Is plasminogen deployed as a virulence factor by Northern Territory group A streptococcal isolates during invasive disease?

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Certification

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

Fiona C. McKay

22 December 2005

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List of Abbreviations

A_{600}, A_{405}	absorbance measured at 600 or 405 nm respectively
ANOVA	analysis of variance
APSGN	acute post-streptococcal glomerulonephritis
bp	base pairs
°C	degrees Celcius
CD	cluster of differentiation
cfu	colony-forming units
cpm	counts per min
C-terminal	carboxy-terminal
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E64	trans-epoxysuccinyl-l-leucylamido-(4-guanidino)butane
εACA	ε-aminocaproic acid
ECM	extracellular matrix
EDTA	ethylenediamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
Fbp54	fibronectin-binding protein 54
Fg	fibrinogen
Fg-R	fibrinogen receptor
FSD	fibrinogen-streptokinase dependent
g	units of force
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAS	group A streptococcus
Glu-plasminogen	native plasminogen bearing an amino-terminal glutamine residue

h	hour
HLA	human leukocyte antigen
IgG	immunoglobulin G
Inv	invasive
K1-5	kringles 1-5
kb	kilobases
kDa	kilodaltons
KLH	keyhole limpet hemocyanin
l	litres
Lys-plasminogen	plasminogen bearing an amino-terminal lysine residue
m	milli
µ	micro
Mga	multiple gene regulator in group A streptococci
min	minutes
M	molar
m	metres
n	nano
NPBP	nephritis plasmin binding protein
N-terminal	amino-terminal
NT	Northern Territory (of Australia)
NTP	N-terminal peptide
PAI-1/-2	plasminogen activator-inhibitor type 1/2
PAM	plasminogen-binding group A streptococcal M-like protein
PBS	phosphate-buffered saline
PCR	polymerase chain reaction

PD	protease domain
PINT	phosphate isotonic sodium buffer containing Tween
Plg	plasminogen
Plr	plasmin receptor
Plg-R	plasminogen receptor
PMSF	phenylmethylsulfonyl fluoride
PPACK	D-Phe-Pro-Arg-chloromethylketone dihydrochloride
protein GRAB	protein G-related α_2-macroglobulin-binding protein
PVDF	polyvinyl difluoride
RT	room temperature
s	seconds
SCID	severe combined immunodeficient
SD	standard deviation
SDH	streptococcal dehydrogenase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SEN	streptococcal surface enolase
ska	streptokinase gene
SpeB	streptococcal pyrogenic exotoxin
STSS	streptococcal toxic shock syndrome
TAE	Tris acetate-EDTA
TBS(T)	Tris-buffered saline (containing Tween)
TE	Tris-EDTA
TEA	tranexamic acid
THY	Todd-Hewitt broth containing 1% yeast

tPA	tissue plasminogen activator
uncomp	uncomplicated infection
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor
UV	ultraviolet
V	volts
vir	virulence (regulon)
Vmax	maximum reaction velocity
wt	wildtype
w/v	weight per volume

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Abstract

Group A streptococcal (*S. pyogenes*; GAS) infection is endemic in the Northern Territory of Australia, and the rates of invasive GAS disease and post-infection sequelae are among the highest reported in the world. Plasminogen is a potent human protease sequestered to the GAS cell-surface by plasminogen and fibrinogen receptors and activated by GAS streptokinase and host plasminogen activators. The critical role of plasminogen in GAS invasion was recently demonstrated in a human plasminogen transgenic mouse model of infection.

The aim of this study was to determine whether plasminogen is deployed as a virulence factor in invasive GAS disease, with particular reference to the Northern Territory of Australia. This question was first approached from an epidemiological perspective, comparing Northern Territory GAS isolates from invasive infections with those from uncomplicated infections for their interaction with the plasminogen system. Plasminogen binding; plasminogen receptor expression and genetic variation; fibrinogen binding; streptokinase expression, activity and genetic variation; streptococcal pyrogenic exotoxin B (SpeB) expression and activity; and acquisition of cell-surface plasmin in human plasma were characterised for 29 GAS isolates of known clinical origin from the Northern Territory. The second approach to determining the role of plasminogen in invasive disease in the Northern Territory was to investigate GAS infection using a human plasminogen transgenic mouse model. A subset of Northern Territory GAS isolates selected for different *in vitro* plasminogen activation characteristics was tested for virulence in this model.

This study revealed that Northern Territory GAS isolates from invasive disease cases acquire more plasminogen than isolates from uncomplicated infections, however they do not produce more streptokinase nor acquire more cell-surface plasmin after incubation in human plasma *in vitro*. Presence of the gene for the plasminogen-binding group A streptococcal M-like protein (PAM) conferred upon GAS isolates a strikingly different profile of interaction with plasminogen, characterised by higher plasminogen binding and plasmin acquisition in plasma. Differences in the catalytic specificity of streptokinase secreted by *pam*-positive and *pam*-negative isolates were identified and their allelic determinants investigated. A new model of GAS cell-surface plasminogen activation is proposed for *pam*-positive isolates. To characterise the role of streptokinase and the cysteine protease SpeB in acquisition of cell-surface plasmin activity in human plasma, such acquisition was compared in GAS deletion mutants for genes encoding these proteins and the corresponding wildtype strains. The dramatic reduction of GAS cell-surface plasmin activity by SpeB may significantly disable GAS invasive potential. Infection studies in the human plasminogen transgenic mouse model revealed the critical role of plasminogen in virulence of some Northern Territory isolates, however *in vitro* plasminogen activation characteristics do not predict clinical phenotype nor virulence in the transgenic mouse model. The results suggest an important and complex interaction between plasminogen and other host and/or bacterial factors in establishing invasive GAS infection in the Northern Territory of Australia.

CHAPTER 1 Review of the Literature

Sanderson-Smith, M.L., McKay, F.C., Ranson, M. and Walker, M.J. (2004) Subversion of the plasminogen activation system by *Streptococcus pyogenes*: mounting evidence to implicate the human protease plasmin in disease processes. Current Trends in Microbiology 1: 75-85.

Walker, M.J., McArthur, J.D., McKay, F.C, and Ranson, M. (2005) Is plasminogen deployed as a *Streptococcus pyogenes* virulence factor? Trends in Microbiology, 13:308-13.

1.1 Group A streptococcal infection

1.1.1 Group A Streptococci (GAS)

Streptococcus pyogenes, (group A streptococci; GAS) is a Gram-positive, beta-hemolytic, non-motile cocci which forms chains and does not form spores. GAS are facultative anaerobes and are catalase-negative. Traditionally described as extracellular pathogens, GAS can also survive intracellularly, contributing to immune evasion and antibiotic resistance (Molinari and Chhatwal, 1998). The Genus *Streptococcus* is divided into groups based on the cell wall or C-carbohydrate, of which GAS possess the *N*-acetyl glucosamine polymers of the "A" antigenic epitope (Lancefield, 1933). The streptococcal group A contains only one species, *S. pyogenes*, originally named according to a different classification system of which the "pyogenic" class included streptococci bearing the group A antigen (Sherman, 1937).

Group A streptococci are further differentiated into over 80 M types on the basis of serological typing of the M protein. M proteins are cell surface alpha-helical coiled-coil proteins with a highly conserved C-terminal half and a hypervariable 30-50 amino acid domain at the N-terminus which confers the serotype (Fischetti, 1989). In recent years sequence analysis of the *emm* genes encoding this protein has revealed 124 *emm*-types (Facklam et al., 2002). *Emm*-typing largely reflects the serological grouping but has allowed characterisation of isolates previously non-typeable by serological methods (Beall et al., 1996; Moses et al., 2003). *Emm* pattern is a grouping into one of five classes (A-E), based on the number of *emm* genes present, the subfamily to which the *emm* genes belong and their relative arrangement on the chromosome (Bessen et al., 1996).

1.1.2 GAS diseases

Diagnostic criteria for the classification of GAS diseases have been defined in detail (Table 1) (Breiman and et al., 1993) and their clinical manifestations reviewed recently (Bisno and Stevens, 1996; Cunningham, 2000). The most common clinical manifestations of GAS infection are impetigo, a localised purulent skin infection, and pharyngitis or "strep throat". These infections are classified as non-invasive, defined by the isolation of GAS from a non-sterile tissue site. Isolation of GAS from a normally sterile tissue site defines an invasive infection. Erysipelas is an invasive infection of the skin and cutaneous lymphatics, with a raised and distinct skin inflammation, while cellulitis extends more deeply into subcutaneous tissues with no distinct demarcation of infected tissue. Necrotising fasciitis is a rapidly spreading infection of the deeper subcutaneous tissues and fascia resulting in extensive necrosis and requiring aggressive surgical debridement. Streptococcal toxic shock syndrome with severe systemic involvement was first described in 1987 and has been attributed in part to the inflammatory response generated during polyclonal T cell activation by GAS superantigens (Cone et al., 1987; McCormick et al., 2001).

1.1.3 Epidemiology of GAS disease worldwide

Recent estimates of the global burden of GAS disease (Table 1.2) demonstrate its importance in morbidity and mortality (Carapetis et al., 2005). There has been a global resurgence in severe invasive infections in developed countries in the last 20 years (Johnson et al., 1992; Stevens, 1996). Despite antibiotic treatment, these infections progress rapidly and are associated with high morbidity and mortality.

Table 1.1 Classification of group A streptococcal infections

Please see print copy for Table 1.1

(Breiman and et al., 1993; Walker et al., 2005)

Early studies of this phenomenon suggested the emergence of highly virulent strains of distinct M-types, especially M1 and M3 (Carapetis et al., 1995b; Cleary et al., 1992; Cockerill et al., 1997; Cockerill et al., 1998; Musser et al., 1993; Musser et al., 1995). However, more recent work in larger strain sets has demonstrated that the high proportion of certain *emm* types in invasive disease reflects their prevalence in non-invasive disease in the general population (Haukness et al., 2002; Johnson et al., 2002). M1T1 and M3 have been isolated from both severe invasive diseases such as necrotising fasciitis, and from uncomplicated infections such as pharyngitis, despite inducing similar mitogenic and cytokine responses *in vitro* (Chatellier et al., 2000; Johnson et al., 1992). Genetic and environmental variation in host immunity contributes to the severity of invasive infection, as discussed in section 1.1.6.

Table 1.2. Estimates of global burden of GAS disease[a]

Please see print copy for Table 1.2

[a]Tabulated from published data (Carapetis et al., 2005).
[b]including acute rheumatic fever, rheumatic heart disease, post-streptococcal glomerulonephritis, and invasive infections.

1.1.4 Epidemiology of GAS disease in developing nations and in the Northern Territory of Australia

In developing nations, the epidemiology of GAS disease is less well-described, and while no resurgence of GAS disease has been reported, the burden of serious GAS diseases is much greater than in developed countries. The high rates of GAS infection in developing nations (and amongst indigenous communities in industrialised countries) may result from increased transmission due to such factors as overcrowded housing, limited medical intervention, and inadequate hygiene and sanitation (Carapetis et al., 1999b). A diversity of *emm* types is often found and a high proportion of isolates are non-typeable by conventional serological methods (Bergner-Rabinowitz and Ferne, 1978; Pruksakorn et al., 2000).

In the Northern Territory (NT) of Australia GAS infection is endemic, with high incidences of impetigo (Gardiner and Sriprakash, 1996) and bacteremia (Carapetis et al., 1999c) and the highest published rate of rheumatic fever in the world (Carapetis and Currie, 1997). Around 81% of the NT is geographically classified as tropical and Aboriginal Australians account for 28.8% of the total population (Australian Bureau of Statistics, 2004). Aboriginal residents of the NT mostly live in remote areas with high rates of poverty, preventable infections and their sequelae (Hoy et al., 1997; Munoz et al., 1992). The incidence of GAS bacteremia amongst Aboriginal people in NT communities is five times that of non-Aboriginal people (Carapetis et al., 1999c).

Many aspects of the epidemiology of GAS infection amongst Aboriginal communities in the NT resemble those of developing nations. Firstly, the high incidence of GAS infection in general (Gardiner et al., 1997; Gardiner and Sriprakash, 1996) and invasive

infection specifically (Carapetis et al., 1999c; Towers et al., 2002), is not correlated with specific M-types. Secondly, the diversity and turnover rate of GAS strains is exceptionally high (Carapetis et al., 1999a). Multiple GAS serotypes are often found within the one lesion (Carapetis et al., 1995a) and up to 14 genetically distinct strains have been reported to circulate within an individual community at any one time (Gardiner and Sriprakash, 1996). Thirdly, streptococcal pharyngitis is rare, with throat carriage consistently less than 5% and often less than 2% (Carapetis et al., 1997). Thus invasive GAS infection and acute rheumatic fever are thought to develop secondary to GAS pyoderma (Carapetis et al., 1999c; McDonald et al., 2004) rather than secondary to GAS pharyngitis as described in urbanised populations (Fiorentino et al., 1997; Haukness et al., 2002; Stollerman, 2001).

1.1.5 GAS virulence factors

The pathogenicity of GAS is attributed to an array of virulence factors. The established and putative GAS virulence factors have been reviewed in detail recently and are summarised in Tables 1.3 (cell-associated factors) and 1.4 (secreted factors) (Bisno et al., 2003; Cunningham, 2000; Hynes, 2004). These factors are controlled by transcriptional regulators, many of which have been identified only within the last decade and whose signalling and interactions have not been fully elucidated. Regulatory strategies include two-component systems such as CovR/CovS, FasBCAX, Ihk/Irr; the stand-alone regulators Mga, Rgg, and RALPs; and those with more complex regulation such as Sag/Pel, RelA-independent regulation and RocA (Hynes, 2004; Kreikemeyer et al., 2003).

Table 1.3. Established or putative cell-associated virulence factors of GAS[a]

Please see print copy for Table 1.3

[a]from recent reviews (Bisno et al., 2003; Cunningham, 2000; Hynes, 2004)
[b](Edwards et al., 2005; Hidalgo-Grass et al., 2004)

Table 1.4. Established or putative secreted virulence factors of GAS*

Please see print copy for Table 1.4

*from recent reviews (Bisno et al., 2003; Cunningham, 2000; Hynes, 2004).

Among the putative virulence factors are those arming GAS with the potent broad spectrum proteolytic activity of human plasmin, sequestered from the host and activated via a system of cell-surface receptors and bacterial and host activators. It has been hypothesised that cell-surface and soluble plasmin mediate the breach of host tissue barriers, escape from fibrin clots and cleavage of specific host immune factors. The GAS factors that putatively mediate or affect this system during infection include plasminogen and fibrinogen receptors, the plasminogen activator streptokinase, the protein G-related α_2-macroglobulin-binding protein (protein GRAB), and the broad-specificity GAS cysteine protease, streptococcal pyogenic exotoxin B (SpeB) and its precursor. Host factors that may affect plasminogen activation during infection include fibrinogen, urokinase- and tissue-plasminogen activators (uPA and tPA), the plasmin inhibitors α_2-antiplasmin and α_2-macroglobulin and the plasminogen activator-inhibitors (PAI-1 and PAI-2).

1.1.6 Host factors predisposing to GAS disease

Host factors also play an important role in susceptibility to GAS disease. Factors such as age, underlying medical conditions and living environment are known to be associated with GAS invasive disease risk in industrialised countries (Factor, 2003; Factor et al., 2005; Hollm-Delgado et al., 2005). Low levels of humoral immunity to virulence factors are associated with development of invasive disease (Basma et al., 1999; Mascini et al., 2000) and with severity of invasive disease (Akesson et al., 2004; Eriksson et al., 1999; Mascini et al., 2000). Host genetic factors such as HLA type and the associated cytokine response to superantigen stimulation also influence the severity of invasive infection (Kotb et al., 2002; Norrby-Teglund et al., 2000).

1.2 Plasminogen

1.2.1 Plasminogen and fibrinolysis

Plasminogen is the zymogen of the broad-specificity trypsin-like serine protease plasmin. It is synthesized mainly in the liver (Raum et al., 1980) and is found in the blood and extracellular fluids at a concentration of approximately 2 μM (Rabiner et al., 1969). Plasmin cleaves the insoluble fibrin polymers of blood clots, representing one of the major anticoagulation mechanisms in humans (Figure 1.1). Fibrinolysis is crucial to many physiological processes such as wound healing and prevention of thrombosis. Accordingly, plasminogen-deficient mice are predisposed to severe thrombosis and fibrin deposition in major organs (Bugge et al., 1995; Ploplis et al., 1995).

1.2.2 Plasminogen structure

Plasminogen is a 92 kDa glycoprotein secreted as a single 790 amino acid chain termed Glu-plasminogen due to the glutamic acid residue at the N-terminus (Sottrup-Jensen et al., 1978; Wiman and Collen, 1977). Human plasminogen activators cleave Arg_{560}-Val_{561} to form the active protease plasmin, a two-chain species stabilized by disulfide bridges (Vassalli et al., 1992). The 25 kDa light chain contains the protease domain while the 65 kDa heavy chain consists of the amino terminal acidic region (N-terminal peptide or NTP; amino acids 1-77), and the lysine-binding sites located on 5 triple-disulfide-bonded kringle domains, K1-5 (Forsgren et al., 1987; Markus et al., 1978; Sottrup-Jensen et al., 1978) (Figure 1.2).

The kringles mediate binding of plasminogen to ω-carboxylic acids such as lysine within its cell-surface receptors and fibrin, ε-aminocaproic acid (εACA) and tranexamic

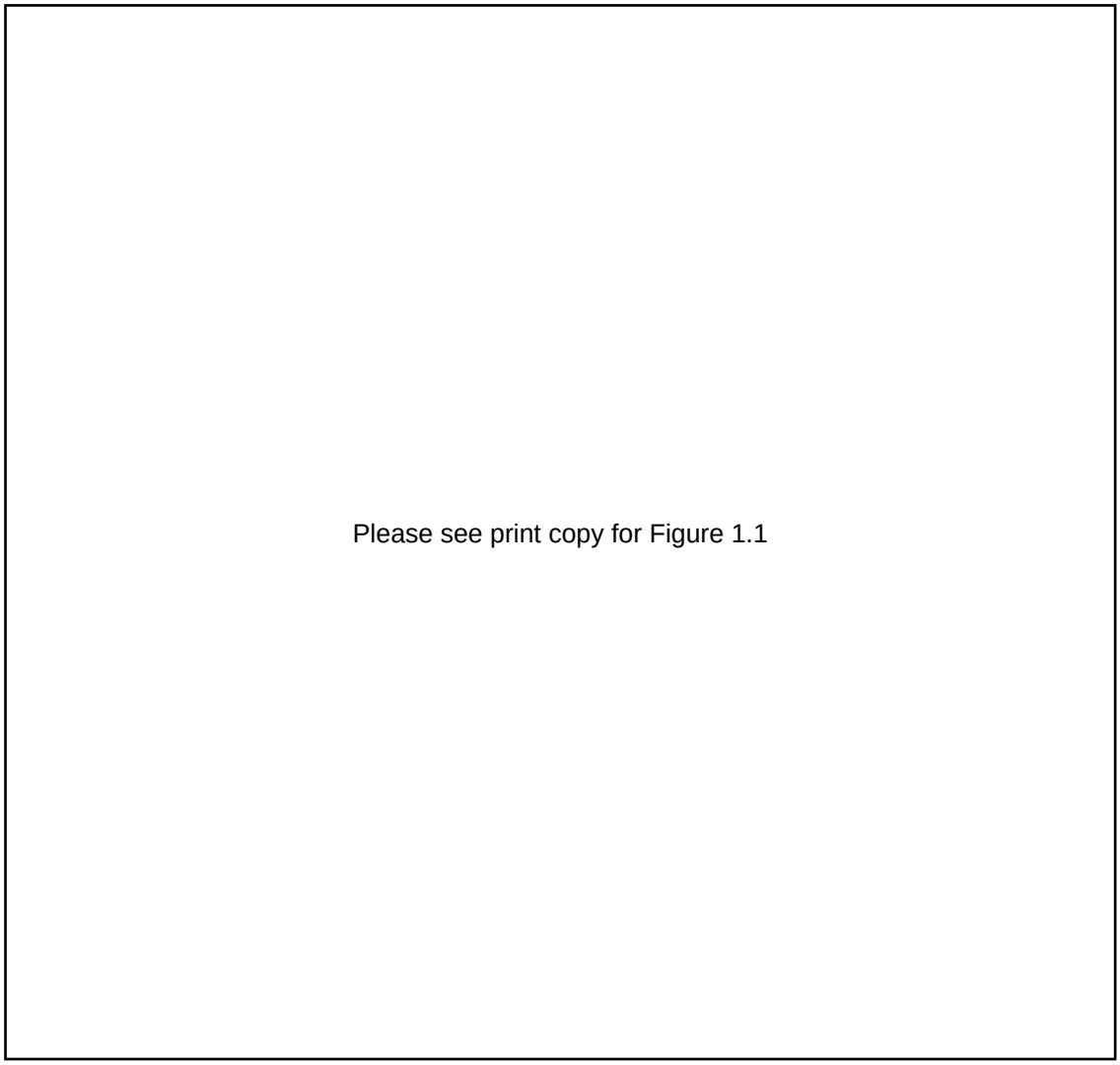

Figure 1.1 Human coagulation and anticoagulation systems. The coagulation cascade consists of factors (some denoted by Roman numerals) that ultimately mediate cleavage of the precursor protein, fibrinogen, to release fibrin monomers that form the insoluble matrix of the blood clot (Bryant, 2003; Mathews and van Holde, 1990). The coagulation factors exist in zymogen forms (denoted in black) that are proteolytically cleaved to active forms (denoted in red). Serine proteases are denoted by asterisks (*). Coagulation may be initiated via the intrinsic pathway, in which exposure of blood at damaged tissue surfaces cause the release of kininogen and kallikrein that activate this pathway. Alternatively, internal trauma to the vasculature causes the release of tissue factor and the activation of factor VII, triggering coagulation via the extrinsic pathway. The pathways converge with the activation of factor X, which activates thrombin. Thrombin cleaves fibrinopeptides A and B from fibrinogen, allowing newly exposed sites within the fibrin monomers to associate to form the fibrin clot. The clot is further stabilized by the activity of factor XIII. The four major anticoagulant systems are shown in blue (Bryant, 2003; Roitt and Delves, 2001). Tissue factor pathway inhibitor (TFPI; 1) is a plasma protease inhibitor that inactivates tissue factor by formation of a complex with tissue factor, factor X* and factor VII* on the surface of platelets or endothelial cells. Antithrombin (2) inactivates thrombin and other serine proteases of the coagulation cascade (factor X*, IX*, XI*and VII) by formation of covalent complexes with the target protease. Antithrombin (AT) is found in plasma and on the surface of microvascular endothelial cells, and its action is enhanced at least 1000-fold by heparin. Thrombomodulin (3) is an integral membrane protein of endothelial cells that binds thrombin and inhibits its ability to generate fibrin, while enabling thrombin-mediated activation of protein C. Activated protein C in association with protein S inhibits thrombin synthesis via inactivation of factor V on platelets. The fibrinolytic system consists of a series of activators and inhibitors that regulate the formation and activity of plasmin (4), a potent serine protease that cleaves fibrin to form soluble degradation products (ie clot lysis) (Bryant, 2003). Figure modified from previous work (Bryant, 2003; Mathews and van Holde, 1990).

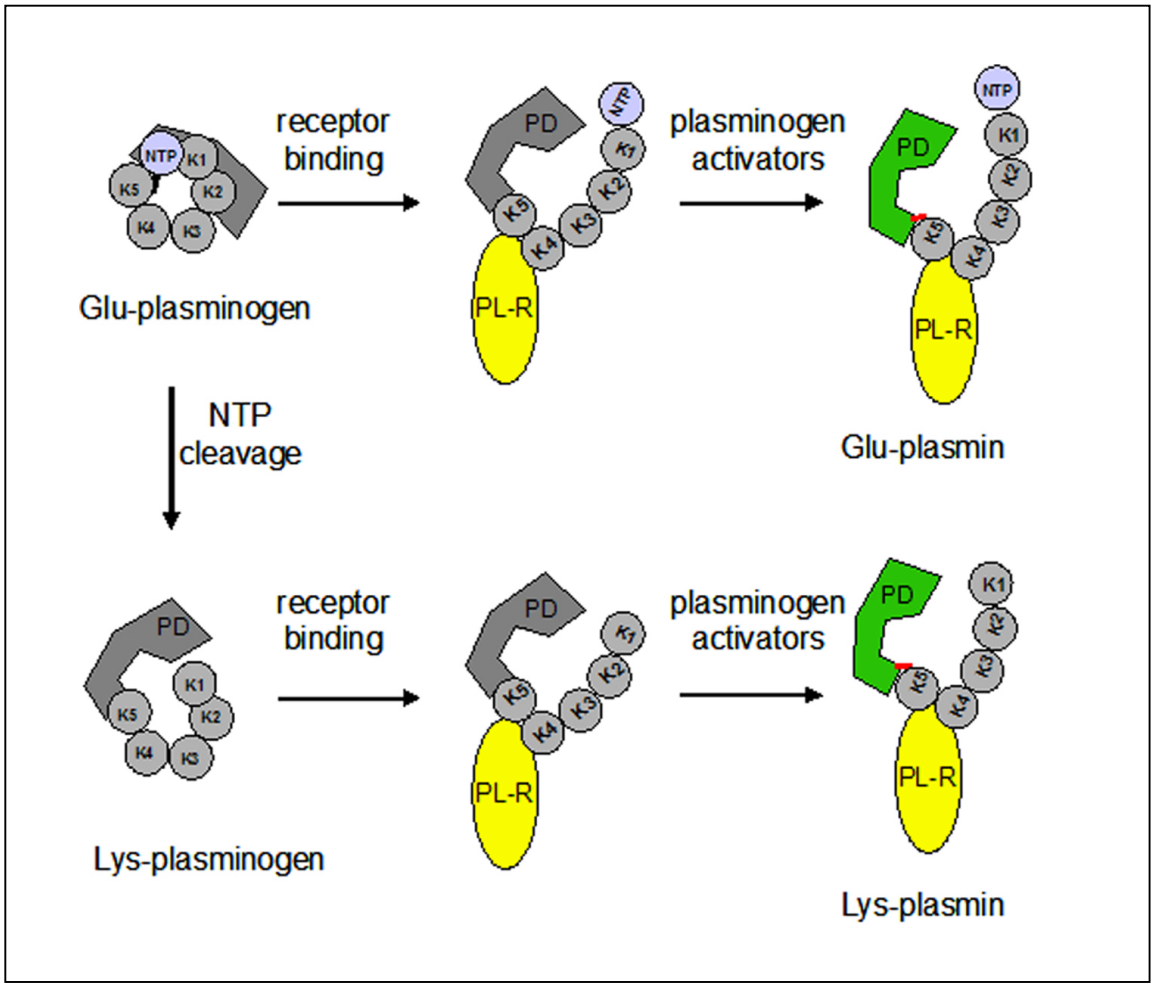

Figure 1.2. Schematic representation of plasminogen structure, receptor binding and activation. Plasminogen is a single chain glycoprotein containing seven distinct structural domains: the amino terminal peptide (NTP) followed by kringles 1–5 (K1-5) and the carboxy terminal serine protease domain (PD). K1, K2, K4 and K5 contain lysine-binding motifs that are responsible for binding to fibrinogen and to plasminogen receptors. Within the circulating zymogen Glu-plasminogen, lysine-dependent interaction/s between the NTP and K5 maintain a mostly closed, activation-resistant form. Both binding to plasminogen receptors (PL-R) and/or cleavage of the NTP to form Lys-plasminogen result in conformational change rendering plasminogen more susceptible to activation. Cleavage of the the Arg_{560}-Val_{561} peptide bond of plasminogen forms the proteolytically active two-chain plasmin (depicted by the green coloured PD linked to the kringle domains via a disulfide bond shown in red).

acid (TEA) (Pollanen et al., 1991). Each kringle contains approximately 80 amino acids each and isolated kringles differ in their affinity for lysine analogues resembling either N-terminal, carboxy-terminal (C-terminal) or internal lysine residues of proteins. For example, the high-affinity lysine binding site K1 displays a preference for C-terminal lysine residues whereas K2 shows a similiar affinity for internal and terminal lysines (Marti et al., 1997). C-terminal lysine residues of plasminogen receptors usually bind to kringles 1, 4 and 5 of plasminogen (Ranson and Andronicos, 2003).

A second species of plasminogen in which the 8 kDa N-terminal peptide is absent is produced by autocatalytic cleavage of the Arg_{68}-Met_{69}, Lys_{77}-Lys_{78} or Lys_{78}-Val_{79} bonds of Glu-plasminogen, and is termed Lys-plasminogen (Figure 1.2) (Collen and Verstraete, 1975; Gonzalez-Gronow et al., 1977; Summaria et al., 1973; Wiman, 1973). Lys-forms of plasmin(ogen) exhibit striking conformational differences, with Lys-plasminogen more readily activated by plasminogen activators and Lys-plasmin displaying a higher specific activity than Glu-plasmin (Claeys and Vermylen, 1974; Lucas et al., 1983; Violand et al., 1978). This may be due to the absence of the lysine residues of the N-terminal peptide in Lys-plasminogen, such as Lys_{50} and Lys_{62}, which in Glu-plasminogen bind the lysine-binding kringle domains intramolecularly to maintain the closed conformation (Christensen, 1984; Cockell et al., 1998). Incubation of Glu-plasminogen with lysine or its analogues induces a change in conformation from a tight right hand spiral to an open U-shaped conformation (Brockway and Castellino, 1972; Castellino et al., 1973; Violand et al., 1978), (Weisel et al., 1994). This conformation is more susceptible to mammalian activators (Banyai and Patthy, 1985).

There are two glycoforms of plasminogen (I and II), both of which possess an *o*-linked tetrasaccharide at Thr_{345}, while plasminogen I also has an *N*-glycosylation site at Asn_{289} (Hayes and Castellino, 1979; Lijnen et al., 1981). The functional consequence of additional glycosylation is a higher affinity for lysine (Sugawara et al., 1984), higher molecular stability in the lysine-induced open conformation (Molgaard et al., 1997) higher susceptibility to activators (Takada and Takada, 1983) and greater enhancement of activation rates by uPA and streptokinase in the presence of fibrin and fibrinogen (Takada et al., 1985b).

1.2.3 Plasmin specificity

Plasminogen can be activated by mammalian activators uPA and tPA or by bacterial activators, including streptokinase of group A, C and G streptococci, staphylokinase of *Staphylococcus aureus* and Pla of *Yersinia pestis* (Lahteenmaki et al., 2001). Activation results in formation of a trypsin-like catalytic triad composed of residues His_{602}, Asp_{645} and Ser_{740} (Robbins et al., 1967). The specificity of plasmin is similar to that of trypsin, hydrolyzing peptide bonds on the C-terminal side of exposed arginine or lysine residues (Keil, 1992). In so doing, plasmin generates new exposed C-terminal lysine residues, or potential new plasmin(ogen) binding sites.

In vivo, plasmin is primarily responsible for the degradation of fibrin polymers, cleaving 11 of 73 arginine residues and 20 of 98 lysine residues in fibrin (Astrup, 1978; Henschen and Lottspeich, 1980). Plasmin degrades components of the extracellular matrix (ECM) such as fibronectin, laminin, vitronectin, and proteoglycans (Richardson et al., 1988; Werb, 1997). A significant portion of the action of plasmin on ECM is indirect; via its activation of matrix prometalloproteases -1, -3, -9 and -14, which cleave

other components of ECM including collagen (Gordon et al., 1993; Werb, 1997). Plasmin also activates and releases latent growth factors such as transforming growth factor-β (Werb, 1997).

1.2.4 Plasminogen activation and its regulation *in vivo*

The two major eukaryotic activators of the plasminogen system, tPA and uPA, cleave the Arg_{560}-Val_{561} peptide bond of plasminogen to form two-chain plasmin (Dano et al., 1985). tPA is a 70 kDa serine protease, secreted mainly by endothelial cells. With a plasma concentration of 5-10 ng/ml, it represents the major circulating plasminogen activator and therefore the major activator in fibrinolysis. tPA contains two kringle domains, the second of which, in combination with the amino-terminal fibrin-binding finger domain, is responsible for high-affinity fibrin binding of tPA (Banyai et al., 1983; Verheijen et al., 1986). uPA is a 50 kDa serine protease secreted by a variety of normal and malignant cells. The concentration in plasma is 3.5 ng/ml, and in urine is 200-300 ng/ml. It is the major cell-bound plasminogen activator, functioning in cellular invasion of the ECM during inflammation, wound healing, tissue remodelling, and tumour metastasis. uPA binds to cell-surfaces via its receptor, uPAR (CD87) (Dano et al., 1985; Lahteenmaki et al., 2001). 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 subject to its own regulatory system (Saksela and Rifkin, 1988).

The potent proteolytic activity of plasmin necessitates regulation of the plasminogen activation system at multiple levels and the inhibitory system possesses some redundancy. Plasminogen binding to specific receptors or extracellular matrix proteins

plays a vital role in localising plasminogen activation at appropriate locations, thus representing and important regulatory step in plasminogen activation (Pollanen et al., 1991). Plasminogen binding facilitates and amplifies plasminogen activation in these precise locations (Ranson and Andronicos, 2003). Firstly, the lysine-dependent binding event disrupts intramolecular plasminogen interactions, exposing the activation domain and resulting in a more activation-susceptible conformation of plasminogen (Andronicos et al., 2000). Secondly, binding to fibrin or the cell surface receptor brings plasminogen into close proximity with it activators (tPA or uPA; bound to each of these surfaces respectively) facilitating increased plasminogen activation (Plow et al., 1995). Thirdly, plasmin-mediated proteolysis of membrane proteins or fibrin exposes C-terminal lysine residues that may function as additional plasminogen binding sites (Plow et al., 1995). Finally, as outlined below, lysine-dependent binding protects plasmin from its circulating inhibitors.

Plasmin in solution is very rapidly inhibited and cleared from the circulation by complexing with the major plasmin inhibitor α_2-antiplasmin. α_2-antiplasmin is found at 60 – 70 μg/ml in the plasma and belongs to the subclass of serine protease inhibitors known as serpins. Serpins possess a flexible exposed binding loop that acts as a "substrate" for the corresponding protease (Carrell and Huntington, 2003; Otlewski et al., 1999). Cleavage of this loop by the protease results in dramatic conformational change in both molecules such that the protease is inactivated and entrapped in an irreversible covalent complex that is rapidly cleared from the circulation (Carrell and Huntington, 2003; Huntington, 2003; Ye and Goldsmith, 2001).

Plasmin binds α_2-antiplasmin reversibly via kringles 1-3 initially, followed by formation of a covalent bind between the active sites of the two molecules. Binding of α_2-antiplasmin to plasmin is lysine-dependent (Longstaff and Gaffney, 1992; Wiman and Collen, 1978); 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). The 725 kDa broad-spectrum protease inhibitor α_2-macroglobulin, at a concentration of 2.5 mg/ml, is the most abundant plasmin inhibitor in plasma. However, plasmin inhibition by α_2-macroglobulin commences only if the concentration of α_2-antiplasmin is compromised locally or systemically (Saksela and Rifkin, 1988; Travis and Salvesen, 1983).

