Generation of Recombinant Factor H Proteins to Control Alternative Pathway Activation in the Kidney

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Abstract

The alternative pathway (AP) of complement activation is implicated in several renal pathologies. These include membranoproliferative glomerulonephritis (MPGN) type II and atypical haemolytic uraemic syndrome (aHUS). Factor H (FH), comprised of 20 short consensus repeats (SCRs), is the primary regulator of the AP at the glomerular basement membrane (GBM) as evidenced by the fact that impaired factor H function is linked to both diseases. The current treatment options for MPGN type II and aHUS are limited and patients ultimately develop renal failure. There is a clear need for kidney targeted therapeutics to control AP dysregulation. I sought to generate novel recombinant FH fusion proteins to replace dysfunctional FH and to deliver the complement regulatory domain of factor H (SCRs 1-5) to the GBM. A protein containing the regulatory SCRs (FH1-5) and one containing the regulatory and cell-surface binding SCRs (FH1-5/18-20) was generated and expressed in a eukaryotic expression system. C3b binding and full complement regulatory function of FH1-5 and FH1-5/18-20 was confirmed by *in vitro* assays. To target the GBM a B cell hybridoma (mAb3) was obtained which secretes an antibody that binds to the NC1 domains of collagen type IV alpha-3 chain, expressed predominately in the GBM. The mAb3 variable regions (Fv) were successfully cloned after elimination of an abundantly expressed endogenous light chain transcript. A mAb3 single chain FV (scFv) was cloned downstream of FH1-5 creating a FH1-5/mAb3 scFv fusion protein. As an alternative a fusion protein incorporating the scFv of the 1G6 monoclonal antibody (FH1-5/1G6 scFv) was generated. Low expression levels of the FH1-5/scFv proteins limited the functional analysis. Binding of both proteins to C3b and binding of FH1-5/1G6 scFv but not FH1-5/mAb3 scFv to bovine and murine NC1 domains was demonstrated using cell culture supernatants. These reagents remain in the early stage of development but have potential to be attractive alternatives for the treatment of aHUS and MPGN type II. In addition to MPGN type II, FH1-5/scFv may find application in the treatment or prevention of anti-GBM nephritis and renal ischaemia-reperfusion injury.

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Abbreviations

aHUS	Atypical Haemolytic Uraemic Syndrome
AMD	Age-related Macular Degeneration
AMI	Acute Myocardial Infarction
AP	Alternative Pathway
APC	Antigen Presenting Cell
BCR	B Cell Receptor
BLAST	Basic Local Alignment Tool
bp	Base pair
BSA	Bovine Serum Albumin
C3NeF	C3 Nephritic Factor
CD59	Cluster of Differentiation 59
cDNA	Complementary DNA
*CFH*L-1	Complement Factor H Like protein 1
*CFH*R-1-5	Complement Factor H Related proteins 1-5
CHO cells	Chinese Hamster Ovary cells
CKD	Chronic Kidney Disease
CP	Classical Pathway
CR1	Complement Receptor type 1
CR2	Complement Receptor type 2
DAB	Diaminobenzidine
DAF	Decay Accelerating Factor
DDD	Dense Deposit Disease
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethylene Glycol Tetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FB	Factor B
FC	Crystallisable Fragment
FCRn	Neonatal Fc Receptor
FCS	Fetal Calf Serum
FH	Factor H
FI	Factor I
FITC	Fluorescein Isothiocyanate
GBM	Glomerular Basement Membrane
GVB	Veronal Buffer with Gelatine
HAE	Hereditary Angioedema

HEL	Hen Egg Lysozyme
HRPO	Horseradish Peroxidase
Ig	Immunoglobulin
IgG	Immunoglobulin G
Ile	Isoleucine
kb	Kilo base pair
kDa	Kilo Dalton
LB medium	Lysogeny Broth / Luria-Bertani broth
LP	Lectin Pathway
mAB	Monoclonal Antibody
MAC	Membrane Attack Complex
MASP-1	Mannose-Associated Serine Protease 1
MASP-2	Mannose-Associated Serine Protease 2
MBL	Mannan-Binding Lectin
MCP	Membrane Cofactor Protein
MHC	Major Histocompatibility Complex
MPGN type II	Membranoproliferative Glomerulonephritis type II
NHS	National Health Service
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
RE	Rabbit Erythrocytes
Rpm	Rotations per minute
RT	Room Temperature
RU	Resonance Units
SCR	Short Consensus Repeat
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SLE	Systemic lupus erythematosus
SOC	Super Optimal broth with Catabolite repression
SOB	Super optimal broth
SPR	Surface Plasmon Resonance
TCR	T cell receptor
TMB	3,3’,5,5’-Tetramethylbenzidine
UUO	Unilateral Uretal Obstruction
UV	Ultraviolet
Val	Valine

VB	Veronal Buffer
VH	Heavy chain variable region
VL	Light chain variable region

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1 Introduction

1.1 The complement system

1.1.1 A brief historical background

Complement is an ancient system of proteins that forms part of innate immunity. The origin of the complement system dates much further back than that of the adaptive immune system. Key constituents of adaptive immunity, like antibodies, can be traced back to sharks and higher vertebrates and may only be about 600 million years old (Kasahara *et al*., 1997, Nonaka and Kimura, 2006). However, homologues of complement component C3 and complement factor B (FB) are found in sea urchins and may be more than 1000 million years old (Smith *et al*., 1999, Nonaka and Kimura, 2006). The "anti-bacterial" property of serum was first described by Hans Buchner in 1889, who named the component alexine (Buchner, 1889). In 1895 Jules Bordet then demonstrated the presence of a heat-stabile (antibodies) and a heat-labile (alexine or complement) anti-bacterial component in serum (Bordet, 1909). The term complement was coined by Paul Ehrlich since he described alexine as a component that complements the function of antibodies (Ehrlich, 1899). Further complement components were identified in the following years, but the alternative pathway and the lectin pathway were only described in latter part of the 20th century (Pillemer *et al*., 1954, Super *et al*., 1989).

1.1.2 Emerging roles of complement beyond pathogen defence

The complement system is now known to comprise more than 50 serum and cell-surface proteins. Their role has traditionally been described as the first line of defence against invading pathogens through the opsonisation of pathogens to enhance phagocytosis, lysis of microorganisms and induction of inflammatory responses through generation of proinflammatory anaphylatoxins (Trouw and Daha, 2011). However, many new roles for complement have emerged over the past two decades. In addition to mediation of innate immunity and inflammation, complement coordinates the adaptive immune response by regulation of B cell and T cell effector functions (Fearon and Locksley, 1996, Heeger and Kemper, 2012). Evidence of the involvement in blood coagulation, apoptosis, tissue regeneration and lipid metabolism implicates complement as a mediator of homeostasis (Rutkowski *et al*., 2010, Ehrnthaller *et al*., 2011, Amara *et al*., 2008) Three pathways that activate the complement cascade have been described (Figure 1); the classical pathway (CP), lectin pathway (LP) and the alternative pathway (AP) (Trouw and Daha, 2011). The pathways share formation of a C3 convertase and a C5 convertase and ultimately result in formation of the membrane attack complex and cell lysis (Trouw and Daha, 2011).

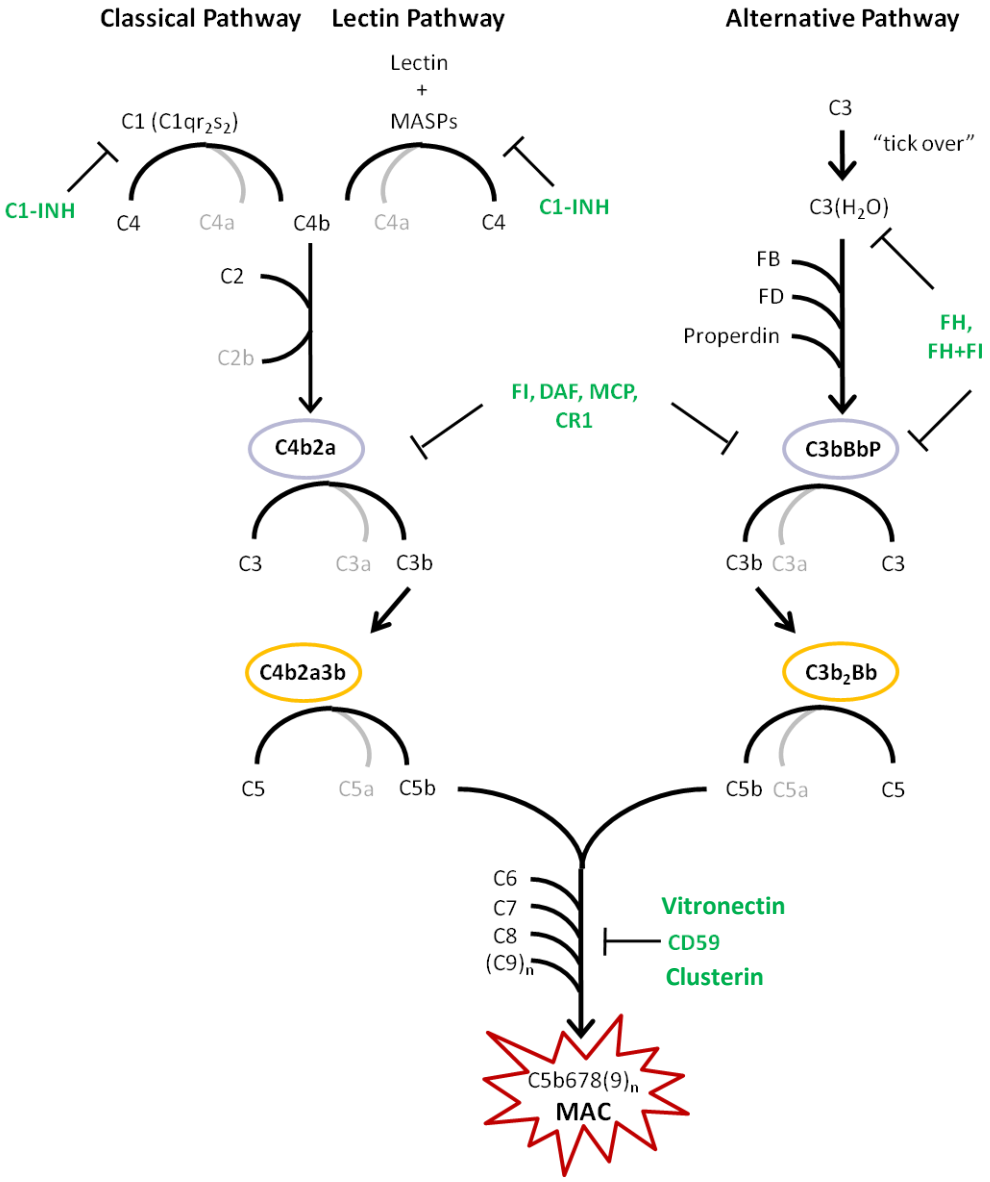

Figure 1: The complement system – activation and regulation

The C1 complex of the classical pathway and the MBL-MASP complexes of the lectin pathway mediate cleavage of C4 into C4a and C4b and C2 into C2a and C2b. C4b and C2a form a C3 convertase (C4b2a). Spontaneous hydrolysis of C3 ("tick over") initiates the AP. Hydrolysed C3 ($C3(H_2O)$) associates with factor B (FB) which is cleaved by factor D (FD) to form the AP C3 convertase C3Bb. Both convertases cleave C3 into C3a and C3b. C3b is used to form the classical and alternative pathway C5 convertases (C4b2a3b and C3bC3bBb) but also to amplify the AP by generation of further C3 convertases. The C5 convertases cleave C5 into C5a and C5b, the latter initiates the formation of the MAC by association with C6, C7, C8 and several C9 molecules. C1-INH controls activation of the classical and lectin pathway, whilst FH and FI are the principal regulators of AP activation and the AP C3 convertase. FI, DAF, MCP and CR1 control both pathways at the C3 convertase level. Formation of the MAC is inhibited by CD59, vitronectin and clusterin.

1.1.3 Classical pathway

Classical and lectin activation pathways are very similar and share the same C3 convertase. To activate the complement cascade the classical pathway requires an antibody bound to an antigen, e.g. a pathogen (Kojouharova *et al*., 2010). The initiation molecules in this pathway are C1q, C1r and C1s which form the C1 complex (one C1q, two C1r and C1s). Initiation takes place when several C1q molecules (which bind to the Fc portion of an antigen associated antibody) as part of the C1 complex aggregate on a pathogen surface. Notably, antibody independent activation can occur on surfaces that bind C1q directly. These include viruses, gram-negative bacteria and DNA (Kojouharova *et al*., 2010). This causes self-activation of C1r which in turn activates C1s. C1s is a serine protease that cleaves C2 and C4 to generate C2a and C4b. Both fragments associate to form a C3 convertase which cleaves C3 into the anaphylatoxin C3a and C3b. The latter is deposited on the pathogen surface and forms the C5 convertase by binding C4b and C2a. The C5 convertase cleaves C5 into C5b and the anaphylatoxin C5a. C5b is the first component of the membrane attack complex (MAC). C5b binds to the pathogen surface and associates with C6, C7 and C8. This complex then allows binding of C9 which inserts into the cell membrane and polymerises to form a lytic pore (MAC). The formation of several MACs ultimately results in the lysis of the pathogen (Trouw and Daha, 2011).

1.1.4 Lectin pathway

The lectin in the lectin pathway serves the same function as C1q in the classical pathway as it forms a complex with three proteases: MASP-1, MASP-2 and MASP-3. Upon binding of the complex to a pathogen surface, the proteases are activated and generate C4b and C2a by cleavage of C4 and C2. The subsequent steps are the same as described for the classical pathway (Degn *et al*., 2007).

1.1.5 Alternative pathway

The AP is a rapid and potent activation pathway that can be initiated by a range of pathogens, including bacteria, fungi and viruses (Pangburn and Muller-Eberhard, 1984). Rapid activation of the AP is facilitated by the spontaneous and continuous hydrolysis of small amounts of C3, a process also described as “tick over” (Lachmann and Halbwachs, 1975, Pangburn *et al*., 1981, Pangburn and Muller-Eberhard, 1983). Hydrolysis of C3 results in a conformational change which allows association with FB. FB bound to C3b is cleaved by factor D (FD) to Bb, forming the specific AP C3 convertase (C3Bb) (Fearon *et al*., 1973). C3b generated by this initial convertase can form further C3 convertases which, stabilised by binding of properdin can result in a positive amplification loop (Schwaeble and Reid, 1999). The AP C5 convertase is formed by association of a second C3b molecule to the AP C3 convertase (C3bBbC3b). The

subsequent steps leading to formation of the MAC are identical to the other pathways. In the absence of an activating surface, C3b is usually deactivated by hydrolysis but may be deposited on host cells. Effective regulation of the AP and differentiation between pathogen and host cell surface are therefore paramount to avoid damage to the host.

1.1.6 Regulation of complement

As described previously, the complement system provides potent measures to defend the host against invading pathogens. To protect the host from complement-mediated damage a number of regulatory molecules are employed in the fluid-phase and on host cell-surfaces (Zipfel and Skerka, 2009). The cell-surface regulators are widely expressed on all cell-types, with some exceptions. Complement receptor type 1 (CR1, CD35) and decay accelerating factor (DAF, CD55) are cell-surface bound and bind C3b when bound to a host-cell surface and can dissociate Bb from the AP C3 convertase (Zipfel and Skerka, 2009). CR1 also functions as a co-factor for FI. The function of the decay accelerating factor (DAF, CD55) is in its name – it accelerates the decay of both classical and AP C3 convertases. CD59 inhibits MAC formation by preventing binding of C8 to C9. Properdin is the best described positive regulator of the complement system. It binds to and stabilises the AP C3 convertase (Schwaeble and Reid, 1999). The classical and MBL pathways are regulated early by the fluid-phase inhibitor of C1 (C1-INH) which blocks function of both the C1 complex and MASP-1 and MASP-2. The anaphylatoxins C3a and C5a can be inactivated by carboxypeptidase N; vitronectin and clusterin prevent insertion of the MAC into the cell membrane. FI is a fluid-phase serine protease which can use membrane co-factor protein (MCP, CD46), factor H (FH), CR1 and C4b binding protein (C4bp) to cleave the α-chains of C3b and C4b (Sim *et al*., 1993). In addition to its FI co-factor activity FH can by itself bind C3b in a similar way as CR1 and DAF. It regulates in the fluid-phase and selectively on host cell-surfaces as it exerts a higher affinity for C3b in the context of heparin and sialic acid residues (Fearon, 1978a).

1.1.7 Complement factor H

1.1.7.1 FH protein family

The human factor H gene (*CFH*) is located on chromosome 1 within the regulators of complement activation (RCA) gene cluster in 1q32 (Rodriguez de Cordoba *et al*., 1999, Diaz-Guillen *et al*., 1999). FH is mainly produced in the liver but other cell types, including proximal tubular epithelial cells in the kidney, produce FH (Gerritsma *et al*., 1997). It is the most abundant member of a family of related proteins that include complement factor H-like protein 1 (FHL-1) and five complement factor H related proteins (FHR1-5) (Zipfel and Skerka, 1994). *CFH*, *CFHL-1* and the five *CFHRs* are located within the same group of genes. FH and

FHL-1, comprised of the first seven SCRs of FH with additional C-terminal amino acid residues, are located on the same gene and generated by alternative splicing (Schwaeble *et al*., 1987, Friese *et al*., 1999).

1.1.7.2 FH structure and function

FH (Figure 2) is a 155 kDa protein which is comprised of 20 SCRs of approximately 60 amino acids each (Kristensen and Tack, 1986). Each of these globular domains contains four cysteine residues that form two disulphide bonds (the first and third, second and fourth cysteine pair up) (Kristensen and Tack, 1986). Further conserved residues add to structure and stability of each SCR unit (Zipfel and Skerka, 1994). FH has two principal functional domains located at the N- and C-terminal. The N-terminal SCRs 1-4 contain the complement regulatory functions. The AP is regulated by FH in three distinct ways. Formation of the AP C3 convertase (C3bBb) is controlled by competition with FB for binding to C3b (Weiler *et al*., 1976, Whaley and Ruddy, 1976). FH accelerates decay of both the AP C3 convertase and the AP C5 convertase (C3bBbC3b) by dissociating FB from the complex (decay acceleration activity). FH also serves as cofactor for the serine protease FI that mediates cleavage of C3b (cofactor activity) (Weiler *et al*., 1976). C-terminal SCRs 19-20 bear the cell-binding domain. The specificity of FH for C3b on non-activating surfaces was first described by Fearon and colleagues. They observed that FH and FI cannot access pathogen associated C3b and that specificity of FH for C3b deposited on non-activating surfaces is mediated through polyanions (Fearon, 1978b, Pangburn and Muller-Eberhard, 1978, Fearon and Austen, 1977). Polyanions are molecules with negative charges at several sites; Examples are heparin and sialic acid. Heparin is a highly-sulphated glycosaminoglycan and has the highest negative charge density known. Sialic acids are a diverse family of monosaccharides with a number of different functions including stabilisation of cell membranes and proteins. Interaction sites of FH with heparin have been located in SCR7 and SCR13 (Blackmore *et al*., 1996, Sharma and Pangburn, 1996). The heparin binding site SCR19 and SCR20 engage with both heparin and sialic acid residues and exert the highest affinity and have therefore been attributed a particularly important role (Blackmore *et al*., 1998, Ram *et al*., 1998). Functionally the presence of polyanions enables FH to compete with FB for C3b and to subsequently dissociate FB from the AP C3 convertase (Pangburn and Muller-Eberhard, 1978, Pangburn and Muller-Eberhard, 1986, Weiler *et al*., 1976, Whaley and Ruddy, 1976). FI requires the presence of a cofactor to cleave C3b to the inactive iC3b. FH, CR1, and MCP (Lublin and Atkinson, 1990, Seya *et al*., 1986, Weiler *et al*., 1976) can act as cofactors and need to be associated with C3b. Linker sequences, on average four amino acids long, connect individual SCRs.

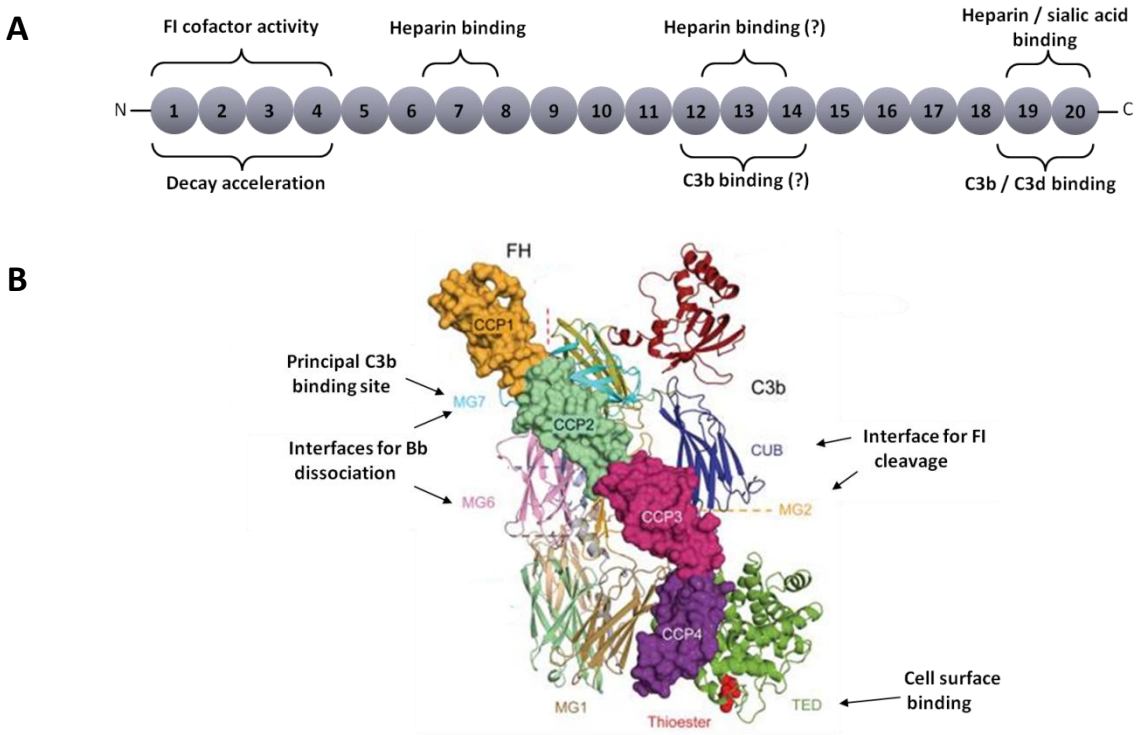

Figure 2: Factor H structure and interaction with C3b

(A) Factor H is comprised of 20 SCRs and has several functional domains. The principal C3b binding site, which confers decay acceleration and FI cofactor activity, is located at the N-terminal. FH also binds C3b and C3d via the C-terminal SCRs 19 and 20. SCRs 12-14 interact weakly with C3b. SCRs 7 and 19-20 strongly bind heparin / sialic acid. The heparin binding domain in SCRs 12-14 is debated since a SCR 12-14 fragment does not interact with heparin. **(B)** Co-crystallisation of FH SCRs 1-5 with C3b allowed the mapping of functional interfaces. FH SCR2 (CCP2) directly interacts with MG7 and MG6 on C3b and forms the interface involved in C3b binding and decay acceleration. SCR3 on FH and CUB and MG2 of C3b form the interface required for FI-mediated cleavage of C3b. The thioester domain (TED) of C3b allows binding of C3b with target surfaces.
Adapted from: (Wu *et al*., 2009).

