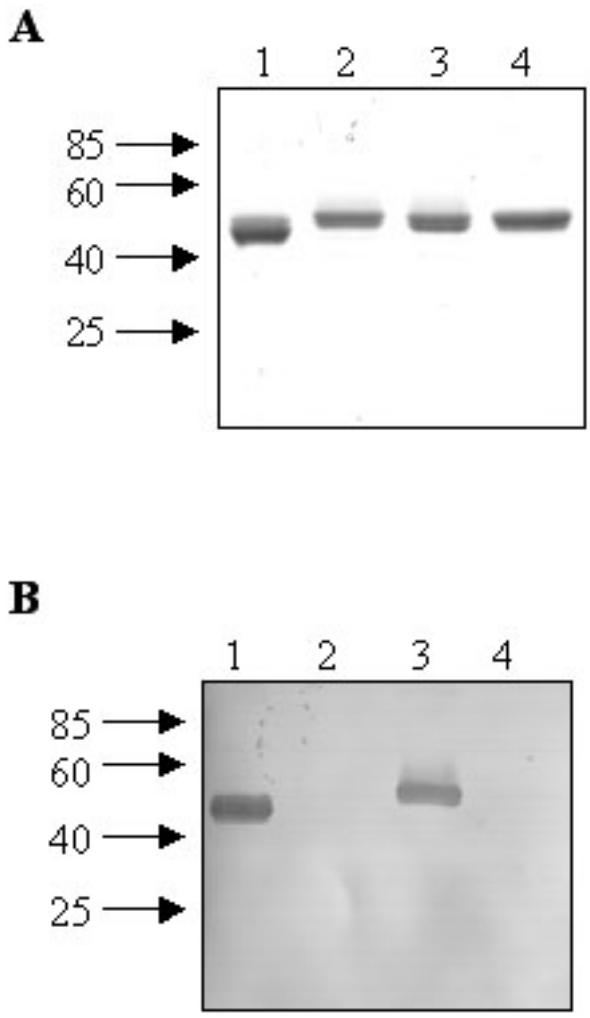


Figure 5.3 Ligand blot analysis of recombinant $PAM_{NS88.2}$ site-directed mutants. **A** 12% SDS-PAGE gel of recombinant $PAM_{NS88.2}$ mutants used for ligand blotting analysis. Lane 1, $PAM_{NS88.2}$; lane 2, $PAM_{NS88.2}[K^{96}K^{101}R^{107}H^{108}]$; lane 3 $PAM_{NS88.2}[K^{96}K^{101}]$; lane 4, $PAM_{NS88.2}[R^{107}H^{108}]$ **B** Ligand blot of purified $PAM_{NS88.2}$ site-directed mutants employing biotinylated glu- plasminogen and neutravidin-HRP. Lane 1, $PAM_{NS88.2}$; lane 2 $PAM_{NS88.2}[K^{96}K^{101}R^{107}H^{108}]$; lane 3, $PAM_{NS88.2}[K^{96}K^{101}]$; lane 4, $PAM_{NS88.2}[R^{107}H^{108}]$ .Molecular weight markers are given in kilo Daltons (kDa).