Another important level of regulation of the plasminogen activation cascade involves inhibition of the plasminogen activators, tPA and uPA. *In vivo* the most significant inhibitors are the serpins plasminogen activator inhibitor 1 (PAI-1), plasminogen activator inhibitor 2 (PAI-2), and protease nexin 1 (Coleman and Benach, 1999; Pollanen et al., 1991). Expression of these molecules is precisely regulated by a number of growth factors, hormones and cytokines (Andreotti and Kluft, 1991; Chandler et al., 1995; Kruithof et al., 1995). Free, active tPA is inhibited by rapid clearance from the circulation or inhibited by PAI-1 and subsequent clearance (Chandler et al., 1997; Kruithof et al., 1995). Free uPA is rapidly cleared while cell-bound uPA complexed to PAI-2 is internalized almost immediately (Al-Ejeh et al., 2004; Kruithof et al., 1995; Ranson and Andronicos, 2003).

In summary, uncontrolled proteolysis by plasmin is prevented through the precise and ccordinated regulation of the plasminogen activation system by cytokine and hormonal

regulation of gene expression, precise localisation of of various components of the system via binding to various ECM proteins and cell-surface receptors, specific inhibition of plasmin and plasminogen activators by α_2-antiplasmin and PAI-1 and PAI-2 respectively, and by rapid clearance of free and inhibited plasminogen activator (Al-Ejeh et al., 2004; Andreasen et al., 1997; Kruithof et al., 1995; Ranson and Andronicos, 2003).

1.3 Plasminogen and GAS

GAS interact with the human plasminogen system at several levels (Figure 1.3). Firstly, GAS bind plasminogen and plasmin directly via cell-surface receptors and indirectly by binding a trimolecular complex of streptokinase, fibrinogen and plasminogen. GAS activate both surface-bound and solution-phase plasminogen via the secreted activator streptokinase. The potent GAS cysteine protease, SpeB, has a significant negative effect on GAS plasmin activity by proteolytic degradation of several of these component proteins; while the SpeB zymogen binds plasminogen. GAS sequester the plasmin inhibitor, α_2-macroglobulin, to the cell-surface via protein GRAB.

1.3.1 Direct plasminogen binding: GAS plasminogen receptors

1.3.1.1 Glyceraldehyde-3-phosphate dehydrogenase (GAPDH/Plr/SDH)

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a 41 kDa glycolytic enzyme initially identified on the cell-surface as a plasmin receptor, thus designated Plr, or streptococcal surface dehydrogenase (SDH) (D'Costa et al., 1997; Pancholi and Fischetti, 1992; Winram and Lottenberg, 1996). GAPDH binds Glu-plasmin, Lys-plasmin and Lys-plasminogen via its C-terminal lysine, but shows almost no affinity for the circulating form, Glu-plasminogen (Broder et al., 1989; Broeseker et al., 1988; DesJardin et al., 1989; Winram and Lottenberg, 1998; Winram et al., 1995). Plasmin bound to GAPDH is protected from circulating α_2-antiplasmin (Broder et al., 1991).

GAPDH is present on the cell-surface in close physical association with (or possibly anchored by) M and M-related proteins. It is released from the GAS cell-surface during

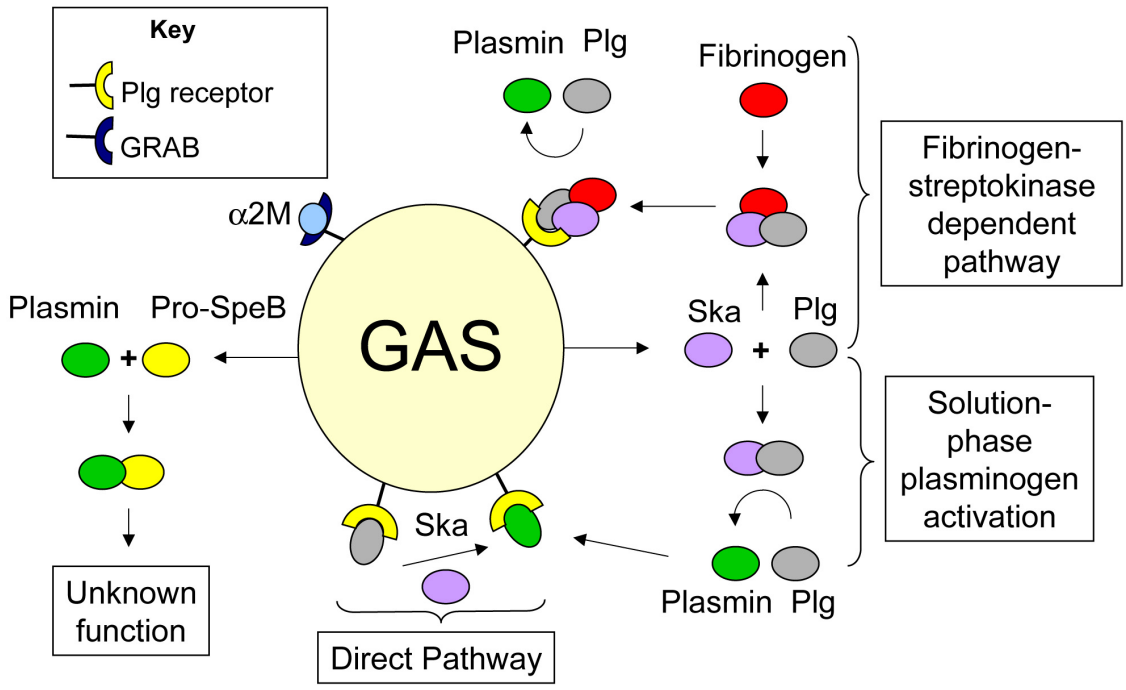

Figure 1.3. Schematic representation of the GAS plasminogen activation system. GAS secrete the plasminogen activator, streptokinase (Ska), which activates (Plg) in solution (solution-phase plasminogen activation). GAS may bind both plasmin and plasminogen directly via cell-surface plasminogen receptors (direct pathway). Directly bound plasminogen may be activated by streptokinase or by host activators (not depicted). In addition, GAS bind a trimolecular complex of streptokinase, fibrinogen and plasminogen, which binds to cell-surface fibrinogen receptors (fibrinogen-streptokinase-dependent pathway). This complex has both plasmin activity and plasminogen-activator activity, and thus is capable of activating additional plasminogen molecules in solution. The protein G-related α_2-macroglobulin-binding protein (GRAB) sequesters the human plasmin inhibitor, α_2-macroglobulin (α_2M). Solution-phase plasmin generated by streptokinase or the trimolecular complex may bind to the secreted zymogen form of SpeB (Pro-SpeB).

iron starvation, potentially releasing bound plasmin to more distant sites (D'Costa et al., 2000; Eichenbaum et al., 1996). Site-directed mutagenesis suggested that GAPDH may not be a major contributor to overall cell-surface plasmin(ogen) binding (Winram and Lottenberg, 1998). In contrast, a recent study demonstrated that prevention of GAPDH export to the cell surface results in lower plasminogen binding to GAS (Boel et al., 2005) suggesting that GAPDH or a co-regulated factor may mediate significant plasminogen binding. Cell-surface GAPDH also binds fibronectin, lysozyme and the cytoskeletal proteins myosin and actin, suggesting a potential role in GAS adherence and colonization (Pancholi and Fischetti, 1992). GAPDH also acts as a pharyngeal cell adhesin by directly binding uPAR on the surface of pharyngeal cells (Boel et al., 2005; Jin et al., 2005).

1.3.1.2 Streptococcal enolase (SEN)

Streptococcal enolase (SEN) is a 45 kDa glycolytic enzyme and high-affinity plasminogen receptor expressed on the surface of GAS of many M types (Pancholi and Fischetti, 1997; Pancholi and Fischetti, 1998). SEN has a 3-fold higher affinity for Lys-plasminogen than Glu-plasminogen and possesses more than one site for interaction with plasminogen (Derbise et al., 2004; Pancholi and Fischetti, 1998). The high affinity for plasminogen is mediated in part by two lysine residues at the C-terminus, Lys_{434} and Lys_{435} (Derbise et al., 2004; Pancholi and Fischetti, 1998). Plasmin and plasminogen bound to SEN are significantly (but not completely) resistant to inactivation by α_2-antiplasmin (Pancholi and Fischetti, 1998).

Plasminogen bound to SEN may act as an adhesin by binding to enolase on the surface of host pharyngeal cells. In addition, subsequent plasminogen activation may

compromise the pharyngeal epithelial barrier via mechanisms such as degradation of tight junctions (Pancholi et al., 2003). Humoral immunity to SEN has been implicated in post-streptococcal sequelae. Anti-SEN antibodies, cross-reactive with human enolase, have been identified in the sera in APSGN and may be implicated in autoimmune disease processes (Fontan et al., 2000; Rattner et al., 1991).

1.3.1.3 Plasminogen-binding group A streptococcal M-like protein (PAM)

Some GAS isolates also possess an M-like protein with plasminogen-binding capacity. *Emm*-like genes belong to the *emm* gene superfamily, including more than 20 members sharing greater than 70% DNA sequence identity at their 5' ends, and encoding proteins which may possess immunoglobulin-binding or antiphagocytic functions (Cunningham, 2000; Hollingshead et al., 1993). The plasminogen-binding group A streptococcal M-like protein (PAM) is a 42 kDa cell-surface protein originally isolated from an M53 strain (Berge and Sjobring, 1993). PAM binds both plasminogen and plasmin with high affinity via a domain of 30 amino acids within the variable N-terminal region. This domain contains two characteristic tandem repeat regions of approximately 13 amino acids in length, designated A1 and A2. These are identical in 9 of 13 amino acid residues, with non-identical residues possessing the same charge and polarity. The A1 repeat region is followed by a highly conserved domain consisting of C repeats which are highly homologous with other M proteins (Berge and Sjobring, 1993; Ringdahl and Sjobring, 2000).

Unlike SEN and GAPDH which bind plasminogen via C-terminal lysine residues, PAM binds plasminogen via its internal lysine residues contained within the A repeat regions and an internal histidine residue in the A1 repeat (Schenone et al., 2000; Wistedt et al.,

1995). The majority of plasminogen binding is mediated by the lysine residue within the A1 repeat (Wistedt et al., 1995). PAM binds to kringle 2 of plasminogen in such a way that the catalytic domain remains accessible to activation by streptokinase and tPA (Berge and Sjobring, 1993; Ringdahl and Sjobring, 2000; Ringdahl et al., 1998). PAM-bound plasmin activity is protected from inactivation by α_2-antiplasmin, which also binds to kringle 2 of plasminogen (Berge and Sjobring, 1993).

The *pam* genotype is largely restricted to *emm* pattern D GAS isolates which display a strong tendency to cause impetigo (Svensson et al., 1999). This association has been consistently observed in isolates from various geographic locations and has lead to the hypothesis that PAM-mediated plasminogen binding may be an important factor in localised skin infection rather than invasive infection (Bessen et al., 2000; Bessen et al., 1996; Svensson et al., 1999). *Emm* pattern A, B and C isolates are generally associated with nasopharyngeal infection, while *emm* pattern E isolates display an affinity for both tissue sites (Bessen et al., 1999; Bessen et al., 1996). A humanised *in vivo* model of impetigo (human skin grafted onto SCID mice) was developed to examine the molecular determinants of GAS tissue tropism. Isolates of *emm* pattern D caused more severe erosion of the epidermal layer than emm pattern A – C isolates (Scaramuzzino et al., 2000). Inactivation of the *pam* gene attenuates virulence in this model, although the relative contribution to virulence of antiphagocytic and plasminogen-binding activities of the PAM protein could not be determined (Svensson et al., 2002).

Invasive disease does not generally develop secondary to skin infection in urbanised populations (Fiorentino et al., 1997; Haukness et al., 2002; Stollerman, 2001), where *emm* pattern D and *pam*-positive isolates are associated with skin infection (Bessen et

al., 1996; Svensson et al., 1999). In the NT where invasive GAS infection develops from pyoderma (Carapetis et al., 1999c; McDonald et al., 2004), the *pam* gene is present in the same proportions in skin and invasive disease isolates (Sanderson-Smith et al., 2004). Thus the contribution of PAM to virulence may be more relevant to invasive disease processes in these communities than in urban populations.

1.3.2 Streptokinase

1.3.2.1 Streptokinase structure, allelic variation and expression

Streptokinase is a 47 kDa protein of 414 amino acid residues with three distinct domains designated α, β and γ based on structural and functional characteristics (Huang et al., 1989b; Wang et al., 1998). Two highly variable regions within an exposed loop of the β domain of the streptokinase protein have been identified, known as V1 (residues 174-244) and V2 (residues 270-290) (Huang et al., 1989a; Johnson et al., 1992; Kapur et al., 1995; Wang et al., 1999). Classification of variants of *ska* is based on sequence differences within the variable region; over 80 variants have been described (Johnston et al., 1992; Kalia and Bessen, 2004; Kapur et al., 1995). Despite these polymorphisms, the overall structure in terms of hydrophobicity, hydrophilicity, antigenic sites, amphipathic regions etc. is strikingly conserved (Kapur et al., 1995).

The streptokinase gene (*ska*) is found in isolates of all GAS M-types, however *ska* alleles cluster with differing levels of streptokinase activity produced in supernatants of the corresponding GAS isolates (Ferretti et al., 1991; Tewodros et al., 1995). Expression of *ska* is growth-phase dependent, appearing early in the logarithmic phase of growth and under nutrient-rich conditions (Donabedian and Boyle, 1998; Johnston and Zabriskie, 1986). The *ska* gene is positively regulated by the FasCAX and Sag/Pel

systems, and repressed by CovRS (Federle et al., 1999; Kreikemeyer et al., 2001; Li et al., 1999b; Malke and Steiner, 2004). Interestingly, a subcluster of highly related *ska* alleles is found in strong linkage disequilibrium with the *pam* gene, which clusters amongst the skin-tropic *emm* pattern D isolates (Kalia and Bessen, 2004; Svensson et al., 1999). PAM and streptokinase cooperate in GAS plasmin acquisition *in vitro,* and both genes are required for full virulence in an *in vivo* model of impetigo (Ringdahl et al., 1998; Svensson et al., 2002). Together, these data support an involvement of *ska* allelic subtypes in tissue tropism at the skin (Kalia and Bessen, 2004).

1.3.2.2 Plasminogen activation by streptokinase

Streptokinase has long been known as a streptococcal spreading factor, conferring the ability to lyse fibrin clots allowing bacterial dissemination (Tillet et al., 1934). Streptokinase is secreted by group A, C and G streptococci. The activity of streptokinase from individual streptococcal isolates is often specific for plasminogen of the mammalian host, with inefficient activation of plasminogen from other host species (Marcum and Kline, 1983; Schroeder et al., 1999; Wohl et al., 1983). 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).

Unlike the host plasminogen activators, streptokinase is not 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 (McClintock and Bell, 1971; Ponting et al., 1992; Tomar and Taylor, 1971). The active site in the 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 (Cederholm-Williams et

al., 1979; Wiman, 1980). Proteolytic activity of the streptokinase-plasminogen complex is not inhibited by α_2-antiplasmin (Cederholm-Williams et al., 1979; Wiman, 1980). A second plasminogen molecule interacts with the streptokinase-plasminogen complex, resulting in its conversion to plasmin. This lysine-dependant step is thought to involve the kringle domains of the plasminogen molecule (Cederholm-Williams et al., 1979; Lin et al., 2000; Wiman, 1980; Young et al., 1998). The streptokinase-plasminogen complex shows a high specificity for plasminogen, and is an extremely efficient activator of the zymogen (Parry et al., 2000).

Molecular models of activation of GAS receptor-bound plasminogen by streptokinase have not yet been elucidated in detail. Plasminogen bound to GAS by receptors bearing either C-terminal or internal lysine residues can be activated by streptokinase (Berge and Sjobring, 1993; Pancholi and Fischetti, 1998). It is known that the extended conformation of Glu-plasminogen (such as is induced by lysine-dependent binding to mammalian plasminogen receptors) (Andronicos et al., 1997; Redlitz et al., 1995) 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.

1.3.3 Fibrinogen-streptokinase-dependent (FSD) plasminogen binding

1.3.3.1 Fibrinogen receptors

Virtually all clinical isolates of GAS bind fibrinogen (Herwald et al., 2003) and fibrinogen binding capacity of GAS was first mapped to the M protein (Kantor, 1965; Whitnack and Beachey, 1985). The antiphagocytic activity of some M proteins is attributed to binding of fibrinogen, which inhibits complement deposition by reducing

the amount of classical pathway C3 convertase bound to the GAS cell-surface (Carlsson et al., 2005; Whitnack and Beachey, 1985). M proteins possessing fibrinogen-binding capacity include M1, M3, M5, M6 and M18 (Akesson et al., 1994; Courtney et al., 1997; Reichardt et al., 1997; Whitnack et al., 1984). M proteins known not to bind fibrinogen include M4, M22 and M60 (Stenberg et al., 1992; Thern et al., 1995; Thern et al., 1998).

Other fibrinogen-binding proteins of GAS include M-related proteins and T proteins (Schmidt and Kohler, 1984; Stenberg et al., 1992; Thern et al., 1998). In addition, certain GAS fibronectin-binding proteins possess a dual affinity for fibrinogen, including protein F and Fbp54, the gene for which is found in 100% of NT GAS isolates tested (Courtney et al., 1994; Delvecchio et al., 2002a; Katerov et al., 1998).

1.3.3.2 Fibrinogen-streptokinase-dependent plasmin acquisition

Acquisition of plasmin activity by some GAS isolates requires human fibrinogen in addition to plasminogen and streptokinase (fibrinogen-streptokinase-dependent (FSD) pathway). According to this model, fibrinogen bound to the GAS cell surface provides a target for the lysine-dependent binding of a preassembled plasminogen-streptokinase complex (Wang et al., 1995a). Subsequent molecular rearrangements render the binding interaction lysine-independent (Wang et al., 1995a). The trimolecular complex formed activates fluid phase plasminogen in the presence of host inhibitors and also possesses plasmin activity (D'Costa and Boyle, 1998). Thus, plasminogen acquisition via the FSD pathway results in the creation of an unregulated surface protease and an immobilised plasminogen activator (Wang et al., 1995b). 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 α_2-antiplasmin (Wang et al., 1995a).

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 FSD 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 (Wang et al., 1995b). This suggests that streptokinase is not only involved as a plasminogen activator, but also as an essential binding co-factor in the FSD pathway (Wang et al., 1995b). Fibrinogen-binding capacity is the limiting factor in the ability of many GAS strains to acquire plasmin activity (Wang et al., 1995b). Deletion of the major fibrinogen protein in GAS isolates significantly reduces plasmin acquisition (Christner et al., 1997).

1.3.4 Interaction of SpeB with the GAS plasminogen system

1.3.4.1 SpeB structure, specificity and expression

Streptococcal pyrogenic exotoxin B (SpeB) is a highly conserved extracellular and cell-surface cysteine protease with broad specificity (Hytonen et al., 2001; Kapur et al., 1993b). SpeB is secreted as 40 kDa zymogen which is processed to a 28 kDa active protease by autocatalysis, trypsin, subtilisin or the mature SpeB protease, and involves several intermediate forms (Hauser and Schlievert, 1990). Autocatalytic activation of the zymogen occurs under reducing conditions by intra- and inter-molecular mechanisms and involves sequential cleavages within the N-terminal pro-sequence domain (Doran et al., 1999).

SpeB binds the ECM component laminin and other glycoproteins independent of both the activity and the substrate binding site of the cysteine protease (Hytonen et al., 2001; Hytonen et al., 2000). Originally identified as a plasmin-binding protein secreted by nephritogenic GAS strains, amino acid sequence analysis and immunological reactivity suggest that the nephritis plasmin binding protein (NPBP) is the zymogen form of SpeB. NPBP binds plasmin in a lysine-dependent manner, suggesting it may be protected from inactivation by α_2-antiplasmin as for other lysine-dependent receptor interactions (Poon-King et al., 1993). Thus the zymogen form of SpeB may confer an unregulated, diffusible plasmin activity capable of targeting the ECM.

SpeB itself cleaves ECM components and activates host metalloproteases (Burns et al., 1996; Kapur et al., 1993b; Tamura et al., 2004) Further, SpeB activates inflammatory cytokines and kinins and cleaves immunoglobulins, suggesting a role for SpeB in GAS invasion, inflammatory disease and immune evasion (Burns et al., 1996; Collin and Olsen, 2001; Herwald et al., 1996; Kapur et al., 1993a; Tamura et al., 2004). However SpeB also degrades GAS virulence factors (Aziz et al., 2004; Kansal et al., 2003) and components of the GAS plasminogen activation system including surface-bound plasminogen and plasmin, streptokinase (Rezcallah et al., 2004), fibrinogen (Matsuka et al., 1999) and fibrinogen-binding proteins such as M1 (Raeder et al., 1998) and protein F (Nyberg et al., 2004). Whether SpeB tips the proteolytic balance in favour of GAS or the host is unclear, however it is of interest that invasive M1 GAS undergoes a phase-shift *in vivo* to acquire a SpeB-negative phenotype (Aziz et al., 2004; Raeder et al., 2000).

The *speB* gene is carried by all GAS strains but the level of expression varies between strains (Hytonen et al., 2001). Expression is controlled by the multiple gene activator *mga*, by *ropA* involved in secretion and folding of SpeB, and by *ropB (rgg),* a transcriptional activator of SpeB expression (Chaussee et al., 1999; Lyon et al., 1998; Podbielski et al., 1996). The expression of SpeB is growth-phase dependent, with maximal expression occurring from late logarithmic phase to stationary phase in response to nutrient limitation (Chaussee et al., 1997; Neely et al., 2003).

1.3.4.2 SpeB and GAS virulence

Reflecting the diversity of GAS and host molecules degraded by SpeB, the role of SpeB in GAS virulence is complex. Studies employing genetic inactivation of SpeB have implicated the protease in phagocytosis resistance, streptococcal dissemination to organs and lethality in a murine infection model (Lukomski et al., 1998; Lukomski et al., 1999; Lukomski et al., 1997). SpeB is associated with disease severity, and appears to exert a synergistic effect on other virulence factors in murine skin infection models (Saouda et al., 2001; Svensson et al., 2000). SpeB inhibits IgG-mediated opsonophagocytosis in human blood, supporting its postulated role in immune evasion (Collin et al., 2002). In contrast, others have found no effect of SpeB inactivation in murine models of invasive infection (Ashbaugh et al., 1998; Ashbaugh and Wessels, 2001), noting that the work by Lukomski's group may have been confounded by diminished expression of the antiphagocytic hyaluronic acid capsule coincident with SpeB inactivation (Ashbaugh and Wessels, 2001; Lukomski et al., 1998; Woischnik et al., 2000). Corroborating the animal work showing diminished SpeB production by invasive M1 clones (Aziz et al., 2004; Raeder et al., 2000), M1T1 isolates from human

disease show an inverse relationship between SpeB expression and severity of invasive disease (Kansal et al., 2000).

The relationship between SpeB production and infection severity in the *in vivo* impetigo model, and the observation that skin isolates of GAS produce more SpeB than nasopharygeal isolates, support a role for SpeB in skin tropism (Svensson et al., 2000). An integrated model of skin infection has been proposed (Svensson et al., 2002) in which GAS proteolytic activity is dominated by SpeB in the initial stages of infection, possibly mediating nutrient acquisition (Chaussee et al., 1997). Once colonisation is established, immune infiltration generates a nutrient-rich environment favouring streptokinase expression and a plentiful source of plasminogen (Donabedian and Boyle, 1998). The fibrinolytic activity of plasmin prevents wound healing, favouring bacterial persistence and spread (Donabedian and Boyle, 1998; Sun et al., 2004).

1.3.5 Role of plasminogen activation in GAS virulence

The importance of plasminogen in streptococcal pathogenicity is illustrated by the biochemical and molecular evolution of streptococcal plasminogen activation. The interaction of streptokinase with plasminogen reflects the mammalian host range of various streptococcal species (Marcum and Kline, 1983; Schroeder et al., 1999; Wohl et al., 1983). DNA sequence analysis of plasmin from various species indicates that sites in plasmin that interact with streptokinase are preferentially targeted for mutation compared to its homologues, trypsin and chymotrypsin, which are not cofactors in bacterial virulence. Amongst the streptococci, intermolecular contact sites in streptokinase that activate human plasminogen are more highly conserved

than other loci in the molecule, or than the same sites in streptokinases that activate plasminogen of other species (Gladysheva et al., 2003).

The species specificity of plasminogen activation and its importance in virulence are exemplified in murine models of GAS infection, where murine plasminogen is resistant to activation by GAS streptokinase (Lijnen et al., 1994) and mice are generally resistant to skin infection with GAS (Li et al., 1999a; Sun et al., 2004). Accordingly, a high streptokinase-producing GAS strain and its streptokinase-deletion mutant show no difference in virulence in a mouse skin infection model (Khil et al., 2003). In contrast, when a source of human plasminogen is present at the infection site, the presence of the streptokinase gene markedly enhances group A streptococcal virulence (Khil et al., 2003; Svensson et al., 2002) Likewise, virulence of streptokinase-producing GAS is enhanced in the mouse by preincubation in human plasma, but not plasminogen-depleted plasma (Li et al., 1999a).

Recently, the development of a humanised plasminogen transgenic mouse model of infection definitively demonstrated the critical role of human plasminogen in group A streptococcal pathogenicity *in vivo*. The presence of a human plasminogen transgene markedly increased mortality following skin infection with GAS, but not following intravenous infection. The susceptibility conferred by the human plasminogen transgene is dependent on streptokinase secretion by GAS, and is characterized by enhanced bacterial dissemination. Fibrinogen may be essential to host defense against group A streptococcal skin infection, possibly due to encapsulation of the bacteria within fibrin networks and prevention of systemic access by occlusion of the local vasculature. Acquisition of fibrinolytic activity by

GAS circumvents fibrin-dependent mechanisms, even if only transiently, thereby enhancing virulence (Sun et al., 2004).

Even in the absence of streptokinase, the ability to focus plasminogen at the group A streptococcal surface via cell-surface receptors enhances virulence *in vivo*. Activation of GAS-bound plasminogen by uPA and tPA has been demonstrated *in vitro* but has not been thoroughly investigated *in vivo* (Berge and Sjobring, 1993; Kuusela et al., 1992). However, a streptokinase-deficient GAS strain with plasminogen-binding capacity showed enhanced virulence when a human plasminogen source was provided, probably due to plasmin formation by host activators (Khil et al., 2003). Human plasminogen is more readily activated by murine tPA than is murine plasminogen (Lijnen et al., 1994). The binding of plasminogen to GAS is known to markedly increase the rate of plasminogen activation by tPA (Kuusela et al., 1992) supporting the hypothesis that cell-surface binding of plasminogen is an important component of group A streptococcal pathogenesis.

To our knowledge, only one study of human clinical isolates of GAS has examined the relationship between plasmin acquisition and invasive capacity. Invasive disease isolates demonstrated higher levels of plasmin than isolates from uncomplicated throat infections, although this difference did not reach significance (Wang et al., 1994). Amongst highly related *emm*49 isolates, those from severe invasive disease showed higher streptokinase expression at the transcriptional level than those from non-invasive disease (Ikebe et al., 2005).

Overall, recent studies point to an important role for plasminogen activation in GAS virulence. It is not known whether a low level of plasminogen activation is sufficient to maintain GAS infection or initiate invasion from a superficial site; or whether isolates with higher plasmin/plasminogen activator activity are more efficient in these processes. The network of molecules involved in the interaction of plasminogen with GAS constitute a complex system with multiple levels of regulation.

1.4 Rationale and aims of this study

The aim of this study was to determine whether plasminogen is deployed as a virulence factor during GAS invasive disease, with particular reference to GAS isolates from the NT of Australia. The high rates of invasive disease in this region manifest secondary to endemic skin infection. Against this background, the postulated importance of plasminogen activation in skin tropism, as well as in the breach of tissue barriers has lead to the hypothesis that plasminogen activation may play an important role in NT GAS invasive disease.

This question was first approached from an epidemiological perspective, comparing GAS isolates from invasive infections with those from uncomplicated infections for their interaction with the plasminogen system *in vitro*. Plasminogen binding; plasminogen receptor expression and genetic variation; fibrinogen binding; streptokinase expression, activity and genetic variation; SpeB expression and activity; and acquisition of cell-surface plasmin in human plasma were characterised in 29 GAS isolates of known clinical origin from the Northern Territory. To further characterise the role of PAM, streptokinase and SpeB in acquisition of cell-surface plasmin activity in human plasma, such acquisition was compared in GAS deletion mutants for genes encoding these proteins and the corresponding wildtype strains.

The second approach to addressing the role of plasminogen in GAS virulence and invasive disease was to examine GAS infection using the human plasminogen transgenic mouse model. A subset of NT GAS isolates selected for different *in vitro* plasminogen activation characteristics was tested for virulence in this model.

CHAPTER 2 Plasminogen binding by group A streptococcal isolates from the Northern Territory of Australia

McKay, F. C., McArthur, J. D., Sanderson-Smith, M. L., Gardam, S., Currie, B. J., Sriprakash, K. S., Fagan, P. K., Towers, R. J., Batzloff, M. R., Chhatwal, G. S., Ranson, M. and Walker, M. J. (2004). Plasminogen binding by group A streptococcal isolates from a region of hyperendemicity for streptococcal skin infection and a high incidence of invasive infection, Infection and Immunity, 72, 364-70.

2.1 Introduction

In the tropical Northern Territory of Australia (NT), GAS skin infection is hyperendemic and the skin is thought to represent the major portal of entry for GAS invasion to sterile sites (Carapetis et al., 1999c; McDonald et al., 2004). The importance of plasminogen binding in GAS skin infection is suggested by the clustering of the high affinity plasminogen receptor PAM amongst isolates of *emm* pattern D, which display a preference for skin as the primary infection site (Bessen et al., 1996; Svensson et al., 1999). The importance of plasminogen acquisition in both skin infection and tissue invasion has been demonstrated in various animal models (Li et al., 1999a; Sun et al., 2004; Svensson et al., 2002).

The aim of this study was to characterise the binding of plasminogen by clinical isolates of GAS from the NT of Australia and to determine from an epidemiological perspective whether there is any relationship between GAS plasminogen acquisition and invasive propensity. We are aware of only one other study of GAS infection employing this approach, which found no significant difference in plasminogen binding between North American clinical isolates of GAS from throat infection and bacteremia (Wang et al., 1994). Invasive GAS disease in industrialised nations generally originates from throat infection rather than skin infection as found in the NT of Australia (Fiorentino et al., 1997). The niche specialisation observed amongst GAS populations supports the hypothesis that the virulence factor profile mediating tissue invasion from the skin and throat may also be divergent (Bessen et al., 1999; Bessen et al., 2005; Fiorentino et al., 1997; Kalia and Bessen, 2004). Thus the plasminogen binding profile of invasive GAS isolates of distinct geographic and epidemiological origin was investigated.

In this study a clonally diverse collection of 29 NT GAS isolates from either invasive disease or uncomplicated infections was assayed for the presence of the high affinity plasminogen receptors, SEN and PAM. DNA sequence analysis of the plasminogen-binding domain of the *sen* gene was performed and the cell-surface expression of SEN investigated. Presence of the *pam* gene in these isolates was noted from the work of Martina Sanderson-Smith, PhD candidate, University of Wollongong. The plasminogen receptor GAPDH was not investigated due to its reported lack of importance in plasmin acquisition by GAS (Winram and Lottenberg, 1998). Radioligand binding assays were used to determine the direct binding of plasminogen to the GAS cell-surface. Plasminogen binding as part of a trimolecular complex by the fibrinogen-streptokinase-dependent (FSD) pathway was determined by measuring plasminogen binding in the presence of streptokinase and fibrinogen (Wang et al., 1995a). Binding of plasminogen by the FSD pathway is thought to be mediated by fibrinogen receptors, thus the capacity of isolates to bind human fibrinogen was also determined (Christner et al., 1997; Wang et al., 1995a).

2.2 Materials and Methods

2.2.1 Protein Methods

2.2.1.1 SDS-PAGE Analysis

Acrylamide gels (0.375 M Tris, pH 8.8, 12% resolving gel) were prepared using the Mini-Protean II or III system with an acrylamide stacking gel (0.125 M Tris, pH 6.8, 4% acrylamide) according to the manufacturer's instructions (Bio-Rad Laboratories Inc, Hercules, California, USA). Samples were boiled for 5 min in SDS-PAGE sample buffer (Appendix 1) and chilled on ice before loading. Unstained molecular weight markers (Precision or Precision Plus Protein™ Unstained Standards, Bio-Rad) were used on all gels, while prestained markers (Benchmark™ Prestained protein ladder, Invitrogen Life Technologies, Carlsbad, California, USA; or Prestained SDS-PAGE Standards, Low or High Range, Bio-Rad) were used for gels to be western transferred. Following electrophoresis at 200 V in running buffer (Appendix 1), gels were prepared for transfer as described below or stained with Coomassie Blue (Appendix 1) for 1 h with gentle agitation and destained overnight. Standard curves of distance migrated against $\log_{10}$ molecular weight of markers were used to determine molecular weight of unknowns.

2.2.1.2 Western Transfer and Immunoblotting

Gels were prepared for electrophoretic transfer by equilibration in methanol transfer buffer (Appendix 1) for 10 min. Polyvinyl difluoride (PVDF) membranes (Immun-blot™ PVDF membrane, 0.2 μm, Bio-Rad) were wetted using 100% ethanol, equilibrated in transfer buffer and the transfer performed using the Bio-Rad Mini Trans-blot assembly according to the manufacturer's instructions for 1 h at 110 V at 4°C.

Membranes were rinsed in Tris-buffered saline (TBS; Appendix 1) and blocked overnight at 4°C in blocking buffer (Appendix 1), followed by 1 h incubation in primary antibody diluted 1 in 2000 in blocking buffer at room temperature. The membrane was washed 5 x 5 min in 50 ml TBS with 0.05% Tween-20 (TBST) and re-blocked for 30 min in blocking buffer. Horseradish peroxidase-conjugated secondary antibody diluted 1 in 2000 in blocking buffer was incubated on the membrane for 1 h at RT. The membrane was washed as above, with a fourth wash in 50 ml TBS, followed by chemilluminescent detection using the Supersignal® Enhanced Chemiluminescent Substrate or the Supersignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, Illinois, USA) according to the manufacturer's instructions. In some instances, antibody concentrations were reduced to minimise background signal for the latter more sensitive substrate. High performance chemiluminescent film (Hyperfilm™, Amersham Biosciences, Piscataway, New Jersey, USA) was developed using GBX developer and fixer solutions (supplied as 5X stock solution from Kodak (Australasia) Pty Ltd, Coburg, Victoria, Australia). Blots were stained with Coomassie Blue following development to visualise transferred unstained molecular weight markers. For Western blot of streptokinase, the membrane was equilibrated in 100 mM Tris, pH 7.6 for 2 min before chromogenic detection with diaminobenzidine (Sigma, St Louis, Missouri, USA) in the presence of hydrogen peroxide (Appendix 1).