These inter-SCR linkers provide the degree of flexibility required for the interaction with multiple ligands but create problems for structural studies using X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy (Perkins *et al*., 2002). Solution scattering and electron microscopy suggest that full length FH is not a linear structure but folded-back on itself (Perkins *et al*., 1991, DiScipio, 1992, Aslam and Perkins, 2001). Studies of individual SCRs and fragments and in particular solution structures of SCRs 1-5 and 16-20 provided further evidence for the folded back conformation (Barlow *et al*., 1992, Hocking *et al*., 2008, Okemefuna *et al*., 2008). X-ray crystallography studies of FH SCRs 1-5 in complex with C3b and FH SCRs 19-20 in complex with C3d have shown that FH can engage a cell surface associated C3b molecule simultaneously with the N- and C-terminal (Wu *et al*., 2009, Morgan *et al*., 2011). The SCRs that are not involved in C3b binding form a tight loop but the functional role of this large portion of FH is currently not fully understood (Schmidt *et al*., 2010). The current models place the apex of the loop, i.e. SCRs 11-13, and the heparin binding SCR7 close to the cell surface. The ability of SCR 13 to bind heparin has been subject to debate. A study by Schmidt *et al* found fragments comprising SCRs 11-14, 13-15, 10-15 or 8-15 only exerted low or no affinity for heparin (Schmidt *et al*., 2008b).

In summary, FH can discriminate effectively against pathogen surfaces and exerts its function by three main mechanisms; C3b capture, acceleration of AP C3 convertase decay and FI cofactor activity. With several regulators and discriminating molecules present there seems to be a high degree of redundancy. However, all these molecules are required to act in concert to provide efficient protection which becomes apparent considering the effects of point mutations in individual regulators.

1.1.7.3 Complement C3 and FH

C3 is a 185 kDa protein that is present in plasma at approximately 1 mg/ml (Lambris, 1988). C3 has 13 domains, including eight macroglobulin domains (MGs), a thioester domain (TED) and a CUB domain (Wu *et al*., 2009). Its central role in the complement system involves the interaction with several other components. The conformational changes required for these interactions are achieved by number of proteolytic cleavage steps. The initial step in the AP is the “tick over” in which the hydrolytic cleavage of the thioester in TED produces an active form of C3, C3(H_2O) which can bind FB to form a fluid-phase C3 convertase (Pangburn *et al*., 1981). The C3 convertases of the CP and AP catalyze cleavage of C3 into C3b (177 kDa) and C3a (9 kDa). This cleavage also induces a conformational change in the TED domain of C3b, enabling interaction with target surfaces (Janssen and Gros, 2006, Janssen and Gros, 2007). Further cleavage of the C3b α-chain is mediated by FI, forming iC3b and C3f. Under reducing conditions the cleavage of FI at two sites becomes apparent – in addition to the intact β-chain fragments

of 68, 43 and 3 kDa can be observed (see Figure 3). Presence of CR1 causes further cleavage of iC3b (or the 68 kDa α-chain fragment) into C3c (27 kDa) and C3dg (41 kDa). Recent crystallography studies have revealed detailed information about C3 and the conformational changes that occur to mediate interaction with other proteins and surfaces (Janssen *et al*., 2005, Janssen and Gros, 2007, Gros *et al*., 2008, Rooijakkers *et al*., 2009, Wu *et al*., 2009). Co-crystallisation of FH SCRs 1-5 and C3b allowed the mapping of four key interfaces, as shown in Figure 2 (Wu *et al*., 2009). Decay accelerating activity has been attributed to SCR1 and SCR2 which interact mainly through hydrophobic areas in MG6 and MG7 on C3b, respectively. These interactions contribute largely to dissociation of Bb from the AP C3 convertase. SCR3 and SCR4 (via TED domain and MG1) have been assigned a supportive role in C3b binding, although the interface between SCR3 and MG2 and CUB also forms FI cleavage sites (Wu *et al*., 2009).

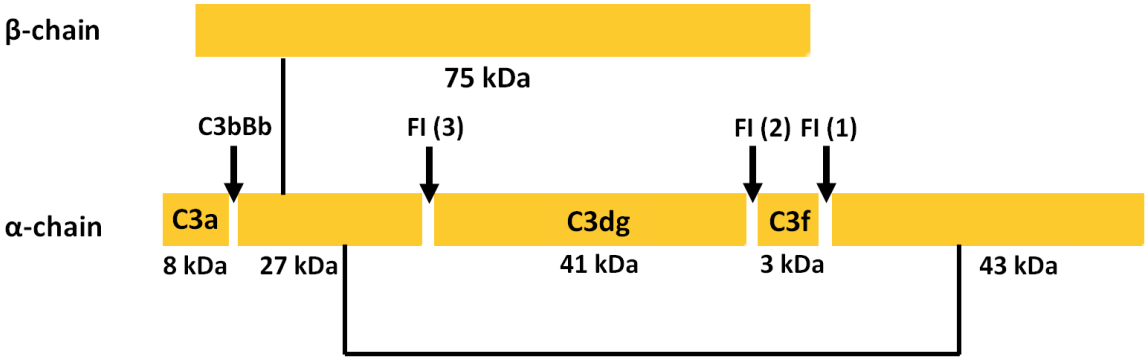

Figure 3: Factor I mediated cleavage of C3b

C3b is generated by cleavage of C3 by the C3 convertase (C3bBb). There are three FI cleavage sites on the C3b α-chain. In the presence of the cofactors FH, MCP and CR1 the α-chain is cleaved at FI (1) and F (2). Fragments of 68 kDa, 43 kDa and 3 kDa are generated. In the presence of CR1 FI can cleave the α-chain at FI (3). This cleaves the 68 kDa fragment into a 27 kDa fragment and a 41 kDa fragment (C3dg). Disulphide bonds are shown as black lines.
Schematic adapted from (Harris, 2000).

1.2 Alternative pathway mediated renal disease

1.2.1 Renal anatomy and function

The kidney eliminates waste products from the body in the form of urine and is a central homeostatic organ. It regulates the composition of the extracellular fluid (ECF) which includes the plasma, interstitial and transcellular fluids to ensure normal physiological function of the cells in the body (Valtin, 1994). Volume, osmolality, pH and ionic composition of the ECF are regulated by the kidneys. The composition of the ECF ultimately dictates the composition of intracellular fluids (ICF) (Valtin, 1994). The kidneys are part of the urinary tract that is comprised of the kidneys, ureters, bladder and the urethra (Figure 4). Urine produced by the kidneys drains into the renal pelvis and is transported to the bladder via the ureters. The bladder is emptied via the urethra. The two functional tissues, the inner medulla and outer cortex, contain more than 1 million functional units called nephrons. Each nephron consists of a glomerulus which is encased by the Bowman's capsule, the proximal tubule, the loop of Henle, the distal tubule and the collecting duct (O'Callaghan, 2006). The unfiltered blood is supplied to the glomerulus by the afferent arteriole which branches out to form the glomerular capillary and converges to form the efferent arteriole to return filtered blood to the main circulation (Figure 5). Urine is the product of ultrafiltration that takes place in the kidney (O'Callaghan, 2006). This process involves the filtration of small metabolites and other substances across selective barriers based on their size, shape and charge (Figure 5). The three components of the glomerular filtration barrier are the glomerular endothelium (including the glycocalyx), the glomerular basement membrane and the podocytes which are the specialised visceral endothelium of the Bowman's capsule (Miner, 2012). The blood first encounters the endothelial glycocalyx, a thick, dynamic layer of proteoglycans, glycoproteins and glycosaminoglycans (GAGs). Part of the glycocalyx components are derived from and anchored to the endothelium and part are sourced from the circulating plasma (Figure 5). The most prevalent GAGs in the glycocalyx of vascular endothelium are heparan sulphate, chondroitin sulphate and hyaluronic acid (Esko JD, 2009). Up to 90% of the proteoglycans are associated with heparan sulphate. The negative charge of the glycocalyx functions as the first barrier by repelling serum proteins. Substances that pass through the glycocalyx reach the fenestrated endothelium which contains numerous pores of approximately 60 nM in diameter. The glomerular endothelium is directly attached to the GBM which is comprised of three layers, the lamina rara externa, lamina densa and lamina internal (Miner, 2012). The lamina externa are densely covered by heparan sulphate and the lamina densa is mainly comprised of type IV collagen.

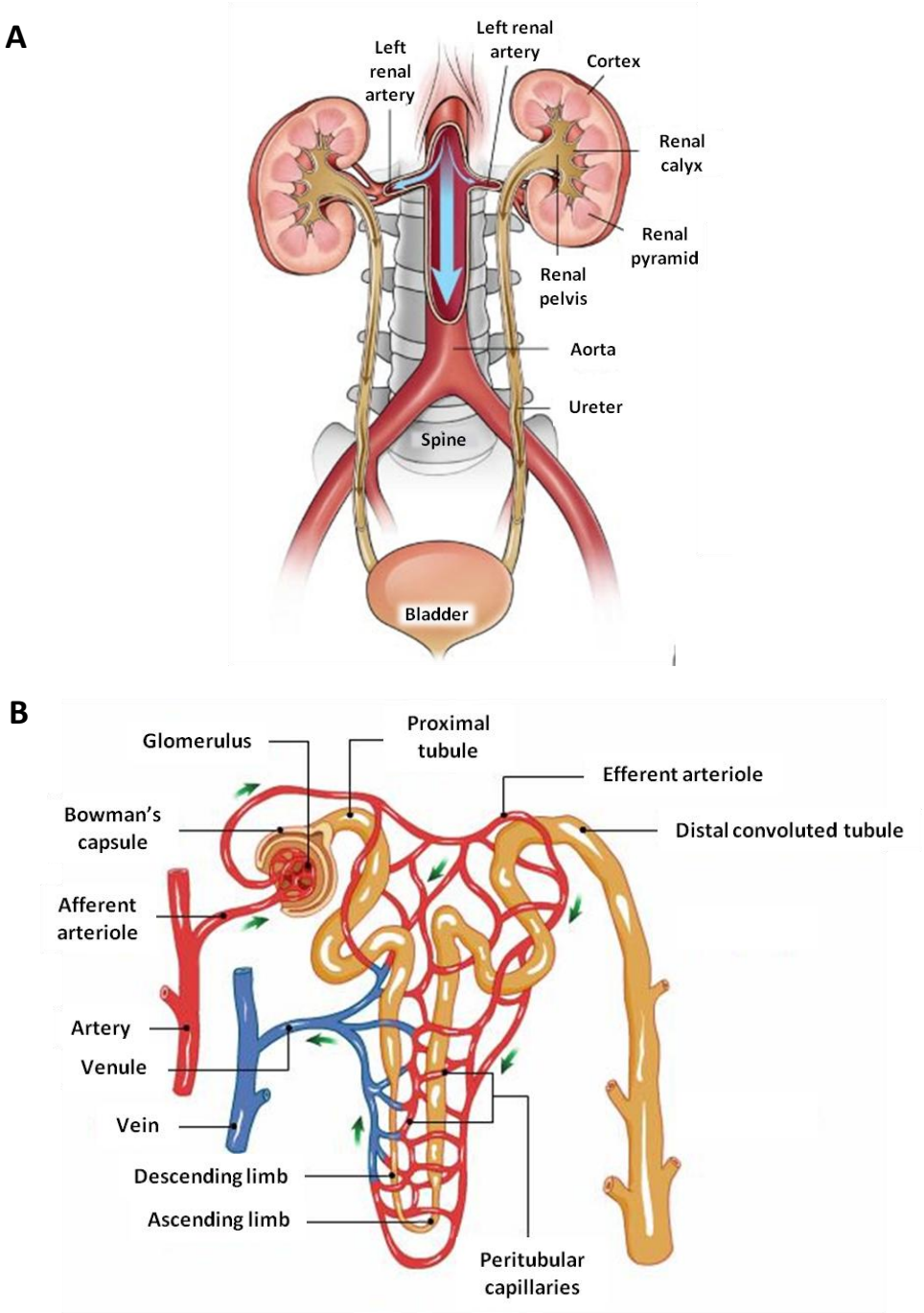

Figure 4: Anatomy of the urinary tract and nephron

(A) The nephrons are located in the cortex and medulla (containing the renal pyramids) of each kidney. Urine produced is collected into the renal pelvis and transported to the bladder via the ureters. The bladder is emptied via the urethra. **(B)** The afferent arteriole supplies blood to the glomerulus and the efferent arteriole transports the filtered blood, via the peritubular arteries, to a vein. The urine leaves the glomerulus via the proximal tubule and is transported to the distal convoluted tubule via the descending and ascending limbs of the loop of Henle.
Adapted from: (Mariam-Webbster, 2012) and (Ganesh, 2012)

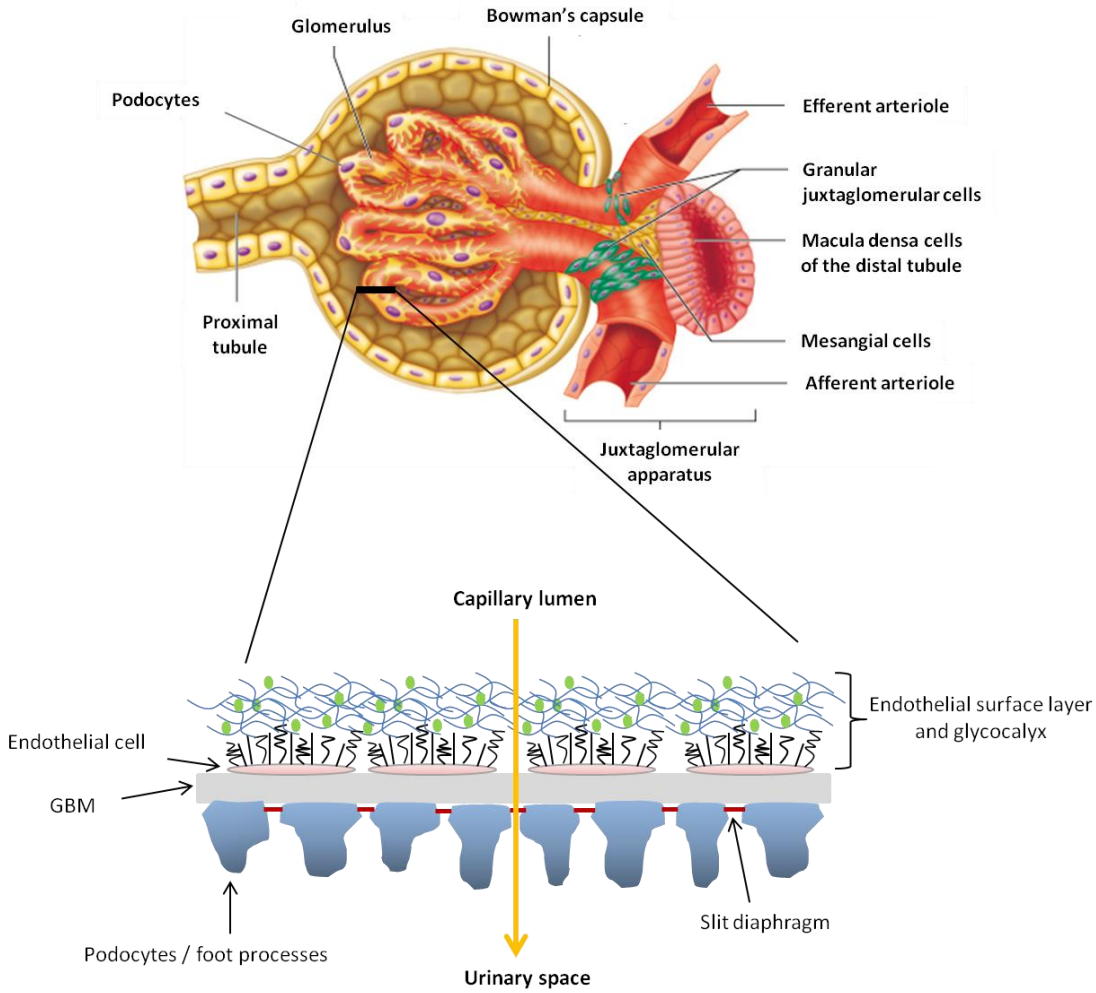

Figure 5: Structures of the glomerulus and the filtration barrier

The glomerulus, encased by the Bowman's capsule, is the central site of blood filtration. Blood enters the glomerulus, a cluster of capillaries, via the afferent arteriole and exits via the efferent arteriole. The glomerular capillary wall is comprised of three layers as indicated by the cross-section. Fenestrated endothelial cells and their glycocalyx, the GBM and podocytes with filtration slits form the filtration barrier that allows passage of proteins and other molecules based on their size, shape and charge. The filtrate that is formed at this barrier is urine.
Adapted from: (Cummings, 2001) and (Miner, 2008)

The GBM forms an important barrier against water by contributing to the resistance to filtrate flow and selectively filters molecules based on their charge and size (Miner, 2008).

1.2.2 Complement and the kidney

Multiple mechanisms are in place to effectively protect host cells from damage, yet there are diseases in which defective regulation of the AP in particular is implicated. Certain sites are deficient in surface bound regulators. In the kidney for example all regulators named above are expressed (Timmerman *et al*., 1996, Zipfel and Skerka, 2009, Ichida *et al*., 1994). However, the GBM is mainly dependent on FH. The high density of heparin sulphate and sialic acid at the GBM provides a good environment for FH to deposit as shown by co-localisation of FH and type IV collagen at the GBM (Miner, 2012, Licht *et al*., 2007). DAF and MCP, the other two regulators of the C3 convertase, are absent. In healthy individuals this is not a problem because FH is sufficient in its function. If FH is dysfunctional or deficient there is an increased risk of complement-mediated damage to the GBM.

1.2.3 Thrombotic microangiopathies

1.2.3.1 Shiga toxin associated haemolytic uraemic syndrome

Typical HUS is distinguished from the atypical form by both the clinical presentation and pathogenesis. It is caused by *E. coli* serotypes O157:H7 and O104:H4 which produce two very similar exotoxins (Shiga toxins Stx1 and Stx2) (Noris *et al*., 2012). Particular susceptibility of the glomerular endothelium to these toxins results from the presence of high concentrations of the cell-surface glycosphingolipid Gb3 (globotriosyl ceramide) (Keir *et al*., 2012). The toxins enter the cell by binding Gb3 via the B subunits. The toxic A subunit can then exert toxicity within the cell (Keir *et al*., 2012).

1.2.3.2 Atypical haemolytic uraemic syndrome

Atypical HUS is a thrombotic microangiopathy that is characterised by haemolytic anaemia, thrombocytopenia, progressive loss of renal function and ultimately renal failure (Kavanagh and Goodship, 2011). Extensive involvement of the complement system in these patients is indicated by low levels of serum C3 and FB but high levels of the C3 breakdown fragment C3d (Benz and Amann, 2009b). There is a strong genetic component in aHUS with loss-of-function mutations that affect the complement regulators FH, FI and MCP (Geerdink *et al*., 2012, Jozsi *et al*., 2006, Fang *et al*., 2008, Kavanagh *et al*., 2008, Richards *et al*., 2003). More than 60 mutations have been described for FH alone. Mutations in the C-terminal SCRs 19 and 20 in FH are particularly associated with aHUS (Jozsi *et al*., 2006). Gain-of-function mutations have been described for C3 and FB (Fremeaux-Bacchi *et al*., 2008, Goicoechea de Jorge *et al*., 2007). Reports also describe a small number of patients with autoantibodies against FH and FI (Zipfel

et al., 2006, Pickering and Cook, 2008b, Strobel et al., 2010, Kavanagh et al., 2012). Although many aspects of the pathogenesis of aHUS remain unclear, it is known that complement attack on the renal endothelium disrupts the cell membrane integrity which results in exposure of collagen. Von Willebrandt factor (vWf) binds to the exposed collagen resulting in platelet binding and further steps of the coagulation cascade (Desch and Motto, 2007). It is clear that aHUS is a complex, multi-factorial condition. Patients presenting with aHUS usually have rare mutations in complement proteins and carry at-risk genetic variants (like polynucleomorphisms) commonly found in the population. Further, a trigger like infection or pregnancy is required to precipitate disease (Kavanagh and Goodship, 2011). There are also sporadic cases of aHUS in which the development of aHUS is acquired and secondary to external events (drug induced, infection). For example infection with *S. pneumoniae* may cause aHUS by increasing susceptibility of endothelial cells to complement-mediated damage. This may be achieved by reduction of the endothelial cell glycocalyx and "hijacking" of FH (Copelovitch and Kaplan, 2008). Endothelial cells are protected from complement attack in different ways. The cell-surface complement regulators DAF, MCP and CD59 are widely expressed, FH and FI are available in the fluid-phase. The glycocalyx provides a further means of protection. The negative charge of this layer prevents binding of circulating serum proteins, but FH would bind to the highly negative heparin and sialic acid residues. Dysfunction of the C-terminal domain of FH underlines the importance of this interaction.