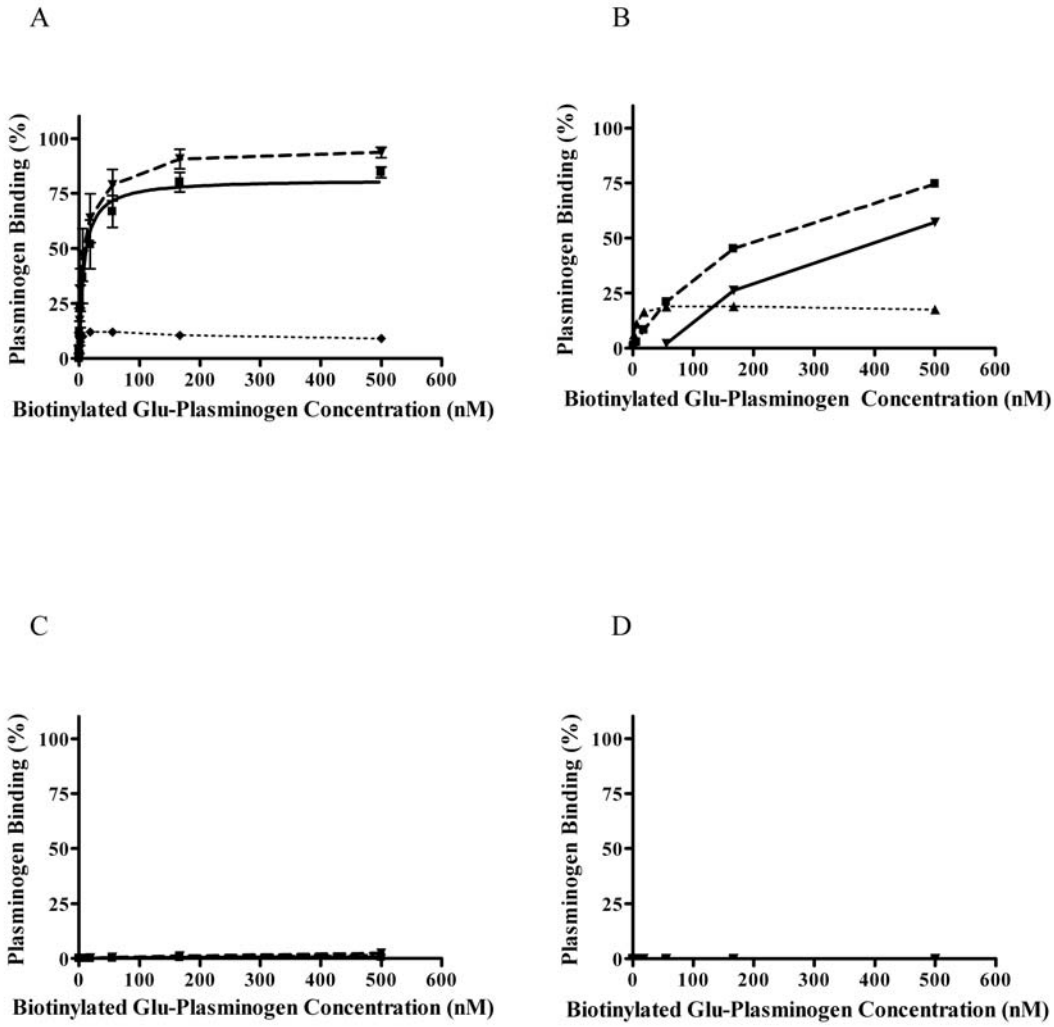


Figure 5.4 Saturation binding analysis of biotinylated glu-plasminogen to immobilised recombinant $PAM_{NS88.2}$ mutant proteins. Biotinylated glu-plasminogen binding to immobilised recombinant protein (**A**, $PAM_{NS88.2}$; **B**, $PAM_{NS88.2}[K^{96}K^{101}]$; **C,** $PAM_{NS88.2}[K^{96}K^{101}R^{107}H^{108}]$; **D,** $PAM_{NS88.2}[R^{107}H^{108}]$) was measured in the absence (▼, total binding) and presence (●, non-specific binding) of a 50 fold molar excess of unlabelled glu-plasminogen. Specific binding (■) was determined by subtracting non-specific binding from total binding at each concentration. A one-site hyperbolic binding function was fitted to the data ($p < 0.05$), from which the binding dissociation constants were determined. Error bars represent the standard error of the mean (SEM; n = 3).