2.2.1.3 Enzyme-linked immunosorbent assay (ELISA)

Microtitre plates were coated with 5 μg/ml antigen in carbonate buffer (Appendix 1) by overnight incubation at 4°C. Plates were washed 3 times with PINT buffer (Appendix 1), incubated with assay blocking buffer (Appendix 1) for 1 h at 37°C, and washed as before. Serial dilutions of test sera in blocking buffer were incubated for 1 h at 37°C,

the plates washed and secondary antibody added at 1 in 2000 in blocking buffer for 1 h at 37°C. Plates were washed 4 times with PINT, developed with *o*-phenylenediamine (Sigma) developing solution (Appendix 1), and the reaction stopped with 1 N hydrochloric acid. Absorbance at 490 nm (A_{490}) was determined using the Spectramax® 250 platereader (Molecular Devices Corporation, Sunnyvale, California, USA; used for all subsequent absorbance determination in 96-well plates). All incubation volumes were 50 µl.

2.2.1.4 Determination of protein concentration

Protein concentrations were determined using the Bio-Rad DC protein assay. The assay was performed according to the manufacturer's instructions (microplate assay protocol) and A_{750} determined. Concentrations of unknown samples were determined from a standard curve of bovine serum albumin (0 - 1.5 mg/ml range) interpolated using SoftMax® Pro software (version 1.2.0; Molecular Devices Corporation).

2.2.1.5 Affinity purification of plasminogen from human plasma

Plasminogen was purified from human plasma using a method modified from that described previously (Deutsch and Mertz, 1970). Frozen plasma was purchased from the Australian Red Cross Blood Service. Lysine sepharose® 4B (Amersham Biosciences) was rehydrated and washed according to the manufacturer's instructions. Plasma was defrosted in ice-cold water, diluted 1 in 2 in ice-cold distilled water and the protease inhibitors EDTA (5 mM) and phenylmethylsulfonyl fluoride (PMSF; 1 mM) added. Excess dilute plasma was added to lysine sepharose in 50 ml centrifuge tubes and incubated with gentle agitation 1 - 2 h on ice at 4°C. Lysine sepharose was centrifuged at 200 x *g*, resuspended in 1 volume of wash buffer (Appendix 1), and

loaded onto a chromatography column. Chromatography was performed at 4°C at a flow rate of 0.5 ml/min using using a peristaltic pump with flow monitor (Econo Pump, Model EP1, Bio-Rad).

The column was washed overnight using wash buffer. Chromatography (protein concentration) was monitored at A_{280} using either the Econo™ UV monitor (Bio-Rad), or by spectrophotometry of wash fractions collected using an automated fraction collector (Model 2110, Bio-Rad). The column was washed until baseline absorbance was reached. Further elution of contaminants was achieved by washing with a high-salt buffer (Appendix 1) until baseline absorbance was reached. Plasminogen was eluted with buffer containing the lysine analogue, ε-aminocaproic acid (Sigma; Appendix 1). Fractions corresponding to the elution peak at A_{280} containing plasminogen were collected and the tail of the elution peak (potentially contaminated with plasmin) was discarded. Plasminogen was dialysed against 10 l of phosphate buffered saline (PBS; Appendix 1) pH 8.0, with two changes over two days at 4°C.

Plasminogen concentration was adjusted to greater than 1 mg/ml by dialysis against solid polyvinyl pyrrolidone-40 (ICN Biomedicals Inc, Aurora, Ohio, USA) at 4°C and aliquots stored at -70°C. Identity of plasminogen was confirmed by Western blotting with an anti-human plasminogen antibody (Calbiochem®, Merck Pty, Kilsyth, Victoria, Australia) and by chromogenic plasmin activity assay in presence of the plasminogen activator, streptokinase. Aliquots were tested for the absence of plasmin by reducing SDS-PAGE, yielding two protein bands for plasmin and one for plasminogen, or by chromogenic plasmin activity assays.

2.2.1.6 Plasmin activity assay

Plasmin activity of human plasma or purified plasminogen was measured using a plasmin-specific chromogenic substrate, H-D-norleucyl-hexahydrotyrosol-lysine-para-nitroanilide diacetate; (Spectrozyme® PL; American Diagnostica, Stamford, Connecticut, USA) in the presence and absence of group C streptokinase (Sigma). The assay was performed at 37°C in 50 mM Tris, pH 7.4, in 96-well plates using a final concentration of 0.4 mM Spectrozyme® PL and monitored at A_{405}. Initial reaction rate (V_{max}) was calculated and compared to a standard curve of plasmin using Softmax® Pro. This assay was modified to determine supernatant streptokinase activity and cell-surface plasmin acquisition by GAS as described in later chapters.

2.2.1.7 Generation and purification of rabbit polyclonal antibody

A New Zealand white rabbit (Merungora, Wauchope, NSW, Australia) was immunised intramuscularly with an emulsion containing approximately 300 μg of a commercially synthesised peptide representing the the carboxy-terminal region of SEN (NH_2-CYKGIKSFYNLKK-COOH; Chiron Mimotopes, San Diego, California, USA). A cysteine residue was added to the amino-terminal end of the peptide for conjugation to keyhole limpet hemocyanin (KLH) by the maleimide method (Chiron Mimotopes). The peptide was resuspended 1:1 in complete Freund's adjuvant (Sigma) for primary immunisation.

The rabbit was boosted with approximately 200 μg of peptide-KLH conjugate suspended in incomplete Freund's adjuvant (Sigma) on days 34 and 84 and antibody responses were monitored by ELISA. Blood was collected by cardiac puncture 9 days after the final immunisation. This experiment was conducted according to the

Guidelines for the Care and Use of Laboratory Animals (National Health and Medical Research Council, Australia) and approved by the University of Wollongong Animal Ethics Committee.

Immune serum was adsorbed to a protein G-Sepharose® 4 Fast Flow™ column (Amersham Biosciences) and washed with PBS, pH 7.4, until A_{280} of the eluant reached baseline. Bound IgG was eluted with 0.1 M glycine, pH 2.3, and adjusted immediately to pH 7 using 3 M Tris HCl, pH 8.4. The IgG fraction was dialysed for 48 h with 2 changes of PBS, pH 7.4 at 4ºC and stored at -70ºC. Purity and identity of IgG was tested by SDS-PAGE, confirming the expected molecular weights for reduced and non-reduced IgG bands (Harlow and Lane, 1988).

2.2.1.8 Labelling of proteins with ^{125}iodine

Proteins (plasminogen and fibrinogen) were labelled with ^{125}iodine (Amersham Biosciences) by the chloramine T method (Hunter and Greenwood, 1962). Glu-plasminogen was purified from human plasma as previously described. Human fibrinogen (essentially plasminogen-free; Sigma) was tested to confirm the absence of contaminating fibronectin by Western blotting with a rabbit polyclonal antibody against human fibronectin (Sigma). The iodination reaction was performed in a siliconised (Sigmacoat; Sigma) glass vial by mixing 100 µl of protein at 1 mg/ml in phosphate buffer (0.05 M sodium phosphate, pH 7.5) with 350 Ci ^{125}iodine and 20 µl chloramine T (Sigma; 1.5 mg/ml in phosphate buffer). The reaction was allowed to proceed for 1 min before stopping with 20 µl of sodium metabisulfite (Sigma; 1.5 mg/ml) in phosphate buffer. Radiolabelling efficiency (% incorporation of radioactivity into protein) was determined by comparing radioactivity of the labelling reaction with that of

trichloroacetic acid-precipitated protein from an equivalent reaction volume. All radioactivity measurements were determined using an automatic gamma counter (Wallac; Perkin Elmer Life Sciences, Boston, Massachusetts) for 30 s per tube. Unincorporated radioactivity was removed and the buffer exchanged to PBS with 0.05% Tween-20 (PBST) by size exclusion chromatography using a PD-10 Sephadex G-25M column (Amersham Biosciences). The reaction was diluted to 2500 - 4500 cpm/µl for binding assays. All labelled proteins were analysed for homogeneity by gel electrophoresis and autoradiography in comparison with non-labelled proteins.

2.2.2 DNA methods

2.2.2.1 Polymerase chain reaction (PCR) amplification of DNA

Fragments of streptococcal genomic DNA were amplified using *Taq* polymerase (Qiagen GmbH, Hilden, Germany) and specific oligonucleotide primers (Sigma Genosys, Sydney, NSW, Australia; see Appendix 1 for reaction mix). PCR was performed using a thermal cycler (Cooled/gradient Palm Cycler version 2.2; Corbett Research, Mortlake, NSW, Australia) with denaturation, annealing and extension conducted at 94°C, 50°C and 72°C respectively. Each temperature was held for 2 min in the first amplification cycle; 1 min for the next 30 cycles; and 1 min for the final cycle with the exception of the extension step, held for 7 min. Oligonucleotide primers used are given in Table 2.1. *Emm* pattern of the isolates was determined by Martina Sanderson-Smith (University of Wollongong) as described previously (McKay et al., 2004).

Table 2.1. Oligonucleotide primers used for analysis of the *sen* gene

Please see print copy for Table 2.1

[a] (McKay et al., 2004)

2.2.2.2 Agarose gel electrophoresis

DNA samples were prepared by dilution in DNA loading buffer (Appendix 1). DNA fragments were resolved by electrophoresis in 1% (w/v) Tris acetate (TAE)-agarose gels in TAE buffer (Appendix 1) at 70 V. Gels were stained with ethidium bromide (approximately 1 μg/ml) for 20 min with gentle agitation, destained for 20 min in water, and visualised using a UV transilluminator (UVP Inc, Upland, California, USA) and photographed using a gel documentation system (NovaLine, Sydney, NSW, Australia). Standard curves of distance migrated against $\log_{10}$ molecular weight of markers were used to determine molecular weight of unknowns.

2.2.2.3 DNA sequence analysis

DNA to be sequenced was first PCR-amplified from chromosomal DNA and purified using either the QIAquick® PCR purification kit (Qiagen) or Wizard® SV PCR Clean-up System (Promega Corporation, Madison, Wisconsin, USA) according to the manufacturers' instructions. DNA sequencing reactions were performed using the BigDye® Terminator cycle sequencing kits version 2.1 or 3.1 (Applied Biosystems, Foster City, California, USA; see Appendix 1 for reaction mixtures) and oligonucleotide primers given in Table 2.1. PCR was performed using a thermal cycler (Palm Cycler version 2.2; Corbett Research) with 25 cycles of denaturation, annealing and extension

conducted at 96 °C for 10 sec, 50 °C for 5 sec and 60°C for 4 min respectively. Unincorporated di-deoxyterminators were removed by ethanol precipitation according to the manufacturer's instructions for each system. DNA pellets were resuspended in loading dye (Appendix 1) and the sequences resolved electrophoretically using the ABI Prism® 377 DNA sequencer (Applied Biosystems). Electropherograms of sequence generated from forward and reverse primers were aligned and a consensus sequence generated using ABI Prism® Autoassembler™ software (Applied Biosystems) or the Staden Package (www://staden.sourceforge.net; (Bonfield et al., 1995).

2.2.2.4 Molecular typing and emm pattern

Emm sequence typing of Northern Territory isolates was performed by Martina Sanderson-Smith and Drs Peter Fagan and Rebecca Towers of the Menzies School of Health Research, Darwin. *Emm* pattern was determined by Martina Sanderson-Smith, and Dr Deborah Bessen of the Department of Microbiology and Immunology, New York Medical College, Valhalla, New York, USA, kindly provided and confirmed some of the *emm* pattern and *emm* sequence data. Presence of the PAM gene was determined by Southern hybridisation by Sandra Gardam, University of Wollongong, and confirmed by sequencing of the A1 and A2 plasminogen-binding repeat region of PAM by Martina Sanderson-Smith. For the purposes of this work, PAM-positive isolates were defined as those positive by Southern hybridisation and containing an A1 and A2 repeat regions with greater than 50% amino acid sequence homology to that of the prototype PAM sequence (Berge and Sjobring, 1993). Methods used for analysis of *emm* and *emm*-like genes have been described elsewhere (McKay et al., 2004). All *vir* typing was performed as described previously by staff of the Menzies School of Health Research, Darwin (Gardiner et al., 1995).

2.2.3 GAS assay methods

2.2.3.1 Source of GAS isolates

Northern Territory GAS isolates were a generous gift from Dr Kadaba Sriprakash and Dr Bart Currie of the Menzies School of Health Research, Darwin, Australia and the microbiology staff of Royal Darwin Hospital and Alice Springs Hospital. GAS were collected from patients either during community clinical visits or hospital visits.

2.2.3.2 GAS culture

GAS isolates were stored at -70°C in cryovials in tryptone soya broth (Oxoid Ltd, Basingstoke, Hampshire, England) containing 15% (v/v) glycerol. GAS were struck out on either defibrinated horse blood agar plates (Biomerieux, Baulkham Hills, NSW, Australia) or agar plates containing Todd Hewitt broth (Difco™, BD Biosciences, Franklin Lakes, New Jersey, USA) supplemented with 1% (w/v) yeast extract (Oxoid; THY) and grown overnight at 37°C. For liquid culture, one colony of GAS from a horse blood agar plate was used to inoculate THY and grown as a stationary culture at 37°C to the appropriate growth phase, unless specified otherwise.

2.2.3.3 Extraction of genomic DNA from GAS

Genomic DNA was extracted from a 2 ml THY overnight culture of GAS using the DNeasy DNA extraction kit (Qiagen) according to the manufacturer's instructions. This extraction included the recommended lysozyme digestion for gram-positive bacteria.

2.2.3.4 Mutanolysin extraction of streptococcal membrane proteins

Overnight cultures (25 ml) of GAS were pelleted by centrifugation at 7,500 x *g* for 20 min and washed twice with 1.25 ml ice-cold Tris-EDTA (TE; 50 mM Tris, pH 8.0, 1

mM EDTA) containing 1 mM PMSF. The cells were resuspended in a mixture containing 250 µl TE with 20% (w/v) sucrose, 25 µl lysozyme (100 mg/ml in TE-sucrose; Sigma) and 12.5 µl mutanolysin (5000 U/ml in 0.1 M K_2HPO_4, pH 6.2). The mixture was shaken for 2 h at 37°C, then centrifuged at 14, 000 x *g* for 5 min and the supernatant collected (Ji et al., 1998). Extracts were boiled in SDS-PAGE sample buffer for 5 min and stored at -20°C until analysis.

2.2.3.5 GAS plasminogen- and fibrinogen-binding assays

GAS were grown overnight in THY as stationary cultures (25 ml) and harvested by centrifugation at 6,000 x *g* for 20 min. The pellets were washed once in PBST (5 ml) and resuspended to a transmittance (600nm) of 10%. Radiolabelled plasminogen or fibrinogen (20 µl) was added to 250 µl of the cell suspension with vortexing and incubated for 45 min at room temperature. Where appropriate, unlabelled streptokinase (10-fold molar excess compared to plasminogen; Sigma), fibrinogen (1.5 fold molar excess compared to plasminogen; Sigma) and/or εACA (10-fold molar excess compared to plasminogen; Sigma) were added to ^{125}I-plasminogen-binding assays. Cells were washed with 1 ml PBST, sedimented by centrifugation at 14,000 x g for 3 min and the supernatant carefully aspirated. Radioactivity of cell pellets was determined using an automatic gamma counter (Wallac) and the results expressed as percentage of input radioactivity. All measurements were determined in triplicate. Plasminogen- and fibrinogen-binding assays of all Northern Territory GAS isolates were performed with the assistance of Dr Jason McArthur, University of Wollongong.

2.2.4 Statistical methods

Statistical analysis was performed using JMP (SAS Institute Inc., Cary, North Carolina, USA). Pearson's χ^2 analysis was used to determine associations between genetic markers and invasive classification. Binding data were analysed for differences between *pam* status and infection classification using two-way analysis of variance (ANOVA). Where data or transformed data did not fit the assumptions for two-way ANOVA, an unpaired Student's *t* test, Welch ANOVA (unequal variances) or Wilcoxan/Kruskal-Wallis Rank Sum test (non-parametric) was used to compare groups separately. A paired Student's *t* test was used to analyse responses of isolates to different experimental conditions. Pearson's or Spearman's correlation coefficients (*r*) were used to describe the relationships between cell-surface binding of radiolabelled proteins for parametric and non-parametric data respectively. The *p* values for some analyses differ slightly from those previously reported due to the subsequent reclassification of isolate NS696 as *pam*-negative based on low sequence identity to the protoype *pam* sequence despite positive Southern hybridisation (McKay et al., 2004). However the overall significance of reported relationships was unchanged.

2.3 Results

2.3.1 Clonal diversity of NT GAS isolates

Characteristics of the 29 clinical isolates of GAS from invasive cases (n = 14) and uncomplicated infections (n = 15) are given in Table 2.2. The isolates showed considerable genetic diversity, with 25 *vir* types, 24 e*mm* sequence types and 4 of the five major *emm* patterns represented. Together the 29 isolates represent 25 genetically distinct strains that are thus not epidemiologically linked. There were 3 small clusters of isolates that were found to be genetically indistinguishable based on the typing methods used. These were NS730 and NS733 (*vir* type 2.2, *emm*ST 90 and *emm* pattern E); NS10, NS13 and NS59 (*vir* type 24, *emm*ST 53 and *emm* pattern D); and NS455 and NS253 (*vir* type 29.1, *emm*ST 52 and *emm* pattern D). In this study *emm* pattern was not significantly associated with invasive disease ($p \leq 0.148$).

2.3.2 Sequence encoding the SEN protein

A 509 bp fragment spanning the 3' end of the *sen* gene (encoding the C-terminal end of the SEN protein) was amplified (Figure 2.1) and the DNA sequence determined. All 29 NT isolates possessed the *sen* gene and the DNA sequence encoding the C-terminal lysine repeat that mediates Glu- and Lys-plasminogen binding was conserved in all isolates (Derbise et al., 2004) (Table 2.3).

2.3.3 Sequence encoding the PAM protein

Of the 29 NT isolates examined, 13 hybridised to a *pam*-specific oligonucleotide probe. Of these, all except NS696 displayed > 50% homology to the prototype *pam*- sequence and thus were designated *pam*-positive (Berge and Sjobring, 1993; McKay et al., 2004).

Table 2.2. Characteristics of the 29 NT GAS isolates

Please see print copy for Table 2.2

See table footnotes overleaf

Table 2.2. Characteristics of the 29 NT GAS isolates (continued)

[a] invasive (inv) or uncomplicated (uncomp) GAS infection classification according to sterile or nonsterile tissue site of infection, respectively.

[b] positive (+) or negative (-) plasminogen-binding group A streptococcal M-like protein (PAM) status according to hybridisation of M or M-like gene from GAS isolate with an oligonucleotide probe homologous to the A1/A2 repeat of the PAM gene by Southern analysis, as well as > 50% predicted amino acid sequence homology to the A1/A2 repeat based on DNA sequence analysis (McKay et al., 2004).

[c] Binding of ^{125}I-plasminogen to GAS in the presence of excess streptokinase and fibrinogen.

[d] *Vir* type was determined as previously described (Gardiner et al., 1995).

[e] emm sequence type was determined using a protocol modified from the *Streptococcus pyogenes emm* sequence database (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm).

[f] emm pattern was determined as previously described (Bessen et al., 1996; Svensson et al., 1999) except that annealing temperatures varied between 56°C and 62°C depending on the strain.

[g] isolates NS244 and NS474 gave an unusual *emm* pattern, in that PCR product resulted only from reaction with primer set 2, rather than sets 1, 2, and 3; or set 1 only as for traditional *emm* pattern ABC.

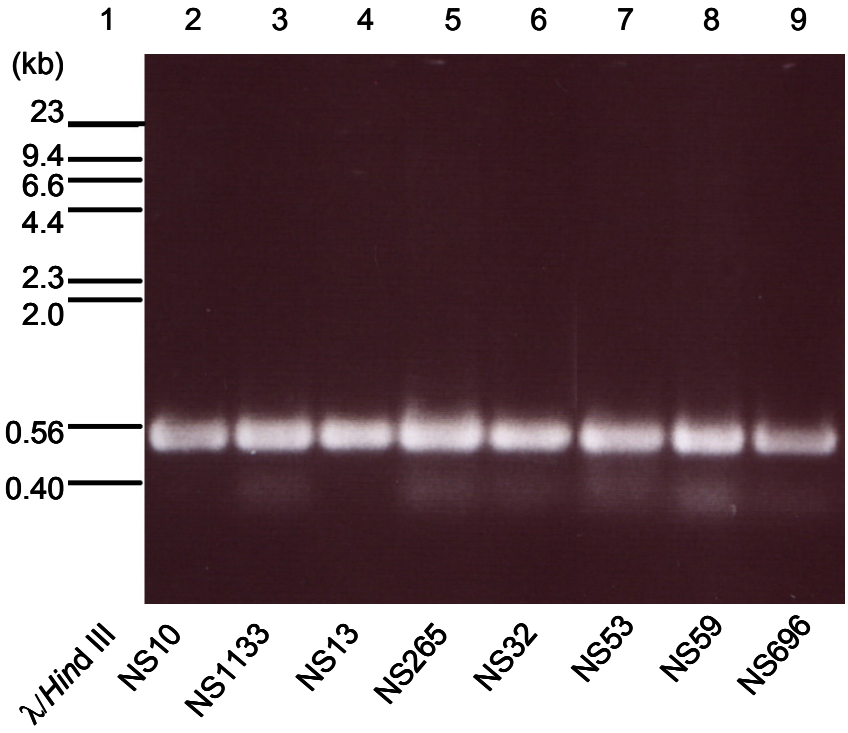

Figure 2.1. Fragment of the *sen* gene amplified from genomic DNA of a representative subset of NT GAS isolates using primers SENF1 and SENR1 and visualised by agarose gel electrophoresis. Lane 1: λ DNA digested with *Hin*d III (Gibco Life Technologies); Lanes 2-9: *sen* fragments amplified from specific GAS isolates with designations shown.

Table 2.3. Translated DNA sequence encoding the carboxy-terminal end of SEN from 29 NT GAS isolates[a].

Isolate	Amino acid sequence encoded by the carboxy-terminal end of *sen* gene
NS192	**N-**…LGKRVQ LVGDDFFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS210	**N-**…LVGDDFFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS414	**N-**…LGKRVQ LVGDDFFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS452	**N-**…GDDFFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS501	**N-**…VLTERLGKRVQ LVGDDFFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS730	**N-**…LVGDDFFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS733	**N-**…VLTERLGKRVQ LVGDDFFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS931	**N-**…ERLGKRVQ LVGDDFFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS179	**N-**…FFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS13	**N-**…TERLGKRVQ LVGDDFFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS88.2	**N-**…EYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS223	**N-**…VLTERLGKRVQ LVGDDFFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS455	**N-**…VLTERLGKRVQ LVGDDFFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS1133	**N-**…ERLGKRVQ LVGDDFFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS14	**N-**…LTERLGKRVQ LVGDDFFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS236	**N-**…LTERLGKRVQ LVGDDFFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS297	**N-**…KRVQ LVGDDFFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS474	**N-**…RVQ LVGDDFFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS488	**N-**…LGKRVQ LVGDDFFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS10	**N-**…LVGDDFFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS32	**N-**…LTERLGKRVQ LVGDDFFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS50.1	**N-**…LTERLGKRVQ LVGDDFFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS53	**N-**…ERLGKRVQ LVGDDFFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS59	**N-**…ERLGKRVQ LVGDDFFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS253	**N-**…VLTERLGKRVQ LVGDDFFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS265	**N-**…ERLGKRVQ LVGDDFFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS696	**N-**…ERLGKRVQ LVGDDFFVTNTEYLAR GIKENAANSILIKVNQ IGTLTETFEAIEMAKE AGYTAVVSHRSGETED STIADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS244	**N-**…ADIAVATNAGQIK TGSLSRTDRI AKYNQLLRIE DQLGEVAQYK GIKSFYNLKK-**C**
NS836	**N-**…QLGEVAQYK GIKSFYNLKK-**C**

[a]amino (N-…) and carboxy (-C) terminal ends of predicted amino acid sequence indicated

Of the 14 isolates from invasive disease cases, 5 were *pam*-positive, while 7 of the 15 isolates from uncomplicated infections were *pam*-positive. *Pam*-genotype of the 29 NT isolates is given in Table 2.2.

2.3.4 SEN expression

2.3.4.1 Generation of a polyclonal antibody against SEN

Antiserum was raised against a 12-mer peptide representing the C-terminal end of the SEN protein encompassing the plasminogen-binding lysine residues K_{434}-K_{435} (Derbise et al., 2004). This antiserum possessed a titre of approximately 60,000 using a definition of seropositivity of A_{490} = 0.1 (Figure 2.2A). Protein G purification of the serum yielded bands of the expected molecular weight for IgG as determined by reducing and non-reducing SDS-PAGE (Figure 2.2B and C) and maintained reactivity against the SEN 12-mer.

2.3.4.2 SEN localisation in GAS cell-wall extracts

Cell-surface expression of SEN was investigated in NT GAS isolates by immunolotting of mutanolysin cell-wall extracts with the anti-SEN peptide antibody described above (Ji et al., 1998). A 47 kDa species was recognised by the anti-SEN antibody in extracts of all 29 NT GAS isolates (Figure 2.3). The discrepancy between the originally reported molecular mass of SEN (45 kDa) and the size of the protein detected in NT GAS isolates may reflect technical differences such as the use of non-reducing SDS-PAGE in this study (Pancholi and Fischetti, 1998). However the molecular mass of SEN predicted from protein sequence databases is 47 kDa (Cole et al., 2005).

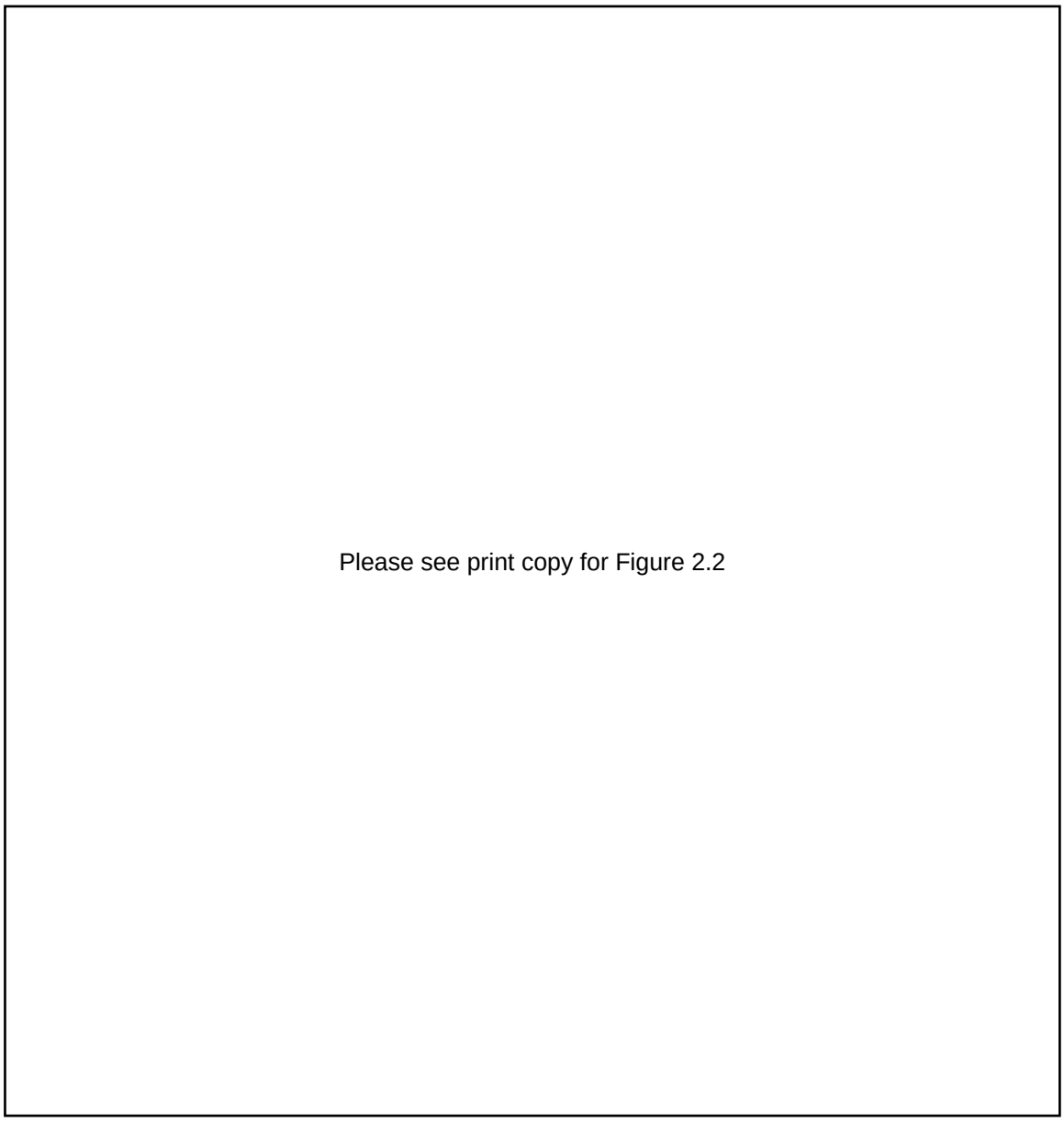

Figure 2.2. Characterisation and purification of a polyclonal antibody raised against a peptide representing the 12 C-terminal amino acid residues of SEN. **(A).** ELISA showing reactivity of antiserum to the 12-mer peptide at successive test bleeds after immunisation (immune 1, 2) compared to preimmune serum. **(B).** Chromatograph of protein G purification of the polyclonal antibody at 280 nm with glycine elution indicated by a sharp peak. Absorbance was measured using the Econo™ UV monitor (BioRad) and is shown in relative units (%). **(C).** SDS-PAGE of protein G-purified antibody preparation under reducing (R) and non-reducing (NR) conditions, showing expected apparent molecular weights (~ 150 kDa for NR conditions; ~ 50 kDa and ~25 kDa for the heavy and light chains respectively under R conditions) (Harlow and Lane, 1988). Migration of high and low range unstained molecular weight standards (BioRad) is shown.

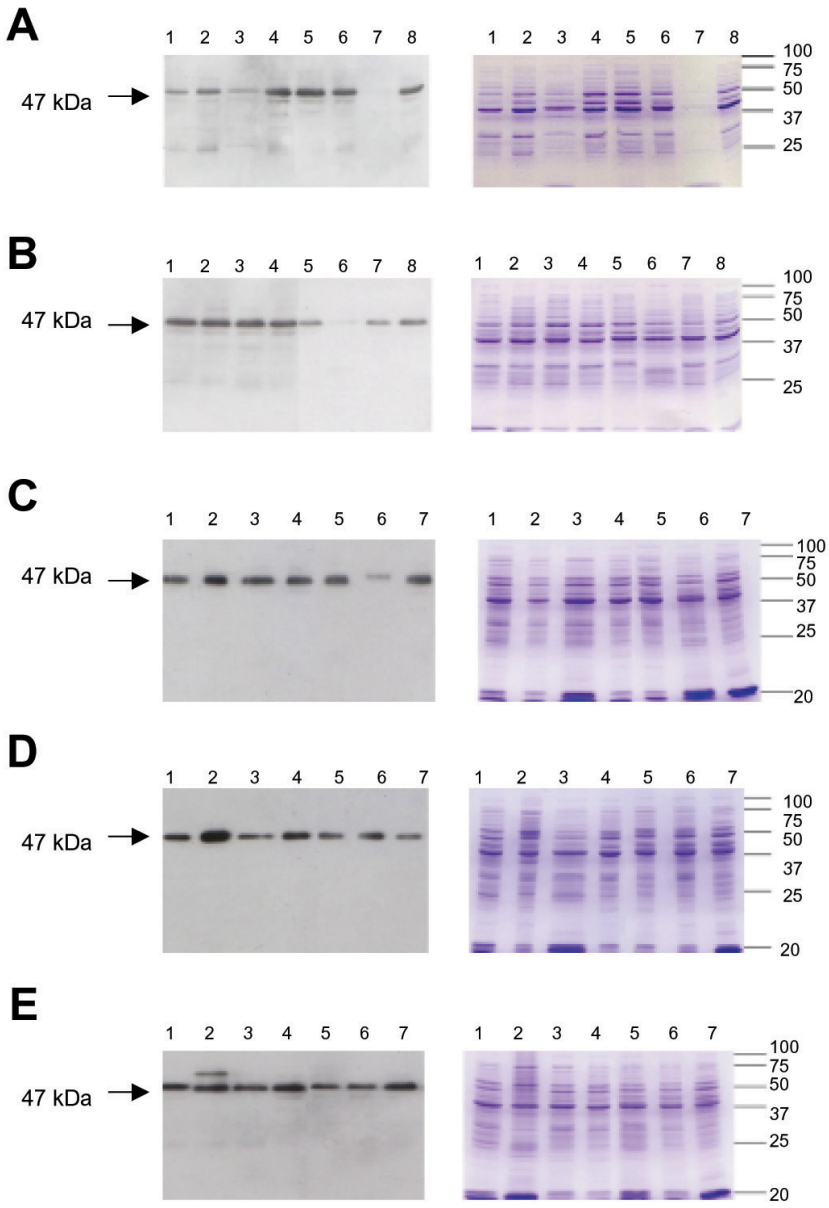

Figure 2.3. Detection of SEN in mutanolysin cell wall extracts of the 29 NT GAS isolates. Immunoblot of mutanolysin cell wall extracts showing a 47 kDa band recognised by a polyclonal antibody raised against the C-terminal 12 amino acid residues of the SEN protein (left). Corresponding 12% non-reducing SDS-PAGE analysis of mutanolysin extracts stained with Coomassie Blue (right). Each lane depicts an extract from an individual isolate with lanes 1 - 4 showing *pam*-negative isolates and lane 5 - end showing *pam*-positive isolates. Isolates NS452 and NS13 were included in the 4th and final lanes respectively of each gel and blot as controls. Protein bands were compared to molecular weight markers (Precision® Broad-range Standards, Bio-Rad). **(A).** Lanes 1 – 8 showing isolates NS14, NS236, NS192, NS452, NS10, NS32, NS88.2 and NS13 respectively. **(B).** Lanes 1 – 8 showing isolates NS210, NS414, NS501, NS452, NS223, NS455, NS1133, and NS13 respectively. **(C).** Lanes 1 – 7 showing isolates NS730, NS733, NS931, NS452, NS50.1, NS53 and NS13 respectively. **(D).** Lanes 1 – 7 showing isolates NS244, NS297, NS179, NS452, NS59, NS253 and NS13 respectively. **(E).** Lanes 1 – 7 showing isolates NS474, NS488, NS836, NS452, NS265, NS696 and NS13 respectively.