1.2.3.3 Animal models for aHUS

Animal models, in particular mice deficient in complement regulators, have proven a valuable tool to understand the pathogenesis of aHUS. There are knockout mice for several murine complement regulators. The first mouse model to resemble human aHUS was generated by a deletion of SCRs 16-20 in *Cfh* (Pickering *et al*., 2007). Mutations in FH that result in aHUS are primarily located at the C-terminal (Sanchez-Corral *et al*., 2002, de Cordoba and de Jorge, 2008). It was therefore anticipated that deletion of the C-terminal SCRs would greatly reduce protection of renal endothelial cells whilst retaining complement regulatory function in the fluid-phase. The *CfhΔ16-20* mice showed better fluid-phase regulation than the $Cfh^{-/-}$ mice, indicated by higher serum C3 levels and mesangial deposition of C3 rather than GBM deposition as seen in $Cfh^{-/-}$ mice (Pickering *et al*., 2007). The histological findings also closely resemble those found in human aHUS. The terminal complement pathway plays a central role in the pathogenesis as *CfhΔ16-20* mice crossed on a $C5^{-/-}$ background are protected from spontaneous aHUS and less susceptible to induced renal injury (de Jorge *et al*., 2011). Overall the data derived from the *CfhΔ16-20* mice suggest that therapy of aHUS should aim to restore complement regulation at the endothelial cell surface. The finding that administration of

human FH reverses complement deposition in *Cfh*$^{-/-}$ mice supports this and implies FH replacement as a suitable therapeutic approach (Fakhouri *et al*., 2010). An autoantibody induced murine model for aHUS was recently described (Miwa, 2012). Wild-type mice that were injected with an anti-FH SCRs 19-20 monoclonal antibody that blocks cell binding of FH showed impaired renal function, aHUS like injury was achieved by injecting this antibody into *DAF* $^{-/-}$ mice (Miwa, 2012).

1.2.3.4 Treatment of aHUS

In aHUS, plasma exchange (PEX) and plasma infusion (PI) are the default therapy and aim to replace deficient or defective regulators (Barz *et al*., 2002, von Baeyer, 2002, Taylor *et al*., 2010). PEX offers the simultaneous removal of defective proteins and autoantibodies. This approach is not effective in all patients and the underlying defect in regulation appears to influence the outcome. Whilst patients with mutations in FH generally respond well to plasma therapy, patients with FI or FB mutations do not appear to benefit (Davin *et al*., 2008, Stratton and Warwicker, 2002, Gerber *et al*., 2003). There are also risks associated with the administration of blood products (Burnouf, 2007). Patients that repeatedly receive plasma can suffer hypersensitivity reactions and develop resistance to therapy, possibly as a result of autoantibody formation (Nathanson *et al*., 2006). Renal transplants are not recommended in patients that have progressed to end-stage renal disease because disease recurrence in the graft is approximately 50%. Graft survival is good in patients with MCP mutations, however in patients with FH and FI mutations graft survival is very poor with graft loss occurring within two years in 80% of patients (Bresin *et al*., 2006, Taylor *et al*., Caprioli and Remuzzi, 2007, Sellier-Leclerc *et al*., 2007). Transplantation of patients with FH autoantibodies appears to offer a longer term solution (Kwon *et al*., 2008, Waters *et al*., 2010). Liver transplantation, in patients with moderate kidney damage, and liver-kidney transplantation have been successful in patients with FH/FI mutations (Saland *et al*., 2009). A new therapeutic, Eculizumab, has been successfully used in several aHUS patients (Nurnberger *et al*., 2009, Gruppo and Rother, 2009, Hadaya *et al*., 2011, Al-Akash *et al*., 2010). The anti-C5 monoclonal antibody blocks formation of the terminal membrane attack complex and therefore protects the glomerular endothelial cells (Rother *et al*., 2007). Eculizumab has been approved in the U.S.A. and Europe for the treatment of aHUS, further clinical trials are ongoing. However, considering the high cost of Eculizumab, it may not be funded in the UK (England) for routine use.

1.2.4 Glomerulonephritis

The term glomerulonephritis covers a range of inflammatory pathologies that concern the glomerulus and is one of the main causes of chronic kidney disease. Glomerulonephritis can occur in the presence or absence of antibodies. Immune complex mediated disease presents

as anti-GBM nephritis (Goodpasture's syndrome), post-infectious glomerulonephritis, IgA nephropathy, type I membranoproliferative glomerulonephritis (MPGN) and also occurs in the in the context of systemic lupus erythematosus (SLE, lupus nephritis) (Couser, 2012). Complement activation, via the CP, AP but also the LP, has been attributed a significant role in several of these conditions (Heeringa and Cohen, 2012). In MPGN type II immune-complexes are absent and the condition is thought to be driven by uncontrolled activation of the AP (Benz and Amann, 2009b).

1.2.4.1 Anti-GBM nephritis

Anti-GBM nephritis or Goodpasture's syndrome is a pulmonary and renal disease that is characterised by rapid progression of glomerulonephritis and lung haemorrhage (Borza *et al*., 2003, Lahmer and Heemann, 2012). The condition is mediated by anti-GBM antibodies and responds to treatment by plasma exchange, cyclophosphamide and steroids. The specific antigen of the autoantibodies has been identified as the α3-chain of type IV collagen which is found primarily in alveolar and glomerular basement membranes (Hellmark *et al*., 1999, Pedchenko *et al*., 2011, Kalluri *et al*., 1995, Weber and Pullig, 1992). The disease is commonly modelled in rodents by injection of GBM extracts or NC1 domains of the α3 chain of type IV collagen (experimental autoimmune glomerulonephritis) or by injection of a heterologous antiserum raised against GBM extract (nephrotoxic nephritis). In nephrotoxic nephritis the initial injury results from binding of the heterologous antibodies to the GBM and is perpetuated by the immune response to the heterologous antibodies (autologous phase). Complement dependence of this model was demonstrated by the protective effect of C3 and C4 deficiency and disease exacerbation by regulator deficiency (Sheerin *et al*., 1997, Sogabe *et al*., 2001, Robson *et al*., 2001). The AP drives pathology during the autologous phase of the model (Thurman *et al*., 2012).

1.2.4.2 Post-infectious glomerulonephritis

Infection with group-A haemolytic streptococci can be followed by post-infectious glomerulonephritis several weeks after the initial infection. It is characterised by mesangial C3 deposition and leucocyte infiltration. Normal serum C4 levels in patients suggest that the pathology is likely driven by the AP (Nasr *et al*., 2013).

1.2.4.3 IgA nephropathy

IgA nephropathy is marked by mesangial staining for IgA, C3 and MAC. Although the serum levels of complement are normal animal models suggest a role for both the LP and the AP (Scivittaro *et al*., 1993, Emancipator, 1994, Emancipator, 2011, Ohsawa *et al*., 2012).

1.2.4.4 Lupus nephritis

Deficiency of complement components C1q, C4 and C2 predisposes to SLE by impaired clearance of apoptotic materials (Robson and Walport, 2001). SLE is a very complex systemic condition and is characterised by the presence of anti-DNA auto-antibodies that form immune-complexes with nucleosomes. These complexes can deposit in the glomeruli and initiate activation of the CP and followed by AP activation result in lupus nephritis (Saxena *et al*., 2011).

1.2.4.5 Membranoproliferative glomerulonephritis type II

MPGN type II is histologically defined by strong proliferation of mesangial cells and thickening of the GBM (Sethi and Fervenza, 2012). Loss of renal function is progressive. In these patients FH is often defective or deficient and serum C3 and FB levels low (Thompson and Winterborn, 1981, Wyatt *et al*., 1982, Levy *et al*., 1986, Fijen *et al*., 1996, Vogt *et al*., 1995, Ault, 2000). FH function may also be impaired by autoantibodies (Goodship *et al*., 2012). A gain of function mutation of C3 that causes resistance to fluid-phase regulation by FH has been described in the context of the MPGN type II (Martinez-Barricarte *et al*., 2010). Approximately 80% of patients present with a C3 nephritic factor (C3NeF), an antibody that binds to and stabilises the C3 convertase allowing increased turnover of C3 (Spitzer *et al*., 1969, Daha *et al*., 1976, Smith *et al*., 2011). The ongoing AP activation results in the generation of C3 metabolites that deposit along the GBM. The dense deposits along the GBM give this disorder the alternative name "Dense Deposit Disease" (DDD) (Benz and Amann, 2009a, Pickering and Cook, 2008a). The structure of the GBM and absence of surface bound regulators provide clues to understanding the pathogenesis of MPGN type II. FH is the prime complement regulator of the GBM and in its absence C3 metabolites are constantly produced in the fluid-phase and deposited along the unprotected membrane. The GBM cannot undergo lysis or apoptosis. Subsequently the complement-coated material is not cleared and further AP activation occurs.

Animals deficient in FH have provided invaluable insight into the pathogenesis of MPGN type II. Complete deficiency of FH in mice and pigs results in MPGN type II like disease (Hogasen *et al*., 1995, Pickering *et al*., 2002). The deposits along the GBM contain iC3b, C5, C6, MAC in pigs and C9 in mice, with C3 metabolites appearing first (Jansen, 1993, Pickering *et al*., 2002). Deposition of MAC, besides C5 and C9, along the GBM has also been shown in human MPGN type II (Levy *et al*., 1986, Falk *et al*., 1983, West *et al*., 2001). It has been suggested that the MAC may be formed in the fluid-phase since $Cfh^{-/-}$ mice show C5 activation in plasma and renal disease is ameliorated by inhibition of C5 with an anti-C5 mAb (Pickering *et al*., 2006). An important role of fluid-phase complement activation to generate C3 metabolites for deposition along the GBM is implicated by the fact that $Cfi^{-/-}$ do not develop DDD (Rose *et al*., 2008).

Studies of the *Cfh*$^{-/-}$ and *Cfi*$^{-/-}$ therefore strongly imply that, opposed to aHUS, treatment of DDD should aim to restore fluid-phase regulation to ultimately prevent deposition of C3 metabolites at the GBM. Some MPGN II patients respond to treatment with anti-platelet agents, for others plasma infusion presents an option. For patients presenting with C3NeF plasmapheresis can alleviate progression (Licht *et al*., 2005). As for aHUS, transplantation is an option that requires careful consideration. Disease recurs in 50% of patients and graft survival is generally poor (Smith *et al*., 2007, Smith, 2008, Neumann *et al*., 2003). The current treatment options for MPGN type II are not satisfactory and there is a need for new therapies that target the uncontrolled AP activation.

1.2.5 The AP in renal ischaemia-reperfusion injury

There are several clinical settings in which the circulation of an organ or tissue is interrupted for an extended period of time and subsequently reperfused. These include organ transplantation, coronary bypass surgery and thrombolytic therapy (Eltzschig and Eckle, 2011, Eltzschig and Collard, 2004). Ischaemia-reperfusion is recognised as key factor in graft survival after transplantation and the tubulointerstitial space is the main site of injury (Troppmann *et al*., 1995). In a hypoxic tissue the production of ATP is interrupted which impacts on the function of ion-pumps and ultimately leads to the influx of water and certain ions. As a result the cells swell and are very susceptible to external stress. A number of complex processes are initiated in the cells and gene expression profiles change dramatically (Vieyra and Heeger, 2010, Correa-Costa *et al*., 2012). Generally the cells enter a proinflammatory state with increased expression of cell adhesion molecules, cytokines and complement components. Complement plays an early role in the pathogenesis of renal ischaemia-reperfusion injury. The anaphylatoxins C3a and, in particular, C5a facilitate pathogenesis in a number ways, including neutrophil attraction, stimulation of local chemokine secretion and mediation of T cell responses (de Vries *et al*., 2003a, Thurman, 2007, Peng *et al*., 2012). A primary role for the AP and terminal pathway components has been demonstrated by the use of knock-out mice for several complement components. *C3*$^{-/-}$, *C5*$^{-/-}$, *C6*$^{-/-}$ and *fB*$^{-/-}$ but not *C4*$^{-/-}$ mice are protected from injury to some extent when subjected to renal ischaemia-reperfusion (Zhou *et al*., 2000, Thurman *et al*., 2003, Thurman *et al*., 2006a, Thurman *et al*., 2007). Deficiency of regulators on the other hand, exacerbates injury (Yamada *et al*., 2004, Turnberg *et al*., 2004). Murine proximal tubular epithelial cells secrete FH but express only Crry on their basolateral side and no surface regulators on the luminal side (Gerritsma *et al*., 1997). Ischaemia-reperfusion alters the expression of Crry and thus renders the tubular epithelium susceptible to damage (Thurman *et al*., 2006a). Renner *et al.* established a protective role for surface bound FH in wild-type mice subjected to ischaemia-reperfusion (Renner *et al*., 2010, Renner *et al*., 2011).

They further showed protection of tubular epithelial cells from AP-mediated lysis in the presence of super physiologic concentrations of FH *in vitro* and prevention of injury after ischaemia-reperfusion by administration of a CR2-FH fusion protein (Renner *et al*., 2011). This and several other studies have demonstrated that complement is a valid therapeutic target in renal ischaemia-reperfusion injury (Pratt *et al*., 2003, Arumugam *et al*., 2003, de Vries *et al*., 2003b, Woodruff *et al*., 2003).

1.3 Therapeutic potential of the complement system

The complement system participates in numerous functions of the body and dysfunction is unsurprisingly implicated in a wide array of pathologies. The therapeutic potential of the complement system has been explored extensively in the last decade resulting in the identification of numerous attractive targets and potential agents (Ricklin and Lambris, 2007a, Wagner and Frank, 2010). The small number of approved therapeutic agents suggests that modulation of complement activity is a challenging undertaking. Some promising agents have failed at the clinical development stage as they do not satisfy the criteria of an "ideal drug" and fail to deliver high efficacy and specificity alongside minimal side-effects and low production cost. This may reflect on the complexity of the cross-talk between complement and other physiological systems but also on the insufficient understanding of the mechanisms involved in complement-mediated conditions. Undesired activation of complement can be modulated by inhibition of pathway components or replacement of dysfunctional components. The key targets are the central components C3 and C5 (see Tables 1 and 2). The majority of agents under development are biologicals or biopharmaceuticals, a newer class of drugs that is largely based on proteins and that has been expanding significantly over the past decade (Bristow *et al*., 2006, Reichert, 2010, Page *et al*., 2012). Native or recombinant proteins, fusion proteins, monoclonal antibodies, small peptides and serine protease inhibitors comprise the range of potential therapeutics. Tables 1 and 2 provide a summary of therapeutics that are approved, in clinical trials or currently undergoing preclinical development. Examples of advanced reagents that are of particular relevance for this project because they find application in AP-mediated conditions are POT-4 / ALP-1, Eculizumab and Mirococept. Compstatin, and its analogue POT-4 / ALP-1, is a small cyclic peptide (13 amino acids) that effectively inhibits the turnover of C3 by both C3 convertases and binds several C3 metabolites including hydrolysed C3, C3b, iC3b and C3c (Sahu *et al*., 1996, Ricklin and Lambris, 2008). It is currently undergoing clinical testing in phase II trials for age-related macular degeneration (AMD) but there are reports of successful application in animal models of other conditions, including sepsis (Silasi-Mansat *et al*., 2010). Mirococept controls, similar to POT-4, the metabolism of C3 and is the latest of several CR1-based approaches. The soluble CR1 receptor (sCR1/TP10) yielded promising results in several animal models but clinical evaluation revealed TP10 did not affect the outcome for female subjects undergoing bypass surgery for acute myocardial infarction (AMI) and trials of TP10 for AMI have been discontinued (Weisman *et al*., 1990, Couser *et al*., 1995, Lazar *et al*., 2004, Lazar *et al*., 2007). Full size soluble CR1 is nevertheless the focus of many studies.

Table 1: Therapeutic mAbs and recombinant proteins to target complement

	Agent	Description	Development stage	Company
Therapeutic antibodies	Eculizumab / Soliris	Humanised anti-C5 mAb	Approved for PNH; phase II for aHUS; phase I for DDD	Alexion Pharmaceuticals
	Pexelizumab	Humanised anti-C5 mAb	Phase III for AMI and CABG	Alexion Pharmaceuticals
	Ofatumumab / Arzerra	Humanised anti-CD20	Approved for CLL; phase III for lymphomas and RA	Genmab A/S
	HuMax-CD38	Humanised anti-CD38	Preclinical, for multiple myeloma	Genmab A/S
	TNX-234	Humanised anti-FD mAb	Latest: phase I wet AMD?	Tanox / Genentech
	TNX-558	Humanised anti-C5a mAb	Preclinical?	Tanox / Genentech
	TA106	Humanised anti-FB mAb Fab fragment	Preclinical, for AMD	Alexion
	Neutrazumab	Anti-C5aR mAb	Phase I (2008) for RA and psoriasis	G2 therapeutics
	Anti-properdin	Humanised anti-properdin mAb	Preclinical, for RA	Novelmed therapeutics
	MEDI7814	Human anti-C5 antibody	Phase I (safety in healthy volunteers)	MedImmune LLC
Recombinant regulators / components	sCR1 / TP10 / CDX1135	Soluble CR1	Phase II for CABG; phase I fro DDD	Avant Immunotherapeutics; Celldex
	sCR1-sLe^x / TP20	Soluble CR1 targeted to inflammation site	Preclinical, for stroke and AMI	Avant Immunotherapeutics
	Mirococept	Soluble CR1 with lipopeptide membrane linker	Phase II for ischaemia-reperfusion in cadaveric renal allograft	King's College London
	TT30	CR2/FH fusion protein, targeting to site of inflammation	Phase 1 for PNH	Alexion
	CAB-2 / MLN-2222	DAF/MCP fusion protein	Phase 1 for CABG (?)	Millenium therapeutics / Xoma
	rhMBL	Recombinant human MBL	Programme terminated after phase I trials for infection in liver transplant / multiple myeloma patients	Enzon Pharmaceuticals

Adapted from: (Wagner and Frank, 2010) and (Ricklin and Lambris, 2013)

Table 2: Small peptides and serine protease inhibitors to target complement

	Agent	Description	Development stage	Company
Small peptides	Compstatin / POT-4 / APL-1	Small peptide C3 inhibitor	Phase II for AMD	Apellis; Alcon / Novartis
	PMX-53	Small peptide C5aR antagonist	Phase II for RA and psoriasis	Arana / Cephalon / Teva Pharmaceuticals
	ARC1905	RNA aptamer, C5 inhibitor	Phase I for AMD	Ophthotech Corporation
	JPE-1375 / JSM-7717	Small peptide C5aR inhibitors	Preclinical?	Jerini AG / Shire
Serine protease inhibitors	C1-INH	Purified from plasma	Approved for HAE; phase I for AMI, CABG, renal transplantation	CSL Behring
	Rhucin / rhC1INH	Recombinant human C1-INH	Phase III for HAE completed	Pharming Technologies B.V.

Adapted from: (Wagner and Frank, 2010) and (Ricklin and Lambris, 2013)

Celldex are currently investigating the use of TP10, under the name CDX-1135, for MPGN type II in phase I trials. A further variant is the sLex linked TP10 which targets to E- and P-selectins displayed on endothelial cells for use in stroke and AMI (Rittershaus *et al*., 1999, Picard *et al*., 2000, Thomas *et al*., 2004). Mirococept targets the first three SCRs of CR1 to the epithelial and endothelial cell surface via a fatty acid tail that can incorporate into the cell membrane. Perfusion of rat kidneys prior to transplantation was shown to improve ischaemia-reperfusion injury even after prolonged storage (Pratt *et al*., 2003, Patel *et al*., 2006). Mirococept is currently undergoing testing in clinical phase IIb trials. Other cell-surface regulators have been produced in soluble forms sMCP and sDAF have been shown to be less active than their physiological counterparts (Wagner and Frank, 2010).

C5, its metabolite C5a and the C5a receptor (C5aR) are appealing targets for therapeutic intervention (Table 1). The most successful candidate to date is the humanised anti-C5 mAb that was developed by Alexion for the treatment of paroxysmal nocturnal haemoglobinuria (PNH) (Thomas *et al*., 1996, Rother *et al*., 2007). PNH is a rare but severe complement-mediated haemolytic anaemia. Eculizumab was shown to effectively reduce haemolysis in PNH patients and consequently reduce the requirement for blood transfusion (Hillmen *et al*., 2004, Hill, 2005, Parker, 2009). As discussed in section 1.2.3.4, Eculizumab has been successfully used in a small number of aHUS patients (Nurnberger *et al*., 2009, Gruppo and Rother, 2009, Hadaya *et al*., 2011, Al-Akash *et al*., 2010). The clinical phase II trials for the treatment of aHUS with Eculizumab are to conclude in December 2012. There are several reports that demonstrate that Eculizumab can have a beneficial effect in a number of other complement-mediated conditions. These include MPGN type II, SLE and rheumatoid arthritis (Bomback *et al*., 2012, Herlitz *et al*., 2012, Hadaya *et al*., 2011, Wang *et al*., 1995). Clinical trials for the use of Eculizumab in MPGN type II and renal transplantation are underway. Eculizumab and POT-4 offer very effective systemic inhibition of the complement system which is desirable in the acute setting as a life-saving intervention.

The effect of long-term suppression of the complement system is not known. Undoubtedly it results in an increased risk for infection. A key concern is the potential effect of complement modulation on innate and adaptive immune responses and other functions of complement. Drugs that target pathway activation or C3/C5 convertase activity may exert adverse immune-compromising effects but would still be suitable for acute phase treatment (Ricklin and Lambris, 2007b). The currently approved agents target the classical and lectin activation pathways or the common lytic pathway leading up to formation of the MAC and fall into this category. Targeting to the site of complement activation or tissue injury has become a new focus in the development of these agents. It offers solutions to problems associated with

systemic drug administration such as immunosuppressive effects, negative effects on other homeostatic functions of complement, dose requirements and half-life (Holers *et al*., 2013). Recombinant regulators are specific, can be targeted and would probably not greatly interfere with normal immunity. These agents are therefore ideal candidates for the treatment of chronic conditions and/or conditions with underlying regulator deficiency or dysfunction. There are several recombinant regulators under development. Mirococept, as discussed above, is a promising candidate that has so far progressed well through the clinical trials. TNX-234 (Tanox, now Genetech) is a humanised monoclonal antibody against Factor D and is currently undergoing testing in Phase I trials for AMD.