5.3 Discussion

A key feature of certain GAS is the ability to invade from cutaneous and mucosal surfaces to deep tissue sites, resulting in serious invasive infections such as necrotising fasciitis and bacteraemia. Amongst the indigenous population of the Northern Territory of Australia, there is a high incidence of GAS infection in general, and invasive infection specifically (Carapetis *et al.* 2005). An epidemiological study of GAS from this region showed that isolates associated with invasive disease bound more plasminogen than isolates from uncomplicated sites of infection (McKay *et al.* 2004). Isolated from a bacteraemic infection in the Northern Territory, GAS strain NS88.2 has been shown to bind high levels of plasminogen when compared to other GAS isolates from this region (McKay *et al.* 2004). The plasminogen binding site of $PAM_{NS88.2}$ is only 52% identical to that of the prototype a1/a2 binding site. In spite of this variation, results presented in chapter 3 of this thesis indicate that $PAM_{NS88.2}$ functions as a high affinity receptor for glu-plasminogen. Results presented here indicate that this high affinity interaction is mediated by the same critical residues as for the prototypical PAM binding site. Plasminogen ligand blotting analysis of $PAM_{NS88.2}$ site-directed mutants suggested that plasminogen binding could only be fully eliminated following mutagenesis of residues Arg^{107} and His^{108}. Solid phase plasminogen binding assays indicated that none of the mutants created here were able to interact with plasminogen in a saturable fashion. However, the binding curve for $PAM_{NS88.2}[K^{96}K^{101}]$ approached, but did not reach saturation. This, together with the interaction of $PAM_{NS88.2}[K^{96}\ K^{101}]$ with plasminogen in the ligand blot analysis suggests that this protein is still able to bind plasminogen, with lower affinity and specificity than the wildtype protein. Plasminogen binding was eliminated only following the mutagenesis of residues Arg^{107} and His^{108}. Thus, in spite of the significant differences between $PAM_{NS88.2}$ and the other

PAM variants characterised in this study, and the apparent phylogenetic difference of $PAM_{NS88.2}$, the interaction of these plasminogen binding proteins appears to be largely dependent on the presence of arginine and histidine residues within a binding site sequence.

The critical role of human plasminogen in the pathogenesis of certain GAS isolates has been demonstrated by the development of the humanised plasminogen transgenic mouse (Sun *et al.* 2004). This murine model is ideal for future studies of the role of the human plasminogen activation system in GAS disease. Previous studies involving the use of PAM deletion isogenic GAS mutants have not conclusively demonstrated a role for PAM in the pathogenesis of GAS invasive infection, in part, due to the finding that GAS with reduced levels of M protein expression show a reduced resistance to phagocytosis (Svensson *et al.* 2002). PAM is almost exclusively associated with *emm* pattern D GAS isolates, which encode multiple *emm and emm* like genes within the chromosome (Hollingshead *et al.* 1993; Svensson *et al.* 1999). For GAS with multiple *emm* genes, each gene product partially contributes to the ability of the isolate to resist phagocytosis (Podbielski *et al.* 1996). Thus, the deletion of *emm* genes may be responsible for any observed decrease in virulence of isogenic mutants when compared to wildtype strains. Construction of an isogenic mutant of the highly virulent GAS strain NS88.2 that expresses one of the site-directed mutant PAM proteins created here may prove useful in a murine model of GAS infection, and clearly indicate the role of PAM in GAS disease. The functional characteristics of M proteins, including plasminogen and fibrinogen binding ability, may depend largely on their coiled-coil alpha helical structure (Phillips *et al.* 1981; Cedervall *et al.* 1997; Wistedt *et al.* 1998). This may also be the case for the antiphagocytic properties of the M protein. The coiled-coil structure

of proteins is based on a recurring heptad periodicity in the primary amino acid sequence. Heptad repeat residues can be designated "a" through to "g", in which residues "a" and "d" are generally non-polar and pack to form the hydrophobic core of the coiled coil (Mason and Arndt 2004). All $PAM_{NS88.2}$ site-directed mutants displayed CD spectra similar to that of wildtype $PAM_{NS88.2}$, and characteristic of coiled-coil alpha helical proteins. Site-directed mutagenesis appears then, to have had limited impact on protein structure, further highlighting the usefulness of these variants in future studies involving isogenic GAS mutants.