2.3.5 Plasminogen binding by GAS

2.3.5.1 Plasminogen purification

Purification of human plasminogen by lysine-sepharose affinity chromatography and its characterisation by SDS-PAGE analysis and specific chromogenic assay is shown in Figure 2.4 (Deutsch and Mertz, 1970). The chromatograph shows elution of purified plasminogen with the lysine analogue, εACA (Figure 2.4A). The molecular weight of purified plasminogen determined by SDS-PAGE analysis approximated that expected (92 kDa), with reducing SDS-PAGE consistently yielding slightly higher apparent molecular weights for plasminogen (97 kDa) than non-reducing SDS-PAGE (90 kDa) (Figure 2.4B). The disparity is likely to result from disruption of the heavily disulfide-bonded structure of the protein under reducing conditions. The dual protein bands in the purified preparation reflect the presence of the two glycoforms of plasminogen in human plasma. Disruption of the disulfide bonds in plasmin under reducing conditions yields two bands representing the light (26 kDa) and heavy (70 kDa) chains of plasmin (reported molecular weights of these species are 25 kDa and 65 kDa respectively (Lahteenmaki et al., 2001)). The commercially available plasminogen control (Sigma) has a significantly lower apparent molecular weight (~ 10 kDa) than that of plasminogen purified from human plasma under non-reducing conditions. This lower molecular weight is similar to that of the plasmin control, possibly reflecting autocatalyic cleavage of the 8 kDa N-terminal peptide to yield Lys-plasminogen. Negligible plasmin activity in the purified plasminogen preparations was confirmed by plasmin assay using a specific chromogenic substrate, while preincubation with streptokinase yielded significant specific dose-dependent plasmin activity (Figure 2.4C).

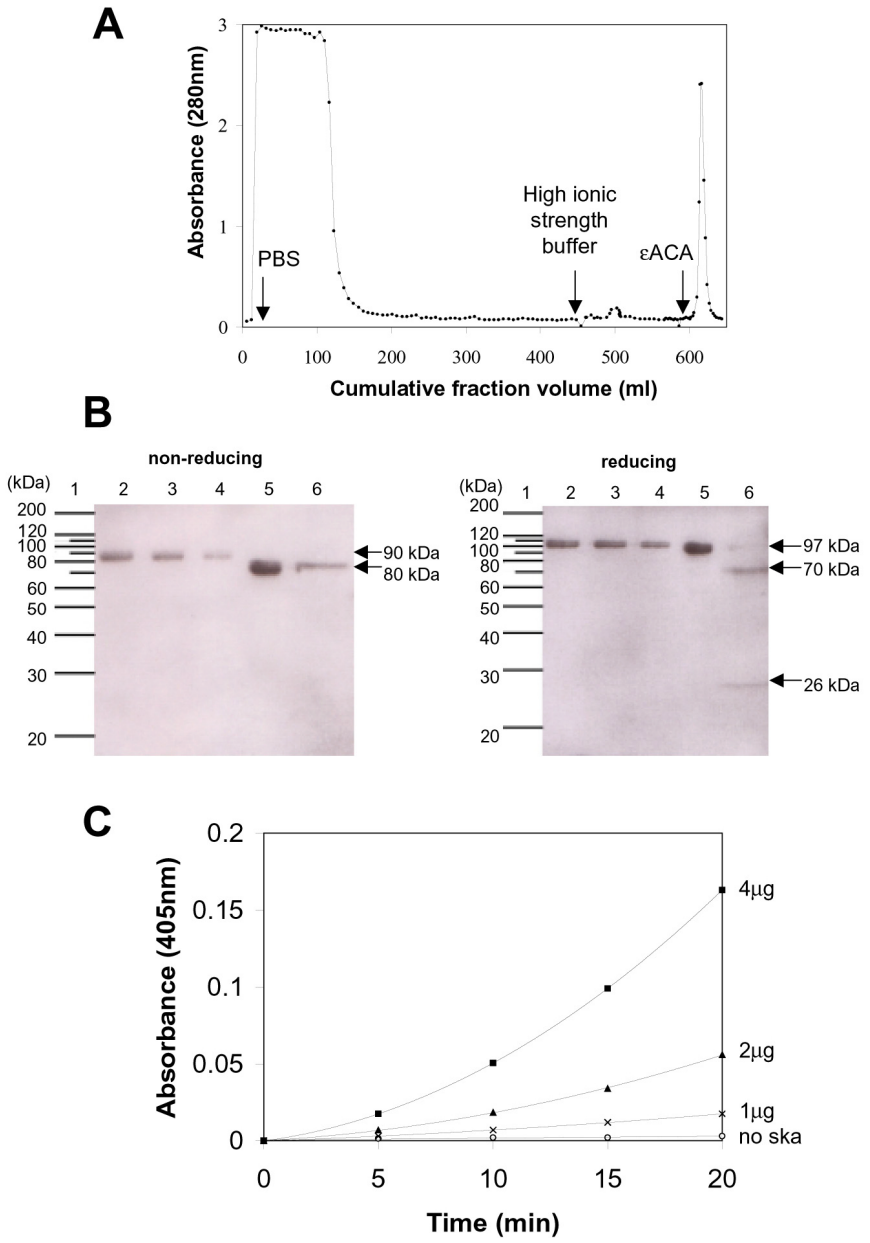

Figure 2.4 Purification and characterisation of plasminogen from human plasma. **(A).** Chromatograph of lysine-sepharose affinity purification of plasminogen at 280nm showing wash steps and elution with the lysine analogue, εACA. **(B).** SDS-PAGE of purified plasminogen under reducing and non-reducing conditions. Lane 1, 10 kDa protein ladder (Gibco Life Technologies) with markers for 110 kDa, 90 kDa, and 70 kDa unlabelled and indented; lanes 2-3, first and second elution fractions (elution peak excluding the tail of the peak); lane 4, pooled elution fractions from an independent plasminogen purification; lane 5, commercially available plasminogen preparation (Sigma); lane 6, plasmin (Roche). **(C).** Cleavage of plasmin-specific chromogenic substrate by purified plasminogen (1, 2 or 4 µg/well) following preincubation with streptokinase (25 units/well) in 150µl determined by kinetic assay at 405 nm. Negligible plasmin activity of 4 µg plasminogen in the absence of streptokinase (no ska) is shown.

2.3.5.2 Plasminogen binding

To investigate whether plasminogen binding is correlated with the invasive phenotype in NT isolates, isolates from invasive disease and uncomplicated infections were compared for this property. Binding by the direct and FSD pathways was measured (Figure 2.5; Table 2.2). In the presence of εACA, a competitive inhibitor of lysine-dependent binding, binding was reduced to 4% of input plasminogen (range 2-11%), except in isolate NS 88.2, which showed high level of lysine-independent binding of plasminogen (17%).

Presence of the *pam* gene had a significant positive effect on plasminogen binding by both the direct ($p \leq 0.031$) and FSD ($p \leq 0.005$) pathways. Invasive isolates bound more plasminogen directly than isolates from uncomplicated infections in the *pam*-positive group ($p \leq 0.048$). In the *pam*-negative group all of the isolates displayed minimal reactivity with plasminogen (7% mean) and there was no difference in direct plasminogen binding capacity between invasive and non-invasive isolates ($p \leq 0.120$).

In the presence of streptokinase and fibrinogen, isolates from invasive disease cases bound more plasminogen than isolates from uncomplicated infections ($p \leq 0.005$ for effect of invasive phenotype in 2-way ANOVA; $p \leq 0.005$ for effect of *pam* genotype). There was an absolute requirement for both fibrinogen and streptokinase to significantly increase plasminogen binding in a subset of isolates (Figure 2.6). The enhancement of plasminogen binding did not occur when streptokinase alone or fibrinogen alone was added ($p \geq 0.05$ for both conditions). Within small clusters of genetically indistinguishable isolates identified, invasive isolates bound more plasminogen than isolates from uncomplicated infection by both direct and FSD pathways in each case.

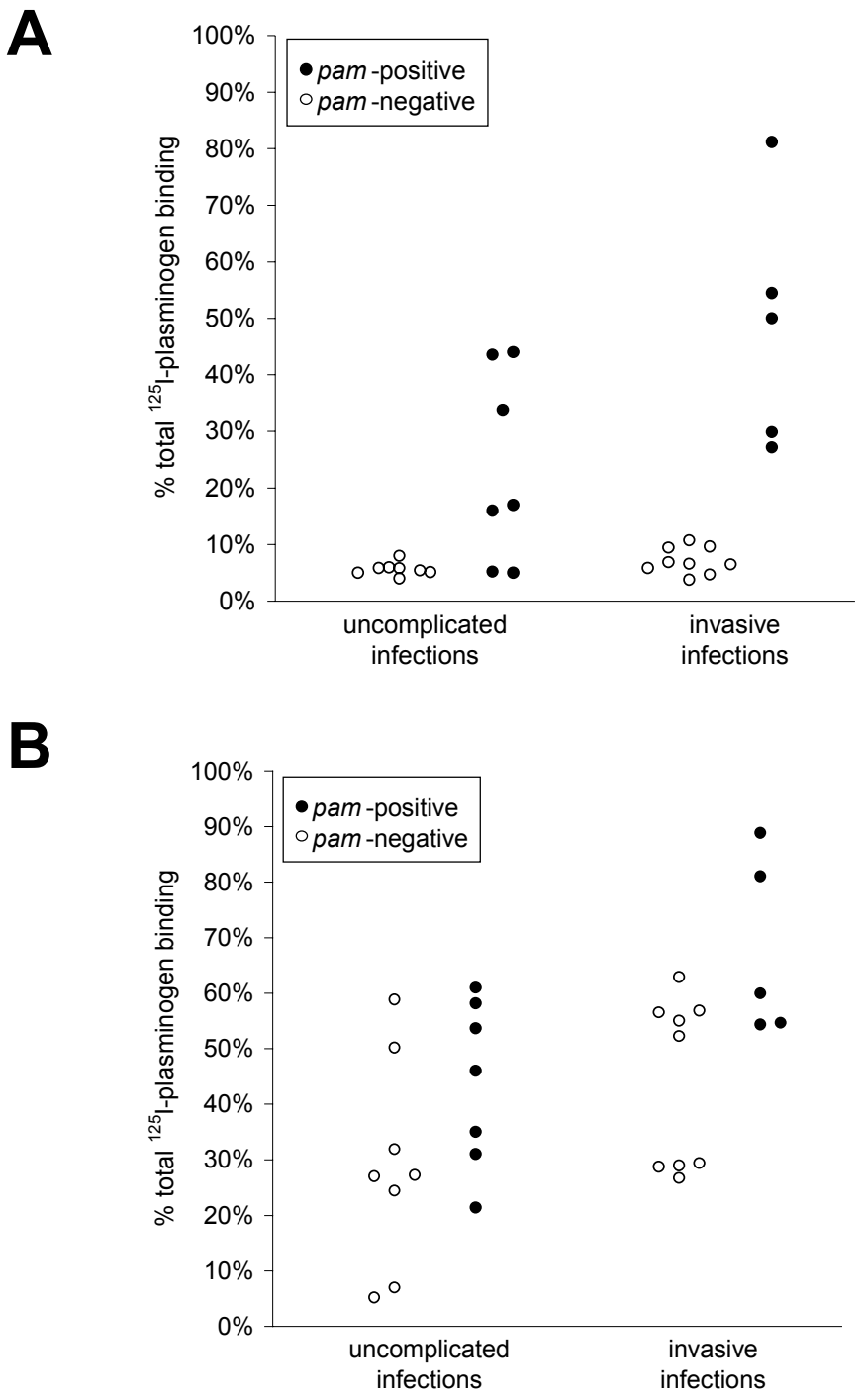

Figure 2.5. ^{125}I-plasminogen binding to *pam*-positive (●) and *pam*-negative (○) GAS isolates from invasive and uncomplicated infections. **(A).** Direct binding of ^{125}I-plasminogen. **(B).** Binding of ^{125}I-plasminogen in the presence of molar excesses of streptokinase and fibrinogen. All estimates were determined in triplicate, and the data are presented as mean values. Standard error of the mean was 0 – 6% of input plasminogen. (Table 2.2 shows values for individual isolates by isolate designation).

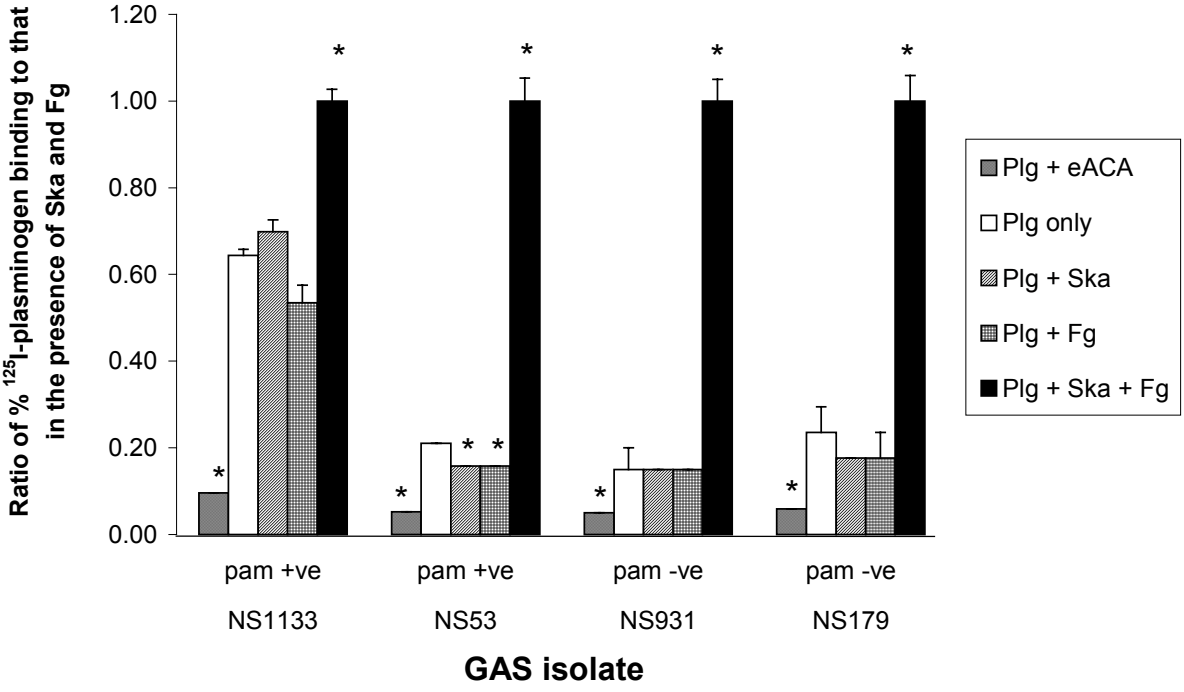

Figure 2.6. Binding of ^{125}I-plasminogen to selected *pam*-positive (pam +ve) and *pam*-negative (pam –ve) GAS isolates under different conditions. Binding is expressed as a ratio of that in the presence of streptokinase and fibrinogen for comparison of the relative effect of various conditions on individual isolates. ^{125}I-plasminogen binding was measured in the presence of the lysine analogue, εACA (Plg + eACA); in the presence of no other added reagents (Plg only); in the presence of streptokinase (Plg + Ska); in the presence of fibrinogen (Plg + Fg); and in the presence of streptokinase and fibrinogen (Plg + Ska + Fg). All reagents were added in molar excess relative to ^{125}I-plasminogen. All estimates were determined in triplicate and data are presented as mean $\pm$ SEM. Significant differences from the binding of ^{125}I-plasminogen alone (Plg only), as determined by unpaired *t* test, are shown (*).

2.3.5.3 Relationships between fibrinogen and plasminogen binding

The relationship between fibrinogen-binding and FSD plasminogen binding by the NT GAS isolates was investigated to determine whether the data is consistent with a model of capture of fibrinogen-streptokinase-plasminogen complexes by fibrinogen binding proteins (Christner et al., 1997; Wang et al., 1995a). Binding of fibrinogen to GAS isolates is shown in Figure 2.7. *Pam*-positive isolates bound more fibrinogen than *pam*-negative isolates ($p \leq 0.028$; Figure 2.7A). There was a correlation between fibrinogen binding and FSD plasminogen binding among *pam*-positive isolates ($r = 0.790$, $p \leq 0.002$; Figure 2.7A). By contrast *pam*-negative isolates do not show such correlation ($r = -0.341$, $p \leq 0.180$); nor was there a correlation between fibrinogen binding and the increase in plasminogen binding resulting from addition of streptokinase and fibrinogen ($r = -0.353$, $p \leq 0.164$)

In *pam*-positive isolates there was a strong correlation between direct plasminogen binding and FSD plasminogen binding ($r = 0.914$, $p \leq 0.0001$; Figure 2.7B), and between direct plasminogen binding and fibrinogen binding ($r = 0.811$, $p \leq 0.001$).

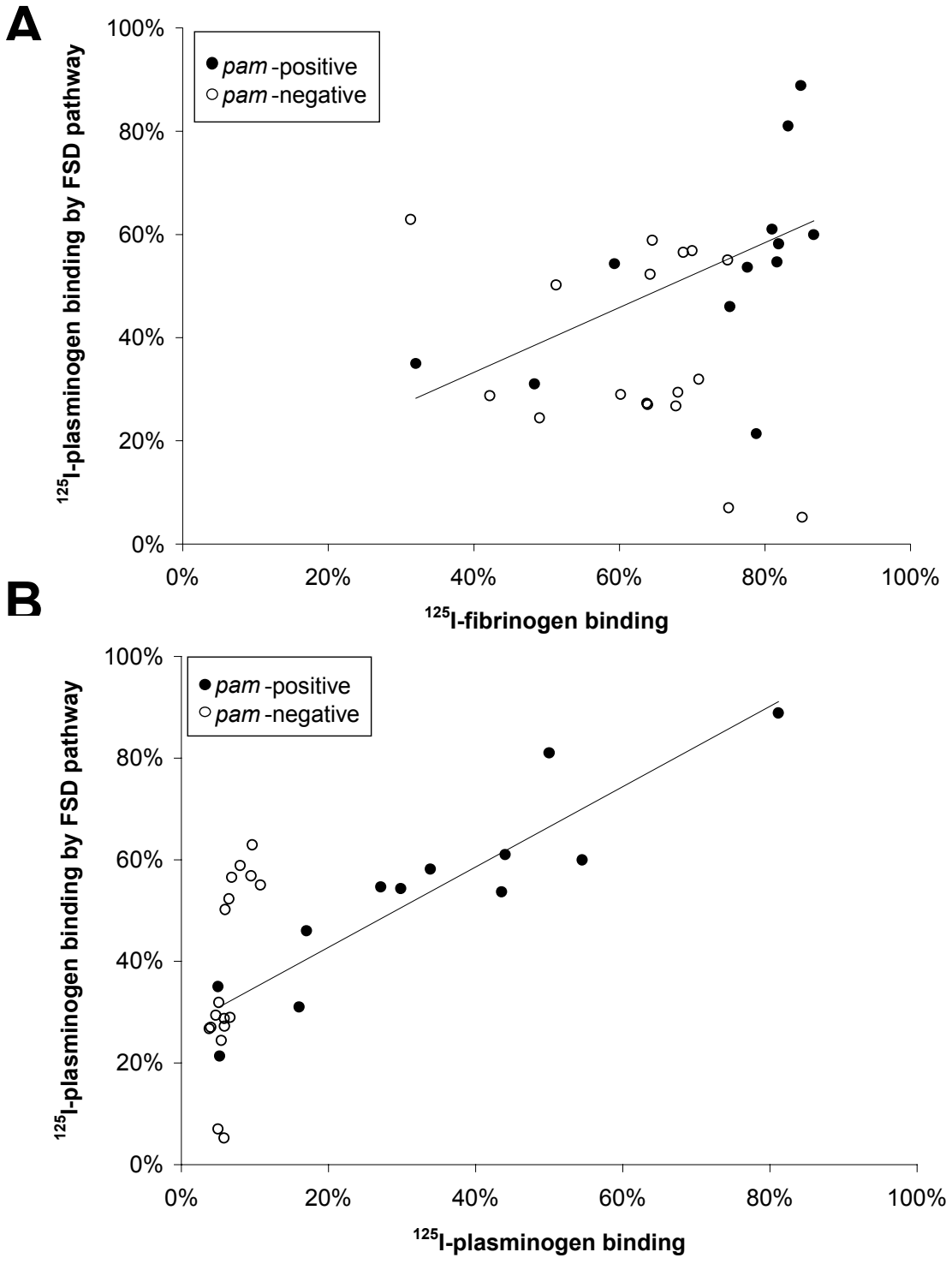

Figure 2.7. Comparison of ^{125}I-plasminogen binding to *pam*-positive (●) and *pam*-negative (○) GAS isolates in the presence of streptokinase and fibrinogen with ability of the isolates to bind ^{125}I-fibrinogen or ^{125}I-plasminogen directly. All estimates were determined in triplicate and data are presented as mean. Correlation curves are shown for relationships with significant correlation. **(A).** Comparison with binding of ^{125}I-fibrinogen by GAS isolates with a correlation curve fitted to *pam*-positive isolates ($r = 0.790$, $p \leq 0.002$). **(B).** Comparison with direct binding of ^{125}I-plasminogen by GAS isolates with a correlation curve fitted to *pam*-positive isolates ($r = 0.914$, $p \leq 0.0001$).

2.4 Discussion

Subversion of the host plasminogen system renders a pathogen capable of degrading ECM proteins and activating a cascade of metalloproteases, conferring the potential to invade host tissue barriers. An important role for plasminogen in the invasive process of GAS has been demonstrated in animal models (Li et al., 1999a; Sun et al., 2004; Svensson et al., 2002), but as yet no definitive epidemiological evidence has supported the hypothesis that the human plasminogen system plays a role in GAS invasive disease. To our knowledge this is the first report to demonstrate a significant relationship between acquisition of plasminogen by human clinical isolates from a range of *emm* sequence types of GAS and propensity to cause invasive diseases.

Of the 29 NT isolates studied, all possessed both the gene for the high-affinity plasminogen receptor SEN and the gene product in cell-wall extracts. Similarly, a previous study has demonstrated cell-surface expression of SEN in a variety of serotypes (Pancholi and Fischetti, 1998). Analysis of the cell-surface proteome of isolates NS13 and NS931 examined in this study demonstrates that SEN is immunoreactive when probed with serum from children living in a remote NT community. This suggests that the protein is expressed during infection *in vivo* (Cole et al., 2005).

Conservation of the sequence of SEN is consistent with its dual function as an essential glycolytic enzyme (Pancholi and Fischetti, 1998). The wildtype SEN protein possesses lysine residues in the C-terminal domain at positions 428, 434 and 435. Recombinant mutagenised forms of SEN generated by replacement of these C-terminal 1, 2 or 3

lysine residues with leucine showed complete loss of plasminogen-binding capacity (Derbise et al., 2004). In addition, an isogenic mutant of GAS expressing SEN with leucine replacement at residues 434 and 435 showed ~40% reduced Lys-plasminogen binding capacity. The retained plasminogen-binding capacity was attributed to either additional plasminogen receptors such as GAPDH or the presence of additional plasminogen-binding sites in surface-expressed SEN compared to the purified recombinant protein (Derbise et al., 2004). Interestingly, crystallographic and functional studies have demonstrated that the plasminogen-binding C-terminal lysine residues in enolase of *S. pneumoniae* are not surface-exposed in the octamer form, rather plasminogen-binding is mediated by an exposed internal 9-residue motif (Bergmann et al., 2005; Ehinger et al., 2004).

In addition to SEN, 12 isolates possessed the gene encoding the other known high-affinity plasminogen receptor, PAM. Expression of PAM in these isolates is currently under investigation by Martina Sanderson-Smith and was not investigated further here. However, the significantly higher Glu-plasminogen binding of *pam*-positive isolates is strongly suggestive of cell-surface expression of PAM (Svensson et al., 1999). Cell-surface expressed and immunoreactive M protein was previously identified in the proteome of the *pam*-positive isolate NS13 (Cole et al., 2005).

Amongst *pam*-positive isolates that are capable of binding plasminogen directly, invasive isolates bind more plasminogen both directly, and by a FSD pathway that is correlated with direct plasminogen binding. Within the small clusters of *pam*-positive isolates indistinguishable by the genotyping methods used (NS13, NS59 and NS10; NS455 and NS253; Table 2.2), invasive isolates always bound more plasminogen than

isolates from uncomplicated infection by both the direct and FSD pathways. Possible reasons for plasminogen binding differences in these clusters might include variation in expression levels or differential post-translational processing or degradation. For *pam*-negative isolates that do not bind substantial amounts of plasminogen directly, invasive isolates bind more plasminogen by the FSD pathway. Together these results suggest that plasminogen acquisition may be an important virulence determinant of GAS. The diversity of *emm* sequence types represented in this collection of isolates suggests that these relationships between plasminogen binding and invasive capacity do not depend on a small number of dominant clones.

The extent of direct plasminogen binding (4-81%) in this study is similar to that found in a previous study (9-69%) (Ullberg et al., 1989). The fact that *pam*-negative isolates show only moderate reactivity with plasminogen despite the presence of the gene for SEN suggests that SEN may not play a major role in acquisition of Glu-plasminogen by these isolates. This is consistent with the reported lower affinity of SEN for Glu-plasminogen (Derbise et al., 2004). GAPDH has no affinity for Glu-plasminogen and would not be expected to mediate any of the direct plasminogen binding observed in this study (Winram and Lottenberg, 1998). Similarly, a previous study demonstrated that ~80% of *pam*-negative isolates bound ≤ 5% of plasminogen, while 70% of *pam*-positive isolates bound ≥ 50% of plasminogen (Svensson et al., 1999). The data from these two isolate collections suggests that PAM is necessary for acquisition of Glu-plasminogen by the direct pathway.

Isolates from invasive disease cases bind significantly more plasminogen by the FSD pathway than isolates from uncomplicated infections. Plasminogen acquisition by the

indirect pathway may therefore be a determinant for the invasive propensity. Plasmin activity owing to the FSD pathway is resistant to inhibition by plasma proteins, and has a half-life of over 4 h as opposed to 20 min for plasmin activity owing to direct plasminogen binding (Wang et al., 1995a). The plasminogen-activation function of the complex also generates plasmin which can bind to surface receptors despite the presence of host physiological inhibitors (D'Costa and Boyle, 1998). An animal model showed a correlation between virulence and acquisition of this activator activity specifically, as opposed to plasmin activity *per se* (Li et al., 1999a). The significant enhancement of plasminogen binding has an absolute requirement for both fibrinogen and streptokinase, consistent with earlier reports supporting the role of streptokinase as a GAS cell-surface plasminogen binding cofactor (Wang et al., 1995a; Wang et al., 1995b).

Thus it must be emphasised that while SEN does not mediate direct binding of Glu-plasminogen, it may contribute significantly to the plasminogen binding observed in this study in the presence of fibrinogen and streptokinase. Plasminogen activation at the GAS cell-surface and in solution would be expected to yield modified forms of plasmin(ogen) such as Lys-plasminogen, for which SEN has higher affinity. Direct binding of Lys-plasminogen and Lys-plasmin to clinical isolates of GAS which do not bind Glu-plasminogen directly has been demonstrated (Derbise et al., 2004; Wang et al., 1994).

The difference in fibrinogen binding between *pam*-positive and *pam*-negative strains, correlation between fibrinogen and PAM-mediated plasminogen binding, and further enhancement of plasminogen binding by a combination of streptokinase and fibrinogen

in *pam*-positive isolates point to the occurrence of another receptor concomitant with PAM. Alternatively, PAM itself may be endowed with dual receptor activity. In contrast to *pam*-positive isolates, neither the basal levels of plasminogen binding, nor the enhancement of the binding by the FSD pathway was correlated with fibrinogen binding to the surface of the *pam*-negative isolates. This underscores the different mechanisms of plasminogen binding between *pam*-positive and *pam*-negative GAS strains.

In summary, these results demonstrate that GAS isolates from NT invasive disease cases belonging to a wide range of *emm* sequence types acquire more plasminogen than isolates from uncomplicated infections by a pathway requiring fibrinogen and streptokinase as cofactors. Among isolates containing the gene encoding PAM, invasive isolates also bind more plasminogen directly. The functional implications of higher plasminogen acquisition by invasive GAS isolates were investigated in a study described in the following chapter.

CHAPTER 3 Plasminogen activation and virulence of group A streptococcal isolates from the Northern Territory of Australia

Cole, J.N., McArthur, J.D., McKay, F.C., Sanderson-Smith, M.L., Cork, A.J., Ranson, M., Rohde, M., Itzek, A., Sun, H., Ginsburg,D., Kotb, M., Nizet, V., Chhatwal, G.S. and Walker, M.J. (2006). Trigger for group A streptococcal M1T1 invasive disease FASEB Journal *(accepted 31 Mar 2006).*

3.1 Introduction

The importance of the human plasminogen system in GAS invasive disease in the NT of Australia is suggested by the finding that invasive disease isolates acquire higher levels of plasminogen than isolates from uncomplicated infection (McKay et al., 2004). This chapter characterises plasminogen activation by the same collection of NT GAS isolates *in vitro* and the relationship to GAS virulence using a humanized plasminogen transgenic murine model of GAS infection.

An important component of plasminogen activation by GAS is the secretion of the plasminogen binding co-factor and activator, streptokinase (Svensson et al., 2002; Wang et al., 1995a; Wang et al., 1995b). Streptokinase is critical for full virulence in GAS skin infection (Svensson et al., 2002) and for the establishment of invasive infection in animal models (Sun et al., 2004). In human infection, isolates from severe invasive disease express higher levels of streptokinase at the transcriptional level than those from non-invasive disease within a clonal *emm*49 isolate collection (Ikebe et al., 2005). In this study, secretion of streptokinase by NT GAS isolates from invasive disease and uncomplicated infection was compared.

Streptokinase activity of GAS supernatants varies with streptokinase genotype of GAS (Tewodros et al., 1995), which is characterized according to a hypervariable genetic region encoding amino acid residues 173 - 316 that represents the plasminogen-docking β domain of the streptokinase protein (Chaudhary et al., 1999; Loy et al., 2001; Musser et al., 1994). Specific allelic variants of streptokinase are coinherited with the plasminogen receptor PAM, a marker for skin tropism amongst GAS isolates (Kalia and

Bessen, 2004). Allelic variation of streptokinase was investigated in this NT isolate collection, and its relationship to plasminogen activation by GAS was investigated.

The potent streptococcal cysteine protease SpeB degrades most of the components of the GAS plasminogen activation system, including surface-bound plasminogen and plasmin, streptokinase (Rezcallah et al., 2004), fibrinogen (Matsuka et al., 1999) and fibrinogen-binding proteins such as M1 (Raeder et al., 1998) and protein F (Nyberg et al., 2004). SpeB is downregulated in invasive GAS variants and its expression is inversely correlated with severity of invasive disease (Aziz et al., 2004; Kansal et al., 2000; Raeder et al., 2000). However, synergy of the two proteolytic systems has also been postulated (Svensson et al., 2002) and the relationship between GAS plasminogen activation and SpeB remains unclear. Secretion of SpeB by NT GAS isolates from invasive disease and uncomplicated infection was compared in this study and the effect of SpeB on GAS plasminogen acquisition was characterised.

Animal models of infection suggest an important role for human plasminogen activation in the invasive process during GAS infection (Khil et al., 2003; Li et al., 1999a; Sun et al., 2004; Svensson et al., 2002). To our knowledge, only one study of human clinical isolates of GAS has examined the relationship between plasmin acquisition and invasive capacity. Invasive disease isolates acquired higher average levels of plasmin when incubated in human plasma than isolates from uncomplicated throat infections, although this difference did not reach significance (Wang et al., 1994). However, overall proteolytic activity of human isolates has been associated with clinical signs of invasion (Hsueh et al., 1998; Talkington et al., 1993). Given that invasive disease isolates of NT GAS bind more plasminogen than isolates from uncomplicated infection, it was

hypothesised that invasive disease isolates would also be capable of higher plasmin acquisition *in vitro*. The ability of NT GAS isolates to acquire cell-surface plasmin activity following incubation in human plasma was investigated. To further characterise the role of streptokinase and SpeB in acquisition of cell-surface plasmin activity in human plasma, acquisition by GAS isogenic deletion mutants for genes encoding streptokinase and SpeB and the corresponding wildtype strains was compared.

The second approach to addressing the role of plasminogen in GAS invasive disease was to examine GAS infection using the human plasminogen transgenic mouse model. A subset of NT GAS isolates selected for different *in vitro* plasminogen activation characteristics was tested for virulence in this model.

3.2 Materials and Methods

3.2.1 DNA methods

3.2.1.1 DNA amplification and sequence analysis

PCR amplification of fragments of the streptokinase (*ska*) and SpeB (*speB*) genes and DNA sequence analysis of *ska* alleles was performed as described in Chapter 2 using primers in Table 3.1. A phylogenetic tree was constructed for the 423 bp nucleotide sequence encoding the variable β-domain of the streptokinase protein of the 29 NT isolates. The tree was constructed with MEGA version 3.1 (Kumar et al., 2004) using the neighbour-joining method with Kimura two-parameter distance measure as previously described for streptokinase partial alleles (Kalia and Bessen, 2004). DNA partial *ska* alleles were translated using Entigen (BioManager by ANGIS; www.angis.org.au).

Table 3.1. Oligonucleotide primers used for DNA analysis

Please see print copy for Table 3.1

[a] (Musser et al., 1994)

3.2.2 GAS assay methods

3.2.2.1 Source of GAS isolates

In addition to the NT isolates described in the previous chapter, North American GAS isolates and their isogenic mutants were used in this study to investigate mechanistic aspects of GAS plasmin acquisition. GAS isolate ALAB49 and its isogenic mutants were a kind gift from Dr Ulf Sjobring and Dr Ulrika Ringdahl (Department of Laboratory Medicine, Section for Microbiology, Immunology and Glycobiology, Lund University, Lund, Sweden). GAS isolate 5448 and its isogenic *speB*-negative mutant, 5448ΔspeB were a kind gift from Dr Victor Nizet (Department of Pediatrics, University of California, San Diego, California, USA) and Dr Malak Kotb (Veterans Affairs Medical Centre, Research Service, Memphis, Tennessee, USA). The wildtype ALAB49 isolate was included with NT isolates in assays of supernatant streptokinase and SpeB and cell-surface plasmin acquisition by GAS.

3.2.2.2 GAS culture

GAS were cultured as previously described in THY. Streptococcal supernatants were harvested for analysis by centrifugation at 400 x *g* and filtration using a 0.2 µm PVDF syringe filter (Millipore, Billerica, Massachusetts, USA) and stored at -70°C until analysis.