The most extensively studied targeting mechanism to the site of complement activation is complement receptor type 2 (CR2 or CD21) (reviewed in detail by (Holers *et al*., 2013). CR2 binds to the C3 fragments C3dg, C3d and iC3b which are generated and deposited at the site of complement activation (van den Elsen and Isenman, 2011). Fusion of the C3 binding domain of CR2 (SCRs 1-4) to a complement regulator thus provides a means of targeting this regulator to the site of complement activation. CR2 fusion proteins that have been tested include CR2-DAF and CR2-CD59 but CR2-Crry is the best characterised example (Song *et al*., 2003, Atkinson *et al*., 2005, Holers *et al*., 2013). CR2-Crry has been tested in a wide array of murine models of conditions in which the complement system is implicated and has demonstrated several benefits of site-specific complement inhibition. The studies included several models of ischaemia-reperfusion injury, lupus nephritis, AMD and arthritis (Atkinson *et al*., 2005, Atkinson *et al*., 2008, Song *et al*., 2007, Sekine *et al*., 2011a, Sekine *et al*., 2011b). In a murine model of intestinal ischaemia-reperfusion injury, CR2-Crry had an increased half-life and provided better protection than an untargeted version of the construct (Crry-Ig) (Atkinson *et al*., 2005). Unlike the untargeted Crry-Ig, CR2-Crry did not increase susceptibility to experimental sepsis (Atkinson *et al*., 2005). Targeted complement inhibition thus avoids the adverse effect of systemic complement inhibition. This was further illustrated in MRL/lpr mice which develop spontaneous lupus. CR2-Crry did not affect complement-mediated clearance of immune-complexes, whilst treatment with Crry-Ig resulted in an increase in circulating immune complexes (Bao *et al*., 2003). To address the emerging evidence of AP involvement in many conditions an AP-specific version of CR2-Crry, CR2-fH, was developed and again tested in several models (Holers *et al*., 2013). These include models of intestinal and renal ischaemia-reperfusion injury, collagen-induced arthritis and spontaneous lupus (Huang *et al*., 2008, Renner *et al*., 2011, Banda *et al*., 2009, Sekine *et al*., 2011a). CR2-fH has so far not been tested in $Cfh^{-/-}$ or $Cfh^{-/-}.\Delta 16\text{-}20$ mice. The successful application of CR2-fH in murine disease models encouraged the development of a human equivalent, TT30 (Fridkis-Hareli *et al*., 2011). In the

treatment of PNH erythrocytes TT30 displayed superior therapeutic properties compared to Eculizumab which systemically blocks C5 activation but allows continued C3 metabolism (Risitano *et al*., 2012). TT30 has also been demonstrated to effectively reduce pathology in a murine model of AMD (Rohrer *et al*., 2012). TA106 (Taligen) is a further CR2 fusion protein comprising SCRs 1-4 of human CR2 and an anti-Factor D antibody fragment. Both TT30 and TA106 are undergoing preclinical testing for the treatment of aHUS and AMD and have yielded encouraging *in vitro* and *ex vivo* data.

The wide-ranging studies of CR2-Crry and CR2-fH / CR2-FH have certainly provided a strong case to move away from systemic inhibition of central complement components to site-specific targeting of complement regulators. Mirococept and sCR1-sLe^x / TP20 are further examples of complement regulators targeted to a specific site by means of a sophisticated targeting mechanism. The identification of suitable targets is challenging, but the advances in antibody engineering have opened up new possibilities to exploit the binding properties of monoclonal antibodies to create novel targeted fusion proteins.

1.4 Monoclonal antibodies for drug targeting

1.4.1 Origin of monoclonal antibodies

B cells are a central cell of adaptive immunity and are distinguished from other lymphocytes by the presence of the B cell receptor (BCR) and molecules like CD19. The three key functions of B cells include the secretion of antibodies (soluble BCR) against specific antigens, development of immunological memory by formation of memory B cells and the presentation of antigen via MHC class I and II molecules (Vaughan *et al*., 2011). The principal of clonal selection is central for the development of a specific adaptive immune response. Lymphoid progenitor cells produce large numbers of T and B cells, each displaying different antigen specificities. Central and peripheral tolerance mechanisms induce and maintain tolerance by deletion of self-reactive cells (Melchers and Rolink, 2006, Meffre, 2011). Activation of a T or B cell occurs after it is presented with its specific antigen and results in proliferation of that specific cell (clonal expansion). An adaptive immune response is initiated when an antigen presenting cell (APC) presents a specific antigen to a naïve T cell in a lymphoid organ. The cell becomes activated and proliferates. Engagement of the BCR is required for a B cell to become activated. B cells can be activated through freely circulating antigen, in particular by antigen associated with C3d or C3dg which will result in co-ligation of BCR and CD19 and lower the activation threshold 10,000-fold (Dempsey *et al*., 1996). Activation of a B cell usually requires help by activated T helper cells. Antigen is captured by the BCR, internalised and displayed by MHC class II molecules on the cell surface. An activated T cell associates the displayed antigen with its T cell receptor (TCR) and provides co-stimulation by binding of CD40L with CD40 on the B cell surface (Frauwirth and Thompson, 2002). This results in secretion of IL-4, IL-5 and IL-6 by the T cell and leads to further activation and proliferation of the B cell (Mosmann, 2000). The expanding B cell clones, now plasma cells, secrete large amounts of antibodies which can act to neutralise and opsonise pathogens and can mediate activation of the classical pathway of complement activation (Calame *et al*., 2003).

1.4.2 Antibody structure and function

Antibodies are soluble immunoglobulins (Ig) that are secreted by plasma cells in response to specific antigen. The five Ig classes include IgG, IgA, IgM, IgD and IgE. Each class has a distinct heavy chain (γ, α, μ, δ, and ε). IgG and IgA have further subclasses (IgG1, IgG2, IgG3, IgG4; IgA1 and IgA_2) (Janeway, 2001). Regardless of class, every antibody is comprised of four polypeptide chains. Two heavy chains (~ 50 kDa each) are joined by two disulphide bonds in the hinge region. Each heavy chain associates with a light chain (~ 25 kDa each) via disulphide bonds in the constant regions (Cohen, 1975). Both heavy and light chains have distinct functional, globular domains of approximately 110 amino acids (Figure 6).

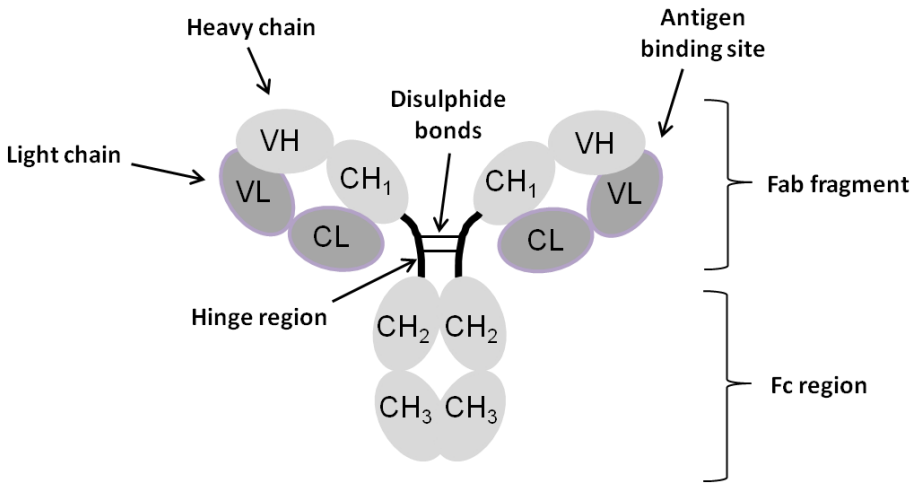

Figure 6: General antibody structure

An antibody is comprised of four polypeptide chains, two identical heavy chains and two identical light chains. The heavy chain has four globular domains: the heavy chain variable domain (VH) and three heavy chain constant domains (CH_1, CH_2, CH_3). The flexible hinge domain connects the CH_1 and CH_2 and also connects the heavy chains via disulphide bonds. The light chain consists of two globular domains: the light chain variable domain (VL) and the light chain constant domain (CL). Each light chain is associated with a heavy chain via disulphide bonds in the CL and CH_1. VL and VH bear the antigen binding site.
Diagram adapted from: (Kalsi et al., 1999, Miner, 2008)

Four such domains, one variable heavy chain domain (VH) and three heavy chain constant domains (CH_1, CH_2 and CH_3), are present in the heavy chain (Janeway, 2001). A flexible linker, the hinge region, is located between CH_1 and CH_2. The light chain is comprised of the light chain variable domain (VL) and the light chain constant region (CL). Antibodies can have one of two light chains, either lambda (λ) or kappa (κ). The amino acid sequences of the N-terminal variable domains, which form the antigen binding site, vary greatly between antibodies (Stewart and Schwartz, 1994). Although each VH and VL is unique it contains conserved, less varied segments called the framework regions. These regions are designated FR1, FR2, FR3 and FR4. The hypervariable or complementarity determining regions (HV1, HV2, HV3 or CDR1, CDR2, CDR3) are located in between the framework regions with HV3/CDR3 showing the highest degree of variability. CDRs of the VH and VL form an antigen binding that is complementary to the surface of a specific antigen (Stewart and Schwartz, 1994). CH_2 and CH_3 form the Fc (Fragment, crystallisable) region. Three key functions of the Fc region have been described. Firstly, it mediates antibody binding to Fc receptors on the cell surface of immune cells including macrophages and neutrophils. Secondly, the classical pathway component C1q can bind to the Fc region of an antibody in complex with an antigen resulting in activation of the complement system. Thirdly, engagement of the Fc with the neonatal Fc receptor (FcRn) facilitates transport of antibodies across certain membranes and body compartments (e.g. the placenta) (Abbas, 2007).

1.4.3 Mechanisms of antigenic diversity

Diversity of the primary antibody repertoire is generated by somatic recombination in the bone marrow (Schatz *et al*., 1992). The genes for variable and constant regions of antibodies are in greater proximity in B cells than in other cells types, allowing for rearrangement of these genes by somatic recombination. The genetic loci of the heavy chain and the λ and κ light chains contain multiple copies of for the V (up to 40) and J (up to 6) genes (Figure 7). In the case of the λ light chain there are several C genes (up to 5) and the heavy chain locus contains several D gene segments (up to 23) and gene segments for each of the immunoglobulin classes, including their respective subclasses (Figure 7) (Abbas, 2007). The VL is comprised of two different gene segments. The V (variable) gene segment and the J (joining) gene segment form the VL exon. Joining of the VL region to the leader sequence and constant region is achieved by splicing of introns that separate these gene segments. The VH contains a third gene segment, the D (diversity) gene segment in addition of the V and J segments. V(D)J-gene rearrangement creates diversity by recombination of different VL gene segments (Figure 8) and adds a further level of diversity at the junctions of joined gene segments by addition and subtraction of nucleotides (Abbas, 2007).

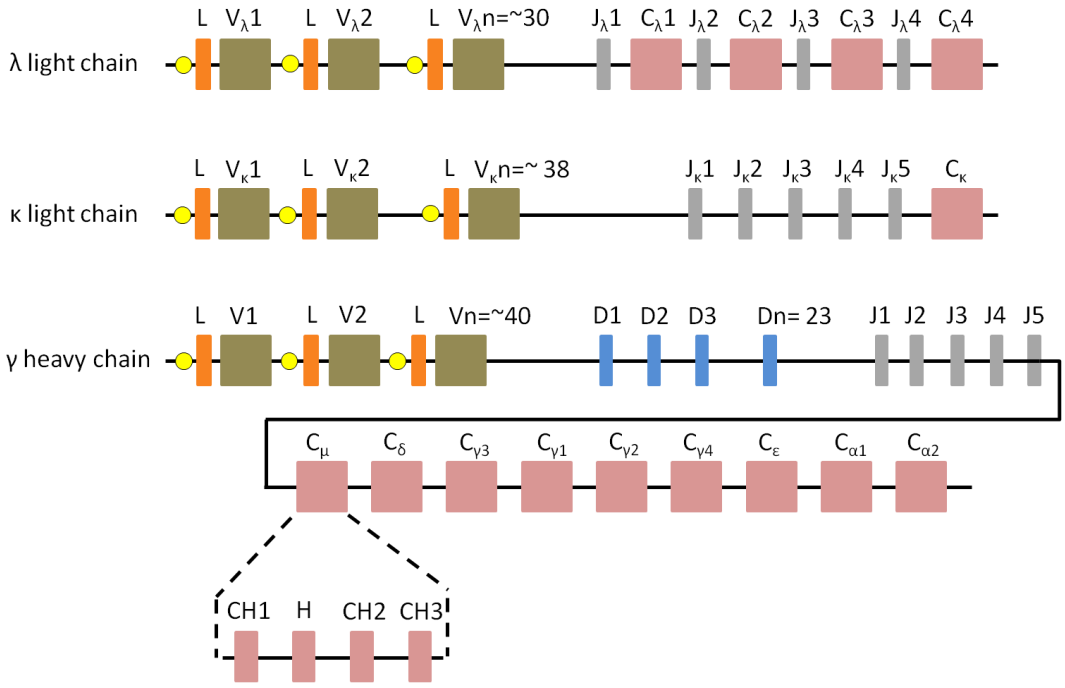

Figure 7: Immunoglobulin heavy and light chain gene structure

The λ light chain has approximately 30 V_λ gene segments, each with its own promoter (yellow circle) and leader sequence, and up to five J_λ and C_λ gene segments. The κ light chain has approximately 38 V_κ gene segments but only one constant region segment. The γ heavy chain gene contains around 40 variable region segments, 23 D and five J gene segments. There is a functional constant region segment for each subclass, each containing segments for CH1, the hinge region, CH2 and CH3.
Diagram adapted from: (Maizels, 2005)

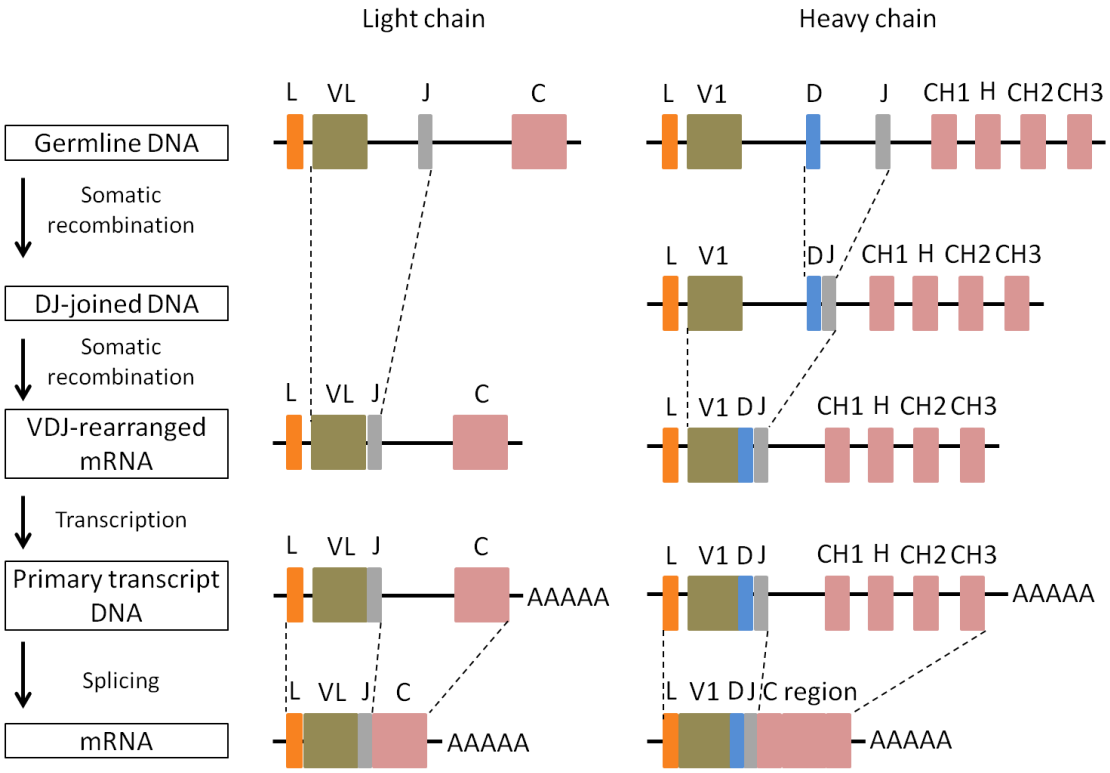

Figure 8: V(D)J rearrangement

The variable regions of antibody light and heavy chains are constructed by rearrangement of the V, D and J gene segments (somatic recombination). One of several V and J segments comprise the exon for a light chain V-region. The rearranged DNA is transcribed into tRNA which is spliced to form the mRNA ready for translation. For the heavy chain a D and a J segment are joined before addition of a V gene segment. As for the light chain, the rearranged DNA is transcribed in to RNA, spliced and translated. Diagram adapted from: (Maizels, 2005)

In addition to these gene rearrangements in the germline DNA, the antibody repertoire can be further diversified in the mature B cell. Three mechanisms have been described. Somatic supermutations are point mutations that occur in the variable region and alter affinity of the secreted antibody. These mutations form the basis of affinity maturation which serves the selection of clones with a higher affinity for a specific antigen. The specificity of the antibody can be altered by introduction of VL fragments derived from pseudogenes. The mechanism of class switching involves the irreversible replacement of the default μ heavy chain with either a γ, α or ε heavy chain (Kracker and Durandy, 2011). Class switching is initiated by cytokines and does not affect the variable regions. These three processes aim to enhance the function of the secreted Ig either with respect to antigen binding or the Fc-mediated effector functions and add to the overall diversity of the available Ig repertoire (Abbas, 2007).

1.4.4 Genetic engineering of antibodies

The technology for the production of monoclonal antibodies was invented by Georges Köhler and Cesar Milstein in 1975 (Kohler and Milstein, 1975). They established a method to immortalise a plasma cell by fusion with a tumour cell to create a monoclonal hybridoma cell line that produces a single type of antibody – a monoclonal antibody (mAb). This key development in the area of antibody research revolutionised the application of antibodies in the research and diagnostic setting within a short period of time and was honoured with a Nobel Prize in 1984. The advantages of this new technology were very soon utilised to develop novel therapeutics. In 1986 the first therapeutic mAb, OKT3, was approved by the Food and Drug Administration (FDA) in the U.S.A.. OKT3 targets the CD3 receptor on T cells and is given to treat acute rejection of kidney, liver and heart transplants in patients that fail to respond to steroid treatment (Goldstein *et al*., 1986, Sevmis *et al*., 2005). The key disadvantage of the early therapeutic mAbs was their immunogenicity. The so called human anti-mouse antibody (HAMA) response impacts on half-life and therefore efficacy but can also increase toxicity in the patient. Developments in the field of molecular biology, in particular recombinant DNA technology, provided the methods for antibody engineering to address the problem of the HAMA response. Chimerisation of antibodies involved the genetic replacement of the murine constant region with human constant regions (Morrison *et al*., 1984, Neuberger *et al*., 1984). To humanise an antibody the CDRs are lifted from the murine template into a human backbone comprising human framework and constant regions (Jones *et al*., 1986, Queen *et al*., 1989). Prominent examples of the first chimeric and humanised therapeutic mAbs are Rituximab (Rituxan, Biogen Idec / Genentech, 1997) and Alemtuzumab (Campath, Genzyme, 2001), respectively. Advances in the generation of transgenic mice ultimately facilitated the production of human mAbs (Taylor *et al*., 1992, Lonberg *et al*., 1994, Green *et al*., 1994).

Special PCR techniques to clone variable regions, e.g. rapid amplification of cDNA (RACE), allowed the establishment of murine and human VH/VL libraries with up to 10^9 specificities (Frohman *et al*., 1988, Orlandi *et al*., 1989, Ohara *et al*., 1989, Loh *et al*., 1989). The tool to fully exploit the value of such libraries, phage display of antibody fragments, was developed by Clackson and co-workers (Clackson *et al*., 1991). This technology does not require the establishment of hybridoma cell lines and requires the immunisation of fewer, if any, animals. The PCR-derived library is cloned into a phagemid plasmid, in-frame with a viral capsid protein and used to transform bacteria. The bacterial culture supernatant contains the phages that display the Fvs. The selection process (panning) involves binding of phages displaying high affinity Fvs to immobilised antigen (e.g. microtitre plate), washing of unbound or weakly bound phages and elution of the strong binders for transformation of bacteria to repeat the process. Each round of panning selects the phages based on the binding properties of the displayed protein and, at the same time, takes forward the according DNA sequence. Panning is a time-saving, efficient process that can be automated and scaled up for high-throughput screening. More than 30 therapeutic mAbs, most humanised or human, are approved for use in Europe and the United States. A similar number is undergoing testing in Phase III clinical trials and several hundred are undergoing pre-clinical testing (Li and Zhu, 2010).

1.4.5 Natural and genetically engineered antibody formats

Affinity, valency (avidity) and size are properties of an antibody that can now be engineered at a molecular level. In addition, functional properties can be modulated. Smaller antibody fragments offer the advantage of better tissue penetration, relevant for cancer therapeutics in particular, and may be easier to produce (Jain, 1990, Yokota *et al*., 1992). Reduction of the antibody to its antigen binding domains, however, greatly impacts on its half-life (Cumber and Wawrzynczak, 1992). The first alternative antibody formats were fragments produced by proteolytic cleavage using either pepsin or papain which will generate Fab or $F(ab')_2$ fragments, respectively (Porter, 1959, Nisonoff *et al*., 1960). Alternative formats also occur naturally. E.g. it has been shown for heavy chain dimers found in camels and sharks and recombinant single-domain antibodies that these molecules are capable of antigen recognition in absence of a light chain (Greenberg *et al*., 1995). A wide array of individual, fused or linked antibody formats with differing properties have been designed (Figure 9).The currently most successful formats in pre-clinical and clinical testing include Fab fragments, scFv fragments and single domain antibodies. Abciximab (Reopro, anti-GPIIb/IIIa receptor), Ranibizumab (Lucentis, anti-VEGF) and Certolizumab (Cimzia, anti-TNFα) are approved therapeutic Fab fragments. Lucentis is the modified Fab of the humanised mAb Avastin (Presta *et al*, 1997; Chen *et al*, 1999).