The finding that $PAM_{NS88.2}$ plasminogen-binding is mediated by similar residues to other PAM proteins indicates that, in spite of significant sequence differences elsewhere, the Arg-His motif endows plasminogen binding M proteins with high affinity receptor ability. This may prove useful in the construction of mutants for use in a murine model of GAS infection when investigating the role of PAM in GAS virulence. Currently, non-polar replacement mutants of GAS can be created using the temperature sensitive plasmid phG9. The vector phG9 contains the temperature sensitive mutant *rep*A gene, erythromycin resistance and the multiple cloning site from the *E. coli* vector pBluescript (Fontaine *et al.* 2003). Using this system, double crossover is required to facilitate allelic replacement in the GAS chromosome. This system avoids the potential polar effects of insertion and deletion mutants, and is useful for integration of genes into the chromosomes of poorly transformable bacteria such as GAS (Biswas *et al.* 1993). Future research could be directed towards the creation of a $PAM_{NS88.2}$ allelic replacement isogenic mutant using this system, which may be used in the humanised plasminogen transgenic mouse for analysis of the role of PAM in serious GAS infection.

6 Conclusions and future research

A major causative agent of skin and mucosal disease in humans, *S. pyogenes* has the capacity to cause invasive infections of the blood and deep tissue. Recent data indicates that over 660,000 cases of GAS invasive infection occur worldwide each year. Almost one quarter of these cases are fatal (Carapetis *et al.* 2005). Invasive infection results from the ability of GAS to migrate from skin and mucosal surfaces to deep tissue sites. The exact mechanisms of this migration have yet to be fully explained, however one hypothesis involves the interaction of GAS with the host plasminogen activation system. The multiplicity of potential virulence factors associated with *S. pyogenes* that interact with the plasminogen activation system necessitates a deeper understanding of the role of plasminogen in GAS virulence. The present study focuses on the high affinity GAS plasminogen receptor PAM. The details of PAM associated GAS virulence remain unclear. As such, the overall aims of this thesis were to examine both the interaction of PAM with plasminogen and the role of PAM in GAS pathogenesis. Specifically, the focus of this study was on determining the level of variation in the *pam* gene of a selection of GAS isolates, the association of variation with GAS epidemiology, and the impact of variation on PAM binding function and host immune recognition. In addition, this study aimed to fully characterise the plasminogen-binding domain of PAM using site–directed mutagenesis of the plasminogen binding domain of PAM, with the final aim of creating a PAM molecule fully devoid of plasminogen binding activity, that has the potential to be used in future research utilising murine models of GAS infection.

PAM is a cell surface exposed, high affinity plasminogen receptor expressed by GAS thought to be associated primarily with impetigo (Svensson *et al.* 1999). However, this study has found that within the Northern Territory of Australia, PAM is associated with

a variety of disease states. Data presented here indicates that the plasminogen binding domain of PAM is highly variable. This variation may have resulted from host selective immune pressure, as it has been clearly demonstrated that whilst the prototypical a1 repeat of PAM is able to elicit the production of opsonophagocytic antibodies in mice, a PAM variant with limited identity to this region is not susceptible to opsonisation by murine anti-a1 sera. Additionally, the protective nature of the a1 domain of PAM has potential implications for the future development of therapeutics.