3.2.2.3 GAS supernatant speB assays

Overnight 12.5 ml GAS cultures were initiated with 250 µl of a 6 h starter culture (2 ml). GAS were grown as stationary cultures for 18 h to achieve stationary phase confirmed by monitoring at A_{600} in the final hour. Supernatants were harvested and stored as described above. For SDS-PAGE, supernatants were boiled in reducing

sample buffer for 5 min. Western transfer and immunoblotting was performed as previously described in section 2.2.1.2 using an affinity-purified rabbit anti-SpeB antibody with purified SpeB protein as a positive control (both from Toxin Technology, Sarasota, Florida, USA).

Cysteine protease activity was measured in GAS supernatants using the chromogenic substrate N-benzoyl-Pro-Phe-Arg-*p*-nitroanilide-hydrochloride (Sigma) as described previously (Hytonen et al., 2001; North, 1994; Raeder et al., 1998). SpeB in GAS supernatants or purified SpeB standards diluted in THY (20 µl) were activated by reduction in a final concentration of 1 mM dithiothreitol (DTT) in PBS for 30 min at 37°C in a total volume of 140 µl. Substrate (20 µl of 2.6 mM) was added and the reaction allowed to proceed at 37°C for 60 min with determination of A_{405} at 5 min intervals. V_{max} was calculated and compared to a standard curve of SpeB using Softmax® Pro. All reactions were performed in triplicate in the presence and absence of the cysteine protease inhibitor, trans-epoxysuccinyl-l-leucylamido-(4-guanidino)butane (E64; 1mM; Sigma) to confirm that activity was attributable to cysteine protease. E64 is a highly selective inhibitor of cysteine proteases that irreversibly inhibits an active thiol group and does not inhibit serine proteases apart from trypsin (Barrett et al., 1982; Katunuma and Kominami, 1995; Sreedharan et al., 1996).

3.2.2.4 GAS supernatant streptokinase assays

GAS were cultured at 37°C in 17.5 ml THY inoculated with 0.25 ml of a 2 ml overnight starter culture. GAS were cultured with shaking for streptokinase assay as described previously (Tewodros et al., 1995) at 150 rpm. Supernatants were collected from

triplicate cultures of 29 NT GAS isolates and ALAB49 at both mid-log (A_{600} = 0.6) and late-log (A_{600} = 1.1) phases of growth.

Streptokinase expression was analysed by Western blot using rabbit immune serum raised against group C streptokinase (Sigma). Polyclonal antibody generation and immunoblotting were performed by Dr Jason McArthur, University of Wollongong. Streptokinase activity was determined indirectly by chromogenic plasmin activity assay of GAS supernatants preincubated with plasminogen. Briefly, supernatants were defrosted on ice and a 20 µl aliquot incubated at 37°C with 100 µl 50 mM Tris, pH 7.5, containing 10 µg/ml plasminogen for 15 min. Spectrozyme® PL (20 µl of 2.5 mM) was added and V_{max} determined by kinetic assay and compared to a standard curve of group C streptokinase (Sigma) using Softmax® Pro. All reactions were performed in triplicate in the presence and absence of plasminogen to confirm that proteolytic activity of supernatants was attributable to plasminogen activation.

3.2.2.5 Concentration of supernatant proteins

GAS supernatants (750 µl) were combined with an equal volume of 10% (v/v) trichloroacetic acid, vortexed briefly and incubated on ice for 30 min. The precipitated proteins were pelleted by centrifugation at 15,000 x *g* for 30 min, the supernatant decanted and the pellet washed with ice-cold 100% ethanol. The mixture was centrifuged and decanted as above and the pellet air-dried for 15 min. Proteins were resuspended in 20 µl of 100 mM Tris, pH 7.5.

3.2.2.6 Plasminogen binding by North American GAS isolates

Plasminogen binding by North American GAS isolates ALAB49 and 5448 and their isogenic mutants was determined as described in section 2.2.3.5 by Prof Mark Walker and Dr Andreas Itzek at the German Research Centre for Biotechnology (GBF), Braunschweig, Germany.

3.2.2.7 Cell-surface plasmin activity assay

Plasmin acquisition in human plasma by GAS isolates was determined using a protocol modified from that described previously (Wang et al., 1994). Frozen plasma was purchased from the Red Cross Blood Bank (Sydney, NSW, Australia), defrosted on ice and pooled. Aliquots of pooled plasma were depleted of plasminogen by incubation at 4°C on ice with excess lysine-sepharose® 4B for 1-2 h with gentle agitation. The extent of plasminogen depletion and the plasmin activity of plasma was determined by chromogenic assay with Spectrozyme® PL in the presence and absence of streptokinase, and by Western blot using a polyclonal rabbit anti-human plasminogen antibody (Calbiochem). Separate aliquots of pooled 100% plasma were pretreated with excess tPA inhibitor, D-Phe-Pro-Arg-chloromethylketone dihydrochloride (PPACK; Calbiochem), at a final concentration of 10 μM immediately prior to assay by preincubation for 1 h on ice with gentle agitation (Kettner and Shaw, 1981; Kunitada et al., 1992; Mohler et al., 1986).

GAS were cultured overnight as stationary cultures in 25 ml THY inoculated with a single colony. GAS were pelleted by centrifugation at 800 x *g*, washed in 50 ml of PBS, pH 7.4 prewarmed to 37°C, and resuspended to A_{600} = 0.7 (corresponding to log phase, in which streptokinase secretion is induced). A 2 ml aliquot of this suspension

was pelleted as above, and resuspended in an equal volume of 100% plasma or plasminogen-depleted plasma at 37°C. GAS were incubated in plasma for 3 h at 37°C, pelleted by centrifugation and washed twice with 1 volume of ice-cold 0.01 M EDTA, 0.1% gelatin in PBS, pH 7.4. GAS were resuspended in 0.1% gelatin in PBS, pH 7.4 to A_{600} = 0.75. Aliquots (100 µl) of this suspension were incubated in triplicate in the presence and absence of 20 µl Spectrozyme® PL, 2.5 mM at 37°C for 60 min in a 96-well plate. The reaction was quenched with 80 µl of 1.75 M acetic acid, the plates centrifuged at 800 x *g* and A_{405} of supernatants determined.

Plasmin activity was determined as the difference between A_{405} in the presence and absence of substrate, thus accounting for differences in the sedimentation efficiency of GAS isolates. Isolate NS931 was included as an internal assay control in every experiment. Each isolate was assayed in at least 2 independent experiments (at least 3 for all NT isolates). Plasmin equivalents and the linear range of the assay (A_{405} = 0 – 0.6) were determined using a standard curve of purified plasmin (Roche Diagnostics, GmbH, Mannheim, Germany).

3.2.3 Infection studies using a human plasminogen transgenic mouse

Male human plasminogen transgenic mice (*Alb*PLG1) were a kind gift from Dr Hongmin Sun and Prof David Ginsburg (University of Michigan, Ann Arbor, Michigan, USA). *Alb*PLG1 differ from the parental strain C57BL/6J by the expression of one copy of the gene for human plasminogen (PLG) under control of the mouse albumin gene regulatory sequences (Sun et al., 2004).

*Alb*PLG1 human plasminogen transgenic mice (Tg+) (Sun et al., 2004) and transgene-negative litter mate controls (Tg-) were infected with GAS isolates at 6-8 weeks of age by subcutaneous injection into the right flank with 100 µl of GAS harvested at mid-log phase (A_{600} = 0.5 in THY) and resuspended in 0.7% saline. Where possible, the dose of GAS required to induce ≥ 40% mortality in wildtype C57BL/6J mice over 14 days was determined experimentally. For isolates NS88.2 and NS696 these doses were 6.4 x 10^6 and 7.4 x 10^6 colony-forming units (cfu) respectively. This dose was used for subsequent infection studies in *Alb*PLG1 transgenic mice. The resistance of mice to skin infection with human pathogenic GAS (Li et al., 1999a) was such that it was not possible to find a lethal dose for some isolates in wildtype mice in this study. In such cases the maximum dose of GAS administrable in 100 µl injection volume was used for infection studies (2.2 x 10^8 and 3.7x 10^8 cfu for isolates NS730 and ALAB49, respectively). Infection studies for each isolate were conducted using 10 *Alb*PLG1 transgenic mice and 10 wildtype littermates and survival was monitored over 14 days. Animal experiments were conducted by Dr Jason McArthur according to the Guidelines for the Care and Use of Laboratory Animals (National Health and Medical Research Council, Australia) and were approved by the University of Wollongong Animal Ethics Committee.

3.2.4 Statistical methods

Enzyme activities were analysed for differences between *pam* genotype and clinical source using two-way analysis of variance (ANOVA). Where data or transformed data did not fit the assumptions for two-way ANOVA, an unpaired *t* test, Welch ANOVA (unequal variances) or Wilcoxan/Kruskal-Wallis Rank Sum test (non-parametric) was used to compare groups separately. Differences in enzyme activities between individual

isolates were determined by unpaired *t* test or Welch ANOVA (raw or transformed data according to test assumptions). Effects of assay conditions were tested by two-way ANOVA as described above or by paired *t* test. Relationships between enzyme activities of the isolates were determined using Pearson's (parametric) or Spearman's rank (non-parametric) correlation. Equality of variances was tested using the O'Brien, Bartlett, Levene and Brown-Forsythe tests and Normality of data tested using the Shapiro-Wilk W test. These tests were performed using JMP (SAS Institute Inc., Cary, North Carolina, USA). Differences in survival of wildtype and transgenic mice was determined by logrank test using GraphPad Prism (GraphPad Software Inc., San Diego, California, USA).

3.3 Results

3.3.1 Streptokinase secretion by GAS isolates

3.3.1.1 Kinetics of streptokinase secretion by GAS isolates

The kinetics of secretion of streptokinase by GAS isolates was measured by determination of streptokinase activity over time in culture supernatants. Supernatant streptokinase activity was measured for two isolates (duplicate cultures) throughout the growth curve to determine the appropriate time at which to sample the entire NT isolate collection. Contrary to a previous analysis (Tewodros et al., 1995), growth rates of NT isolates differed significantly (see Figure 3.1 for examples), suggesting a standardised time-point analysis would be inappropriate for this collection. In addition, despite very similar growth kinetics for duplicate cultures of NS931, there was considerable variation in streptokinase activity (Figure 3.1B). Thus cultures were sampled for streptokinase activity at optical densities of $A_{600} = 0.6$ and $A_{600} = 1.1$ to reflect mid- and late-log phases of growth respectively and triplicate cultures were sampled for accurate estimation of average streptokinase activity. Culture densities for each corresponding supernatant collected are given in Appendix 2.

3.3.1.2 Streptokinase activity in NT isolates

Streptokinase activity was assessed for 29 NT GAS isolates and the impetigo isolate, ALAB49. The results demonstrate a striking difference between streptokinase activity of isolates possessing the gene for PAM (*pam*-positive) and those which do not (*pam*-negative (Figure 3.2A). Significantly higher streptokinase activity was demonstrated in the supernatants of *pam*-negative isolates from both invasive and uncomplicated infections compared to *pam*-positive isolates at both growth phases ($p = 0.0004$ for

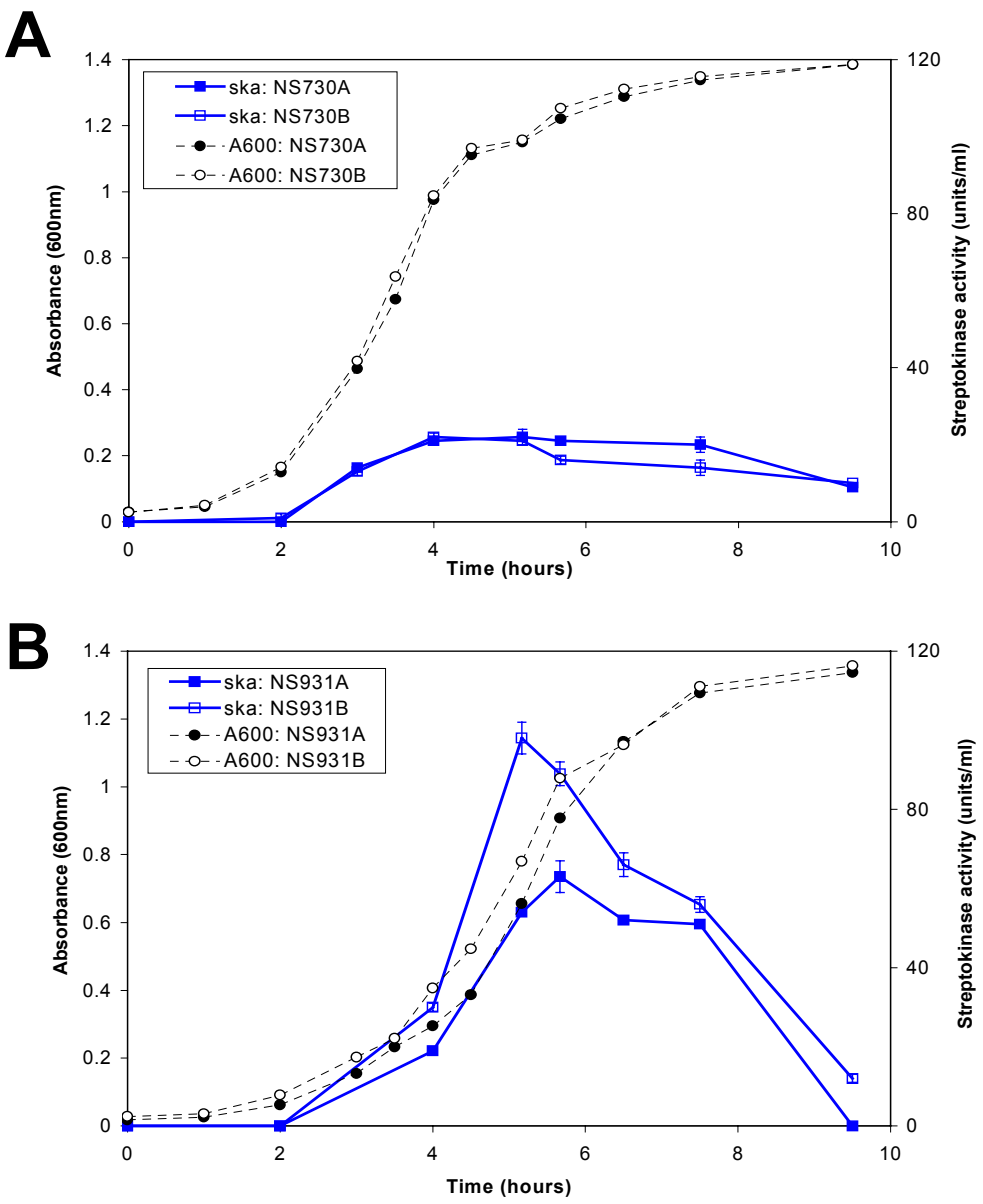

Figure 3.1. Kinetics of streptokinase secretion by sample NT GAS isolates: **(A)** Isolate NS730; **(B)** Isolate NS931. Culture densities of duplicate cultures (A, B) were monitored at an absorbance of 600nm (left Y axis; dotted lines) and culture supernatants sampled for assay of streptokinase activity at the time points shown. Streptokinase activity of GAS supernatants (units/ml; right Y axis; curves shown in blue) was measured indirectly using a chromogenic plasmin substrate (American Diagnostica) following preincubation with plasminogen, and compared to a standard curve of streptokinase (Sigma). Streptokinase activity of duplicate cultures (A, B) was measured ± plasminogen to confirm that proteolytic activity was attributable to plasminogen activation. Error bars denote the standard deviation of triplicate measures.

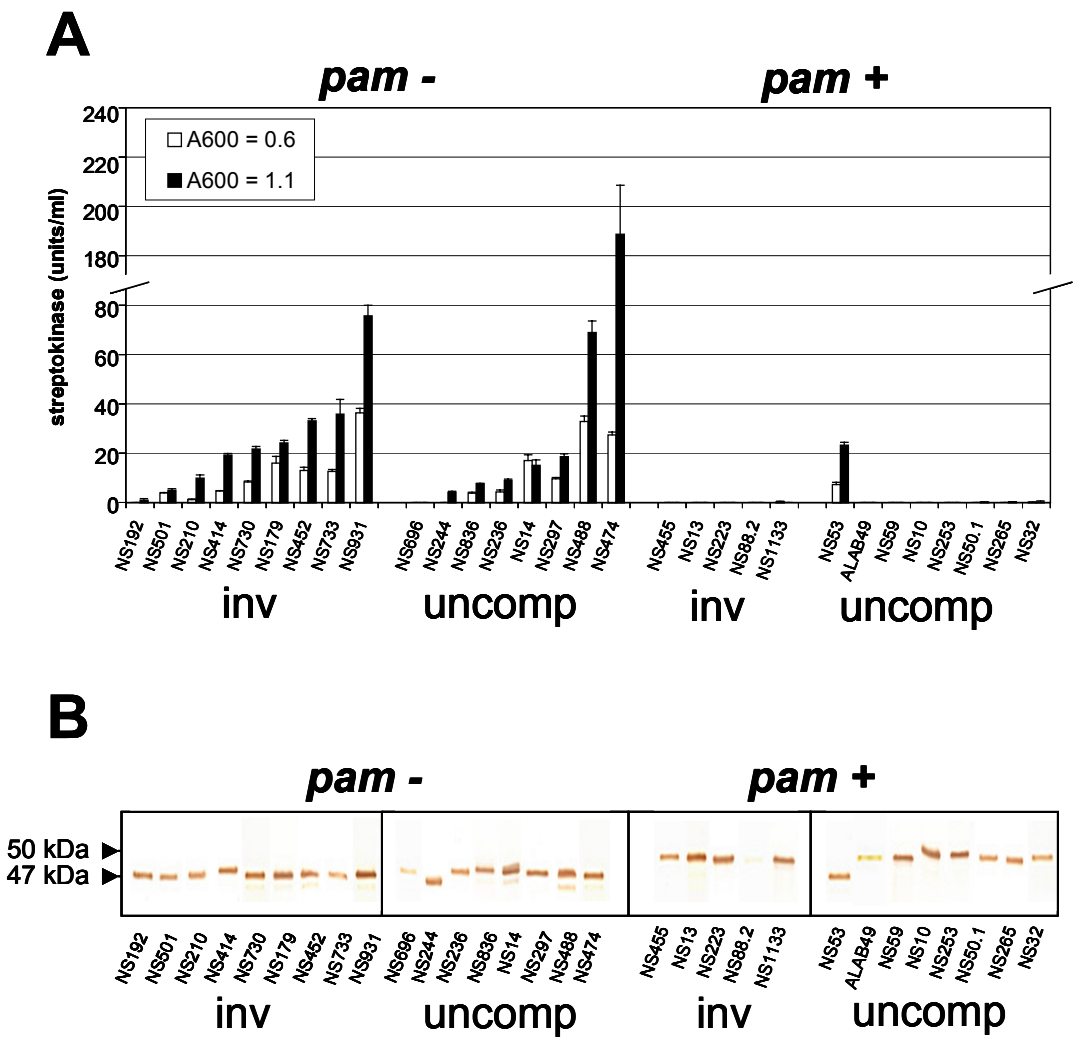

Figure 3.2 Streptokinase activity and protein in supernatants of 30 clinical isolates of GAS. **(A).** Streptokinase activity in GAS supernatants at mid-log (A_{600} = 0.6) and late-log/early stationary (A_{600} = 1.1) phase was measured indirectly using a chromogenic plasmin substrate (American Diagnostica) following preincubation with plasminogen, and compared to a standard curve of streptokinase (Sigma). Data represent mean ± SEM of streptokinase activity in triplicate cultures of GAS isolates corrected for an internal positive control sample in each assay. **(B).** Western blot of mid-log phase GAS supernatants using rabbit immune serum raised against group C streptokinase demonstrating secretion of streptokinase protein by all isolates.

mid-log phase; $p < 0.0001$ for late-log phase). Indeed, with the exception of isolate NS53, streptokinase activity could not be detected in the supernatants of *pam*-positive isolates. Streptokinase activities at mid-log phase and at late log/early stationary phase for the 30 isolates were highly correlated ($r = 0.908$; $p < 0.0001$). There was no difference between isolates from invasive disease and uncomplicated infection in streptokinase activity at either phase of growth ($p \geq 0.921$ for mid-log phase; $p \geq 0.089$ for late-log phase).

3.3.1.3 Effect of cysteine protease on streptokinase activity

SpeB degrades streptokinase, and while it is not expected to be induced in mid-log phase, it may be present as a contaminant from stationary-phase starter cultures *in vitro* (Rezcallah et al., 2004; Svensson et al., 2002). Assays were performed to determine whether the lack of soluble streptokinase activity of *pam*-positive isolates was due to degradation by SpeB. Of 6 isolates examined, all produced significant levels of SpeB at stationary phase with the exception of NS88.2 (Table 3.2). Inhibition of SpeB proteolytic activity did not result in the appearance of soluble streptokinase activity for three NT *pam*-positive isolates. Consistent with SpeB degradation of streptokinase in stationary phase cultures, inhibition of SpeB in cultures of *pam*-negative isolates NS730 and NS931 resulted in an enhancement of streptokinase activity which was pronounced as the culture entered stationary phase (NS730, $p = 0.001$; trend for NS931, $p = 0.052$).

3.3.1.4 Streptokinase protein in GAS supernatants

All GAS isolates secreted detectable levels of streptokinase protein into supernatants at both growth phases and there was no marked difference in expression levels between pam-positive and pam-negative isolates (Figure 3.2B). Isolate NS88.2 displayed

reduced streptokinase protein expression in supernatants compared to other isolates (Figure 3.2B). Thus, the lack of soluble streptokinase activity in pam-positive isolate supernatants was not attributable to proteolytic degradation of streptokinase protein. This confirms the previous observation that SpeB inhibition has no effect on supernatant streptokinase activity of pam-positive isolates. Pam-positive isolates produced streptokinase of larger apparent molecular weight (~50 kDa) compared to the streptokinase of pam-negative isolates (~47 kDa). NS53 was a notable exception amongst pam-positive isolates with a streptokinase protein of molecular weight ~47 kDa.

3.3.2 Streptokinase allelic variation

3.3.2.1 Phylogenetic analysis of streptokinase partial alleles

The relatedness of streptokinase alleles of the 29 NT GAS isolates was investigated by phylogenetic analysis of nucleotide sequences encoding the highly variable β-domain of streptokinase (Figure 3.3). Streptokinase alleles from a recent phylogenetic analysis of a large international isolate collection containing ALAB49 were included to place the NT isolates in the context of previously described allelic subclusters (Kalia and Bessen, 2004). The *ska* alleles of all *pam*-negative NT isolates fell within cluster 1, with the exception of NS696, which fell within cluster 2a and was the only *emm*1 isolate in this collection (Figure 3.2B) (McKay et al., 2004). The majority of *pam*-positive isolates possessed a cluster 2b *ska* allele. A notable exception was the *pam*-positive isolate NS53 which, like the *pam*-negative isolates, possessed a cluster *ska* 1 allele that encodes streptokinase protein of ~47 kDa.

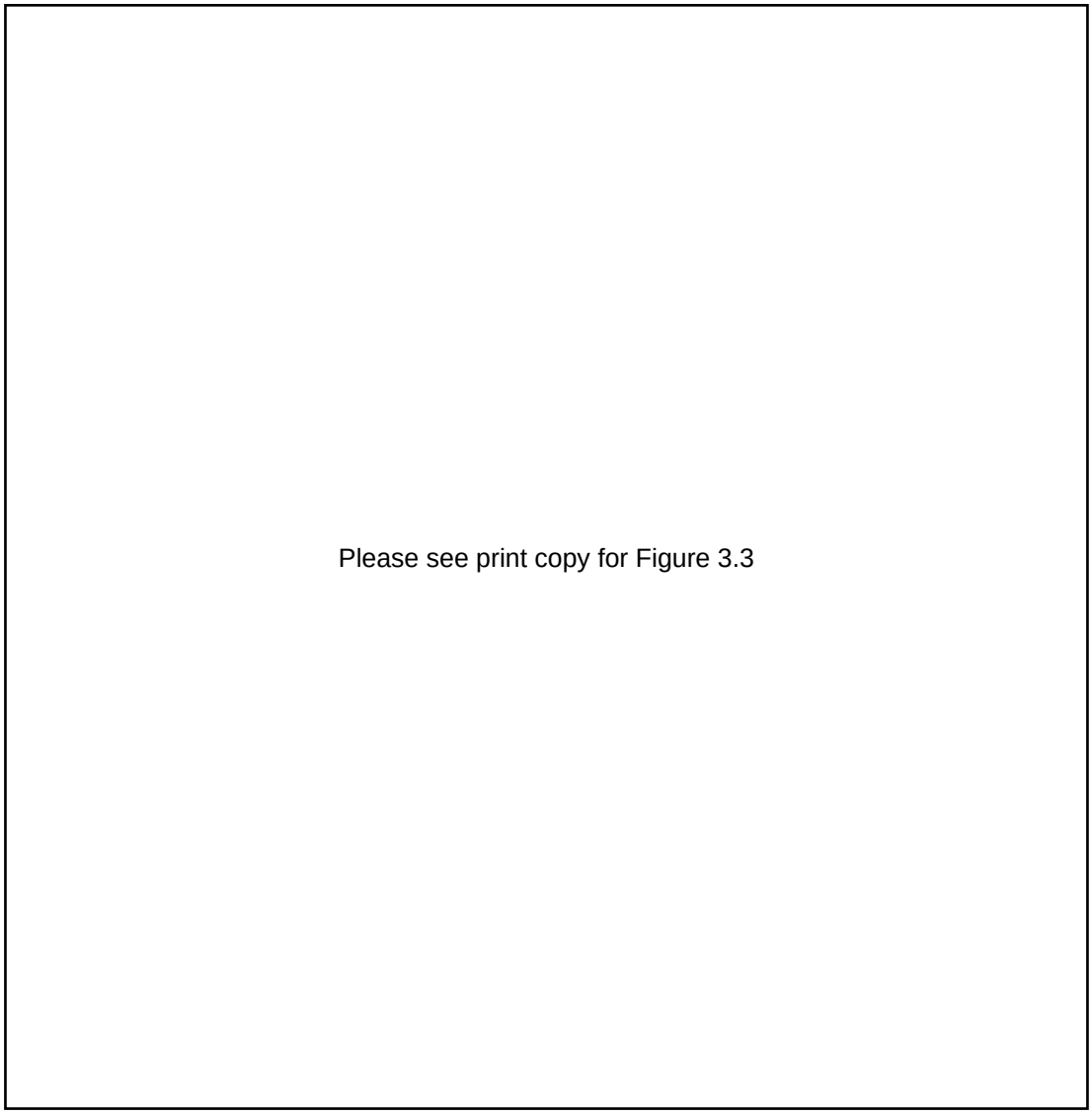

Figure 3.3. Phylogeny of the β-domain of streptokinase for 29 NT GAS isolates. **(A).** Domain structure of streptokinase showing the amino acid (aa) sequence positions of the three major domains of streptokinase denoted α, β and γ (Wang et al., 1998). Secondary structural domains of the β domain determined by crystallographic analysis of group C streptokinase are shown. These include β sheets (β1, aa 158-169; β2, aa 183-8; β3, aa 192-195; β4, aa 214-226; β5, aa 232-236; β6, aa 241-245; β6' aa 251-255, β6", aa 260-264; and β7, aa 266-278), an α helix (α3,4, aa 196-210) and two exposed loops (the 170 loop, aa 170-182 and the 250 loop, aa 251-264 encompassing the β6' and β6" sheets) (Wang et al., 1999) (Figure continued overleaf).

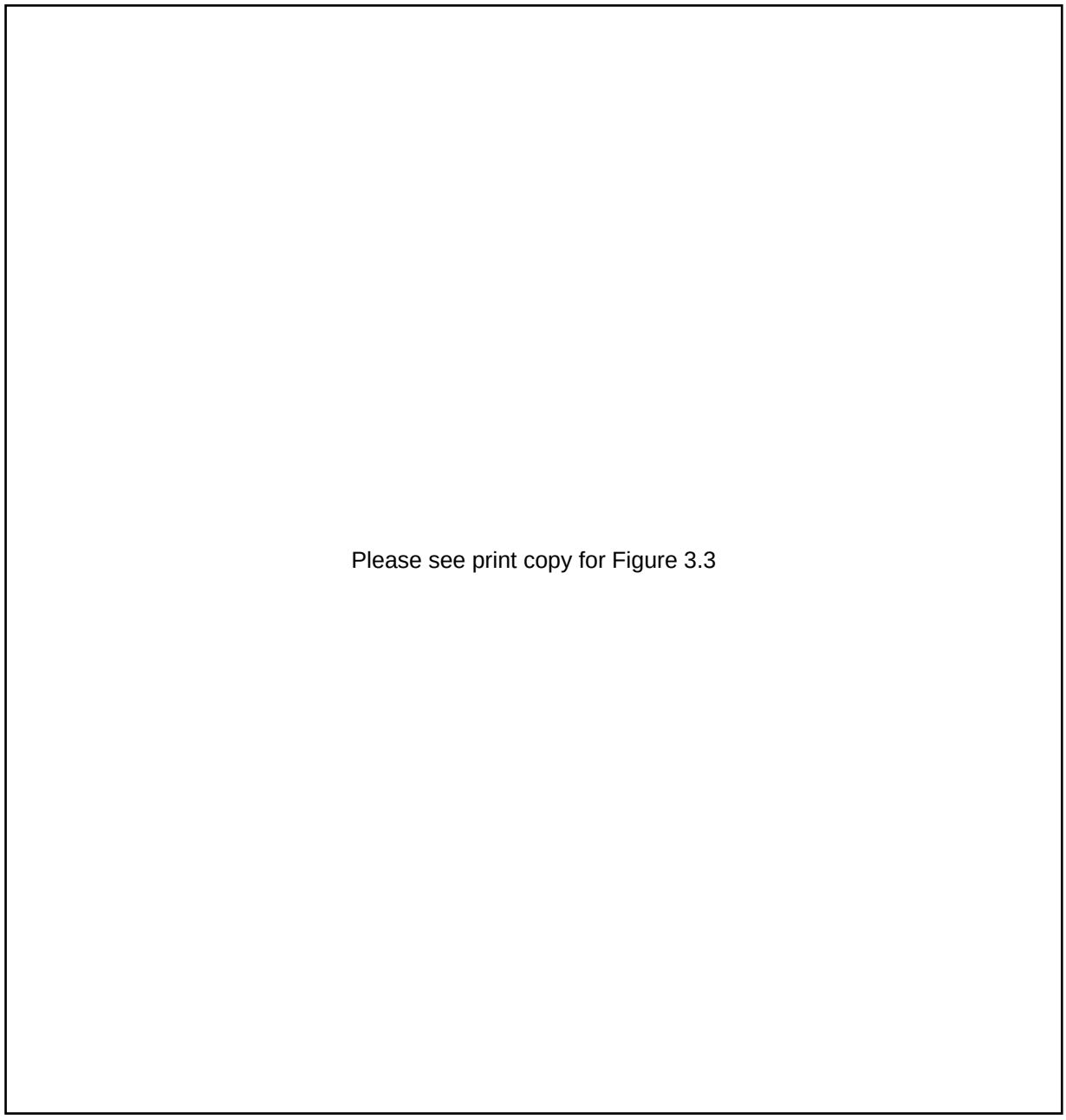

Figure 3.3. (continued) **(B).** Phylogenetic tree for a 423 bp variable region encoding the β-domain of streptokinase for the 29 NT isolates (shown in bold). Alleles from a previously published phylogenetic analysis are included for comparison (prefix "ska"; GenBank accession numbers AY234261 to AY234324) including allele ska41 of isolate ALAB49 (Kalia and Bessen, 2004). *pam*-positive (red) and *pam*-negative (blue) genotypes are denoted for NT isolates and for ska alleles for which at least one corresponding isolate possesses that *pam* genotype. *ska* alleles in black indicate that *pam* genotype of all isolates possessing this allele is unknown. Bootstrap values of ≥ 90% (500 replicates) are indicated and the scale bar indicates 0.05 substitution per site.

3.3.2.2 Amino acid sequence comparison of ska allelic subclusters

To identify differences between solution-phase active and inactive streptokinases, amino acid sequences of the β domains encoded by streptokinase partial alleles of the 30 GAS isolates were aligned (Table 3.3). The *ska* 1 (solution-phase active) and *ska* 2b (solution-phase-inactive) alleles displayed approximately 60% amino acid sequence homology. Amino acid sequences were also compared with homologous domains of group C streptokinase (Wang et al., 1999) which is most closely related to streptokinase encoded by *ska* 2a alleles (Kalia and Bessen, 2004). The commercially available group C streptokinase from isolate H46A is active in solution and its *skc* allele was included for comparison (Kabi, Sweden) (Lizano and Johnston, 2005). While the *ska* 2a allele examined in this study (for isolate NS696) did not display plasminogen activator activity in solution, at least one *ska* 2a allele has been demonstrated to possess solution-phase activity (Lizano and Johnston, 2005). The *ska* 2a and *skc* alleles (solution-phase active) possessed approximately 90% amino acid sequence homology to *ska* 2b alleles (solution-phase-inactive). Amino acid residues unique to positions within the streptokinase β domain encoded by *ska* 2b alleles were identified and their properties compared to corresponding residues encoded by *ska* 1 and *ska* 2a alleles (Table 3.4). These amino acids may be responsible for the lack of solution-phase activity of streptokinases encoded by the *ska* 2b allelic subcluster.

3.3.3 SpeB activity of GAS isolates

The relationship between SpeB activity and clinical manifestation was investigated. All isolates possessed the gene for SpeB (data not shown); and all isolates produced detectable levels of cysteine protease activity, with the exception of NS192, NS414, NS244 and NS88.2 (Figure 3.4A). Similarly, all isolates produced the 28 kDa mature

Please see print copy for Table 3.3

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Table 3.4. Unique amino acid residues of streptokinase β domains encoded by *ska* cluster 2b alleles compared to corresponding residues encoded by *ska* 1 and 2a subclusters[a, b].

Please see print copy for Table 3.4

[a]All amino acids listed are polar, hydrophilic species.
[b]Net charge on the amino acid at physiological pH indicated in brackets (+, positive; -, negative; 0, neutral)
[c](Wang et al., 1999)

SpeB protein with the exception of NS414, NS244 and NS88.2 (Figure 3.4B). There was no difference in SpeB activity of supernatants between between isolates from invasive disease and uncomplicated infection (Figure 3.4A; p = 0.696); or between PAM-positive and PAM-negative isolates (Figure 3.4A; p = 0.837). Amongst PAM-negative isolates, there was a moderate positive correlation between SpeB activity in stationary phase and streptokinase activity at mid-exponential phase (p = 0.026; r = 0.539).