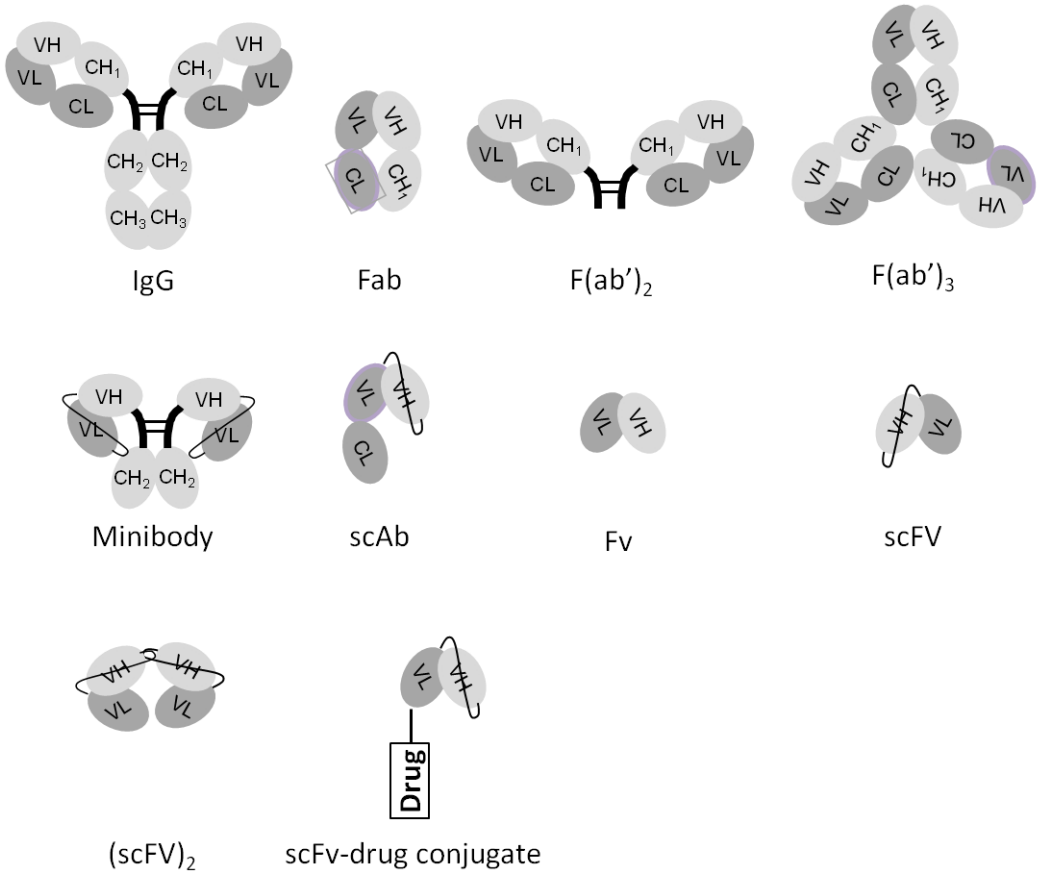

Figure 9: Natural and engineered antibody formats

Antibodies can be engineered to a variety of formats. For some applications a small fragment like a Fab fragment, a single chain antibody, variable fragment (Fv) or scFv may be desired. Multiple Fab fragments, minibodies or scFv dimers ($(scFv)_2$) can be bispecific. Fusion proteins of e.g. a scFv and a drug or other protein components are an attractive format for targeting of a drug to a specific cell type.
Adapted from: (Liu *et al.*, 2008)

1.5 Hypothesis and project aims

The AP is a very potent activation pathway of complement and requires tight regulation to protect host cells from damage. Dysfunctional or overwhelmed AP regulation is implicated in several renal pathologies, including aHUS, MPGN type II, anti-GBM nephritis and ischaemia-reperfusion injury (Sethi and Fervenza, 2012, de Cordoba and de Jorge, 2008, Borza *et al*., 2003, Chowdhury *et al*., 2003). Currently only very few therapeutics address the dysregulation of the AP directly. FH is a central regulator of the AP and plays a particularly important role in the protection of the kidney, especially the glomerulus, from AP-mediated damage (Ault, 2000, Licht and Fremeaux-Bacchi, 2009). The objective of this project was to generate and functionally assess recombinant FH-based therapeutics that are either targeted to glycosaminoglycans at the host cell surface or the NC1 domains of the collagen type IV α3-chain in the glomerular basement membrane.

Hypothesis 1:

FH is a large glycoprotein that is difficult to produce in therapeutically relevant quantities. I hypothesise that a recombinant FH protein comprised of the regulatory domain (SCRs 1-5) or the regulatory domain and the cell-binding domain (SCRs 1-5 and SCRs 18-20) will have comparable functional characteristics as native FH. Production of these lower molecular weight proteins is also anticipated to be easier compared to the full length protein. The specific aims are therefore:

1. Generate two recombinant FH proteins, one incorporating SCRs 1-5 and the other SCRs 1-5 and 18-20 (FH1-5 and FH1-5/18-20) to allow C3b and cell-surface binding.
2. Assess the complement regulatory function of FH1-5 and FH1-5/18-20 using *in vitro* assays.

Hypothesis 2:

Treatment of *Cfh*$^{-/-}$ mice with serum-derived human or murine FH has been shown to restore serum C3 levels and alters glomerular deposition of C3 fragments (Paixao-Cavalcante et al., 2009, Fakhouri et al., 2010). The advantages of targeting of FH to the site of complement activation (CR2-FH/TT30) have been demonstrated in numerous studies (Huang et al., 2008, Rohrer et al., 2010, Fridkis-Hareli et al., 2011). I hypothesise that fusion of the regulatory domain of FH to an antibody fragment (scFv) against a glomerular antigen (collagen type IV) will provide a useful means to target FH to the site of complement activation and deposition. To achieve this, the specific aims were to:

3. Characterise the binding properties of the candidate antibodies for targeting, mAb3 and 1G6.
4. Clone the variable regions of mAb3.
5. Generate FH1-5 and mAb3 scFv / 1G6 scFv fusion proteins
6. Assess antigen binding function of the FH1-5/scFv fusion proteins

2 Materials and methods

2.1 General reagents, materials and equipment

2.1.1 Chemicals

General laboratory chemicals were purchased from Sigma-Aldrich (Dorset, UK) or Fisher Scientific (Loughborough, UK) and were analytical or comparable grade.

2.1.2 Laboratory equipment

For DNA electrophoresis agarose gels were run in the gel tank system from Cleaver Scientific (London, UK) and ethidium bromide staining was visualised using the Alpha Innotech Imager (Santa Clara, USA). The Mighty Small II Gel System (Amersham, Little Chalfont, UK) was used for SDS-PAGE. Transfer of protein from polyacrylamide gels to nitrocellulose was carried out in a water-cooled transfer system from Amersham (Little Chalfont, USA). PCR reactions were carried out using the PTC-200 DNA Engine Thermal Cycler (MJ Research, USA). Tubes up to 2 ml volume were centrifuged using the Mini Eppendorf 5424 bench top centrifuge (Hamburg, Germany). Volumes between 2 ml and 50 ml were centrifuged using the Sanyo Harrier 18/80 (Osaka, Japan) or the Thermo Scientific Sorvall Legend RT (Massachusetts, USA). High speed centrifugation were performed on the Beckman Coulter Avanti J-25 (Brea, USA) using the appropriate rotor. The Äkta Prime System (GE Healthcare) was used for protein purifications. The absorbance was measured at the appropriate wavelengths on the Labsystems Multiskan Spectrum microplate spectrophotometer (Thermo, USA) or the SpectraMAX 190 microplate reader (Molecular Devices, USA).

2.1.3 Vectors and bacterial strains

The mammalian expression vector *pDEF* (originally named *pDR2EF1α*) was used for expression of the recombinant proteins in CHO cells. Both ampicillin and hygromycin resistance genes are contained in pDEF to allow for selection in bacterial and mammalian cell culture, respectively. For molecular cloning *E. coli* strains TOP10 (Invitrogen, UK) or DH5α (NEB, USA) were transformed with plasmid DNA or ligation reactions.

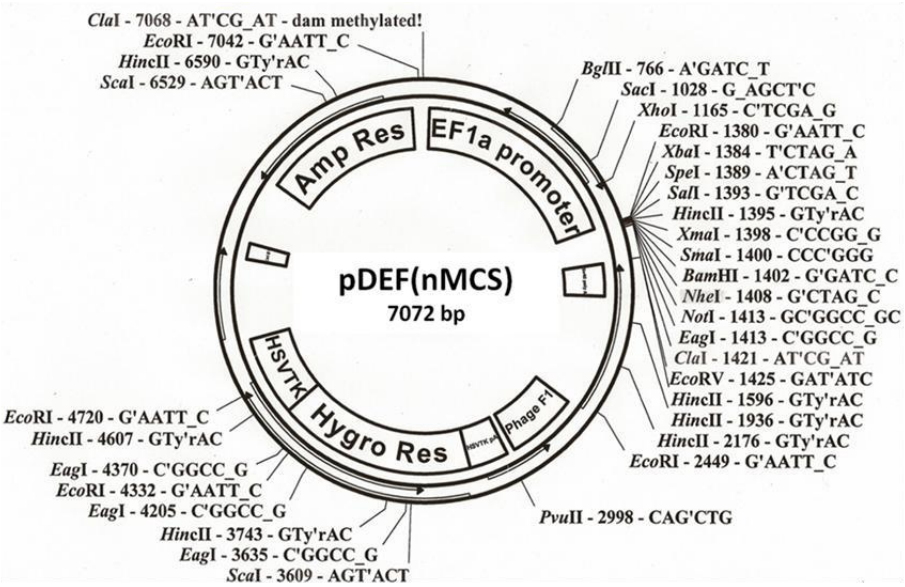

Figure 10: pDEF (nMCS) vector map

pDEF (nMCS) is a mammalian expression vector that contains ampicillin and hygromycin B resistance genes for bacterial and eukaryotic selection, respectively. The multiple cloning site is located downstream of the EF1a promoter.

2.1.4 Mammalian cell lines

Chinese hamster ovary (CHO) cells, wild-type K1 were obtained from CACC. OX24 was gifted by B. Spiller, Cardiff and the mAb3 hybridoma cell line was obtained from Jörgen Wieslander, Lund.

2.1.5 Enzymes

All DNA restriction enzymes (*SalI*, *NheI*, *KpnI*, *XbaI*, *BciVI*) and the appropriate restriction buffers were purchased from New England Biolabs.

2.1.6 Antibodies

All commercially available antibodies and those generated in-house are listed in the table below.

Table 3: Monoclonal and polyclonal antibodies

Antibody	Manufacturer	Western blot	ELISA
Goat anti-human FH	Comptech, concentration not specified	1/5000	1/5000 (detection)
OX24, mouse anti-human FH	In house, 1 mg/ml	1/1000	10 µg/ml (capture/coat)
Bovine anti-goat Ig, HRPO conjugated	Jackson, concentration not specified	1/1000	1/2000
Goat anti-rabbit Ig, FITC conjugated	Jackson, concentration not specified	n/a	n/a
Goat anti-mouse IgG	Jackson, concentration not specified	1/1000	n/a
Sheep anti-mouse Ig	Jackson, concentration not specified	1/2000	1/5000

2.1.7 Proteins

Table 4: Human complement proteins

Name	Manufacturer	Stock concentration
Human factor H	Comptech, USA	1 mg/ml
Human C3b	"	1 mg/ml
Human C3	In house purification	0.8 mg/ml
Human FI	Comptech, USA	0.1 mg/ml
Human factor D	"	0.1 mg/ml
Human factor B	"	1 mg/ml

2.1.8 Sequencing, gene synthesis and primer design

Eurofins MWG Operon (Ebersberg, Germany) was used for DNA sequencing and synthesis of genes and oligonucleotide primers. All primers used for the project are listed in Table 5. The sequences of all recombinant genes that were generated and the genes that were commercially synthesized (mAb3 and 1G6 scFv) are in Appendix B.

Table 5: Oligonucleotides

Primer Name	Sequence (5' to 3')	Template / application
T7	TAATACGACTCACTATAGGG	Sequencing pCR4 (TOPO cloning)
T3	ATTAACCCTCACTAAAGGGA	"
pDEF NF	CTCAAGCCTCAGACAGTGGTGC	Sequencing / colony PCR pDEF MCS
pDEF NR	CTGCATTCTAGTTGTGGTTTGTCC	"
SCRIkozsp*SalI*	ATAGCTGTCGACGCCACCATGAGACTTCTAGCAA	FH SCRs 1-5 *SalI* Kozak forward
SCR5his*KpnI*	CAGTCGATGGTACCATGATGATGATGATGATGTTTCAAG GTACATCTC	FH SCRs 1-5 Histag *KpnI* site reverse
F327fwd	ACATATGGTACCACCTCCTGTGTGAATCC	FH SCRs 18-20 *KpnI* forward
F327rev	TGTTATGCTAGCTTATCTTTTTGCACAAGTTGG	FH SCRs 18-20 *NheI* reverse
1G6fwd*KpnI*	ATATGAAGGTACCCTGGTGAAGCCTGGGGCTT	Forward primer PCR 1G6 scFv from PCR2, introducing *KpnI*
1G6rev*NheI*	TAGACTGGCTAGCTTATTTTATTTCCAGCTTG	Reverse primer PCR 1G6 scFv from PCR2, introducing *NheI*
RACEMOG1^	TATGCAAGGCTTACAACCACA	cDNA synthesis mAb3 heavy chain
CKFOR^	CTCATTCCTGTTGAAGCTCTTGAC	cDNA synthesis mAb3 light chain
MOCG12FOR^	CTCAATTTTCTTGTCCACCTTGGTGC	Reverse primer 5' RACE mAb3 heavy chain
CKMOsp^	CTCATTCCTGTTGAAGCTCTTGACAATGGG	Reverse primer 5' RACE mAb3 light chain
Light RT*	CGCGGATCCGAAGATAGGATGGAGCTGG	Alternative primer cDNA synthesis mAb3 light chain
Heavy RT*	GTCCACCGTGGTGCTGCTGGC	Alternative primer cDNA synthesis mAb3 heavy chain
MKVP1*	ATGGAGWCAGACACACTCCTGYTATGGGT	Mouse kappa light chain leader sequence
MKVP2*	ATGAAGTTGCCTGTTAGGCTGTTGGTGCTG	"
MKVP6*	ATGAGGTKCYYTGYTSAGYTYCTGRGG	"
MKVP8*	ATGTGGGGAYCTKTTTYCMMTTTTTCAATTG	"
MKVP11*	ATGGAAGCCCCAGCTCAGCTTCTCTTCC	"
VL-IIA*	GATGTTKTGATGACCCAAACT	Mouse kappa light chain FR1
mAb3VHf*KpnI*	CATCATCATGGTACCCAGGTGCAGCTGCAGCAGTC	PCR mAb3 heavy chain forward, introducing *KpnI*
mAb3VHrlinker	GGAGCCGCCGCCGCCAGAACCACCACCACCTGAGGAGA CTGTGAGAGTGGGTGCCT	PCR mAb3 heavy chain reverse, introducing $(G_4S)_3$ linker
mAb3VLflinker	GGCGGCGGCGGCTCCGGTGGTGGTGGATCCGACATTGT GATGACCCAAACTC	PCR mAb3 light chain forward, introducing $(G_4S)_3$ linker
mAb3VLr*NheI*	GCTAGCTTATTTTATTTCCAGCTTGGTCCCC	PCR mAb3 light chain reverse, introducing *NheI*
mAb3CDR2rev	GACTCCACTGTACCGGTAGGATGCCAG	Reverse primers for 5' RACE of specific mAb3 light chain cDNA
mAb3CDR3rev	CGTGAGAGGATAGCTGTTATATTGCTG	Synthesis of specific light chain cDNA from mAb3 mRNA
mAb3 0312VHfwd	CCGCAAGCGGTACCCAGGT	Amplification of synthesized mAb3 gene from pCR2
mAb3 0312VLrev	GCCGCAGGTGCTAGCTTATT	"

^ As described in (Kontermann and Dübel, 2010)

***** As described in (Debat *et al*., 2001)

2.2 DNA manipulation and cloning

2.2.1 Site-directed mutagenesis

Mutagenesis primers were designed to change Val62 to isoleucine by site-directed mutagenesis in FH1-5 in the pDEF expression vector:

Eva1 fwd 5'-TATAGATCTCTTGGAAATATAATAATGGTATGCAGGAAG-3'

Eva1 rev 5'-CTTCCTGCATACCATTATTATATTTCCAAGAGATCTATA-3'

Site-directed mutagenesis was performed as described in the manufacturer's manual (Quikchange II XL, Stratagene). XL-10 gold cells were transformed with control plasmids (pWhitescript and pUC18) and grown on LB-ampicillin plates supplemented topically with 20 mg/ml X-gal substrate and 20 mM IPTG. XL-10 gold cells transformed with the mutated pDEF DNA were grown on LB-ampicillin plates. Introduction of the point-mutation was confirmed by DNA sequencing.

2.2.2 DNA ligation

To generate the recombinant DNA constructs, restriction digested DNA inserts (PCR products, synthetic DNA fragments or fragments derived from other plasmids) were ligated with vector DNA that was digested with the same restriction enzymes using a T4 DNA ligase. The concentration of purified insert / vector DNA was determined by spectrophotometry at 260 nM (Nandrop; Thermo Scientific, UK). A range of molar ratios of insert and vector (100 ng) were used for each ligation (1:1, 3:1 and 10:1). To calculate the correct ratios the following formula was used:

$$\boldsymbol{insert\ (ng)} = \frac{\boldsymbol{vector\ (ng)} \times \boldsymbol{insert\ (kb)}}{\boldsymbol{vector\ (kb)}} \times \mathbf{1\ (3\ or\ 10)}$$

For each ligation reaction the appropriate amounts of vector and insert were mixed with 1 µl T4 DNA ligase buffer, 1 µl T4 DNA ligase. H_2O was added to bring the final reaction volume to 10 µl, if required.

2.2.3 Plasmid transformation and DNA purification

Aliquots of TOP10 (Invitrogen, UK) or DH5α (New England Biolabs) were transformed with plasmid DNA (~ 100 ng) or ligation reaction (5 µl) by incubating cells and DNA for 30 min on ice before heat-shocking for 45 s at 42 °C. The heat-shock was followed by 10 min incubation on

ice. After addition of 500 µl super optimal broth (SOB) supplemented with glucose (SOC medium) the cells were allowed to recover for 1 h at 37 °C at 220 rpm.

SOB

2% Bacto-tryptone
0.5% Yeast extract
8.56 mM NaCl
2.5mM KCl
H_2O to 1000 mL

SOC

SOB supplemented with:

10mM $MgCl_2$
20mM glucose

2.2.4 Restriction enzyme digest

Restriction digests of plasmid DNA or PCR amplified DNA fragments were performed for verification of plasmid DNA or to prepare DNA for ligation. Restriction was carried out according to enzyme manufacturer's instructions (NEB), in 10 µl or 20 µl reaction volumes using 1-5 µg of DNA obtained using plasmid preparation kits (Qiagen). To minimize the self-circularisation of digested plasmid DNA the 5' phosphate groups were removed by treatment with Antarctic Phosphatase (NEB, UK). Usually 10 units of Antarctic phosphatase and 2 µl of reaction buffer were added to 1 µg of purified, digested plasmid DNA in a final reaction volume of 20 µl. The reaction was incubated for 20 min at 37 °C and the enzyme heat-inactivated for 5 min at 65 °C.

2.2.5 RNA preparation and cDNA synthesis

Messenger RNA (mRNA) was prepared from mAb3 hybridoma cells using the GenElute Direct mRNA Mini-prep kit (Sigma). The synthesis of the first-strand cDNA of the mAb3 heavy and light chain mRNA was either carried out using the Roche 5' RACE kit or M-MLV RT, RT buffer and RNAsin from Promega. In brief, 1 µg of mRNA was heated at 65 °C for 5 min and then added to the following components as described in Table 6.

Table 6: Typical reaction for cDNA synthesis

Volume	Component
5.0 µl (1 µg)	mRNA
5.0 µl	RT buffer (5x)
0.6 µl (25U)	RNAsin
6.0 µl (0.5 µg)	RACEMOG1 or Heavy RT / CKFOR L or Light RT
2.5 µl (10 mM each)	dNTPs
4.9 µl	Nuclease-free water
24.0 µl	Total volume

This mixture was warmed to 42 °C for 3-4 min before addition of 1 µl of M-MLV reverse transcriptase (200 U). The incubation was continued for 60 min followed by heat-inactivation of the reverse transcriptase for 10 min at 70 °C.

2.2.6 Polymerase chain reaction

Polymerase chain reaction (PCR) was performed to amplify DNA fragments from plasmid DNA either for downstream cloning or to confirm presence of an insert of the correct size in transformed bacterial clones (colony PCR) (Saiki *et al*., 1992). Standard PCR was also used to amplify heavy and light chain variable region from first strand cDNA. A *Taq* polymerase (Standard Taq, NEB) was used for colony PCR. A proof-reading *Pfu* polymerase (Phusion Taq, NEB) or a blend of proof-reading polymerases (Expand High Fidelty PCR System, Roche) was used for amplification of DNA destined for downstream cloning. The composition of a typical 50 µl PCR reaction is shown in Table 7 and the standard cycling parameters are shown in Table 8.

Table 7: Composition of a typical PCR reaction

Ingredients	50 µl reaction
10x reaction buffer	5.0 µl
$MgCl_2$	5.0 µl
dNTPs (25 mM) each	1.0 µl
Forward primer	1.0 µl (5 pmol)
Reverse primer	1.0 µl (5 pmol)
DNA polymerase	0.5 µl
dH_2O	36.5 µl

Table 8: Standard PCR cycling parameters

Step	Temperature	Duration (min)	No. of cycles
Initial melting	95 °C	5	1
Melting	95 °C	1	35
Annealing	58 °C	1	
Extension	72 °C	1-2 min / kb	
Final extension	72 °C	10	1
Storage	4 °C	∞	1

2.2.7 Screening of colonies

Bacterial colonies were screened for the presence of the transformed plasmid and an insert of the correct size by colony PCR (as described in 2.2.6). For this each colony was picked off the LB plate with a sterile pipette tip and suspended in 20 µl sterile water in a 0.2 ml tube. The suspended bacterial material was boiled for 5min and then centrifuged for 5 min at 1000 x g to separate the DNA from the denatured protein. 5 µl of the supernatant was used as template for the colony PCR. To amplify any DNA inserted into the multiple cloning site (MCS) of pDEF, primers upstream (pDEF NF) and downstream (pDEF NR) of the MCS were used.