The high levels of sequence divergence seen within the plasminogen binding domain of PAM has only limited impact on the functional properties of naturally occurring PAM variants. Of the five variants of PAM characterised here, all were found to act as high affinity, physiologically relevant receptors for glu-plasminogen. The arginine and histidine residues in both a1 and a2 repeat domains of PAM are highly conserved, thus, the maintenance of binding function in spite of binding site sequence variation appears to be the result of conservation of these residues. Plasminogen typically binds to receptors via C-terminal or internal lysine residues, as exemplified by previously characterised GAS plasminogen receptors SEN and GAPDH. Binding of plasminogen to SEN occurs via residues Lys^{434} and Lys^{435} at the C-terminus of the SEN molecule, whilst binding of plasminogen to GAPDH occurs via C-terminal lysine Lys^{334} (Broder *et al.* 1991; Pancholi and Fischetti 1998). In contrast, plasminogen binding to PAM has been attributed to internal lysine residues Lys^{98} and Lys^{111} (Berge and Sjobring 1993; Wistedt *et al.* 1995; Wistedt *et al.* 1998). However, site-directed mutagenesis studies conducted using both recombinant PAM_{NS13} and $PAM_{NS88.2}$ proteins highlight the importance of arginine and histidine residues in the binding of plasminogen by PAM. For both proteins, plasminogen binding was only fully abrogated by the mutation of

binding site arginine (PAM_{NS13}: Arg^{101} and Arg^{114}; $PAM_{NS88.2}$: Arg^{107}) and histidine (PAM_{NS13}: His^{102} and His^{115}; $PAM_{NS88.2}$: His^{108}) residues to alanine. Furthermore, it was found that the arginine and histidine mutants of these molecules were unable to bind plasminogen even when binding site lysine residues were left intact. It has previously been shown by X-ray crystallography that residues Arg^{101} and His^{102} in a peptide encompassing the a1 repeat of PAM interact with recombinant kringle 2 of plasminogen in a lysine-like fashion (Rios-Steiner *et al.* 2001). It is thus conceivable that a similar interaction occurs in the full length PAM molecule. This could be confirmed by X-ray crystallographic analysis of the full length PAM molecule with kringle 2 of plasminogen. Such an experiment also has the potential to clarify the role of lysine residues in this interaction, as mutation of binding site lysine residues in both PAM_{NS13} and $PAM_{NS88.2}$ decreased the affinity of these molecules for plasminogen. Nevertheless, it is clear that the binding of plasminogen by PAM is primarily mediated by arginine and histidine residues, and that lysine is not a pre-requisite for this interaction. This has significant implications for the identification of novel plasminogen binding receptors in future studies.

The abrogation of plasminogen binding by $PAM_{NS88.2}$, which is associated with the invasive GAS strain NS88.2 may prove useful in the construction of mutants for use in a murine model of GAS infection. The critical role of human plasminogen in the pathogenesis of certain GAS isolates has been demonstrated by the development of the humanised plasminogen transgenic mouse (Sun *et al.* 2004). This murine model is ideal for future studies of the role of the human plasminogen activation system in GAS disease. Such studies have the potential to conclusively establish the role of PAM dependent plasminogen acquisition by GAS in establishing invasive infection.

Recent findings that the acquisition of plasminogen by *S. pyogenes* may be crucial for the virulence of certain strains of GAS, and the ability of multiple GAS proteins to facilitate this process, necessitates a deeper understanding of the mechanisms via which GAS interacts with plasminogen. Results presented in this thesis are consistent with the hypothesis that acquisition of plasminogen confers a selective advantage on certain strains of GAS, and further highlights the importance of the high affinity plasminogen receptor PAM in this process. This study provides data to show that whilst highly variable, PAM proteins possess a binding site that facilitates high affinity plasminogen binding via highly conserved arginine and histidine residues. The maintenance of binding function in spite of high levels of sequence divergence is indicative of the physiological significance of this interaction, and the ability of GAS to subvert the host plasminogen activation system. In addition to this, the results of this thesis are consistent with host immuno-selective pressure as a potential mechanism for divergence in the functional domain of PAM. Finally, the construction of a site-directed mutant of $PAM_{NS88.2}$, which is fully abrogated for plasminogen binding, provides an ideal candidate for future studies to elucidate the role of PAM in GAS invasive infection. The multifactorial nature of GAS-host interactions, and the apparent advantage conferred on GAS by its ability to interact with the host plasminogen activation system, has widespread implications for both the study and treatment of GAS pathogenesis and infection.