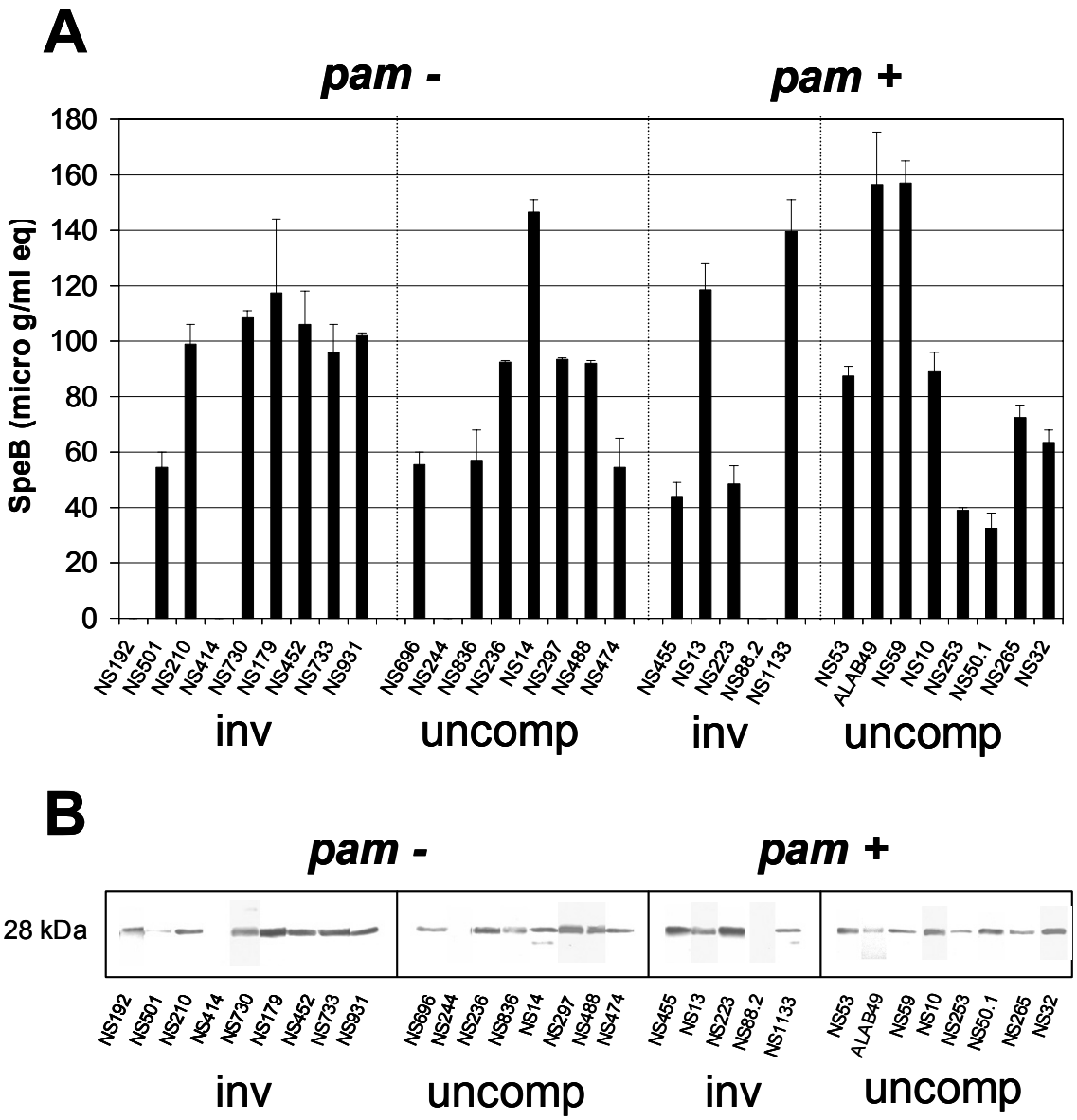

Figure 3.4. Expression and activity of secreted SpeB of NT GAS isolates and ALAB49. (**A).** Cysteine protease activity in GAS supernatants from stationary phase measured by kinetic assay using the chromogenic substrate, N-benzoyl-Pro-Phe-Arg-*p*-nitroanilide-hydrochloride (Sigma). Data represent mean ± SEM of cysteine protease activity measured in triplicate for each of two independent cultures of every isolate, expressed as μg/ml equivalent using a standard curve of purified SpeB (Toxin Technology). (**B).** Immunoblot of GAS stationary phase supernatants probed with rabbit polyclonal anti-SpeB antibody (Toxin Technology). Immunoblotting of purified SpeB with the same anti-SpeB antibody showed a protein of the same apparent molecular weight as that detected in GAS supernatants (data not shown).

3.3.4 Cell-surface plasmin activity assay

3.3.4.1 Assay optimisation

An assay of acquisition of cell-surface plasmin by GAS isolates following incubation in human plasma was modified from that described previously (Wang et al., 1994) and validated in our laboratory. Substrate specificity and assay limits were determined, control plasminogen-depleted plasma prepared and the concentration and time of plasma incubation optimised.

3.3.4.1.1 Substrate specificity and assay linearity

The substrate specificity of SpeB is not well-defined, however SpeB is known to cleave peptide bonds on the C-terminal side of lysine residues during autocatalytic processing (Doran et al., 1999; Nomizu et al., 2001). Plasmin also hydrolyzes peptide bonds on the C-terminal side of exposed arginine or lysine residues (Keil, 1992), thus the specificity of the plasmin-specific chromogenic substrate H-D-norleucyl-hexahydrotyrosol-lysine-para-nitroanilide diacetate (American Diagnostica) was tested. This substrate was cleaved by plasmin but not by plasmin under reducing conditions (DTT) that disrupt the secondary structure of the disulfide-bonded two-chain protease (Figure 3.5A). The substrate was not recognised by SpeB, either alone or under reducing conditions that activate the enzyme. Using identical assay conditions with a cysteine protease-specific substrate, N-benzoyl-Pro-Phe-Arg-*p*-nitroanilide-hydrochloride (Sigma), SpeB induced significant substrate cleavage under reducing conditions (ie when activated) (Figure 3.5B). SpeB without activation or when inhibited by E64 showed no proteolytic activity. Interestingly, plasmin also mediated limited proteolysis of the cysteine protease substrate, but not under reducing conditions which inactivate plasmin (Figure 3.5B).

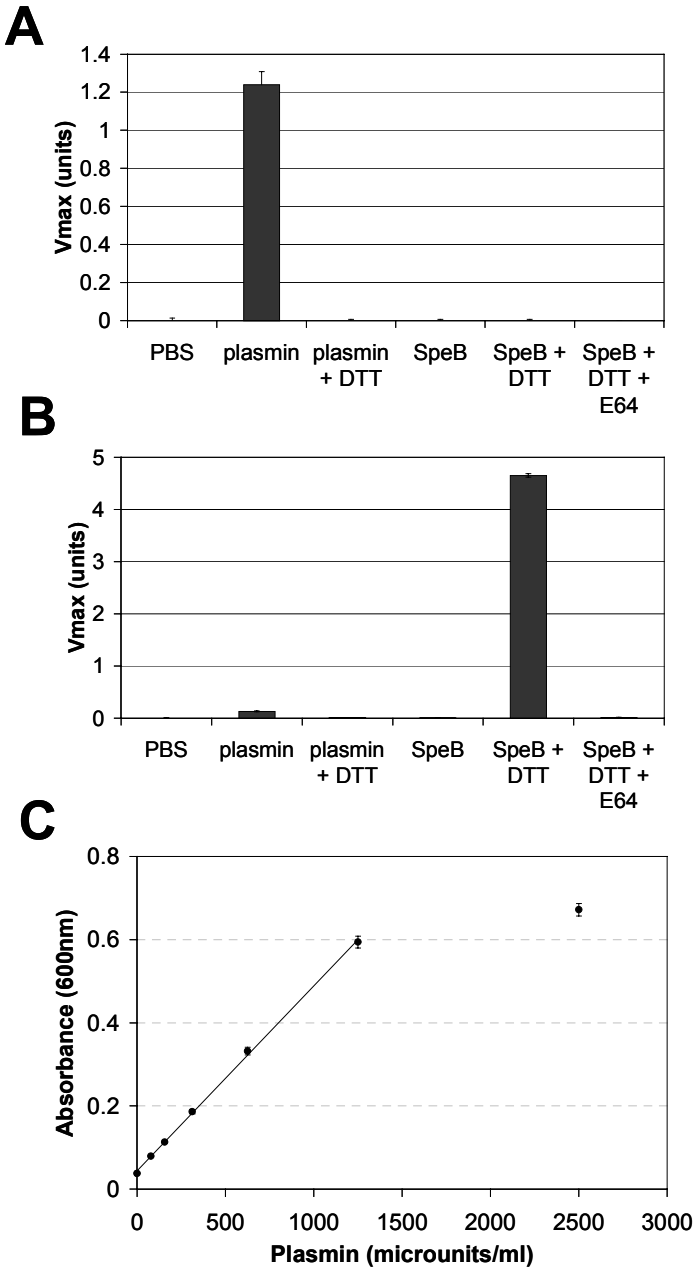

Figure 3.5. Substrate specificity and linearity of plasmin assays using a chromogenic substrate. **(A).** Proteolytic cleavage of the plasmin-specific chromogenic substrate (H-D-norleucyl-hexahydrotyrosol-lysine-para-nitroanilide diacetate; American Diagnostica) by plasmin (Roche) and SpeB (Toxin Technology) $\pm$ the reducing agent dithiothreitol (DTT) which disrupts plasmin structure but activates SpeB. Active SpeB in the presence of the cysteine protease inhibitor, E64 (Sigma) is also shown. Proteolytic cleavage at A_{405} was determined by kinetic assay and is expressed as Vmax (arbitrary units) using Softmax Pro (Molecular Devices) **(B).** Identical assays were conducted using a cysteine protease substrate (N-benzoyl-Pro-Phe-Arg-*p*-nitroanilide-hydrochloride; Sigma). **(C).** Dose-response curve of plasmin at A_{405} using the plasmin-specific chromogenic substrate in an endpoint assay developed for GAS cell-surface plasmin acquisition assays. Linearity of the assay to A_{405} = 0.6 is depicted by the regression line (r^2 = 0.9992) fitted to the linear portion of the curve.

Using an endpoint assay optimised for GAS cell-surface plasmin acquisition assays employing the plasmin-specific chromogenic substrate, a plasmin dose-response curve was linear to A_{405} = 0.6 (Figure 3.5C).

3.3.4.1.2 Plasminogen depletion of plasma

Plasma depleted of plasminogen was prepared by incubation with lysine-sepharose for use as a control in GAS cell-surface plasmin acquisition assays. SDS-PAGE analysis and immunoblotting of 100% plasma and plasminogen-depleted plasma with an anti-plasminogen antibody confirmed the selective depletion of plasminogen (Figure 3.6A). Plasminogen depletion of plasma was not complete, however the amount of activatable plasminogen was reduced by 18-fold ($p < 0.0001$) (Figure 3.6B). Both depleted and 100% plasma possessed some inherent plasmin activity in the absence of streptokinase.

3.3.4.1.3 Optimisation of plasma concentration and incubation time

While several studies have examined GAS plasmin acquisition in dilute human plasma (4% - 30%) (D'Costa and Boyle, 1998; D'Costa and Boyle, 2000; Wang et al., 1994), GAS also acquire significant cell-surface plasmin when incubated in 100% plasma (Wang et al., 1995b). Acquisition of cell-surface plasmin by GAS isolates was compared following incubation in 30%, 50% and 100% plasma, and in 100% plasma depleted of plasminogen (Figure 3.7A). There was a significant effect of plasma concentration on plasmin acquisition ($p = 0.049$) however similar patterns of acquisition were seen for the 30%, 50% and 100% plasma. To reflect physiological conditions a plasma concentration of 100% was chosen for further assays.

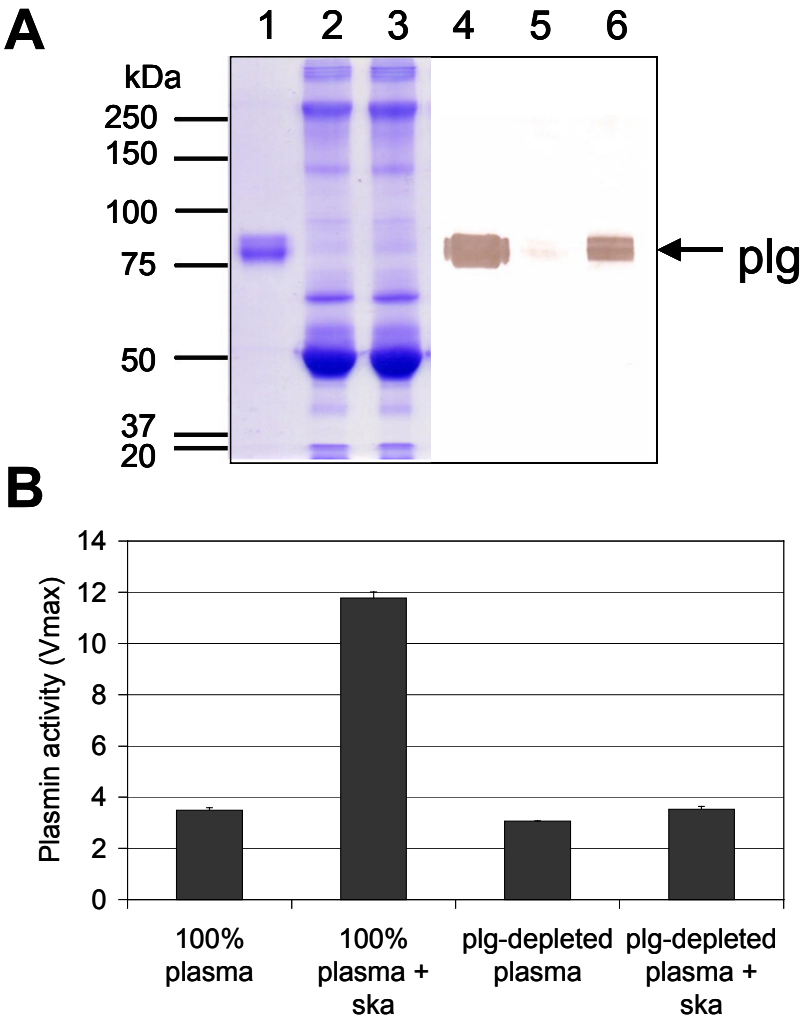

Figure 3.6. Plasminogen depletion of human plasma by incubation with lysine-sepharose. **(A).** SDS-PAGE (lanes 1-3) and immunoblot (lanes 4-6) of 100% plasma and plasminogen-depleted plasma showing purified human plasminogen (plg; lanes 1,4); plasminogen-depleted plasma (lanes 2, 5) and 100% plasma (lanes 3, 6) each diluted 20-fold. Immunoblotting was performed using a rabbit polyclonal antibody against human plasminogen (Calbiochem). **(B).** Kinetic chromogenic assay of plasmin activity of 100% plasma and plasminogen-depleted plasma (plg-depleted) in the presence and absence of streptokinase (ska; 6.25 units/ml final concentration; Sigma) expressed as Vmax (arbitrary units) using Softmax Pro (Molecular Devices).

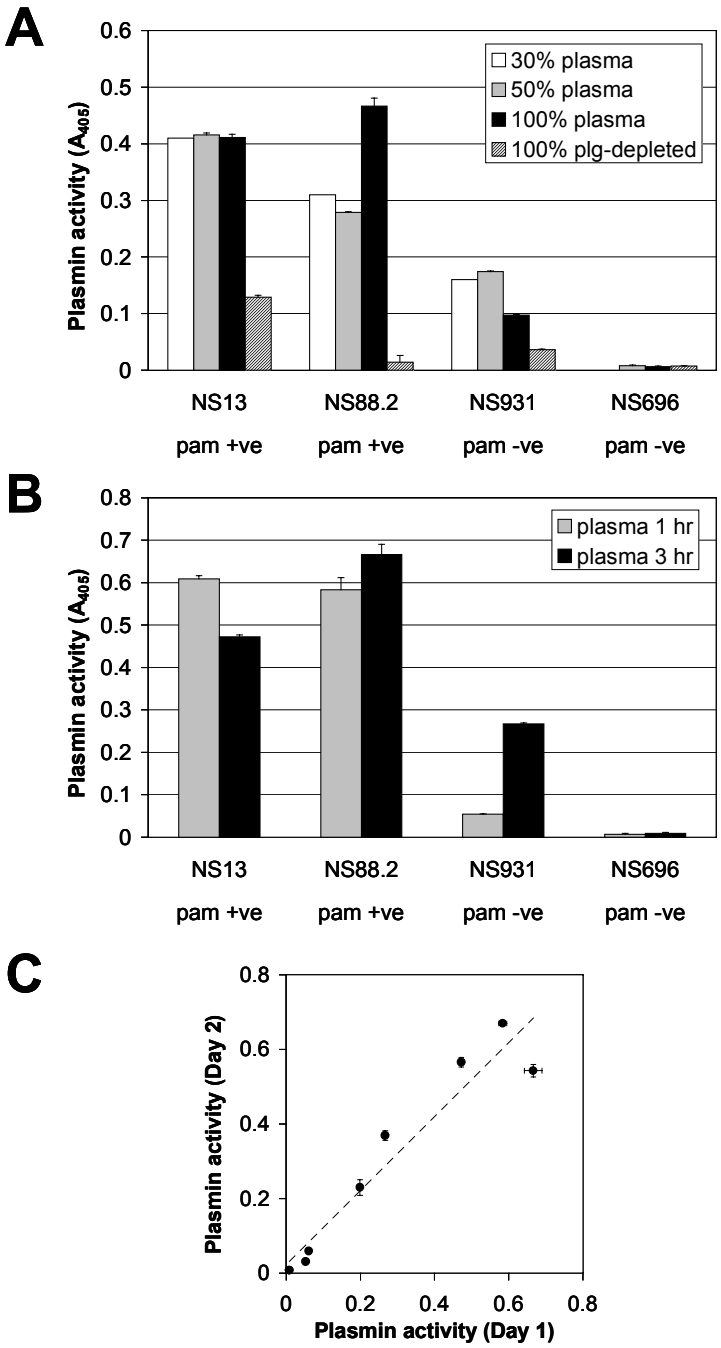

Figure 3.7. Optimisation of an assay for cell-surface plasmin acquisition by GAS isolates incubated in human plasma. **(A).** Cell-surface plasmin acquisition by GAS isolates following incubation in varying concentrations of plasma or plasminogen-(plg-) depleted plasma measured by plasmin assay using a specific chromogenic substrate at 405nm. All plasma incubations were conducted for 3 h with the exception of 30% plasma, incubated for 5 h. **(B).** Cell-surface plasmin acquisition by GAS isolates following incubation in 100% plasma for 1 or 3 h. **(C).** Correlation between cell-surface plasmin acquisition by GAS isolates following 3 h incubation in 100% plasma measured in two independent experiments ($r = 0.959$, $p = 0.0002$). Isolates assayed were NS13, NS455, NS474, NS53, NS696, NS730, NS88.2 and NS931.

Similar patterns of cell-surface plasmin acquisition by the four GAS isolates were seen after one and three h of incubation in human plasma (Figure 3.7B). An incubation of three h was chosen for future experiments due to the higher overall plasmin acquisition by isolates at this timepoint. Plasmin acquisition by 8 isolates incubated in 100% plasma for 3 h was reproducible with highly significant correlations between results obtained in two independent experiments (average coefficient of variation = 15%; correlation r = 0.959, p = 0.0002) (Figure 3.7C).

3.3.4.2 Cell-surface plasmin acquisition by 30 GAS isolates

Acquisition of cell-surface plasmin by the 30 GAS isolates was measured following incubation in human plasma. Paradoxically, *pam*-positive isolates acquired significantly higher levels of plasmin activity at the cell surface compared to the *pam*-negative isolates (Figure 3.8A; p = 0.0002). Regardless of *pam* genotype, there was no difference between isolates from invasive disease and uncomplicated infection in the ability to acquire cell-surface plasmin in plasma (Figure 3.8A; p = 0.450). Amongst *pam*-negative isolates, there was a significant correlation between supernatant streptokinase activity and cell-surface plasmin acquisition in human plasma (p = 0.038, r = 0.506). Overall, inhibition of tPA (using PPACK; Calbiochem) reduced plasmin acquisition by isolates in plasma (p = 0.013); however for selected isolates such as NS13 and NS10 plasmin acquisition was significantly increased (Figure 3.8B). This may relate to the kinetics of plasmin loss from the cell-surface of these isolates, potentially slowed by the decreased plasmin activation. In the presence of the tPA inhibitor, *pam*-positive isolates were still able to acquire higher levels of cell-surface plasmin than *pam*-negative isolates (p = 0.0007).

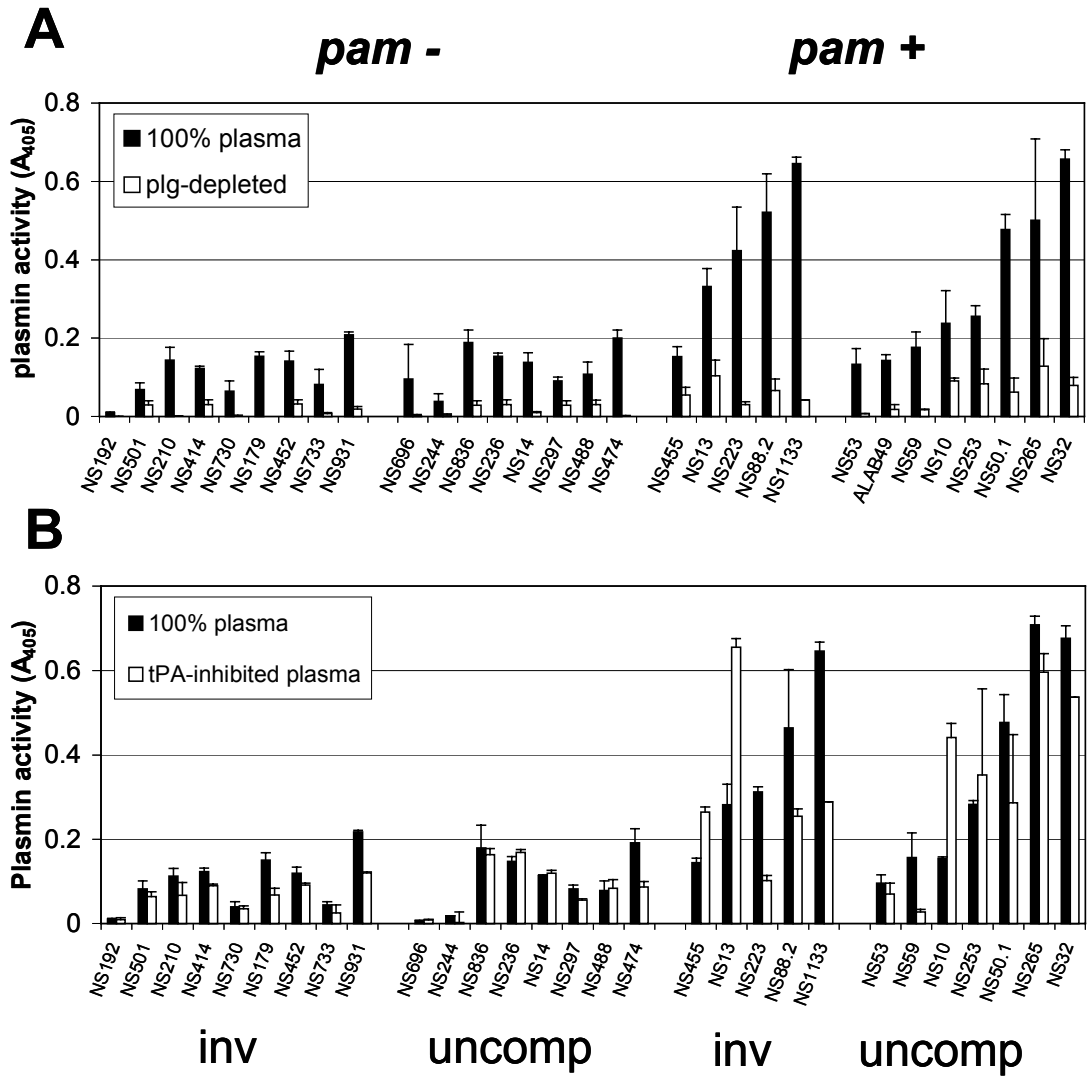

Figure 3.8. Cell-surface plasmin acquisition by GAS isolates following incubation in human plasma. Isolates were incubated in 100% plasma, 100% plasma pretreated with tPA inhibitor (PPACK; Calbiochem) or plasminogen-depleted plasma (plg-depleted) for 3 h, washed extensively, and surface-associated plasmin determined at 405nm following incubation of GAS in a plasmin-specific chromogenic substrate. **(A).** 100% plasma compared to plg-depleted plasma. Results are presented as mean $\pm$ SEM of three independent experiments for each isolate. **(B).** 100% plasma with or without pretreatment with tPA inhibitor. Results are presented as mean $\pm$ SEM of two independent experiments for each isolate.

3.3.4.3 Cell-surface plasmin acquisition by isogenic deletion mutants

The effect of streptokinase and SpeB on cell-surface plasminogen binding and plasmin acquisition of *pam*-positive and *pam*-negative GAS isolates was investigated using isogenic deletion mutants of these genes (Table 3.5, Figure 3.9). The absence of SpeB and streptokinase in supernatants of the respective deletion mutants was confirmed by others in our laboratory (data not shown). In addition, immunoblotting analysis of concentrated supernatants of isolate 5448 revealed that secreted streptokinase could only be detected when SpeB was inhibited (using E64) or absent (in the *speB*-negative mutant) (Cole et al., *submitted*). The isogenic *ska*-negative mutant of PAM-positive isolate ALAB49 (Ringdahl et al., 1998; Svensson et al., 2002) acquired significantly less cell-surface plasmin than the wild-type (Figure 3.9B; $p = 0.019$). This suggests that despite the apparent lack of activity in solution, streptokinase contributes to cell-surface plasmin acquisition of *pam*-positive isolates in plasma. Isogenic *speB*-negative mutants of ALAB49 and 5448 acquired significantly more plasmin than the wild-type ($p = 0.019$; $p = 0.004$ respectively); while ALAB49 *speB*- also bound more plasminogen

Table 3.5. North American clinical isolates of GAS and their isogenic mutants

Please see print copy for Table 3.5

[a]produces high levels of active SpeB (Svensson et al., 2000);[b]binding of human plasminogen almost equivalent to wildtype (Svensson et al., 2002); [c]binding of human plasminogen and human fibrinogen, streptokinase production and β haemolysis reported unaltered from wild-type (Svensson et al., 2000); [d]produces high levels of active SpeB (Kansal et al., 2003); [e]severe invasive disease classified as those with STSS and/or necrotising fasciitis (Chatellier et al., 2000).

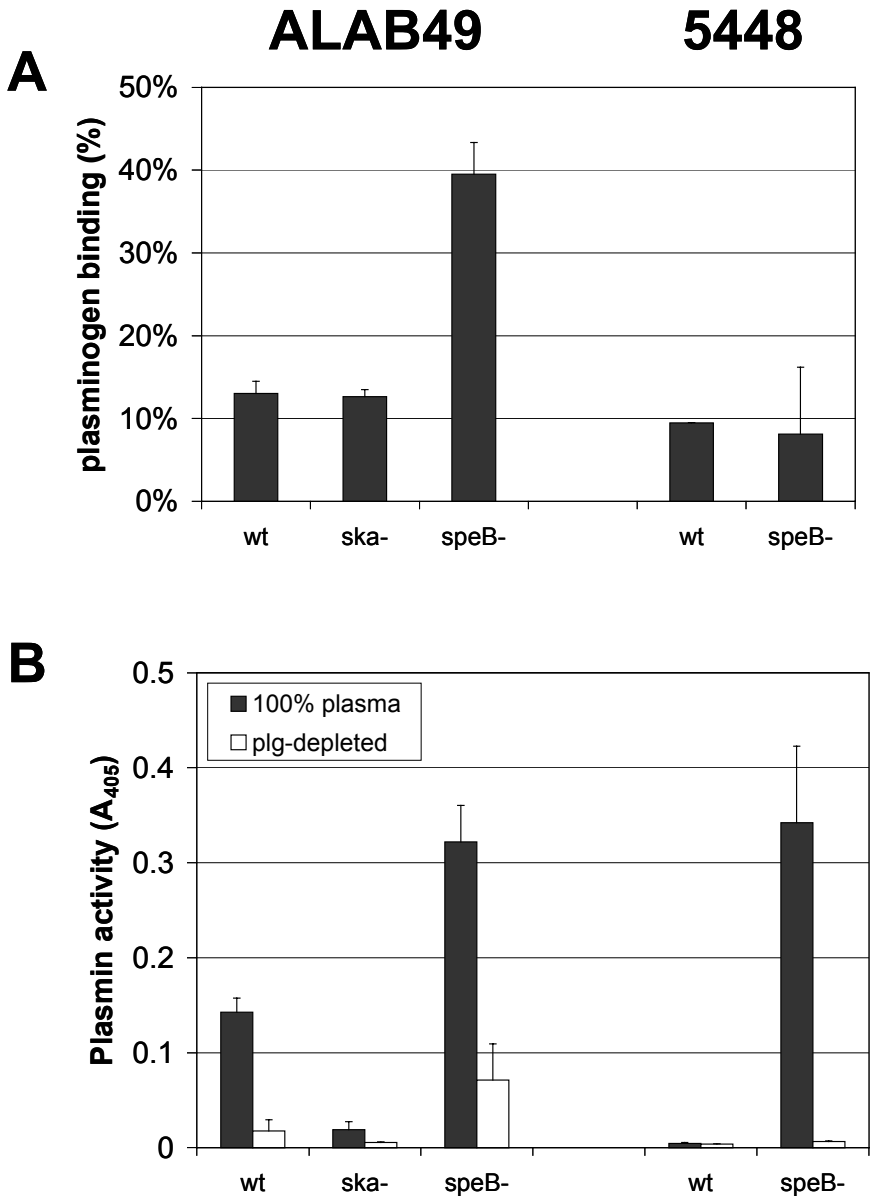

Figure 3.9. Cell-surface plasminogen binding and plasmin acquisition by North American GAS isolates and isogenic *ska*- and *speB*-negative mutants. **(A).** ^{125}I-plasminogen binding to GAS isolates expressed as % radioactivity offered. Data are presented as mean $\pm$ SD of triplicate determinations. **(B).** Cell-surface plasmin acquisition by GAS isolates. Isolates were incubated in 100% plasma or plasminogen-depleted plasma (plg-depleted) for 3 h, washed extensively, and surface-associated plasmin determined at 405nm following incubation of GAS in a plasmin-specific chromogenic substrate. Results are presented as mean $\pm$ SD of two independent experiments for each isolate.

(Figure 3.9A; $p < 0.0001$). This suggests that SpeB degradation of GAS plasminogen activation components (including the plasminogen receptor PAM of ALAB49 and streptokinase of 5448) reduces the acquisition or stability of GAS cell-surface plasmin.

3.3.5 Correlations between *in vitro* plasminogen activation parameters

One of the aims of the *in vitro* studies was to determine whether components of the GAS plasminogen system are correlated within this isolate collection. A summary of correlations between *in vitro* parameters of interest is given in Table 3.6. The relationship between cell-surface plasmin acquisition and its component parts (streptokinase, plasminogen binding and fibrinogen binding) was assessed. Isolates were separated into *pam*-positive and *pam*-negative subgroups for this analysis due to significant differences in the mechanisms of plasmin(ogen) binding and activation (Berge and Sjobring, 1993; McKay et al., 2004; Wang et al., 1994; Wang et al., 1995a; Wang et al., 1995b).

For *pam*-negative isolates, there was a moderate correlation between supernatant streptokinase activity and cell-surface plasmin acquisition ($p = 0.038$, $r = 0.506$; mid-log phase streptokinase activity) supporting previous suggestions that streptokinase availability is the limiting factor in cell-surface plasmin acquisition (Wang et al., 1994). Streptokinase is required by *pam*-negative isolates not only as a plasminogen activator, but also as a co-factor for cell-surface plasminogen binding within the trimolecular complex (Wang et al., 1995b). If streptokinase is the limiting factor in cell-surface plasmin acquisition in plasma, it is not surprising that there was no relationship between plasmin acquisition in plasma and the ability to bind plasminogen in the presence of excess streptokinase and fibrinogen as measured in this study ($p = 0.498$, $r = - 0.177$).

Nor was there a relationship between cell-surface plasmin acquisition and fibrinogen binding (which allows assembly of the fibrinogen-streptokinase-plasminogen trimolecular complex at the cell-surface ($p = 0.185$, $r = 0.061$) (Wang et al., 1995b).

Table 3.6. Correlations[a] between *in vitro* plasminogen activation and its putative components measured in this study for 30 GAS isolates.

Parameter 1	Parameter 2	Group	*p*	*r*
Plasmin acquisition in 100% plasma	Streptokinase activity-mid log	PAM+	NA[b]	NA
		PAM-	0.038	0.506
	Streptokinase activity-late log	PAM+	NA[b]	NA
		PAM-	0.055	0.473
	Direct plasminogen binding[c]	PAM+	0.366	0.287
		PAM-	NA[d]	NA
	Fibrinogen binding	PAM+	0.015	0.678
		PAM-	0.815	0.061
	FSD plasminogen binding	PAM+	0.417	0.259
		PAM-	0.498	-0.177
	SpeB activity	PAM+	0.280	-0.324
		PAM-	0.183	0.339
	tPA-independent plasmin acquisition[e]	All	<0.0001	0.831
	tPA-dependent plasmin acquisition[e]	All	0.007	0.487
tPA-independent plasmin acquisition	Streptokinase activity-mid log	PAM-	0.043	0.496
	Streptokinase activity-late log	PAM-	0.144	0.370
tPA-dependent plasmin acquisition	Direct plasminogen binding	PAM+	0.846	0.063
		PAM-	0.286	0.275
	FSD plasminogen binding	PAM+	0.443	0.245
		PAM-	0.147	0.368
	Fibrinogen binding	PAM+	0.174	0.420
		PAM-	0.428	-0.206

[a]Correlations were performed using Spearman's rank test as the majority of data was not Normally distributed (as determined by the Shapiro Wilk W test) ; significant correlations are highlighted in red.
[b]NA; not applicable due to the absence of supernatant streptokinase activity for all but one *pam*-positive isolate, NS53.
[c]all correlations with ligand binding parameters were calculated for the 29 NT isolates for which they were measured (Chapter 2).
[d]NA; not applicable due to the absence of significant direct plasminogen binding by *pam*-negative isolates.
[e]tPA-dependent plasmin acquisition was calculated as the reduction in plasmin acquisition in the presence of tPA inhibitor compared to the 100% plasma condition. tPA-independent plasmin acquisition was calculated as that acquired in the presence of the tPA inhibitor.