2.2.8 Rapid amplification of 5' cDNA ends (5' RACE)

To amplify the unknown 5' ends of the heavy and light chain variable regions 5' RACE (Rapid Amplification of cDNA Ends) was used (Frohman *et al*., 1988). The principle of this type of anchor PCR is explained in Figure 11. Amplification of mAb3 variable domains from the first strand cDNA was carried out using a 5' RACE kit (Roche). For the heavy chain the gene-specific primer primes at the end of the heavy chain constant region 1 (MOCG12), for the light chain at the end of the constant region (CKMOsp). For all PCR reactions of the heavy and light chain cDNA the anchor primer provided in the 5' RACE kit and a proof-reading polymerase was used.

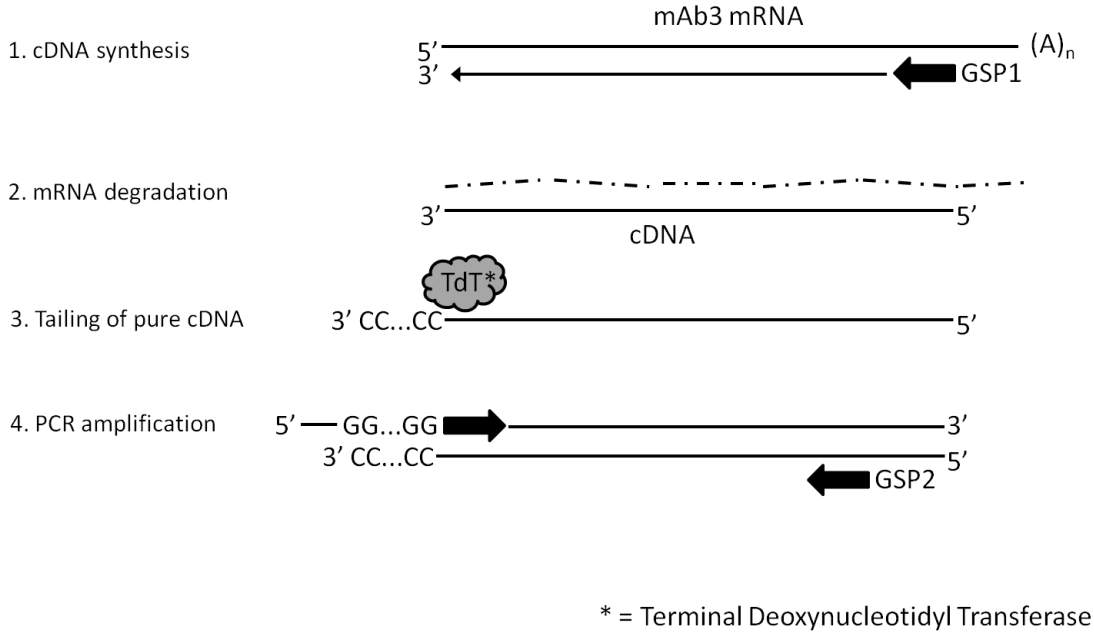

Figure 11: Rapid amplification of 5’ cDNA ends (5’ RACE)

In the first step the first strand cDNA is synthesised from mRNA or total RNA using a gene specific primer (GSP1) reverse transcriptase. An RNAse mix is added in the second step to degrade the mRNA / RNA. In the third step a homopolymeric dCTP tail is added to the 3’ end of the first strand cDNA using a terminal deoxynucleotidyl transferase (TdT) to allow amplification of the unknown 5’ end. The poly-dCTP tail serves as priming site for the anchor primer which is used with a second gene specific primer (GSP2) to amplify the area of interest, including the complete 5’ region by PCR.

2.2.9 Agarose gel electrophoresis

Plasmid DNA and PCR products were analysed by agarose gel electrophoresis. Depending on the size of the DNA fragments, 0.8-1.5% agarose gels were prepared with TAE buffer. After cooling ethidium bromide was added to a final concentration of 0.5 µg/ml into the melted agarose for visualisation of the DNA under ultraviolet (UV) light. The set gels were run in TAE buffer after samples were loaded (with loading dye, 6x from NEB). Each gel was run at 100-120 V for approximately an hour or until the dye front had migrated approximately half way through the gel. The DNA was visualised using the Alpha Innotech gel imaging system (Santa Clara, USA). For gel purification of DNA fragments, the band of the corresponding size was excised using a scalpel and processed with a gel purification kit (Qiagen).

Tris-acetic acid ethylenediamine tetraacetic acid (TAE, pH 7.2)
40 mM Tris base
40 mM Glacial Acetic acid
10 mM EDTA

DNA sample buffer (6x)
60 mM Tris-HCl (pH 7.6)
0.15% Orange G
60% Glycerol
60 mM EDTA

2.2.10 Bacterial cell culture

E. coli cultures were grown in 5 ml cultures in LB medium (Fisher Scientific) or on LB-agar (Fisher Scientific) plates supplemented with 100 µg/ml ampicillin. Plasmid DNA was isolated using Qiagen Mini Plasmid Prep kits.

2.3 Mammalian cell culture

2.3.1 Maintenance of cells

All cells were maintained in humidified incubator with 5% CO_2 supply at 37 °C. Cells were grown in standard plastic cell culture plates (6, 12, 24 or 96 wells) or flasks (25, 75 or 175 cm^2) from Greiner Bio-one, Germany. Cells were counted using a haemocytometer (Neubauer, Germany). Adherent cell lines were split when they reached confluence, cell lines expressing recombinant proteins were left to grow and secrete protein for 10 days or until significant cell death was observed. Adherent cells were detached with trypsin / EDTA (PAA) or 0.01% EDTA only after washing with PBS. After successful detachment of the cells the trypsin was neutralised by addition of culture medium. The cells were centrifuged for 3 min at 300 x g, resuspended in culture medium and a fraction (usually 10%) returned to the flask containing fresh culture medium. Suspension cell lines were split every 3-4 days by centrifugation as above, usually 10% of the cells were returned to culture flask.

Wild-type CHO

CHO cells were maintained in Ham's F-12 medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 0.1 units/ml penicillin and 0.1 mg/ml streptomycin (all PAA).

Stably transfected CHO cell lines

Stably transfected CHO cell lines were maintained like the wild-type cells with addition of 200-300 µg/ml hygromycin B.

Hybridomas

Hybridoma cell lines (OX24, mAb3) were cultured in RPMI supplemented with 10% low immunoglobulin fetal calf serum, 2 mM L-glutamine, 0.1 units/ml penicillin, 0.1 mg/ml streptomycin, 1 mM non-essential amino acids, 1 mM sodium pyruvate (all PAA) and 50 µM β-mercaptethanol (Sigma-Aldrich).

2.3.2 Liquid nitrogen storage of cells

For storage cells were washed and resuspended in FCS with 10% DMSO and transferred into cryovials. Cells were frozen at -70 °C before transfer to liquid nitrogen storage. For recovery of cells from liquid nitrogen storage the vials were thawed rapidly in a 37 °C water bath and added to 15 ml of pre-warmed culture medium. After centrifugation for 3 min at 300 x g the cell pellet was resuspended in medium and transferred to a 25 cm^2 culture flask.

2.3.3 Stable transfection

CHO cells were transfected using the jetPRIME transfection reagent (PolyPlus) according to the manufacturer's instructions. In brief, $1x10^5$ cells were seeded in the wells of a 6-well plate in 2ml of wild-type growth medium 24 h before transfection. The transfection reagents were then combined with 2 µg plasmid DNA and added to the cells. A control well treated with the transfection reagent only was included in each transfection experiment. After 24 h the growth medium was replaced with growth medium containing 500 µg/ml hygromycin B and exchanged every two days. The concentration of hygromycin was determined by preparation of a kill curve (Figure 12). After 7-10 days colonies of drug-resistant clones were visible and individual colonies isolated using cloning discs (Sigma-Aldrich). For this, colonies were marked on the well bottom with a pen, the cells then carefully washed wish PBS and the cloning discs (soaked in trypsin-EDTA) layed over each colony. After 3-5 minutes the cloning discs were removed and placed in 24 well plates containing 300 µl selective growth medium. Protein expression levels of each clone were tested by enzyme-linked immune-sorbent assay (ELISA) when the cells were confluent. The selected clones were scaled up in larger cell culture flasks and aliquots stored in liquid nitrogen. During collection of supernatant for purification of recombinant protein the culture medium was supplemented with 200 µg/ml hygromycin B.

2.3.4 Antibiotic killing curve wild-type CHO cells (hygromycin B)

To determine the lowest concentration of hygromycin B that effectively kills all wild-type CHO cells an antibiotic killing experiment was carried out in 24-well plates. $8x10^4$ cells were seeded in wells of a 24-well plate and grown overnight in the absence of antibiotic. On the following day the medium was changed for medium supplemented with hygromycin B at 100-1000 µg/ml. The medium was changed every two days over a period of 10 days. On day 10 survival of the cells was assessed. For this the cells were detached with trypsin-EDTA and after addition of 1 ml of culture medium transferred to a 15 ml tube for centrifugation. The cell pellet was resuspended in PBS and the concentration of each sample adjusted to 10^6 cells/ml. The sample was mixed at a 1:1 ratio with trypan blue (0.4% w/v) and counted with a haemocytometer. The viable, unstained cells and stained dead cells were counted separately and the percentage of viable cells determined using the formula below:

$$Viable\ cells\ = \frac{Total\ viable\ cells\ per\ ml}{Total\ cells\ per\ ml} \times 100$$

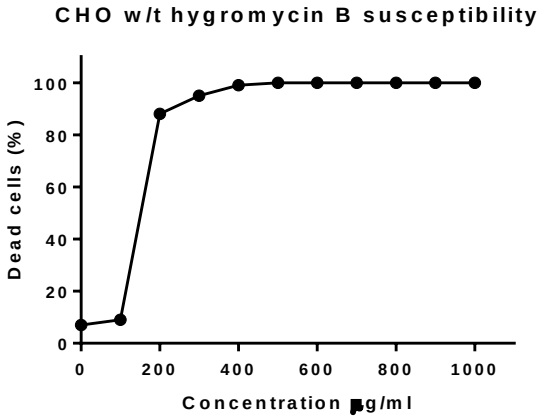

Figure 12: Wild-type CHO hygromycin B kill curve

Survival of wild-type CHO cells in the presence of different concentrations of hygromycin B was assessed at day 10. Cell were removed from the culture flask and cell death assessed by trypan blue staining using a haemocytometer.

2.4 Biochemical techniques

2.4.1 Sandwich ELISA

For enzyme linked immunosorbent assay (ELISA) 96-well plates were used (Nunc Maxisorp). The plates were coated with the capture antibody or protein at 5-10 µg/ml in 200 mM carbonate buffer or PBS and incubated overnight at 4 °C. After three washes with wash buffer (PBS, 0.1% Tween-20) to remove unbound protein the wells were blocked with block solution (PBS, 0.1% Tween 20, 1% BSA) for 2 h, followed by three washes. Each sample (100 µl) was then added to the wells and diluted with block solution as required and incubated room-temperature for 1-2 h. For detection of recombinant protein expression in cell culture supernatants wild-type cell culture supernatant was included as negative control. After three washes the detection antibody, diluted in block solution, was added and the plate incubated for 1 h at RT. Following three washes the HRPO-conjugated secondary antibody, diluted in block solution, was added and the plate incubated for 30 min at room-temperature. After five washes with wash buffer and two washes with PBS, 100 µl of TMB substrate was added and colour was allowed to develop for up to 10 min. The reaction was stopped by addition of 10% H_2SO_4 and the absorbance measured at 450 nm. The ELISA data was usually presented as the mean ± standard deviation of duplicate or triplicate wells after subtraction of the secondary only control reading.

For the heparin binding ELISA Nunc Maxisorp 96-well plates were coated overnight with poly-L-lysine (MW 30 – 150 kDa, Sigma 81339) at 50 µg/ml in H_2O at 4 °C. On the next day, the plate was washed twice with wash buffer 100 µl of 25 µg/ml heparin (Sigma, 4784) in H_2O were added to each well and incubated for 2 h at RT. After washing the wells were blocked as described previously and following a further wash the FH proteins were added at 0-100 nM and incubated at room temperature for 1 h. Bound FH was detected as described previously.

Coating buffer (Carbonate buffer pH 9.6)
24 mM $NaHCO_3$
13.5 mM Na_2CO_3

Wash buffer
PBS, 0.05% Tween20

Block buffer
PBS, 0.05% Tween20 + 1% BSA

TMB substrate solution
For 10 ml of 3,3,5,5-tetramethylbenzidine (TMB) substrate solution were freshly 9.9 ml phosphate-Citrate buffer, 100 µl TMB stock [10 mg/ml in dimethylsulfoxide (DMSO)] and 3 µl 30% H_2O_2 were mixed freshly for each experiment.

Phosphate-Citrate buffer pH 5.0
51.4 mM Na_2HPO_4
24.3 mM Citrate (anhydrous citric acid)

Stop solution
10% H_2SO_4

2.4.2 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried based on the method described by Laemmli (Laemmli, 1970). Samples were mixed with non-reducing or reducing sample-buffer and boiled for 5 min at 95 °C. Freshly prepared 10% resolving gels and 4% stacking gels were used for all experiments, with the exception of the decay acceleration assay in which 5% resolving gels were used. A stained broad range protein ladder (Page Ruler plus, Fermentas) was used as size standard. Gels were electrophoresed in running buffer at a maximum voltage of 300 V and constant 30 mA per gel. Coomassie blue staining was carried out for 10 min and destaining as required.

Table 9: Composition of SDS-PAGE gels

	4% stacking gel	10% resolving gel	5% resolving gel
1 M Tris pH 6.8	0.5 ml	-	-
1.5 M Tris pH 8.8	-	2.5 ml	2.5 ml
30% bis/acrylamide	0.67 ml	3.3 ml	1.7 ml
dH2O	2.4 ml	4.1 ml	5.7 ml
10% SDS	40 µl	100 µl	100 µl
10% APS	30 µl	50 µl	50 µl
TEMED	5 µl	10 µl	10 µl
Total volume	3.6 ml	10 ml	10 ml

Cell lysis buffer
1% NP40 (Fluka Biochemika, Buchs, Switzerland)
150 mM NaCl
50 mM Tris pH 8.0
Supplemented with Roche Complete Protease Inhibitor Tablet (Basel, Switzerland) immediately before use

Protein non-reducing sample buffer (2x)
0.01% bromophenol blue
125 mM Tris-HCl pH 6.8
4% SDS
20% Glycerol

2x protein reducing sample buffer
Non-reducing sample buffer as above and 5% 2-mercaptoethanol.

Gel buffers
Stacking gel buffer: 1 M Tris pH 6.8
Separating gel buffer: 1.5 M Tris pH 8.8

Running buffer (pH 8.3)
25 mM Tris base
192 mM Glycine
0.1% SDS

Coomassie blue staining solution (1 L)
2.5 g Coomassie brilliant blue
500 ml Methanol
425 ml H_2O
75 ml Glacial Acetic acid

Destain solution (1 L)
100 ml Methanol
100 ml Glacial Acetic acid
800 ml H_2O

2.4.3 Immunoblotting

Immunoblotting was carried out according to the method described by Towbin *et al*, with several modifications (Towbin *et al*., 1979). To transfer protein from the SDS-PAGE gels to nitrocellulose the transfer cassette was assembled (sponge, 3MM blotting paper, gel, nitrocellulose, 3MM blotting paper, sponge). The cassette was immersed in transfer buffer in a transfer tank and transferred at 100 V for 1.5 h. The transfer tank was cooled with circulating cold water. After transfer nitrocellulose membranes were blocked in block solution (5% milk / PBS) for a minimum of 30 min at room temperature whilst agitated. Primary antibody was diluted in block solution and incubated for 60 min at room temperature. Following three washes in wash buffer (PBS / 0.05% Tween 20) membranes were blocked again and then incubated with secondary antibody added diluted in block solution for 30-60 min at room temperature. The membranes were finally washed three times for 5 min. Detection was carried out by chemiluminescence. For this the washed nitrocellulose membranes were covered in ECL reagent for immunoblotting (Pierce, UK), placed in an X-ray cassette and covered with overhead projector film. Autoradiography film (Kodak) was used to capture the signal. The films were developed manually or using an automated developer.

Transfer buffer
25 mM Tris base
192 mM Glycine
20% Methanol

Phosphate Buffered Saline (PBS, pH 6.8)
8.1 mM Na_2HPO_4
1.5 mM KH_2PO_4
137 mM NaCl
2.7 mM KCl

Wash buffer
PBS, 0.05% Tween20

Block buffer
PBS, 0.05% Tween20 + 5% fat free milk (w/v)

2.4.4 Chromatography

All protein purification steps were carried out according to manufacturer's manuals (GE Healthcare) or as outlined for each type of purification below. Samples were applied by means of a peristaltic pump whilst wash and elution steps were carried out using an Äkta Prime system (GE Healthcare). The protein concentration of collected fractions was measured by spectrometry (Nanodrop, 280 nm) and samples were then analysed by SDS-PAGE. Selected fractions were subsequently pooled and buffer-exchanged into PBS with 0.1% NaN_3 either by dialysis overnight using dialysis tubing (Sigma), spin columns (Sartorius, Vivaspin) with appropriate molecular weight cut-off or by use of desalting columns (5 ml Desalting column, GE Healthcare).

2.4.4.1 Monoclonal antibody purification

Both mAbs (mAb3 and OX24) were purified on 5 ml Protein G columns (GE Healthcare) according to manufacturer's instructions. In brief, hybridoma cell culture supernatants were collected, centrifuged at 2000 x g and diluted to 50% in 2x PBS and filtered through a 0.2 µm filter. The column was prepared by replacing the storage solution (20% ethanol) with at last 20 ml sterile-filtered H_2O to avoid precipitation of buffer salts. The column was then equilibrated with 20-30 ml PBS. The diluted cell culture supernatant was applied to the column at flow rates of up to 1 ml/min. Unbound protein was removed from the column by application of 10-20 ml of PBS at 1 ml/min. Bound mAb was eluted from the column with low pH elution buffer and collected into 80 µl of neutralising buffer. Collected fractions were analysed by SDS-PAGE followed by Coomassie blue staining and immunoblotting. The purified antibody was buffer exchanged into PBS with 0.01% $NaNa_3$ and stored at 4 °C or stored at -20 °C after addition of glycerol to 50%.

2x Phosphate Buffered Saline (PBS, pH 6.8)
- 16.2 mM Na_2HPO_4
- 3 mM KH_2PO_4
- 174 mM NaCl
- 5.4 mM KCl

Elution buffer
- 0.1 M glycine, pH 7.2

Neutralisation buffer
- 1 M Tris HCl

2.4.4.2 Immobilised metal ion chromatography (IMAC)

Histidine-tagged proteins were purified on 5 ml HiTrap chelating columns (GE Healthcare) according to the method by Hewitt with modifications as described below (Hewitt *et al*., 1997). CHO cell culture supernatants containing secreted histidine-tagged protein was collected, centrifuged at 2000 x g and diluted to 50% in binding buffer and filtered through a 0.2 µm filter. The column was prepared by replacing the storage solution (20% ethanol) with at last 20 ml sterile-filtered H_2O to avoid precipitation of buffer salts. The column was then charged with Ni^{2+} ions by application of 5 ml of 0.1 M $NiSO_4$ to the column, followed by equilibration with 20-30 ml binding buffer. The diluted cell culture supernatant was applied to the column at flow rates of up to 1 ml/min. Unbound protein and weakly bound non-specific proteins were washed from the column with 10-20 ml of binding buffer at 1 ml/min. The specifically bound protein was eluted from the column by application of an imidazole gradient (0-500 mM). The gradient was achieved by gradual mixing of binding and elution buffers over a total volume of 10 or 20 ml at 1 ml/min by the automated purification system. Presence of the protein of interest in the elution fractions was established by SDS-PAGE followed by Coomassie blue staining and immunoblotting.

Binding buffer
0.5 M NaCl
0.1 M Tris-HCl
40 mM imidazole

Elution buffer
0.5 M NaCl
0.1 M Tris-HCl
500 mM imidazole

2.4.4.3 Purification of C3 from human plasma

Human C3 was required for the fluid-phase C3 convertase assay and purified from human plasma by fractional precipitation using polyethylene glycol (PEG) followed by two chromatography steps. This method is described in detail by Harris *et al* (Harris, 2000).

20 ml of whole blood derived from a healthy donor was collected into 1 ml of 100 mM EDTA, mixed and centrifuged for 20 min at 2000 x g. The plasma was removed and diluted 1/10 in PBS containing 5 mM EDTA and 4% PEG6000 to final volume of 25 ml. Four tubes containing 25 ml were centrifuged for 30 min at 10,000 x g. The supernatant was then carefully removed and 30% PEG6000 in PBS/5 mM EDTA was added to a final concentration of 10%. The tubes were again centrifuged for 30 min at 10,000 x g. The supernatant was discarded and the resulting pellet resuspended in binding buffer for the anion exchange column (20 mM Tris, 5 mM EDTA, pH 8.9) by vortexing and pipetting.

The anion exchange column (HiTrap Q HP, GE Healthcare) was equilibrated with binding buffer and the resuspended serum fraction containing C3 was applied to the column in batches of 5 ml. After application of the suspension to the column, the column was washed with binding buffer and eluted using a 20 ml linear NaCl gradient from 0 mM to 500 mM. The collected fractions were analysed by SDS-PAGE and immunoblotting to identify fractions containing C3. Fractions containing C3 were pooled and applied to a gel filtration column (HiPrep Sephacryl 16/60 S100, GE Healthcare) to allow buffer exchange into PBS and separation of contaminating lower molecular weight bands.