7 Appendix 1

Media

LB (1 L)

Tryptone ..... 10 g
NaCl ..... 10 g
Yeast Extract ..... 5 g

LB agar (500ml)

Agar ..... 7.5 g
LB Broth ..... 500 ml

SOC medium

Tryptone ..... 2.0 %
Yeast Extract ..... 0.5 %
NaCl ..... 10 mM
KCl ..... 2.5 mM
$MgCl_2$ ..... 10 mM
Glucose ..... 20 mM

Storage of E.coli cells

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

Southern hybridisation reagents

Denaturation solution

NaOH ..... 20.0 g/L
NaCl ..... 87.5 g/L

Neutralisation solution (pH 7.5)

Tris-HCl ..... 78.78 g/L
NaCl ..... 175.32 g/L

20 x SSC buffer (pH 7.0)

NaCl ..... 175.32 g/L
Sodium citrate ..... 88.23 g/L

2 x Wash solution

2 x SSC buffer
0.1% SDS

0.5 x Wash solution

0.5 x SSC
0.1% SDS

Maleic acid buffer
Maleic acid ..... 11.6 g/L
NaCl ..... 8.77 g/L

Blocking solution
Maleic acid buffer
5% skim milk powder

Washing buffer
Maleic acid buffer
Tween 20 ..... 3.0g/L

Detection buffer (pH 9.5)
Tris-HCl ..... 15.7 g/L
NaCl ..... 5.84 g/L

Pulsed field gel electrophoresis reagents

TSE buffer (pH 8.0)
Tris-HCl ..... 10 mM
NaCl ..... 1.0 M
EDTA ..... 50 mM

TE buffer (pH 8.0)
Tris-HCl ..... 10 mM
EDTA ..... 50 mM

Cell lysis solution
Tris-HCl (pH 7.6) ..... 6 mM
EDTA (pH 7.5) ..... 100 mM
NaCl ..... 1 M
Brij 58 ..... 0.5 % (v/v)
Deoxycholate ..... 0.2% (v/v)
Sodium lauroyl sarcosine ..... 0.5% (v/v)
Lysozyme ..... 1 mg/ml
Mutanolysin ..... 100 U/ml
RNase ..... 20 µg/ml

Deproteination solution
Proteinase K ..... 1 µg/ml
Sodium lauroyl sarcosine ..... 1% (v/v)
EDTA (pH 7.5) ..... 500 mM

Recombinant protein purification reagents

Cell lysis buffer (pH 8.0)

Tris-HCl .....25 mM
Lysozyme .....10 mg/ml

Native elution buffer (pH 8.0)

NaH_2PO_4 .....50 mM
NaCl .....300 mM
Imidazole.....250 mM

Plasminogen purification reagents

Salt wash buffer (pH 7.5)

Na_2HPO_4 .....0.05 M
NaCl .....5 M
EDTA .....5 mM
PMSF .....1 mM

EACA elution buffer (pH7.5)

1× PBS (pH 7.5)
ε-amino caproic acid .....200 mM

SDS-PAGE reagents

SDS-PAGE running buffer (8×)

Tris base .....15 g/L
Glycine.....72 g/L
SDS .....5 g/L

Non-reducing sample buffer (2×)

Tris-Cl (pH 6.8).....90 mM
Glycerol .....20 % (v/v)
SDS .....2 % (v/v)
Bromophenol blue.....0.02 % (w/v)

Reducing sample buffer (2×)

Dithiothrietol.....100 mM
Non-reducing sample buffer (2 ×)