As streptokinase activity was used as the quantitative measure of streptokinase availability, *pam*-positive isolates could not be analysed for the contribution of streptokinase to plasmin acquisition. For *pam*-positive isolates, there was no relationship between cell-surface plasmin acquisition and ability to bind plasminogen, either directly ($p = 0.366$; $r = 0.287$) or by the FSD pathway ($p = 0.417$; $r = 0.259$). Thus it is possible that streptokinase is also limiting for plasmin acquisition by *pam*-positive isolates in plasma. Interestingly, however, there was a correlation between cell-surface plasmin acquisition and the ability to bind fibrinogen ($p = 0.015$, $r = 0.678$). Fibrinogen may have important role in plasminogen activation by *pam*-positive GAS, as described in a later section (3.4.1.1).

Studies using isogenic mutants demonstrate that SpeB significantly lowers cell-surface plasmin acquisition, while inhibition experiments demonstrate SpeB degradation of streptokinase. Thus a negative correlation between cell-surface plasmin acquisition and SpeB secretion was hypothesised. No such correlation was identified, possibly indicative of the different growth conditions used for supernatant collection/activity measurements (mid-log phase for cell-surface plasmin; mid- and late-log for streptokinase; stationary phase overnight cultures for SpeB). The timing of SpeB induction and its effect on cell-surface plasmin and streptokinase may also differ between isolates.

Plasmin acquisition by GAS in plasma was also divided into tPA-dependent plasmin acquisition (that lost by inhibition of tPA) and tPA-independent plasmin acquisition (that remaining following tPA inhibition). Total plasmin acquisition was strongly correlated with tPA-independent plasmin acquisition ($p < 0.0001$; $r = 0.831$) and

moderately correlated with tPA-dependent plasmin acquisition ($p = 0.007$; $r = 0.487$). This reflects that each of these pathways makes a significant contribution to the overall plasmin acquisition, with the tPA-independent pathway contributing a greater proportion (average 84% for all isolates). There was a moderate correlation between tPA-independent cell-surface plasmin acquisition and streptokinase at mid-log phase for *pam*-negative isolates (for which streptokinase could be measured), supporting the hypothesis that streptokinase mediates cell-surface plasmin acquisition in the absence of tPA. There were no significant correlations between tPA-dependent plasmin acquisition and ligand binding parameters tested, possibly reflecting the myriad of potential interactions both enhancing and competing with this process in plasma.

Finally, it must be noted that the ratio of isolates studied to parameters measured was prohibitive for factor analysis of this data. The use of multiple pairwise comparisons (Table 3.6) risks the identification of false-positive correlations, thus it would be necessary to confirm these relationships in larger independent cohort. However, the significant correlations identified are supported by empirically derived models of GAS plasminogen binding and activation, suggesting that they reflect true biological relationships (McKay et al., 2004; Wang et al., 1994; Wang et al., 1995b).

3.3.6 Virulence of GAS in the humanised plasminogen transgenic mouse model of infection

The importance of plasminogen for virulence of GAS was determined by monitoring survival of human plasminogen transgenic mice and wildtype controls following infection with clinical isolates of GAS. GAS isolates were selected for infection studies to reflect the diversity of clinical isolates with respect to plasminogen binding, *pam*

genotype, streptokinase activity, streptokinase alleles and SpeB activity determined *in vitro* (Table 3.7). Representative *pam*-positive isolates derived from invasive (NS88.2) and uncomplicated infection (ALAB49) and *pam*-negative isolates from invasive (NS730) and uncomplicated infection (NS696) were used in this experiment. The presence of the transgene for human plasminogen markedly increased the virulence of *pam*-positive invasive disease isolate NS88.2 (Figure 3.10A; $p < 0.01$). In contrast, all human plasminogen transgenic mice and wildtype mice survived infection with the *pam*-positive impetigo isolate ALAB49 (Figure 3.10B). All wildtype mice survived infection with either of the *pam*-negative isolates (Figure 3.10C, D). The presence of the human plasminogen transgene did not increase virulence of the invasive disease isolate NS730 (Figure 3.10D), but significantly increased the virulence of isolate NS696 (Figure 3.10C; $p < 0.03$).

Table 3.7. Characteristics of the GAS isolates used in infection studies[a]

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[a]summarised from data previously presented

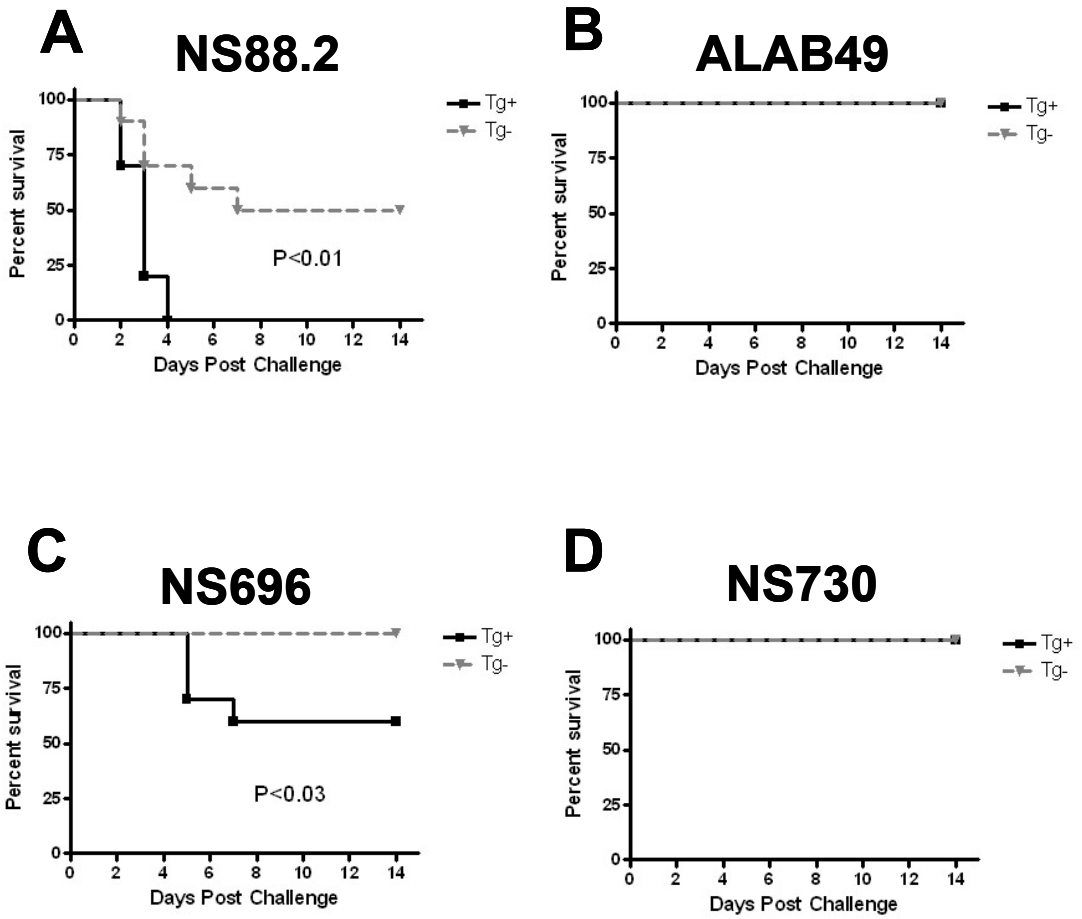

Figure 3.10. Virulence of GAS isolates in a humanized plasminogen transgenic model of infection. Survival of human plasminogen transgenic *Alb*PLG1 mice (Tg+; n = 10) and wildtype C57BL/6J mice (Tg-; n = 10) was monitored following subcutaneous infection with GAS isolates: **(A)** NS88.2 (*pam*-positive); (**B)** ALAB49 (*pam*-positive), (**C)** NS696 (*pam*-negative); or (**D)** NS730 (*pam*-negative). Difference between groups determined by logrank test is shown.

3.4 Discussion

In this study plasminogen activation by GAS from a region of endemic disease was characterised with respect to clinical source and *pam* genotype. It was hypothesised that the higher plasminogen binding capacity of invasive-disease isolates would be reflected in increased plasmin acquisition and virulence of GAS. *Pam* genotype was significantly correlated with streptokinase genotype and activity and cell-surface plasmin acquisition; a model of plasminogen activation by GAS secreting streptokinase of various allelic subclusters is presented. The relationship between *in vitro* plasminogen activation and clinical source was not as predicted, however a relationship between SpeB and cell-surface plasmin acquisition was identified. Virulence of NT isolates in the humanised plasminogen transgenic mouse model is discussed with respect to GAS plasminogen activation *in vitro*.

3.4.1 Plasminogen activation by GAS isolates *in vitro*

3.4.1.1 Effect of pam genotype on streptokinase activity and plasmin acquisition

Striking differences were observed in plasminogen activation between *pam*-positive and *pam*-negative isolates. *Pam*-positive isolates produce little or no detectable streptokinase activity. A previous report similarly identified that isolates with high plasminogen binding capacity did not produce detectable levels of streptokinase activity, although the *pam* genotype was not investigated (Tewodros et al., 1995). *Pam*-positive isolate NS53 was an interesting exception, possessing streptokinase activity and an *ska* 1 allele. The possibility that the lack of streptokinase activity of *pam*-positive isolates resulted from degradation of streptokinase by SpeB in *pam*-positive isolates was

excluded by SpeB inhibition experiments. In addition, immunoblotting revealed the presence of intact streptokinase protein amongst *pam*-positive isolates.

All GAS isolates possessing a subcluster 2b *ska* allele expressed intact soluble streptokinase protein with ~ 3 kDa higher apparent molecular weight. The cause of the higher apparent molecular weight of streptokinase is not clear, however ~40% amino acid sequence difference of the β domains encoded by *ska* 2b and *ska* 1 alleles provides a potential source of variability. Complete *ska* genes of different allelic subclusters vary in predicted molecular weight of the encoded protein (Table 3.8).

Table 3.8. Predicted molecular weights of full-length streptokinase proteins encoded by examples from each of the *ska* allelic subclusters.

GAS isolate	*ska* allelic subcluster	Predicted molecular weight (Da)	Source (GenBank Accession)
89-465	1	49,395	AY234133
M1 GAS	2a	47,327	AE004092 (complete genome)
ALAB49	2b	50,343	AY234134

In addition, all isolates bearing subcluster 2b alleles were *pam*-positive and displayed very inefficient plasminogen activator activity in solution compared to isolate possessing other subcluster *ska* alleles. This suggests that the plasminogen activation kinetics differ between the allelic variants of streptokinase. Despite the lack of soluble streptokinase activity, *pam*-positive isolates acquire significantly higher levels of plasmin in plasma. This may suggest a role for host plasminogen activators in plasmin

acquisition as observed previously (Khil et al., 2003), however even when the major host activator tPA is inhibited, *pam*-positive isolates can acquire significant amounts of cell-surface plasmin. This suggests that streptokinase also plays a major role in cell-surface plasmin acquisition by *pam*-positive isolates. It could be argued that the traces of active plasmin present in plasma *in vitro* may bind PAM directly and mediate plasmin acquisition by *pam*-positive isolates. However, the majority of cell-surface plasmin acquisition in plasma is mediated by streptokinase for at least one *pam*-positive isolate, ALAB49, as evidenced by comparison with its streptokinase-negative isogenic mutant. Conversion in plasma of ALAB49 cell-surface-bound plasminogen to plasmin has previously been demonstrated to be *ska*-dependent (Svensson et al., 2002). Thus despite being inactive in solution, streptokinase from a 2b allelic subcluster demonstrates significant activity at the surface of a *pam*-positive isolate. Together these results suggest that streptokinase plays an important role in plasmin acquisition by both *pam*-negative and *pam*-positive isolates.

Interestingly, tPA inhibition increased cell-surface acquisition by a small number of isolates. Plasmin is capable of degrading fibrinogen and the level of cell-surface plasmin ultimately decreases with time (D'Costa and Boyle, 2000; Wang et al., 1995a; Wang et al., 1995b). It is possible that tPA inhibition slows this process, such that higher cell-surface plasmin was observed for these isolates.

The lack of solution-phase activity of streptokinase encoded by *ska* 2b alleles supports our previously proposed model for plasminogen activation by GAS isolates possessing subcluster 2b streptokinase alleles (Walker et al., 2005). According to this model, the closed solution-phase conformation of plasminogen is resistant to activation by

streptokinase of subcluster 2b alleles. While other subcluster forms of streptokinase can activate plasminogen in the closed conformation, it has been suggested that subcluster 2b forms of streptokinase have adapted for interaction with plasminogen in the open conformation induced by binding to PAM (Kalia and Bessen, 2004). If subcluster 2b forms of streptokinase only form streptokinase-plasminogen activator complexes when bound to PAM, this would account for the apparent lack of streptokinase activity in the supernatants of *pam*-positive strains.

However further investigations in our laboratory have revealed that supernatants from the *pam*-positive GAS isolate NS13 possessing a subcluster 2b *ska* allele do not activate plasminogen in the presence of recombinant PAM, SEN or GAPDH, but in the presence of fibrinogen albeit at a slow rate compared to subcluster 1 alleles (McArthur et al., 2005). It remains possible that plasminogen binding to native cell-surface expressed receptors differs from plasminogen binding to soluble recombinant receptors, and that the original model for a permissive role of PAM in plasminogen activation by streptokinase of the 2b alleles is correct. Studies of cell-surface plasminogen activation by *pam*-positive GAS isolates in the presence and absence of fibrinogen may resolve this question.

Alternatively, this data suggests that the receptor binding (either to C-terminal lysines of SEN/GAPDH or the internal lysines of PAM) and the subsequent induction of the open conformation of plasminogen are insufficient to allow plasminogen activation by streptokinase encoded by (at least one of) the *ska* 2b alleles. Rather, fibrinogen is required as an activation cofactor. Enhancement by fibrinogen of plasminogen activation by streptokinase is well-described, however the mechanism by which this

occurs is poorly understood (Camiolo et al., 1980; Takada et al., 1985a). Fibrinogen does not possess C-terminal lysine residues, thus the initial interactions with plasminogen are thought to be mediated by internal lysine residues, while high-affinity interactions depend on plasmin-catalysed cleavage of fibrinogen and generation of C-terminal lysine residues (Christensen, 1984; Christensen, 1985; Henschen and Lottspeich, 1980). The activation rates of Glu-plasminogen, Lys-plasminogen and mini-plasminogen (containing only the protease domain and kringle 5) by streptokinase are all enhanced by fibrinogen (Chibber et al., 1985; Takada et al., 1985a; Takada et al., 1988), suggesting that the role of fibrinogen lies not only in the induction of an open conformation of plasminogen. Whether the role of fibrinogen in plasminogen activation by streptokinase of 2b alleles mediates or simply enhances the activation to detectable levels is not known. It is important to note that the majority of structural and mechanistic analyses of plasminogen activation by streptokinase have used commercially available group C streptokinase (Takada et al., 1985a; Takada et al., 1988; Wang et al., 1998), thus extrapolation of the mechanism of action to streptokinases of other allelic subclasses is speculative.

The role of the β domain of streptokinase in plasminogen activation has not been fully elucidated. Extensive biochemical analyses of the binding affinities between individual domains of plasminogen and streptokinase suggest that while all three streptokinase domains bind the plasminogen protease domain tightly, the β-domain additionally interacts with plasminogen kringle 5 (Loy et al., 2001). The extended conformation of plasminogen has a higher affinity for streptokinase due to availability of additional lysine binding sites in plasminogen (Boxrud and Bock, 2000). It has been proposed that the initial step in activation of free plasminogen involves a lysine-dependent interaction

between the β-domain of streptokinase and kringle 5 of plasminogen that induces an open conformation of plasminogen, allowing binding of all three streptokinase domains to the plasminogen catalytic domain (Figure 3.11B, 3.12) (Conejero-Lara et al., 1998; Loy et al., 2001). Within the β domain, residues Arg_{248}-Glu_{249}, Lys_{256}-Lys_{257} and Glu_{281}-Lys_{282} have been implicated in the creation of the active site in the initial "partner" plasminogen in the 1:1 activator complex (Chaudhary et al., 1999). Lys_{256}-Lys_{257} in the exposed "250-loop" are also involved in docking and processing of the second "substrate" plasminogen (Chaudhary et al., 1999) and deletion of this loop reduces the affinity of the streptokinase-plasminogen activator complex for the kringles of the substrate plasminogen by three-fold (Dhar et al., 2002). Both formation of the initial activator complex and binding of the second substrate plasminogen are lysine-dependent events mediated by kringle(s) of plasminogen (Lin et al., 2000).

Residues unique to the β domain of streptokinase with very inefficient solution-phase plasminogen activator activity (subcluster 2b) were identified (Figure 3.11). Of the 4 unique amino acids common to all *ska* 2b alleles, Ser_{210} and Thr_{153} correspond to similarly uncharged residues in other *ska* subclusters. However Arg_{161} of the β1 sheet of *ska* 2b alleles is the only positively charged residue at this position among the encoding *ska* allelic subclusters, while Glu_{209} of the α3,4 helix is uniquely negatively charged, and replaces Lys_{209} of the other subclusters. Statistical models of positive selection pressure on streptokinase have identified residues 161 and 210 among those subject to diversifying selection within the β domain (Kalia and Bessen, 2004). Interestingly, all 4 residues unique to *ska* 2b lie within a region of the streptokinase protein encompassing amino acids 144 - 218, which is the major focus of sequence heterogeneity (Johnston, 1991). In a recent study deletion of amino acids 124 – 237 of streptokinases of an *ska* 1

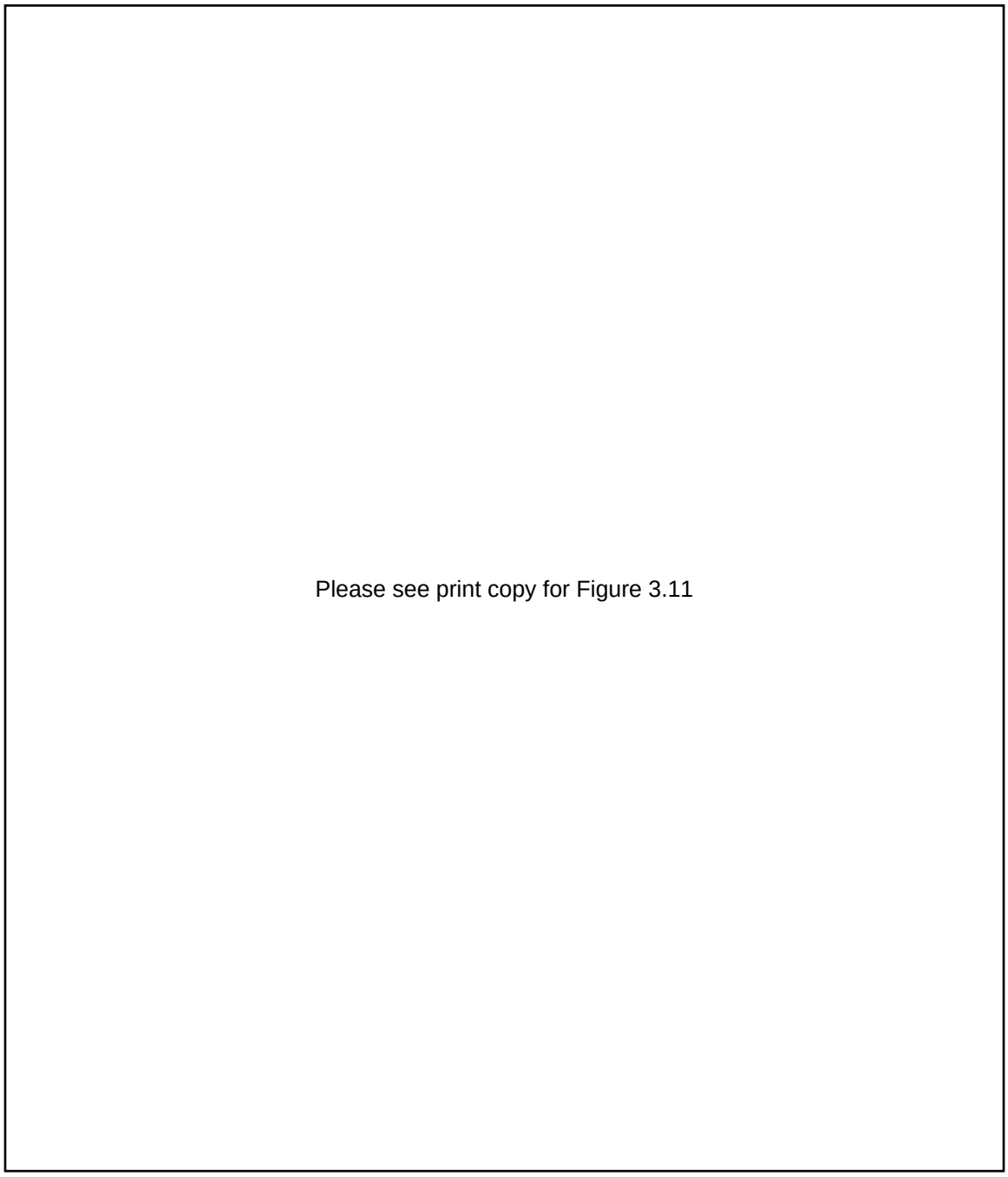

Fig 3.11 Crystal structure of the β domain of group C streptokinase showing homologous residues unique to *ska* 2b alleles, resolved **(A)** in isolation (Wang et al., 1999) or **(B)** as part of the streptokinase molecule complexed with the catalytic domain of human plasminogen (Wang et al., 1998). The β domain is involved in docking the "substrate" plasminogen (not shown) in the concavity generated by the so-called "β-grasp" structure of the 3 domains of streptokinase, such that the activation bond of the substrate is perfectly positioned for cleavage by the catalytic domain of the activator complex (Chaudhary et al., 1999). Stereoview (Wang et al., 1998) and ribbon diagrams (Wang et al., 1999) are reproduced from original papers.

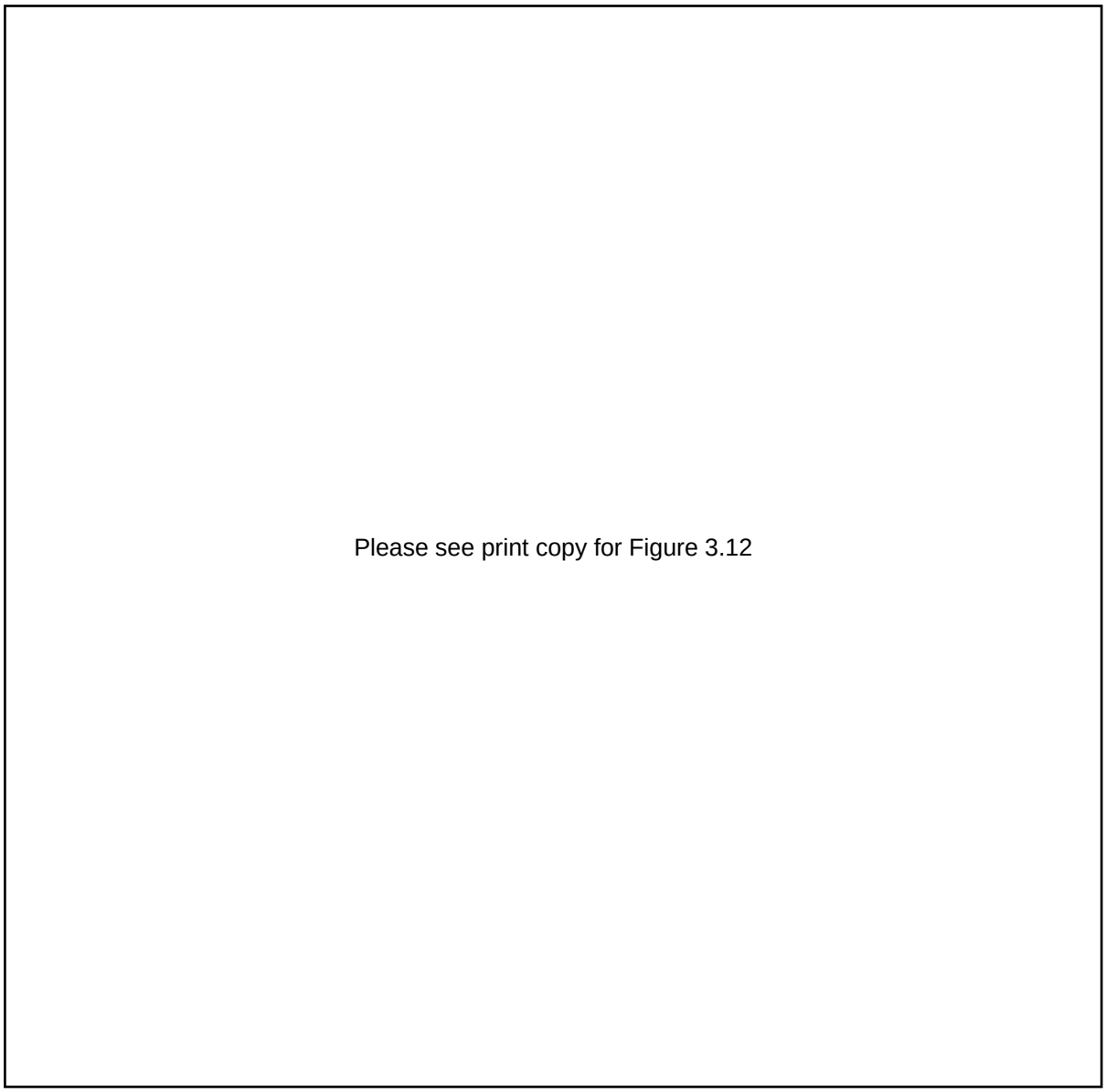

Figure 3.12. Schematic diagram depicting an integrated model of cell surface plasmin acquisition by various GAS isolates. Plasminogen contains seven distinct structural domains, designated the amino-terminal peptide (NTP) followed by kringles 1–5 (K1-5) and the C-terminal serine protease domain (PD). K1, K2, K4 and K5 contain lysine-binding motifs that are responsible for binding to fibrinogen and to plasminogen receptors. Lysine-dependent interaction/s between the NTP and K5 are also responsible for maintaining the circulating zymogen in a mostly closed, activation-resistant form. Efficient conversion of plasminogen to its activated twin-chain form plasmin (depicted by loss of the NTP and by the green coloured PD linked to the kringle domains via a disulphide bond shown in red) by mammalian or microbial activators requires a binding-induced conformational change in plasminogen to an open, activation-susceptible form (Loy et al., 2001; Parry et al., 2000; Ranson and Andronicos, 2003). **(A).** Plasminogen bound directly to the cell surface via PAM, or other plasminogen receptors (PL-R) may be activated to plasmin by host activators. **(B).** Streptokinase contains three structural domains designated the α, β and γ domains. The β domain interacts with K5 to induce the open conformation of plasminogen, allowing high-affinity binding of all 3 domains to the PD forming the streptokinase-plasminogen activator complex. Generally *pam*-negative strains secrete subcluster 1 or 2a allele forms of streptokinase (Kalia and Bessen, 2004) which can readily interact with solution-phase closed plasminogen (Loy et al., 2001). This results in a free activator complex that can convert substrate plasminogen molecules to plasmin that

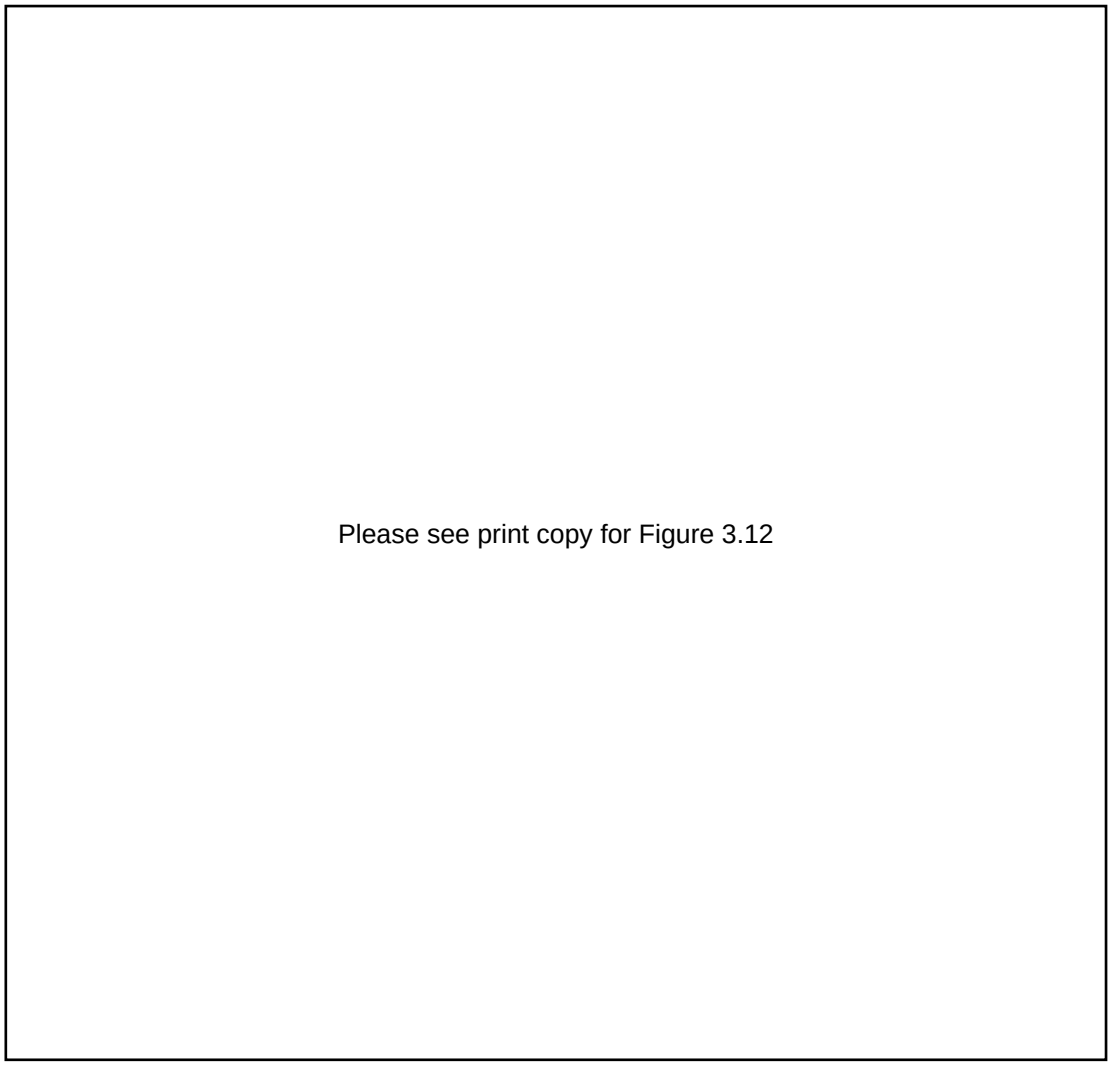

Figure 3.12. (cont) readily bind the cell surface directly via plasmin(ogen) receptors (e.g. SEN, GAPDH) (D'Costa and Boyle, 1998). Alternatively, the activator complex can bind to fibrinogen to form the fibrinogen-streptokinase-plasminogen trimolecular complex. This can then bind to the cell surface via fibrinogen receptors (F-R) and act on bound or free substrate plasminogen producing plasmin. The conversion of streptokinase-plasminogen to streptokinase-plasmin within the 1:1 activator complex occurs spontaneously (Boxrud and Bock, 2004). **(C)** Most *pam*-positive strains secrete subcluster 2b allele forms of streptokinase (orange) which require fibrinogen as a cofactor to form plasmin. The streptokinase-fibrinogen-plasminogen activator complex can convert solution-phase substrate plasminogen to plasmin. Alternatively, the complex may bind to the GAS cell-surface via plasminogen binding to fibrinogen and/or PAM, creating an exceptionally stable plasminogen activator which may activate either bound or free substrate plasminogen producing plasmin. Plasmin may bind the cell-surface via PAM, SEN, or GAPDH. In all scenarios, plasmin bound to receptors, streptokinase or within a trimolecular complex is resistant to inhibition by α2-antiplasmin. Modified from a previously published model (Walker et al., 2005).

and a 2a allele did not disable plasminogen activation by these species, and the authors suggested that this region plays no direct role in plasminogen activation (Lizano and Johnston, 2005). However, studies with individual domains of streptokinase indicate that each in isolation is capable of slow plasminogen activation, and that the presence of the β domain actually antagonises active site formation by the α and γ domains (Loy et al., 2001). Thus it cannot be assumed that full-length streptokinase does not require any of these 4 residues, for example to overcome steric hindrance introduced by the presence of the entire β domain, or to assist in the docking of substrate plasminogen, compared to the deletion mutants generated previously (Lizano and Johnston, 2005).

As a working hypothesis, it is proposed that either Asn/Glu_{161} of the β1 sheet and/or Lys_{209} of the α3,4 helix encoded by *ska* 1 and 2a alleles are essential for solution-phase activation of plasminogen, and that their replacement with differently charged residues reduces or abolishes plasminogen activation. Induction of an open conformation of plasminogen does not render it susceptible to activation by streptokinase of *ska* 2b alleles, suggesting that this is not the essential function conferred by residues 161 or 209 of the other subclusters. It is tempting to speculate that Lys_{209} encoded by subclusters 1 and 2a is required for one of the lysine-dependent interactions either generating the active site in the first plasminogen molecule, or is permissive for docking of the substrate plasminogen (Boxrud and Bock, 2000; Chaudhary et al., 1999; Lin et al., 2000). Studies using site-directed mutagenesis to replace these residues in solution-phase active and inactive streptokinases are suggested. It remains possible that the critical residues lie outside the β domain and that further sequence comparison of the α or γ domains will be required. Fibrinogen partially reverses the loss of activator

potential of the *ska* 2b alleles, through unknown mechanisms which are not related to induction of the open conformation of plasminogen.

3.4.1.2 Model for cell-surface plasminogen activation by GAS isolates according to pam genotype and streptokinase allelic subcluster

In the current study, plasmin acquisition by *pam*-positive isolates in plasma was not correlated with plasminogen binding by the isolates, but was correlated with fibrinogen binding. In addition, the higher fibrinogen-binding by *pam*-positive isolates and the correlation between fibrinogen binding and direct plasminogen binding for these isolates points to the occurrence of a fibrinogen receptor coinherited with PAM; or that PAM itself has dual receptor activity (Chapter 2). This data supports an important role for fibrinogen in the remarkably high-levels of cell-surface plasmin acquisition by GAS isolates possessing PAM and streptokinase encoded by subcluster 2b alleles, compared to *pam*-negative isolates.