2.4.5 Immunofluorescence

To assess *in vivo* binding of mAb3 female Balb/c mice were injected with 20 µg of antibody (mAb3, 8D1, HEL) in 100 µl sterile saline or saline only via the tail vein and sacrificed after 1 h. 8D1 (kind gift from Prof D.-B. Borza, Vanderbilt University) is an isotype-matched positive control antibody that recognises the same antigen as mAb3, i.e. the NC1 domain of type IV collagen (Zent *et al*., 2006). HEL is a high affinity hen-egg lysozyme antibody (IgG1, kindly donated by Dr A. Knight, Newcastle University) and was included as isotype control. Kidneys, spleen, liver and lung were removed, frozen in isopentane and transferred to liquid nitrogen. Cryosections of OCT mounted kidneys were produced at -20 °C and sections stored at -80 °C. For visualisation of bound antibody a FITC-conjugated rabbit anti-mouse Ig antibody was used as described below.

To assess *in vitro* binding, kidney sections of the PBS control mouse were used. For staining the slides were warmed at room-temperature for 30 min and fixed in ice-cold acetone for 5 min. After air-drying the sections were encircled with a liquid block pen and washed twice for 2 min in PBS. Sections were then blocked for 1 h in 20% rabbit serum/PBS. The primary antibodies at 3 µg/ml (mAb3, 8D1, HEL) or PBS only were added and slides incubated for 1 h at RT. After three 5 min washes in PBS a FITC-conjugated rabbit anti-mouse Ig polyclonal antibody (Jackson Immuno Research) was added at 1/500 and the slides incubated for 1 h at RT, in the dark. After three 5 min washes in PBS coverslips were mounted in anti-fade mounting medium (Flouromount, Sigma) and allowed to set overnight at 4 °C.

2.5 Assays of complement regulator function

2.5.1 Calculation of the extinction coefficient

The molar extinction coefficient is an estimate of how much light a protein absorbs at a given wavelength and can be used to accurately determine the concentration of a purified protein at 280 nm. This coefficient can be derived from the amino acid sequence because it depends on the number of aromatic residues present. The equation (see below) therefore takes into account the specific extinction coefficients of tyrosine, tryptophan and cystine (Gill and von Hippel, 1989). Cystine, two cysteine residues dimerised by disulphide bonds, has an extinction coefficient of 125. The formula below therefore assumes all disulphide bonds in the protein of interest are oxidised. The coefficients of tyrosine and tryptophan are 1490 and 5500, respectively. The molar extinction coefficient (E) of a given protein is the sum of the multiplication products of the number (X) of tyrosine, tryptophan or cystine residues with their respective coefficients:

$$E\ (protein) = \big(X\ (Tyr) \times E(Tyr)\big) + \ \big(X\ (Trp) \times E\ (Trp)\big) + \big(X(Cys) \times E\ (Cys)\big)$$

2.5.2 Factor I cofactor assay

The ability of the recombinant FH proteins to serve as cofactor for the FI-mediated cleavage of C3b was tested in a fluid-phase assay according to the protocol described by Pechtl *et al*. (Pechtl *et al*., 2011). Human serum-purified C3b, FI and were purchased from Complement Technology. For the negative control 3 µg of C3b was mixed with 0.045 µg FI in a total reaction volume of 20 µl. The recombinant proteins and for the positive control full length FH was added at concentrations ranging from 0.02-0.5 µM. Equimolar concentrations were determined using absorption coefficients based on confirmed or theoretical molecular weights (see Table 10). The reactions were incubated for 20 min at 37 °C, mixed with 2x reducing SDS-PAGE sample buffer and analysed by SDS-PAGE on 10% gels followed by Coomassie blue staining of the gels.

2.5.3 Solid phase decay acceleration assay

FH1-5 mediated accelerated decay of the AP C3 convertase was measured by surface Plasmon resonance as described in Schmidt *et al*, 2011 with some modifications (Schmidt *et al*, 2011). First C3b (Complement Technology) was immobilised on an S-carboxymethylated dextran chip (CM5, GE Healthcare) by amine coupling. For this 0.5 mg/ml C3b in 50 mM acetate buffer was coupled to the chip using a coupling kit (Amine Coupling Kit, GE Healthcare). C3b was flowed over the chip until about 130 resonance units were achieved. A reference chip was treated in

exactly the same way but in the absence of protein. All proteins were gel-filtrated into running buffer (HEPES-buffered saline, 1 mM $MgCl_2$, 0.05% surfactant P20). The convertase was formed on the C3 coated chip by flowing over a mixture of FB (0.5 µM) and FD (60 nM) for 120 s at 10 µl/min. The C3 convertase was allowed to decay for 210 s before injection of FH1-5 or the control proteins FH1-4, FH1-4 R83S and FH19-20 (produced by E. Wong, Newcastle University) at 0.5 µM and 0.17 µM for 60 s. FH19-20 was used at 1 µM. After each injection the chip surface was regenerated for 45 s with 1 µM full length FH (Complement Technology) followed by 1 M NaCl for 45 s. The extinction coefficient used to calculate accurate molar concentrations is listed in Table 10.

Table 10: Recombinant FH proteins and extinction coefficients

Protein name	Extinction coefficient
FH1-5	59580
FH1-4	47870
FH19-20	27430
FH1-4 R83S	47870
Full length FH	246800
C3b	176700

2.5.4 Fluid-phase C3 convertase assay

The effect of the recombinant FH proteins on the activity of the AP C3 convertase was tested in a fluid-phase assay as described by Wiesmann *et al* (Wiesmann *et al*., 2006). For this 0.4 µM C3 (purified in house) and 0.02–0.5 µM of the recombinant FH proteins or full length FH (Complement Technology) for the positive control were mixed in PBS in a volume of 20 µl. For the negative control no decay accelerator was added and the volume replaced by PBS only. The mixes were incubated for 15 min at 37 °C before addition of FB and FD (both Complement Technology) to final concentrations of 0.4 µM and 0.04 µM, respectively, in a final reaction volume of 30 µl. The FB/FD mix was supplemented with Mg^{2+}/EGTA to give a final concentration of 33 mM. The incubation at 37 °C was continued for a further 30 min and stopped by addition of 30 µl 2x reducing SDS-PAGE buffer. To analyse the C3 cleavage pattern the samples were subjected to SDS-PAGE on 5% polyacrylamide gels followed by immunoblotting. C3 fragments were detected with a rabbit polyclonal anti-human C3 antiserum and a HRP-conjugated goat anti-rabbit Ig polyclonal antiserum.

2.5.5 Haemolytic assay

The haemolytic assay used is a basic assay to determine the ability of the recombinant FH proteins to protect sheep or rabbit erythrocytes from complement-mediated lysis (Harris, 2000). For each reaction (total volume 200 µl), 60 µl (0, 20, 40, 80 and 100 µl for standard curve) fresh normal human serum (diluted to 1/25 in Mg-EGTA) was mixed with 20 µl BSA,

FH1-5 or FH1-5/18-20 (in Mg-EGTA) to final concentrations of 0-100 nM. 20 µl of Mg-EGTA was added to each reaction or to final volume of 100 µl for the standard curve. Rabbit erythrocytes in Mg-EGTA (100 µl, $1*10^6$) were then added and the 96-well plate incubated in a 37 °C water bath for 30 min. Controls were included for spontaneous lysis (100 µl Mg-EGTA, 100 µl rabbit erythrocytes) and 100 % lysis (100 µl H_2O, 100 µl rabbit erythrocytes). After incubation, 100 µl ice cold saline was added to all reactions (H_2O to 100% lysis control) and unlysed cells were pelleted by centrifugation of the plates at 400 x g. 100 µl of the supernatants were then carefully aspirated with a pipette and transferred to a 96-well microtitre plate and the absorbance measured at 414 nm. Percentage lysis was calculated as shown:

$$\%\ Lysis = \frac{A_{414}(sample) - A_{414}(spontaneous\ lysis\ control)}{A_{414}(100\%\ lysis\ control) - A_{414}(spontaneous\ lysis\ control}$$

<u>100 mM EGTA:</u>
38.04 g EGTA in 0.5 L water,
1 M NaOH added slowly until EGTA dissolved and pH was 7.5,
H_2O added to a final volume of 1L

<u>5x VB (veronal buffered saline):</u>

Either from Oxoid complement fixation tablets or:

85 g NaCl and 3.75 g barbitone in 1 L H_2O
5.75 g barbituric acid in 600 ml hot water
Two buffers were mixed and the volume brought to 2 L with H_2O
pH 7.4-7.6

<u>10% (w/v) gelatine:</u>
Dissolve 10 g gelatine in 100 ml hot water.

<u>GBV (isotonic veronal buffered saline with gelatine):</u>
10 ml 10% (w/v) gelatine was dissolved in boiling water bath
Added to 200ml 5x VB
Volume brought to 1 litre with H_2O

<u>Mg-EGTA (0.5 L):</u>
50 ml 100 mM EGTA
35 ml 100 mM $MgCl_2$
104 ml GVB
311 ml 5% (w/v) D glucose
pH 7.4-7.6

3 Generation and functional testing of FH1-5 and FH1-5/18-20

3.1 Introduction

Mutations or autoantibodies affecting the N-terminal regulatory domain or the cell binding domain at the C-terminal are implicated in the development of MPGN type II and aHUS, respectively (de Cordoba and de Jorge, 2008, Jozsi *et al*., 2007, Goodship *et al*., 2012, Benz and Amann, 2009b). The therapies currently available to treat these conditions are limited. There are few therapies in use that specifically address the dysregulation of complement. Evaluation of murine models of aHUS and MPGN type II has allowed invaluable insights into the pathogenesis of the conditions and provided rationale for the development of therapeutics. Mice deficient in *Cfh* develop renal disease that resembles human MPGN type II (Pickering *et al*., 2002). Serum C3 is depleted and the GBM stains strongly for C3. Deposition of C3 is an early pathological feature and deficiency of *Cfi* protects $Cfh^{-/-}$ mice from developing disease (Rose *et al*., 2008). These findings confirmed that the pathophysiology of MPGN type II depends primarily on increased fluid-phase metabolism of C3. This is in agreement with the findings in patients – C3 nephritic factor, gain of function mutations in *FB*/*C3* and *CFH* mutations that cause dysfunction of regulatory domains or deficiency of *CFH* all affect fluid-phase regulation and therefore C3 metabolism (Zhang *et al*., 2012). Therapy of patients with a definite defect in fluid-phase regulation, e.g. C3 nephritic factor, should therefore aim to restore fluid-phase regulation of the AP. Mice that lack the N-terminal SCRs of *Cfh* ($Cfh^{-/-}$*.Δ16-20*) develop renal disease that resembles human aHUS (Pickering *et al*., 2007). $Cfh^{-/-}$.Δ16-20 show higher serum C3 levels than $Cfh^{-/-}$ mice and mesangial C3 deposition. The terminal pathway appears to play a more distinct role in the pathogenesis of aHUS than MPGN type II since C5 deficiency protects $Cfh^{-/-}$.Δ16-20 mice from developing disease (de Jorge *et al*., 2011). Absence of a functional cell binding domain at the C-terminal is central to pathogenesis of aHUS. Therapy of aHUS should therefore aim to restore protection of the renal endothelium from C3 deposition and / or MAC formation.

To test the implications of these animal studies the relevant reagents have to be generated. Previous studies have demonstrated that the SCRs 1-4 of FH are sufficient for fluid-phase regulation of C3 metabolism (Schmidt *et al*., 2008a). The N-terminal of FH bears both the C3b binding site for decay acceleration function and *CFI* cofactor activity (Figure 13). Therefore a recombinant protein comprised of SCRs 1-5 of FH, FH1-5, was designed as fluid-phase regulator of the AP. A second protein, FH1-5/18-20, contains the regulatory SCRs 1-5 and SCRs 18-20, that interact with C3b and GAGs on cell surfaces. SCRs 5 and 18 serve as extension of the hexa histidine linker. The histidine tag allows purification of the construct from cell culture supernatants. Inclusion of SCR5 also allows detection with the monoclonal antibody OX24. Current models of FH in complex with cell surface-bound C3b suggest a “folded-back”

conformation (Aslam and Perkins, 2001, Okemefuna *et al*., 2008). N-terminal SCRs (1-4) bind C3b and C-terminal SCRs (19-20) of FH engage both C3b and GAGs (Figure 13). The remaining SCRs (5-18) form a tight loop that allows SCRs 6-8 to interact with the cell surface (Figure 13) (Schmidt *et al*., 2010). Fusion of SCRs 18-20 directly downstream of SCR 5, linked by a hexa histidine-tag, is supported by the structural data available.

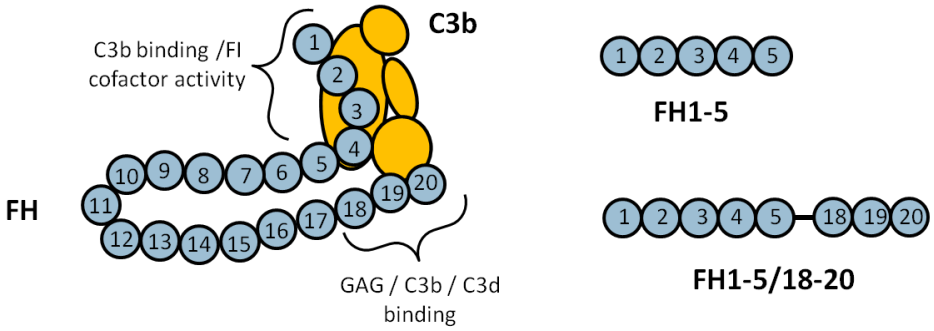

Figure 13: Schematic of FH bound to C3b and proposed recombinant FH proteins
As shown on the left side, native full length FH binds to surface bound C3b via the N-terminal SCRs 1-4 and the C-terminal SCRs 19-20. At the same time the C-terminal and SCR7 can bind to heparin displayed on host cell surfaces. FH1-5 incorporates the first 5 SCRs, bearing cofactor and decay acceleration function, of the full length protein. In FH1-5/18-20 the C-terminal cell binding domain is fused to SCR 5 via a hexa histidine tag.

This chapter has two key aims:

1. Generate two recombinant FH proteins, FH1-5 and FH1-5/18-20.
2. Assess the *in vitro* AP regulatory function of both proteins.

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3.2 Results

3.2.1 Generation of FH1-5 and FH1-5_Ile62 expression vectors

The DNA construct for expression of FH1-5 was generated by K.J. Marchbank. For this the sequence encoding SCRs 1-5, including the native signal peptide, were amplified by PCR from the full length sequence of FH (in pBluescript) and inserted into the mammalian expression vector pDEF. The resulting construct was named S21. A disease-protective polymorphism of FH has been described that changes valine at position 62 to isoleucine (Hageman *et al*., 2006, Tortajada *et al*., 2009). Functionally the Ile62 variant exerts higher affinity for C3b than the Val62 variant and enhanced cofactor activity. Site-directed mutagenesis was used to introduce this amino acid change (Figure 14). In the subsequent progression of the project this construct was not utilised any further due to time constraints.

3.2.2 Generation of the FH1-5/18-20 expression vector

The S21 construct (FH1-5) was used to generate the FH1-5/18-20 expression vector (cloning strategy Figure 15). The generation of the F3 plasmid used for cloning of FH1-5/18-20 is dealt with in chapter 5. SCRs 18-20 were amplified by PCR with primers introducing a *KpnI* restriction site at the 5' end and a stop codon and *NheI* restriction site at the 3' end. A single band of the correct size (575 bp) was obtained (Figure 16.A). The PCR product was purified and digested with *KpnI* and *NheI* restriction enzymes. The expression vector pDEF (F3, Chapter 5), containing SCRs 1-5 of FH fused to an antibody fragment via a hexa histidine tag, was also digested with *KpnI* and *NheI*. This allowed replacement of the antibody fragment with SCRs 18-20 downstream of SCRs 1-5 and the histidine-tag. The digested PCR product and vector were subjected to gel electrophoresis, followed by gel extraction. Vector and DNA insert were ligated using a T4 ligase. Chemically competent cells were transformed with the ligation reaction and grown on selective LB-agar plates (ampicillin). The transformants were screened for presence of the correct insert by colony PCR with primers flanking the multiple cloning site (Figure 16.B). Two of the 11 colonies screened revealed a band of the correct size (~1800 bp). These two colonies were grown up and the DNA isolated. To confirm the correct sequence of the two clones (designated P5 and P6), DNA was sequenced (Eurofins DNA Operon, Germany) using the same flanking primers as used for the colony PCR. At this point the S21 plasmid DNA was also checked by DNA sequencing. The forward and reverse sequencing reactions returned the correct, in-frame sequence for P5, P6 and S21 (see Appendix A). P5 and S21 were subsequently used for transfection of CHO cells.

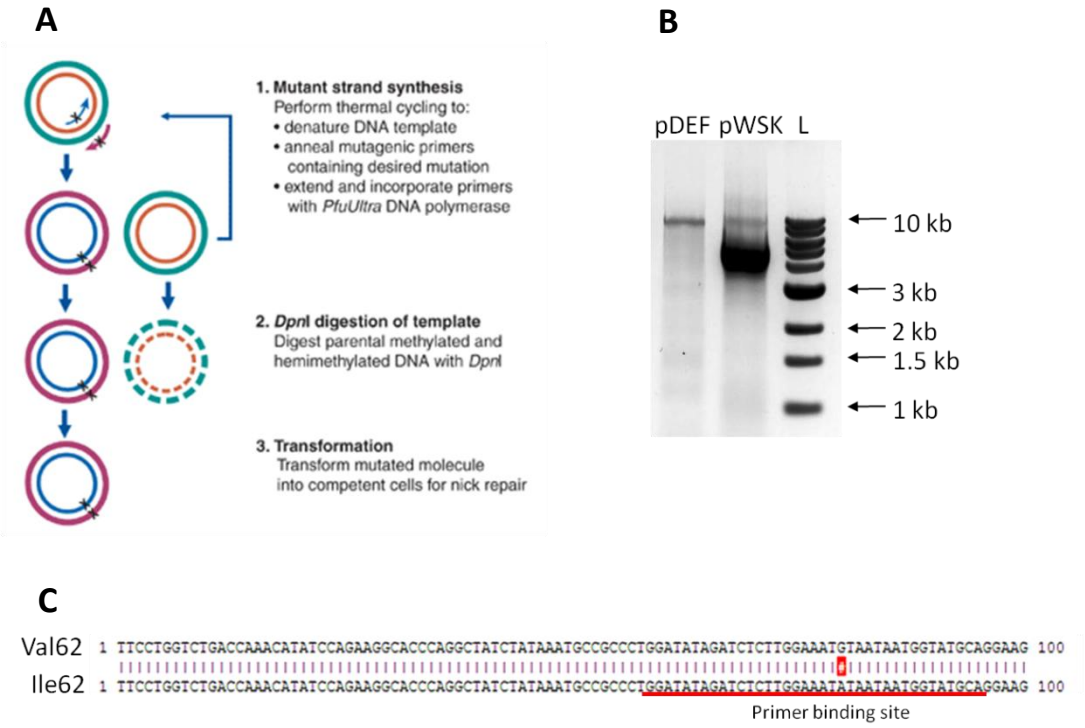


Figure 14: Generation of the FH1-5_Ile62 DNA construct

(A) Introduction of a point-mutation by site-directed mutagenesis requires the design of a pair of long mutagenesis primers which bind to the template DNA and contain a single base pair change in the centre of the primer sequence. In the mutagenesis PCR reaction, the primers hybridise to the template and the entire plasmid is synthesised. Template DNA is methylated and can be degraded with *DpnI*. *E. coli* can then be transformed with the mutated plasmid. **(B)** Lane 1 shows the product of the pDEF S21 mutagenesis PCR and lane 2 the reaction using the smaller control plasmid pWSK. **(C)** The DNA of three clones transformed with the pDEF S21 mutagenesis PCR mix was sequenced and confirmed in two clones. To exemplify the sequence alignment of one clone is shown.

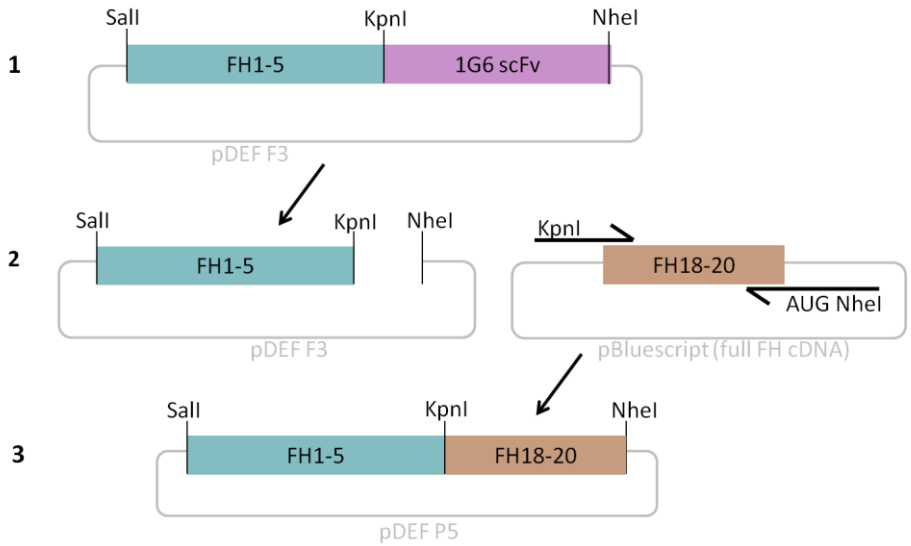


Figure 15: Cloning scheme pDEF P5 (FH1-5/18-20)

To generate FH1-5/18-20 pDEF F3 was digested with *KpnI* and *NheI* to remove the 1G6 scFv fragment. FH SCRs 18-20 were amplified with primers introducing a *KpnI* site at the 5' end and a stop codon and *NheI* site at the 3' end. The PCR product was digested with *KpnI* and *NheI* and ligated with the *KpnI*/*NheI* digested pDEF F3 to form construct P (correct DNA sequence of clone P5 was confirmed later).