Rapid coomassie blue stain

Coomassie blue R-250 .....1 g
Methanol .....200 ml
Glacial acetic acid .....50 ml
Distilled H_2O.....to 500 ml

Rapid destain

Methanol ..... 400 ml
Glacial acetic acid ..... 100 ml
Distilled H_2O ..... to 1 L

Final destain

Glacial acetic acid ..... 100 ml
Glycerol ..... 40 ml
Distilled H_2O ..... to 1 L

12% acrylamide gels (per gel)

40% bis acrylamide ..... 1.55 ml
1.5 M Tris-HCl (pH 8.8) ..... 1.25 ml
10% SDS ..... 50 µl
Distilled H_2O ..... 2.25 ml
TEMED ..... 25 µl
10% APS ..... 25 µl

10% acrylamide gels (per gel)

40% bis acrylamide ..... 1.25 ml
1.5 M Tris-HCl (pH 8.8) ..... 1.25 ml
10% SDS ..... 50 µl
Distilled H_2O ..... 2.4 ml
TEMED ..... 25 µl
10% APS ..... 25 µl

4% Stacking gels (per gel)

40% bis acrylamide ..... 225 µl
0.5 M Tris-HCl (pH 6.8) ..... 600 µl
10% ..... 25 µl
Distilled H_2O ..... 1.6 ml
TEMED ..... 12.5 µl
10% APS ..... 12.5 µl

Western and ligand blotting reagents

Transfer buffer

Tris base ..... 3.03 g/L
Glycine ..... 14.4 g/L
Methanol ..... 10% (v/v)

PBST

PBS (1×)
Tween 20 ..... 0.05% (v/v)

10% blocking solution

PBS (1×)
10% skim milk powder

1% blocking solution

PBS (1×)
1% skim milk powder

DAB developing solution

Diaminobenzidine ..... 25 mg
100 mM Tris-HCl, (pH 7.5) ..... 50 ml
H_2O_2 ..... 30 µl

Solid phase microtitre assay reagents

PiNT (pH 7.5)

Na_2HPO_4 ..... 50 mM
NaCl ..... 150 mM
Tween-80 ..... 0.05% (v/v)

Agarose gel electrophoresis reagents

TAE buffer (50 ×, pH 8.0)

Tris base 242g/l
Glacial acetic acid ..... 57.1 ml
EDTA (0.5 M, pH 8.0) ..... 100 ml
Distilled H_2O ..... to 1 L

Agarose gel

TAE (1 ×)
Agarose ..... 1% (w/v)

DNA loading dye

Bromophenol blue ..... 0.005 g
Glycerol ..... 7.5 ml
TE buffer (pH 8.0) ..... 2.5 ml

DNA sequencing reagents

TBE (10 ×, pH 8.3)

Tris base ..... 16.2 g/L
Boric acid ..... 8.25 g/L
EDTA ..... 1.245 g/L

DNA sequencing gels (48 cm)

Urea ..... 28.8 g
40% Acrylamide ..... 8.48 ml
TBE (10 ×) ..... 8 ml
MilliQ water ..... to 80 ml
10% APS ..... 400 µl
TEMED ..... 55 µl

DNA sequence loading dye

Formamide ..... 250 µl
EDTA (25 mM)/blue dextran (50 mg/ml) ..... 50 µl

General buffers

TE buffer (pH 8.0)

Tris-HCl ..... 10 mM
EDTA ..... 1 mM

PBS (10 ×, pH 8.0)

NaCl ..... 80 g/L
KCl ..... 2 g/L
Na_2HPO_4 ..... 11.15 g/L
KH_2HPO_4 ..... 2 g/L

TBS (pH8.0)

Tris-HCl ..... 50 mM
NaCl ..... 150 mM

10mM phosphate buffer

Na_2HPO_4 ..... 0.336 g/L
NaH_2PO_4 ..... 1.02 g/L

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9 Publications

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