An integrated model is proposed for GAS cell-surface plasminogen acquisition by isolates possessing *ska* alleles of the various subclusters (Figure 3.12). For isolates possessing *ska* 2b alleles, it is proposed that PAM is important for sequestering Glu-plasminogen to the GAS cell-surface via kringle 2 (Figure 3.12A, C). Fibrinogen receptors (possibly including PAM itself) colocalise high levels of fibrinogen at the cell-surface, providing the necessary cofactor for activation of plasminogen by the *ska* 2b streptokinase secreted by these isolates (McArthur et al., 2005) (Figure 3.12C). The trimolecular complex of plasminogen, fibrinogen and streptokinase is generated and may bind to the cell-surface by either PAM or fibrinogen receptors (McKay et al., 2004). If PAM does possess dual receptor activity, both plasminogen and fibrinogen

within the complex could potentially bind PAM, stabilising its cell-surface location. This complex generates plasmin that can bind directly to surface receptors PAM, SEN or GAPDH (D'Costa and Boyle, 1998). In addition, the presence of plasmin generates Lys-plasmin(ogen) which has a higher affinity for SEN (Derbise et al., 2004). Thus the higher plasminogen- and fibrinogen-binding capacity of these isolates results in higher levels of plasmin acquisition.

This contrasts with *pam*-negative isolates (Figure 3.12A, B) that require both fibrinogen and streptokinase (encoded by non-*ska* 2b alleles) in order to bind significant levels of plasminogen to the cell-surface via fibrinogen-binding proteins (McKay et al., 2004). The activator complex generates plasmin that can bind directly to surface receptors SEN or GAPDH (D'Costa and Boyle, 1998). The absence of PAM and lower fibrinogen binding capacity of these strains results in a lower level of plasmin acquisition.

3.4.1.3 In vitro plasminogen activation and clinical source

As described in another GAS population, there was no relationship between clinical source of isolates and secreted streptokinase activity *in vitro* (Tewodros et al., 1995). Amongst those isolates in which streptokinase activity could be detected (*pam*-negative), there was a correlation between secreted streptokinase activity and plasmin acquisition in plasma. The reduction in plasmin acquisition of a streptokinase-negative mutant of ALAB49 and the significant plasmin acquisition by *pam*-positive isolates despite tPA inhibition also suggests the importance of streptokinase in plasmin acquisition by *pam*-positive isolates. Corroborating a previous study in North American isolates (Wang et al., 1994), we found no difference in plasmin acquisition in plasma between isolates from invasive and uncomplicated infections.

These observations may suggest that streptokinase secretion and plasmin acquisition are not important in establishing invasive infection, or that only a low threshold of cell-surface plasmin is required for infection. However, the NT isolates compared in this study were of diverse *emm* sequence types, *vir* types and *emm* patterns (McKay et al., 2004), and thus might be expected to differ significantly in expression of other virulence determinants. In contrast, a recent study of highly related *emm*49 isolates revealed higher streptokinase expression at the transcriptional level amongst isolates from severe invasive disease than those from non-invasive disease (Ikebe et al., 2005). In addition, host factors play a significant role in determining the severity of GAS infection (Kotb et al., 2002; Norrby-Teglund et al., 2000). For example, levels of humoral immunity to GAS virulence factors have been reported to influence susceptibility to invasive infection by clonal isolates (Basma et al., 1999). Anti-streptokinase antibodies and streptokinase resistance are found at exceptionally high levels amongst NT and other indigenous Australian communities where GAS infection is endemic (Blackwell et al., 2005; Urdahl et al., 1996). Thus the level of streptokinase secretion and plasmin acquisition *in vitro* may not be reflective of available streptokinase and plasmin acquisition during infection *in vivo*. Overall, it is likely that establishment of invasive infection results from complex multifactorial interactions between the diverse repertoire of GAS virulence factors including streptokinase, fibrinogen and plasminogen receptors, and the human host.

The finding that cell-surface plasmin acquisition in 100% plasma does not differ between isolates from invasive and uncomplicated infection does not preclude a role for the previously observed higher plasminogen binding in invasive disease. Plasminogen binding assays were performed using plasminogen at concentrations below those found

in human plasma, and may be reflective of tissue niches where plasminogen is more scarce, such as the surface of healthy skin.

3.4.1.4 SpeB, clinical source, and cell-surface plasmin acquisition

There was no relationship between secreted SpeB activity and propensity to cause invasive disease amongst the NT GAS isolates examined. An inverse relationship between SpeB and severity of invasive infection was found in a clonal M1T1 isolate collection (Kansal et al., 2000), thus clonal differences may explain the lack of relationship observed in the NT collection. Alternatively, factors influencing the establishment of invasive disease and the severity of invasive infection may differ. Three isolates possessing the gene for SpeB produced neither detectable levels of SpeB activity nor protein, consistent with the reported existence of stable SpeB-negative phenotypes (Aziz et al., 2004; Kansal et al., 2000; Raeder et al., 2000). Interestingly isolate NS192 produces a mature 28 kDa SpeB protein with no detectable proteolytic activity.

SpeB degradation of streptokinase in early stationary-phase cultures was demonstrated by SpeB inhibition experiments, while the degradation of PAM is suggested by increased plasminogen binding of the *speB*- isogenic mutant of *pam*-positive isolate ALAB49. Isogenic mutants revealed a profound influence of SpeB on the acquisition and/or stability of cell-surface plasmin of both a *pam*-positive and a *pam*-negative isolate. The potential effect of SpeB on virulence mediated by the plasminogen activation system is discussed in the following section.

3.4.2 Virulence of GAS isolates in the human plasminogen transgenic mouse model of infection

Experiments in the human plasminogen transgenic mouse model confirm a critical role for human plasminogen in GAS virulence. Of the *pam*-positive clinical isolates examined, NS88.2 (from an invasive infection) and ALAB49 (from uncomplicated infection) acquired relatively high and low levels of cell-surface plasmin in plasma respectively. NS88.2 showed markedly enhanced virulence in the presence of the human plasminogen transgene, underscoring the specificity of GAS for human plasminogen. ALAB49 showed no such enhancement, and while this result may suggest a dose effect of cell-surface plasmin acquisition in this animal model, the results from the *pam*-negative isolates suggest otherwise. Of the *pam*-negative isolates examined, NS696 (from an uncomplicated infection) and NS730 (from invasive infection) acquired similarly low levels of cell-surface plasmin in plasma to that of ALAB49 and were also avirulent in wildtype mice. In contrast, in the human plasminogen transgenic mouse, NS696 acquired virulence potential while NS730 remained avirulent. NS696 is the only *emm*1 isolate examined in this model and is the only one of the 30 isolates examined possessing the *ska* 2a streptokinase allele. Whether the products of either of these genes function synergistically with human plasminogen to enhance virulence is unknown, however the recently described induction of vascular leakage by released fibrinogen-binding M1 protein could potentially provide a rich source of plasminogen at the infection site (Herwald et al., 2004). Combined, these observations suggest a complex interaction of the plasminogen system with other GAS virulence determinants and highlight the benefit of the human plasminogen transgenic mouse model as more sensitive for discerning differences in GAS pathogenesis.

The interaction of SpeB with the plasminogen activation system may play a significant role in this model. SpeB mediates streptokinase and plasminogen receptor degradation and radically lowers cell-surface plasmin acquisition by GAS isolates in *vitro*. Isolates producing the lowest levels of SpeB showed enhanced virulence in the human plasminogen transgenic mouse, while isolates producing large amounts of SpeB did not. These observations are consistent with a model in which SpeB reduces invasive potential by disabling plasminogen activation. However, other studies suggest a synergy between the two systems during infection at the skin, where SpeB is required for full virulence (Svensson et al., 2002). Interestingly in this study a correlation between SpeB activity at stationary phase and streptokinase activity at mid-log phase was observed. To reconcile these observations, it is hypothesised that SpeB is important for establishing local infection (Saouda et al., 2001; Svensson et al., 2000). However loss of SpeB activity in a subpopulation (Aziz et al., 2004; Raeder et al., 2000) allows these bacteria to focus plasmin activity at the GAS surface (Figure 3.9B). The acquisition of fibrinolytic activity by this subpopulation allows escape from the fibrin networks containing the infection and invasion to sterile sites.

While the presence of the human plasminogen transgene did not induce virulence in ALAB49 in this model, the importance of plasminogen activation for virulence of this strain has been demonstrated in a humanized model of streptococcal impetigo, in which the skin is superficially wounded before infection (Svensson et al., 2002). This may be clinically relevant in the case of NT GAS infection which often manifests secondary to scabies lesions (Wong et al., 2002). Finally, the source of the clinical isolate (invasive or uncomplicated infection) does not predict virulence in the human plasminogen

transgenic mouse model, further suggesting the importance of additional host factors in clinical outcome.

In summary, the results demonstrate that NT GAS isolates acquire and activate plasminogen from plasma at different levels according to *pam* genotype, via streptokinases of defined allelic subtype, size and catalytic specificity. The plasminogen system is critical for virulence of some clinical isolates of GAS in the human plasminogen transgenic mouse model, but is not entirely predictable from *in vitro* measures of plasminogen activation. SpeB causes a radical reduction in cell-surface plasmin activity that may impact on virulence of GAS in the presence of human plasminogen. The complex interaction of the plasminogen activation system with other host and bacterial factors is a key component of GAS virulence.

CHAPTER 4 General discussion and future work

4.1 General discussion

4.1.1 Summary of the major aims and findings of this study

In the tropical NT of Australia, GAS infection and its sequelae are significant contributors to morbidity and mortality, especially amongst the indigenous community (Carapetis and Currie, 1997; Carapetis et al., 1996). The high rate of GAS invasive disease in the NT is not caused by dominant clones nor preceded by GAS pharyngitis as in urbanised populations (Johnson et al., 1992). Rather, this high rate exists against a background of hyperendemic skin infection with rapid turnover of GAS strains and ample opportunity for genetic exchange with other GAS strains and streptococcal species (Carapetis et al., 1995a; Carapetis et al., 1999a; Carapetis et al., 1999c; Fiorentino et al., 1997; Towers et al., 2003; Towers et al., 2004). The putative importance of the GAS-plasminogen system in skin infection and in invasive processes prompted the current study of the role of plasminogen in invasive NT GAS disease (Kalia and Bessen, 2004; Svensson et al., 1999; Svensson et al., 2002).

This study demonstrated that while NT GAS isolates from invasive disease cases acquire more plasminogen than isolates from uncomplicated infections, they do not produce more streptokinase nor acquire more cell-surface plasmin after incubation in human plasma *in vitro*. However, infection studies in the human plasminogen transgenic mouse model revealed the critical role of plasminogen in virulence of some NT isolates. The dramatic reduction of GAS cell-surface plasmin activity by SpeB may significantly disable GAS invasive potential. In addition, novel aspects of plasminogen activation by *pam*-positive isolates were characterised and a new model of GAS cell-surface plasminogen activation proposed for these isolates.

4.1.2 Importance and limitations of the study

There are several important advantages and limitations inherent in the *in vitro* studies conducted. Firstly, the characterisation of a large isolate group dictates that a small number of time-points or conditions be assessed. While every effort was made to mimic physiologically relevant conditions, these snapshots may not provide an accurate representation of an entire phenomenon nor timecourse during infection *in vivo*. For example, the stability of cell-surface plasmin over time could not be assessed for each isolate. The kinetics of SpeB induction may differ among these genetically diverse isolates along with their demonstrated variation in growth kinetics, with concomitant variability in the degradation of cell-surface plasmin. These factors could not be accounted for within the study design. Thus it remains unclear whether some other measure of persistence, maximal acquisition or a certain threshold of cell-surface plasmin *in vitro* may be associated with virulence *in vivo*. However, *in vitro* functional experiments identified the striking variation of catalytic activity of streptokinase with *pam* genotype in this study, highlighting the utility of the study design.

Secondly, NT GAS isolate collections, including invasive disease-causing subgroups, exhibit remarkable clonal diversity (Carapetis et al., 1999c; Hassell et al., 2004; McKay et al., 2004). Thus it is impossible to control for the effects of other virulence factors when assessing the role of GAS plasminogen activation in virulence of NT GAS isolates. It is possible that the clonal diversity within this collection obscures the true biological effect of plasminogen activation on virulence inferred from clinical source of the isolates in *in vitro* studies. Studies of clonal isolates (within a single *emm*-type) have identified relationships between streptokinase expression and invasive disease, and between SpeB expression and infection severity (Ikebe et al., 2005; Kansal et al., 2000).

In contrast, studies of other clonally diverse collections have found no relationship between plasminogen activation and GAS disease manifestation (Tewodros et al., 1995; Wang et al., 1994), similar to that found in this study. Similarly, comparisons of other Northern Australian isolate collections have failed to find relationships between invasive propensity and presence/genotype of genes encoding virulence factors including the M protein, fibronectin binding proteins PrtF1, SfbI, SfbII and Fpb54; the toxin NAD glycohydrolase; or the pyrogenic exotoxins SpeA, SpeB and SpeC (Bricker et al., 2005; Carapetis et al., 1999c; Delvecchio et al., 2002a; Delvecchio et al., 2002b; Norton et al., 2004). To the author's knowledge, the only reported molecular differences between Australian GAS isolates from invasive disease and uncomplicated infections are the higher plasminogen binding identified in this study and the increased prevalence of the *prtfII* gene among invasive disease isolates (Delvecchio et al., 2002a; McKay et al., 2004). Interestingly, one of the two PrtfII subtypes, PFBP, is also a fibrinogen-binding protein (Ramachandran et al., 2004a; Ramachandran et al., 2004b) and thus a potential receptor for the fibrinogen-plasminogen-streptokinase trimolecular complex.

The third limitation inherent in comparison of plasminogen activation by NT isolates of differing clinical source is the difference in virulence generated by variable susceptibility of the host to infection or to particular disease manifestations. Genetic variability of HLA type and humoral immunity to virulence factors such as the M protein, SpeA and SpeB influence GAS disease severity or susceptibility (Basma et al., 1999; Eriksson et al., 1999; Holm et al., 1992; Kotb et al., 2002). With frequent GAS exposure, many NT residents develop humoral immunity to streptokinase; with almost one quarter of Aboriginal adults exhibiting streptokinase resistance capable of completely neutralising therapeutic doses of streptokinase (used as a thrombolytic

treatment) (Blackwell et al., 2005; Urdahl et al., 1996). The implications of streptokinase resistance for virulence of NT GAS have not been investigated.

The clinical profile of invasive GAS disease in the NT also implicates compromised host physiology in GAS invasive potential. In a recent case series of necrotising fasciitis from the NT of Australia 79% of patients had concurrent chronic diseases such as type II diabetes, alcoholism, hypertension, steroid-treated autoimmune disease, cirrhosis, renal impairment etc; while 64% had pre-existing wounds (Hassell et al., 2004). Amongst cases of bacteremia, 90% of adult patients had at least one predisposing factor, compared with 40% for children. No evidence of particularly virulent strains was found, and it was concluded that a major risk factor for GAS bacteremia amongst Aboriginal people in the NT is high-level exposure to a wide range of GAS strains (Carapetis et al., 1999c). In the current study, however, NT clinical isolates displayed markedly different virulence potential in genetically identical hosts (both wildtype and human plasminogen transgenic mice) suggesting that there are important differences between isolates in ability to invade host tissue.

4.1.3 Therapeutic implications of the current study for NT GAS infection

While an important role of human plasminogen activation in GAS virulence is suggested by the current study and a growing body of international work (Boyle and Lottenberg, 1997; Lahteenmaki et al., 2005; Sun et al., 2004), targeting of this system in the prophylaxis and treatment of GAS infection has not been directly addressed. Plasmin inhibition by agents such as aprotinin, or competitive inhibition of plasminogen binding with lysine analogues such εACA are problematic given the fundamental and diverse role of plasmin(ogen) in normal physiological processes such as migration of

immune cells, wound healing and tissue remodelling, and particularly fibrinolysis (Dano et al., 1999; Plow et al., 1999; Pollanen et al., 1991). In fact, both aprotinin and εACA are currently used in the prophylaxis and treatment of post-surgical bleeding (Koscielny et al., 2004; Ray et al., 2005; Ray and O'Brien, 2001).

One potential prophylactic option is the development of vaccines based on components of the GAS plasminogen activation system, such as GAS plasminogen and fibrinogen receptors, and streptokinase. Development of neutralising antibodies against these virulence factors could potentially disable GAS invasive potential (Basma et al., 1999; D'Costa and Boyle, 1998; Holm et al., 1992), along with the objectives of boosting protective opsonic antibody and T-cell responses to GAS (McMillan et al., 2004; Pruksakorn et al., 1992). However, certain components of the GAS-plasminogen system may prove unsuitable vaccine candidates. Anti-SEN antibodies and anti-GAPDH antibodies have been implicated in autoimmune disease processes (Cunningham, 2000; Fontan et al., 2000; Rattner et al., 1991; Takasaki et al., 2004). Boosting of immune responses against streptokinase may prove problematic in acute post-streptococcal glomerulonephritis, in which streptokinase deposition and the ensuing immune response potentially contribute to glomerular pathology (Nordstrand et al., 1998; Nordstrand et al., 1999). Autoreactive T-cell responses against cardiac antigens are elicited by vaccination with M protein (Cunningham, 2000; Kehoe, 1991). Thus at this early stage of research on GAS plasminogen activation, there is no obvious potential therapeutic target arising from the current work. However, given the importance of GAS plasminogen activation in virulence, it is hoped that a greater understanding of this system will aid in the development of more effective prophylaxis and treatment of GAS infection.

4.2 Future work

4.2.1 Future animal modelling of NT GAS invasive infection

In the human plasminogen transgenic mouse model of infection two of the four isolates showed enhanced virulence in the presence of the transgene, highlighting the importance of human plasminogen in GAS invasion. Indeed, human plasminogen was an absolute requirement for fatal infection with isolate NS696, demonstrating enhanced sensitivity of this model to differences between isolates. However, two of the four isolates including a necrotising fasciitis isolate, showed no virulence in the presence or absence of the transgene. The other non-virulent isolate in this model is an impetigo isolate which has shown significant virulence in the humanized model of impetigo, in which human skin grafted onto SCID mice is superficially wounded prior to infection with GAS (hu-skin-SCID model) (Scaramuzzino et al., 2000; Svensson et al., 2002). It would be of interest to examine the blood of mice infected with avirulent isolates to examine whether there is a deficiency in transition to the blood by these isolates, or whether the isolates breach the vasculature but express an exotoxin profile that is ineffectual in establishing severe disease in this model.

One important determinant of the severity of GAS infection is the host inflammatory response to GAS superantigens, which is associated with HLA type in human infection (Chatellier et al., 2000; Kotb et al., 2002; Norrby-Teglund et al., 2000; Norrby-Teglund et al., 2002). Congenic mice which differ only in the MHC locus also display markedly different susceptibility to GAS infection, due in part to differences in the inflammatory cytokine response of T cells to superantigens presented by the different MHC (Goldmann et al., 2005b). Differences in innate immunity also play an important role in

differential GAS infection susceptibility between MHC haplotypes (Goldmann et al., 2005a; Goldmann et al., 2005b; Medina et al., 2001). Humanising of the MHC class II (DR6) and CD4 renders mice exquisitely sensitive to staphylococcal superantigen (10-100 fold lower concentrations) compared to control mice in a model of superantigen-induced sepsis (Yeung et al., 1996). To examine the role of plasminogen in virulence of isolates that do not show virulence in the human plasminogen transgenic mouse model of infection, a hybrid model is suggested in which the human plasminogen transgene is expressed against a superantigen-susceptible MHC background. This could potentially be achieved using either susceptible murine MHC such as $H2^k$ or by producing double transgenic mice bearing recently identified susceptible human haplotypes (Goldmann et al., 2005b; Kotb et al., 2002). Such a model may help elucidate the relative importance and/or synergy of GAS superantigen expression and plasminogen activation in the development of invasive disease by clonally diverse GAS isolates.

Another important question to be addressed is the effect of streptokinase resistance in the NT on GAS virulence. *In vitro* studies have demonstrated that anti-streptokinase antibodies inhibit the assembly of GAS cell-surface plasminogen activator (D'Costa and Boyle, 1998). However, the role of these antibodies at various stages of infection *in vivo* is unknown, and infection studies using isolates which are virulent in the human plasminogen transgenic mouse are suggested, with and without preimmunisation with streptokinase.

4.2.2 Future research questions surrounding GAS-plasminogen activation and its role in virulence

This study showed that streptokinase encoded by *ska* 2b alleles does not activate plasminogen in solution and further work in our laboratory has suggested a requirement for fibrinogen as a cofactor in plasminogen activation by this subclass of streptokinase proteins. Unique amino acid residues of streptokinase β domains encoded by *ska* 2b subcluster alleles compared to the β domains encoded by *ska* 1 and 2a alleles were identified.

In the first instance it will be necessary to determine whether the unique β domain encoded by *ska* 2b alleles is responsible for the novel catalytic activity of the encoded protein. Construction of chimeric proteins, formed by replacement of the entire *ska* 1 or *ska* 2a allele with the *ska* 2b allele within the *ska* gene and expression of the resulting gene product is suggested. Expression and analysis of plasminogen activation in solution of the chimeras would assist in determining whether the β domain encoded by *ska* 2b imparts "solution-phase resistance" to the activity of streptokinase.

If this is found to be the case, site-directed mutagenesis of the *ska* gene is suggested to replace the individual residues encoded by *ska* 1 and *ska* 2a alleles with the unique amino acid residues of streptokinase β domains encoded by *ska* 2b subcluster alleles (as discussed in section 3.4.1.1). Expression and analysis of plasminogen activation in solution of the mutant gene products would assist in determining any role of these residues in both the solution-phase resistance and the fibrinogen dependence of *ska* 2b-encoded streptokinase activity.

One of the major findings of this study was the dramatic reduction of cell-surface plasmin by SpeB, and the observation that low SpeB-secreting isolates are more virulent in the human plasminogen transgenic mouse model than high SpeB-secreting isolates. This relationship has been investigated further in our laboratory for the invasive M1T1 isolate, 5448. Results of that study suggest that while SpeB is important for establishing localised infection, the loss of SpeB in a subpopulation of bacteria allows the accumulation of cell-surface plasmin and systemic dissemination in the human plasminogen transgenic mouse (Cole et al, *submitted*). It is of significant interest to determine whether this is an *emm*-type-specific phenomenon, or common to other GAS clones, particularly in the NT. It will also be of interest to identify the environmental or GAS quorum signal triggering this switch in transcriptional regulation.

A clear exception to this infection model is the invasive-disease isolate NS88.2 which induces mortality in both wildtype and human plasminogen transgenic mice, and yet produces no detectable SpeB *in vitro*. It is hypothesised that NS88.2 uses an alternate exotoxin profile to initiate tissue damage and establish local infection. The requirement of SpeB for establishment of local infection may also involve evasion of host defense (Collin et al., 2002; Eriksson and Norgren, 2003; Voyich et al., 2003) and NS88.2 may employ alternate pathways for evasion of local host defence.

Factors affecting the regulation of streptokinase and SpeB have only begun to be elucidated in the last decade. M1 transcriptomes (for isolate MGAS 5005) have revealed that SpeB is among the genes upregulated during phagocytosis by polymorphonuclear leukocytes to evade killing by these cells (Voyich et al., 2003). Upon incubation in whole blood, SpeB is downregulated, while streptokinase is upregulated (Graham et al.,

2005). Human blood is relatively poor in free amino acids and streptokinase may be essential for nutrient acquisition in this peptide-rich environment (Graham et al., 2005). Several models of GAS infection have suggested that SpeB is induced during nutrient depletion while streptokinase is induced during nutrient-rich stages of infection once the inflammatory process is established (Rasmussen and Bjorck, 2002; Svensson et al., 2002). Further work is required to clarify the role and regulation of plasmin and SpeB in the various stages of infection from the different tissue niches.

Finally, the relative effects of SpeB and GAS-bound plasmin on secreted host immune mediators have not been compared. Recently a trypsin-like serine protease secreted by GAS was shown to cleave IL-8 and prevent neutrophil chemotaxis to the GAS infection site, resulting in severe invasive infection (Edwards et al., 2005; Hidalgo-Grass et al., 2004). It is not known whether the trypsin-like serine protease plasmin may similarly cleave IL-8. It would be of interest to compare a panel of cytokines and chemokines important in the host response to GAS infection for proteolytic degradation by plasmin and SpeB.

4.2.3 Final comments

The search for bacterial virulence factors associated with NT invasive disease continues and the regulation of the GAS-plasminogen activation system appears to be critical for virulence of at least a proportion of NT isolates (McKay et al., 2004; Ramachandran et al., 2004a). Current research efforts in Australia are also directed towards the development of a safe multivalent M-protein based vaccine for the Aboriginal population (Batzloff et al., 2005; Brandt et al., 2000). For now, there remains an urgent need for improved hygiene and housing, scabies control and antibiotic treatment of

GAS skin disease amongst NT indigenous populations to more effectively prevent the devastating consequences of endemic GAS infection and its sequelae (Carapetis et al., 1997; Carapetis et al., 1999b; Shelby-James et al., 2002).

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APPENDICES

Appendix 1: Buffers and reaction mixtures

Protein Analysis

SDS-PAGE

Sample buffer (2X)

Tris HCl	60 mM
SDS	2% (w/v)
Glycerol	10% (w/v)
Bromophenol blue	0.01% (w/v)
2-β-mercaptoethanol (for reducing buffer)	5% (w/v)

Used as a 1 x working solution

SDS Running buffer (10X; pH 8.3)

Tris Base	30 g/l
Glycine	144 g/l
SDS	10 g/l

Used as a 1 x working solution

Coomassie Blue Stain

Methanol	40% (v/v)
Glacial acetic acid	10% (v/v)
Coomassie Blue R250	0.1% (w/v)

Destain

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

Western Transfer and Immunoblotting

Electrophoretic Transfer Buffer (pH 8.3)

Tris Base	3.03 g/l
Glycine	14.4 g/l
Methanol	20% (v/v)

Tris Buffered Saline (TBS; pH 7.5)

NaCl	8 g/l
KCl	0.2 g/l
Tris Base	3 g/l
Tween-20 (for wash buffer, TBST)	0.05% (v/v)

Blocking and Antibody Buffer

Bovine serum albumin	1%

Dissolved in TBS

Diaminobenzidine chromogenic detection system for Western blot

diaminobenzidine	0.5 mg/ml
H_2O_2	0.02% (v/v)

Dissolved in 100mM Tris, pH 7.6

ELISA

Carbonate Coating buffer (pH 8.5)

$NaHCO_3$	50 mM

PINT assay buffer

Na_2HPO_4	7.1 g/l
NaCl	8.76 g/l
Tween 80	0.05% (v/v)

Blocking buffer

BSA	1% (w/v)

Dissolved in PINT buffer

Developing solution

Na_2HPO_4	1.14 g/l

Adjusted to pH 5.0 with solid citric acid.

Immediately before use, the following added:

o-phenylene diamine	0.4 g/l
H_2O_2	3% (w/w)

Plasminogen purification

Plasminogen elution buffer

PBS, pH 7.5	1X
ε-aminocaproic acid	26.2 g/l

Phosphate-buffered saline (PBS)

NaCl	8 g/l
KCl	0.2 g/l
NaH_2PO_4	1.44 g/l
KH_2PO_4	0.24 g/l

Column wash buffer

PBS	1X
EDTA	5 mM
PMSF	1 mM*

** From a stock solution in isopropanol prepared immediately before addition*

High-salt wash buffer

NaH_2PO_4	6.0 g/l
NaCl	29 g/l
EDTA	5 mM
PMSF	1 mM*

** From a stock solution in isopropanol prepared immediately before addition*

DNA analysis

Polymerase chain reaction

PCR reaction mix (50µl)

dH_2O	31.5 µl
dNTPs* (2.5mM)	5 µl
$MgCl_2$ (25mM)	5 µl
Taq buffer (Sigma; contains 15mM $MgCl_2$)	5 µl
Forward primer (25 µM)	1 µl
Reverse primer (25 µM)	1 µl
Streptococcus pyogenes genomic DNA	1 µl
Taq polymerase (5 units/µl)	0.5 µl

** stock solution contains 2.5 mM each of dATP, dCTP, dGTP, dTTP*

Agarose gel electrophoresis

Tris acetate (TAE) buffer (10X)

Tris base	48.4 g/l
Glacial acetic acid	1.14% (v/v)
EDTA (0.5 M, pH 8.0)	2% (v/v)

Used as a 1X working solution

DNA loading dye (6X)

Bromophenol Blue	0.25% (w/v)
Xylene cyanol	0.25% (w/v)
Glycerol	30% (v/v)

DNA sequencing

DNA sequencing reaction for BigDye 2.1

Terminator Ready reaction Mix (v2.1)	4.0 µl
DNA template	1 µl
Primer (1pmol/µl)	1.6 µl
dH_2O	6.6 µl

DNA sequencing reaction for BigDye 3.1

Ready reaction Mix (v3.1)	0.5 µl
Dilution buffer (see below)	2 µl
Primer (1pmol/µl)	1.6 µl
DNA template	1 µl
dH_2O	4.9 µl

DNA sequencing dilution buffer (5X) for BigDye 3.1

Tris, pH 9.0	400 mM
$MgCl_2$	10 mM

DNA loading buffer for sequencing gel electrophoresis

Deionized formamide	5 volumes
25 mM EDTA, pH 8.0 containing 50 mg/ml blue dextran	1 volume

Appendix 2: GAS culture conditions for streptokinase supernatant collection

Table 1. GAS culture optical density at 600nm at mid-log phase supernatant collection for streptokinase analysis.

pam genotype	Clinical source[a]	Isolate	Absorbance (600nm)				
			Culture 1	Culture 2	Culture 3	Mean	SD
-	inv	NS179	0.63	0.69	0.55	0.62	0.07
-	inv	NS192	0.59	0.60	0.65	0.61	0.03
-	inv	NS210	0.65	0.62	0.62	0.63	0.02
-	inv	NS414	0.63	0.62	0.54	0.60	0.05
-	inv	NS452	0.58	0.58	0.60	0.58	0.01
-	inv	NS501	0.60	0.61	0.61	0.61	0.01
-	inv	NS730	0.59	0.52	0.58	0.56	0.04
-	inv	NS733	0.61	0.57	0.52	0.56	0.04
-	inv	NS931	0.58	0.61	0.64	0.61	0.03
-	uncomp	NS14	0.55	0.58	0.66	0.60	0.06
-	uncomp	NS236	0.81	0.66	0.57	0.68	0.12
-	uncomp	NS244	0.63	0.60	0.56	0.60	0.04
-	uncomp	NS297	0.55	0.60	0.64	0.60	0.04
-	uncomp	NS474	0.64	0.58	0.58	0.60	0.03
-	uncomp	NS488	0.59	0.60	0.58	0.59	0.01
-	uncomp	NS696	0.63	0.60	0.69	0.64	0.04
-	uncomp	NS836	0.58	0.63	0.56	0.59	0.04
+	inv	NS1133	0.64	0.58	0.55	0.59	0.04
+	inv	NS13	0.57	0.67	0.68	0.64	0.06
+	inv	NS223	0.74	0.64	0.77	0.71	0.07
+	inv	NS455	0.57	0.60	0.60	0.59	0.02
+	inv	NS88.2	0.63	0.58	0.61	0.61	0.03
+	uncomp	NS10	0.56	0.62	0.63	0.60	0.04
+	uncomp	NS253	0.64	0.67	0.59	0.63	0.04
+	uncomp	NS265	0.58	0.56	0.64	0.59	0.04
+	uncomp	NS32	0.55	0.57	0.54	0.55	0.02
+	uncomp	NS50.1	0.63	0.62	0.60	0.62	0.02
+	uncomp	NS53	0.50	0.55	0.50	0.52	0.03
+	uncomp	NS59	0.64	0.61	0.62	0.62	0.02

[a]clinical source of the isolate; invasive (inv) or uncomplicated infection (uncomp).

Table 2. GAS culture optical density at 600nm at late-log/early stationary phase supernatant collection for streptokinase analysis.

pam genotype	Clinical source[a]	Isolate	Absorbance (600nm)				
			Culture 1	Culture 2	Culture 3	Mean	SD
-	inv	NS179	1.11	1.11	1.21	1.14	0.06
-	inv	NS192	1.03	1.04	1.07	1.05	0.02
-	inv	NS210	1.07	1.08	1.08	1.08	0.01
-	inv	NS414	1.14	1.13	1.12	1.13	0.01
-	inv	NS452	1.24	1.21	1.25	1.23	0.02
-	inv	NS501	0.90	0.90	0.94	0.91	0.02
-	inv	NS730	1.11	1.16	1.19	1.15	0.04
-	inv	NS733	1.31	1.28	1.33	1.31	0.02
-	inv	NS931	1.13	1.10	1.12	1.12	0.01
-	uncomp	NS14	1.03	1.15	1.09	1.09	0.06
-	uncomp	NS236	1.19	1.17	1.06	1.14	0.07
-	uncomp	NS244	0.99	1.15	1.05	1.07	0.08
-	uncomp	NS297	1.15	1.17	1.17	1.16	0.01
-	uncomp	NS474	1.25	1.19	1.29	1.25	0.05
-	uncomp	NS488	1.21	1.20	1.21	1.20	0.01
-	uncomp	NS696	1.09	1.10	1.15	1.11	0.03
-	uncomp	NS836	1.06	1.10	1.12	1.09	0.03
+	inv	NS1133	1.15	1.12	1.11	1.13	0.02
+	inv	NS13	1.02	1.07	1.09	1.06	0.03
+	inv	NS223	1.22	1.09	1.29	1.20	0.11
+	inv	NS455	1.04	1.03	1.18	1.08	0.09
+	inv	NS88.2	1.16	1.14	1.13	1.14	0.01
+	uncomp	NS10	1.21	1.20	1.09	1.17	0.06
+	uncomp	NS253	1.08	1.12	1.10	1.10	0.02
+	uncomp	NS265	1.02	1.04	1.08	1.05	0.03
+	uncomp	NS32	1.20	1.22	1.13	1.18	0.05
+	uncomp	NS50.1	1.09	1.11	1.16	1.12	0.04
+	uncomp	NS53	1.10	1.14	1.06	1.10	0.04
+	uncomp	NS59	1.14	1.13	1.23	1.17	0.06

[a]clinical source of the isolate; invasive (inv) or uncomplicated infection (uncomp).

Table 3. Summary of GAS culture optical density at 600nm supernatant collection for streptokinase analysis for each of the groups analysed.

GAS isolate group[a]	A_{600} at mid-log phase collection		A_{600} at late-log/early stationary phase collection	
	Mean	SD	Mean	SD
pam+	0.61	0.06	1.12	0.06
pam -	0.60	0.05	1.13	0.09
inv	0.61	0.05	1.12	0.10
uncomp	0.60	0.05	1.13	0.07
pam + inv	0.63	0.06	1.12	0.07
pam - inv	0.60	0.04	1.12	0.11
pam + uncomp	0.59	0.05	1.13	0.06
pam - uncomp	0.61	0.06	1.14	0.07

[a]*pam* genotype: positive (*pam*+) or negative (*pam*-); clinical source of the isolate; invasive (inv) or uncomplicated infection (uncomp).

Appendix 3: Publications

Please see print copy for Appendix 3