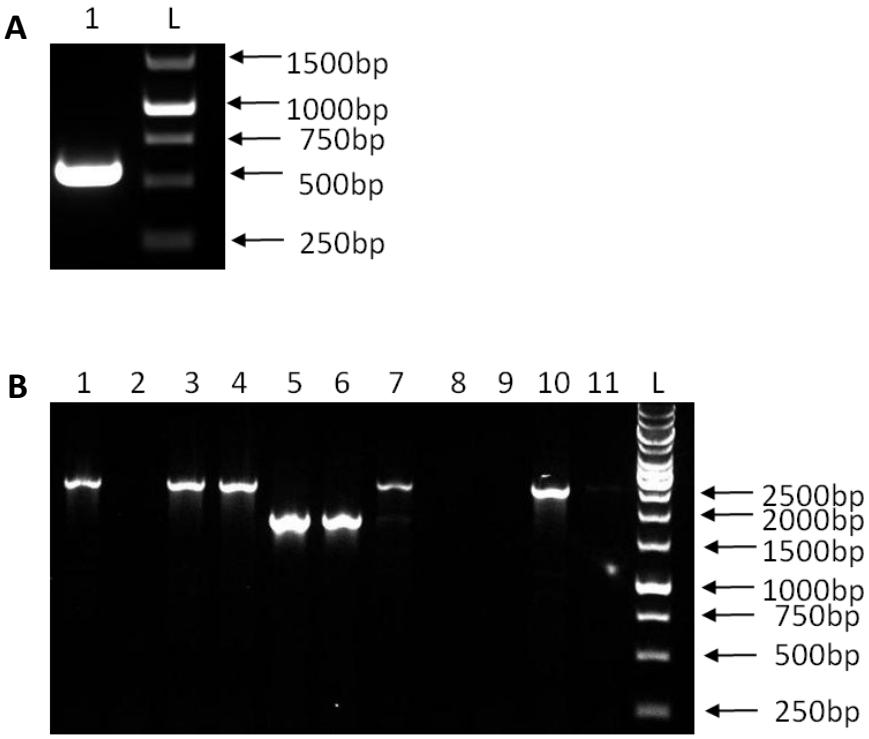


Figure 16: Amplification of FH SCRs 18-20 and colony screen of FH1-5/18-20

(A) SCRs 18-20 were amplified by PCR (lane 1). The forward primer (F327fwd) was designed to introduce a *KpnI* restriction site and the reverse primer (F327R) a stop codon and *NheI* restriction site. **(B)** The *KpnI*/*NheI* digested PCR fragment (SCRs 18-20) was ligated with the gel-purified *KpnI*/*NheI* digested pDEF F3 containing FH SCRs 1-5 and the histidine-tag. Colonies that resulted from transformation of competent E. coli were analysed by colony PCR using forward and reverse primers that flank the pDEF multiple cloning site (pDEF NF and pDEF NR). Only lanes clones 5 and 6 produced a product of the correct size, the other bands are non-specific. L = ladder.

3.2.3 Expression of FH1-5 and FH1-5/18-20 in CHO cells

For production of FH1-5 and FH1-5/18-20, CHO cells were transfected with plasmid DNA. Cells were subjected to selective pressure with hygromycin and individual clones isolated using cloning discs and grown in 12-well plates. Expression of the proteins was tested by immunoblotting and ELISA (Figures 17.A and 17.B). Immunoblotting of cell culture supernatant of confluent cells (transfectant pool) revealed bands of the correct size for both proteins (Figure 17.A). FH1-5 (S21) and FH1-5/18-20 (P5) have a predicted molecular weight of 36kDa and 56 kDa, respectively. SDS-PAGE was performed under reducing conditions. SCRs 1-5 contain 10 disulphide bonds and SCRs 18-20 a further 6. Intact disulphide bonds cause the proteins to have a tighter conformation. This allows faster migration through the gel. In the ELISA (Figure 17.B) clones S21.5 and P5.1 showed high expression levels and culture of these clones was scaled up to allow collection of supernatant for histidine-tag purification.

3.2.4 Histidine-tag purification of FH1-5 and FH1-5/18-20

A hexa histidine-tag was introduced to both proteins to allow purification by immobilised metal ion chromatography (IMAC). In FH1-5 the histidine-tag is located at the C-terminal, in FH1-5/18-20 it serves as a linker between SCRs 1-5 and SCRs 18-20 and is thus an internal histidine-tag. Location of the histidine tag can affect the affinity of the tag for nickel and the two proteins may therefore require different binding and elution conditions. In the absence of imidazole in the binding and wash buffers significant non-specific binding of protein from the CHO cell culture supernatant occurs. This has been established in previous work in the laboratory and therefore binding conditions (40 mM imidazole in binding buffer), used routinely for purification of recombinant proteins from CHO cell culture supernatant, were used. The cell culture supernatant was diluted to 50% with binding buffer, adjusting salt and imidazole concentration to that of the binding and wash buffer (0.5 M NaCl, 40 mM imidazole). Under these conditions both proteins bound well to the nickel-charged column but eluted at different imidazole concentrations with little contaminating protein (Figure 18 and Figure 19). FH1-5 required approximately 100% of the gradient (equivalent to 0.5 M imidazole). FH1-5/18-20 eluted at approximately 80% of the gradient (equivalent to 0.4 M imidazole) and showed more contaminating protein than FH1-5. Contaminating protein derived from the CHO cells or FCS was removed by desalting of the collected fractions and reapplication of the pooled fraction to a nickel charged column (data not shown).

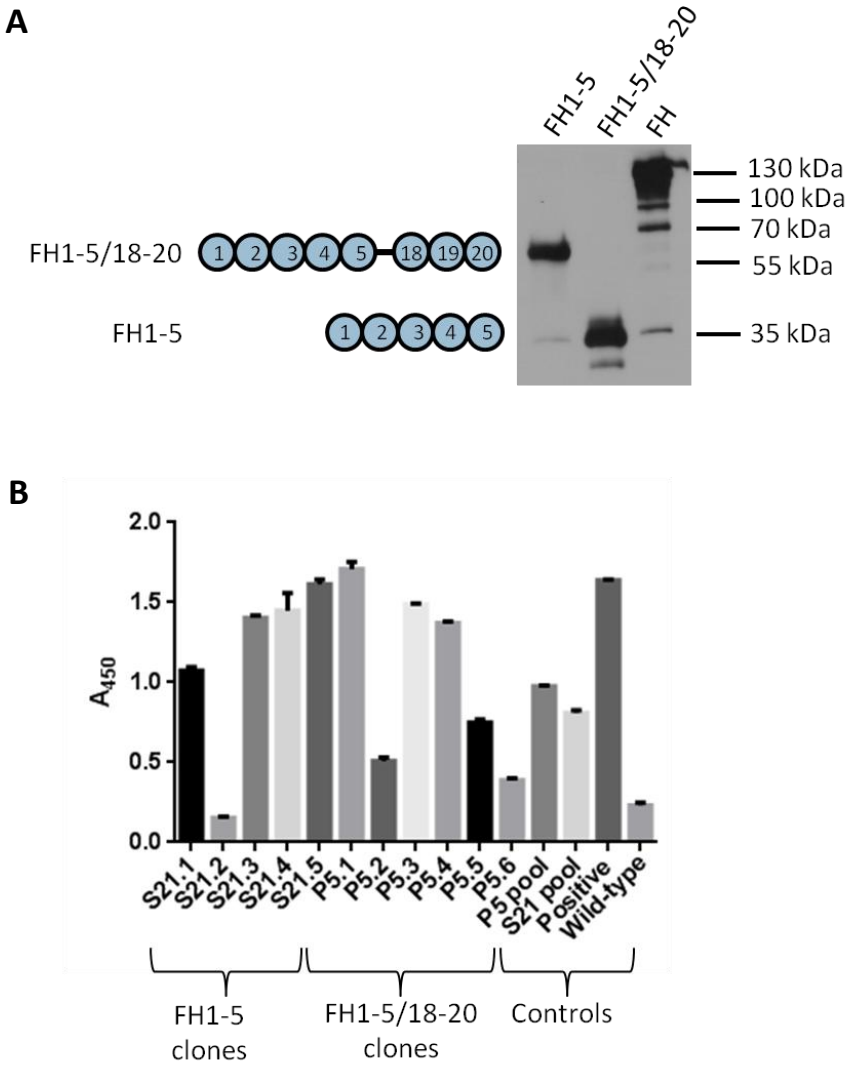


Figure 17: Expression screen of CHO cell clones transfected with FH1-5 and FH1-5/18-20

(A) Supernatant of the P5 and S21 transfectant pools were analysed by non-reducing SDS-PAGE followed by immunoblotting (anti-human FH and HRPO-conjugated donkey anti-goat IgG). **(B)** After selection of individual clones, expression levels were checked using supernatants of confluent flasks. Five clones transfected with S21 (FH1-5) and six clones transfected with P5 (FH1-5/18-20) were analysed by sandwich ELISA. Proteins were captured with OX24 and detected with a polyclonal anti-human FH polyserum and HRPO-conjugated donkey anti-goat Ig secondary. Wild-type CHO cell supernatant was included as negative control and full length FH as positive control. Supernatants from the S21 and P5 transfectant pools were included to allow comparison with selected clones.

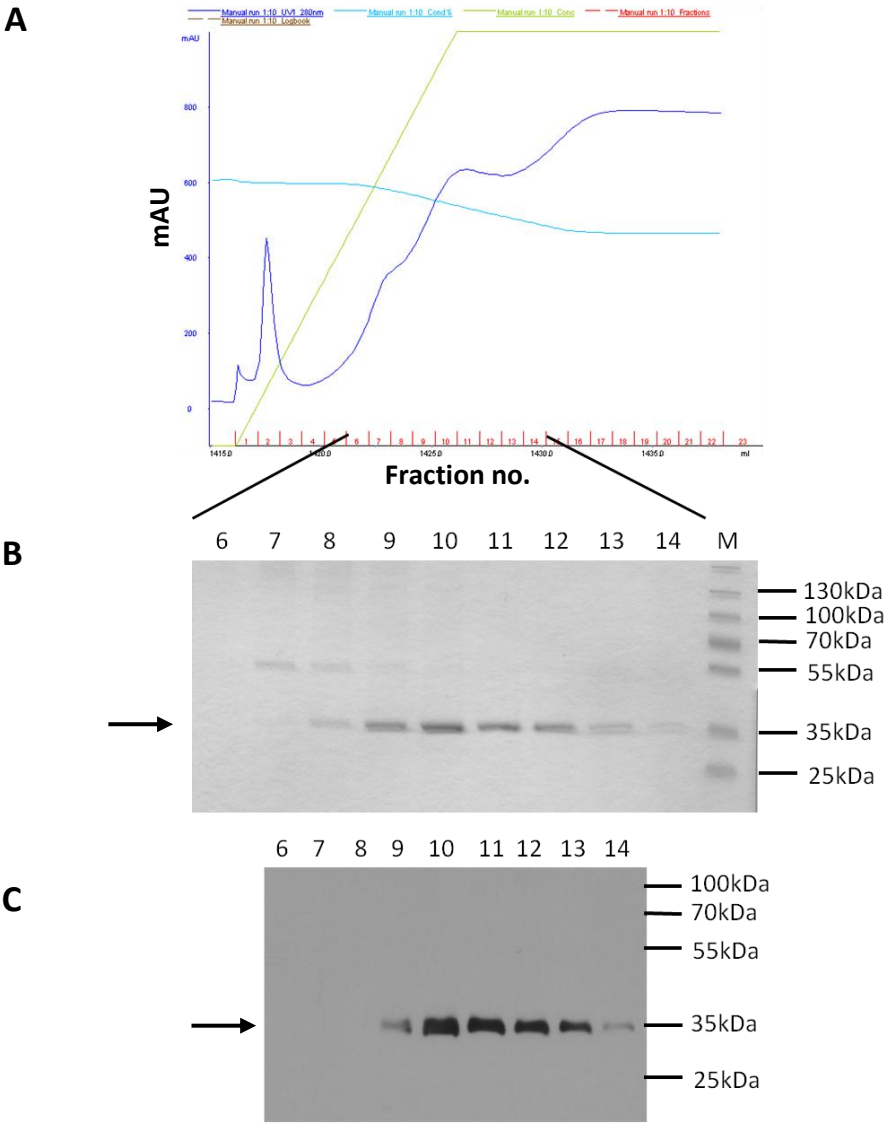


Figure 18: Histidine-tag purification of S21.5 (FH1-5) from CHO cell culture supernatant

(A) FH1-5 was purified from CHO cell culture supernatant by application to and elution from a nickel charged column. The elution gradient (green line) was increased from 40 mM to 0.5 M imidazole over the first ten fractions. Two absorbance peaks occurred within the gradient, between fractions 6 and 14. This chromatography trace is representative of several purifications. **(B)** These fractions were analysed by reducing SDS-PAGE followed by Coomassie blue staining. Under reducing conditions FH1-5 migrates to its predicted molecular weight of 36 kDa. **(C)** The identity of the 36 kDa band was confirmed by immunoblotting (anti-human FH and HRPO-conjugated donkey anti-goat IgG).

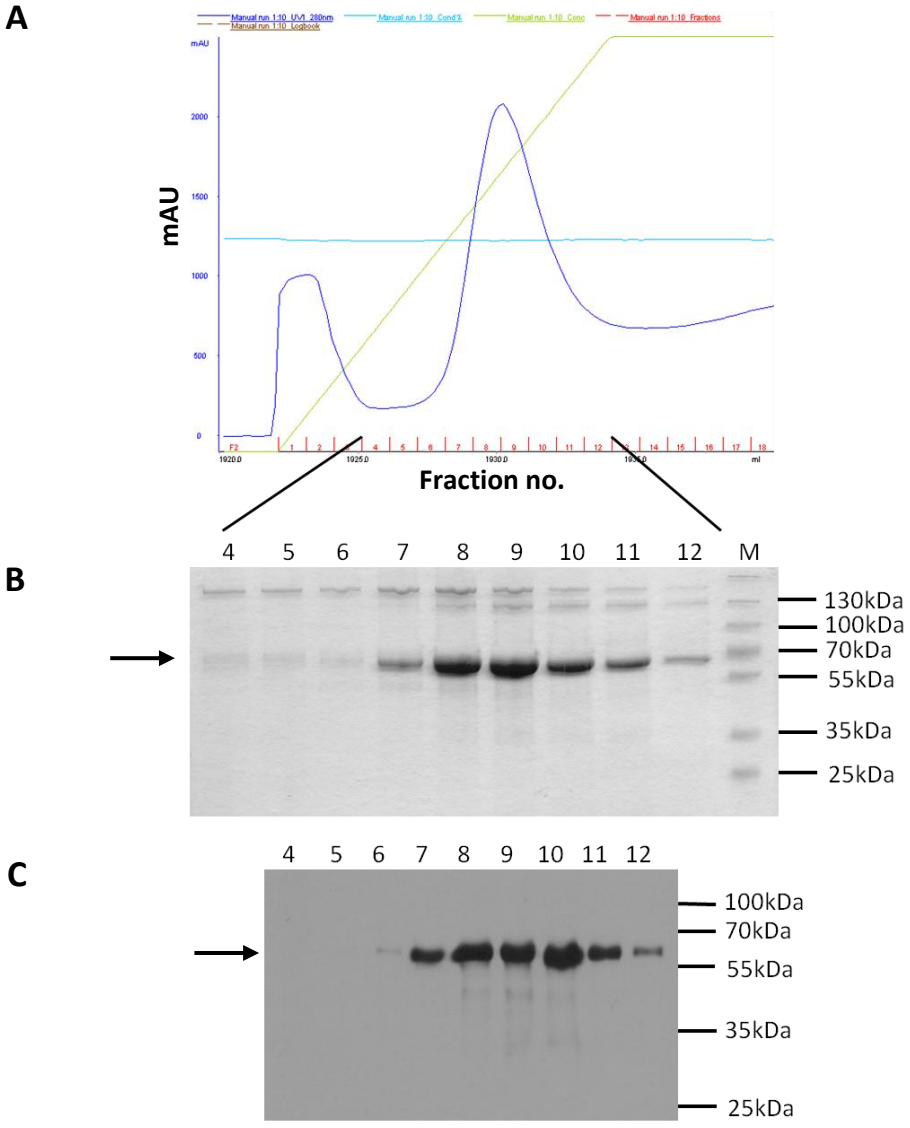


Figure 19: Histidine-tag purification of FH1-5/18-20 from cell culture supernatant

(A) FH1-5/18-20 was purified from CHO cell culture supernatant by application to and elution from a nickel charged column. The elution gradient (green line) was increased from 40 mM to 0.5 M imidazole over the first 12 fractions. A single absorbance peaks occurred within the gradient, between fractions 7 and 11. This purification is representative of several purifications carried out. **(B)** These, and fractions before and after, were analysed by reducing SDS-PAGE followed by Coomassie blue staining. Under reducing conditions FH1-5/18-20 migrates to its predicted molecular weight of ~57 kDa. **(C)** The identity of the 57 kDa band was confirmed by immunoblotting (anti-human FH and HRPO-conjugated donkey anti-goat Ig).

3.2.5 Binding to C3b and heparin

As initial functional screen the ligand binding of the recombinant proteins was established by sandwich-ELISA (Figure 20). Both proteins are expected to bind to C3b. C3b was coated on microtitre plates and binding of the purified proteins detected with a polyclonal anti-human FH polyclonal antiserum (Figure 20.A). Full length FH was included as positive control. All three proteins bound to C3b, with decreasing avidity in the order full length FH, FH1-5/18-20 and FH1-5. To test function of the cell binding domain of FH1-5/18-20, heparin was used as model ligand. For the assay heparin was applied to polylysine-L coated microtitre plates. The purified proteins were added and binding detected as for the C3b binding ELISA (Figure 20.B). FH1-5 has no cell binding domain and was therefore included as negative control.

3.2.6 Purification of C3 from human serum

Human C3 was required for the fluid-phase C3 convertase assay. It was purified from serum of a healthy donor by fractional precipitation (see methods) and two chromatography steps (Figure 21). The C3 containing precipitation fraction was first applied to an anion-exchange column and C3 was eluted with a NaCl gradient (Figure 21.A). The collected elution fractions were analysed by SDS-PAGE analysis followed by Coomassie blue staining or immunoblotting to identify C3 containing fractions (Figure 21.B). Fractions 13 and 14 contained the most C3 as indicated by a high molecular weight band under non-reducing conditions (180 kDa) and two bands for the α-chain (~110 kDa) and the β-chain (~68 kDa) under reducing conditions (Figure 21.B). The positively identified fractions were subjected to gel filtration to separate intact C3 from cleavage products present in the same fraction (Figure 21.C). C3 was then stored at -70 °C until use in the assay.

3.2.7 Fluid-phase FI cofactor assay

FI requires a cofactor like FH to mediate cleavage of the C3b α-chain. The ability of FH1-5 and FH1-5/18-20 to serve as cofactor for FI was tested in a standard fluid-phase cofactor assay (Pechtl *et al*., 2011). C3b, FI and a cofactor (full length FH, FH1-5 or FH1-5/18-20) were incubated and cleavage of the C3 α-chain analysed by reducing SDS-PAGE of samples. Proteins were visualised by Coomassie-blue staining of the gels. Both proteins exert cofactor activity comparable to that of full-length FH (Figure 22.A).

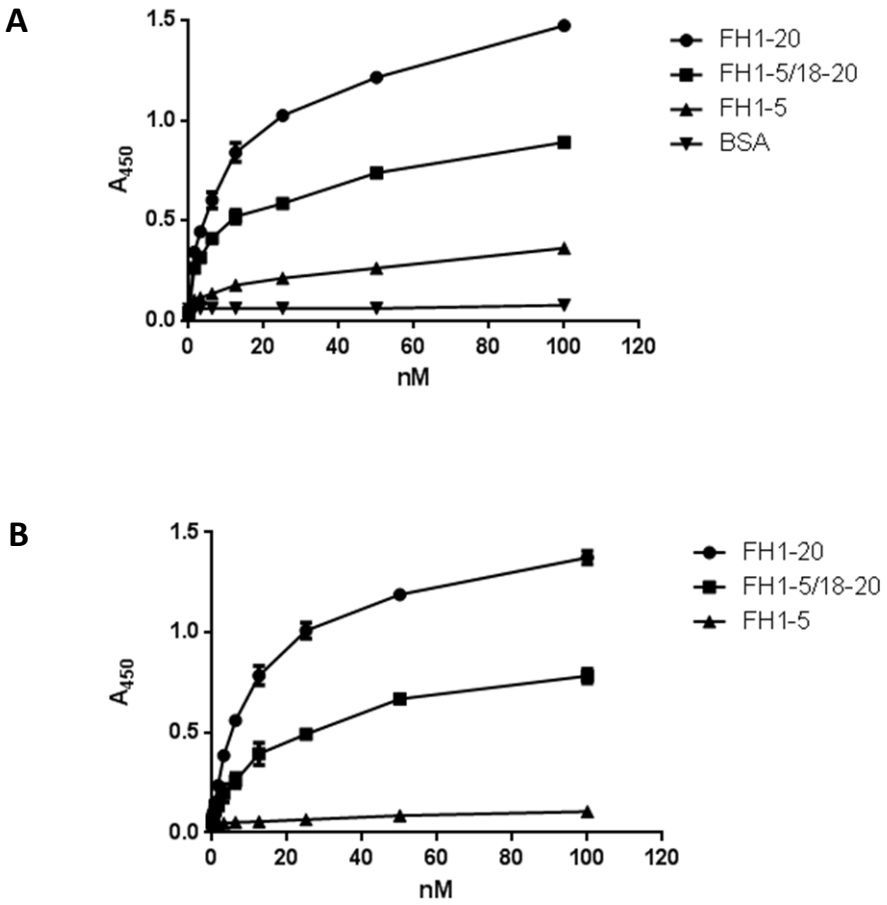


Figure 20: Binding of FH1-5 and FH1-5/18-20 to C3b and heparin

(A) Binding of FH1-5 and FH1-5/18-20 (0-100 nM) to C3b coated on microtitre plates was detected with a polyclonal anti-human FH antiserum and a HRPO-conjugated donkey anti-goat Ig. Full length FH was included as positive control and BSA as negative control. Samples were applied in triplicates. Shown is one representative experiment of three. 3. **(B)** GAG binding of FH1-5/18-20 was tested by detecting binding to heparin coated on microtitre plates. Full length FH was included as positive control, FH1-5 as negative control. Detection was carried out as for the C3b binding. Samples were applied in triplicates. Shown is one representative experiment of three.

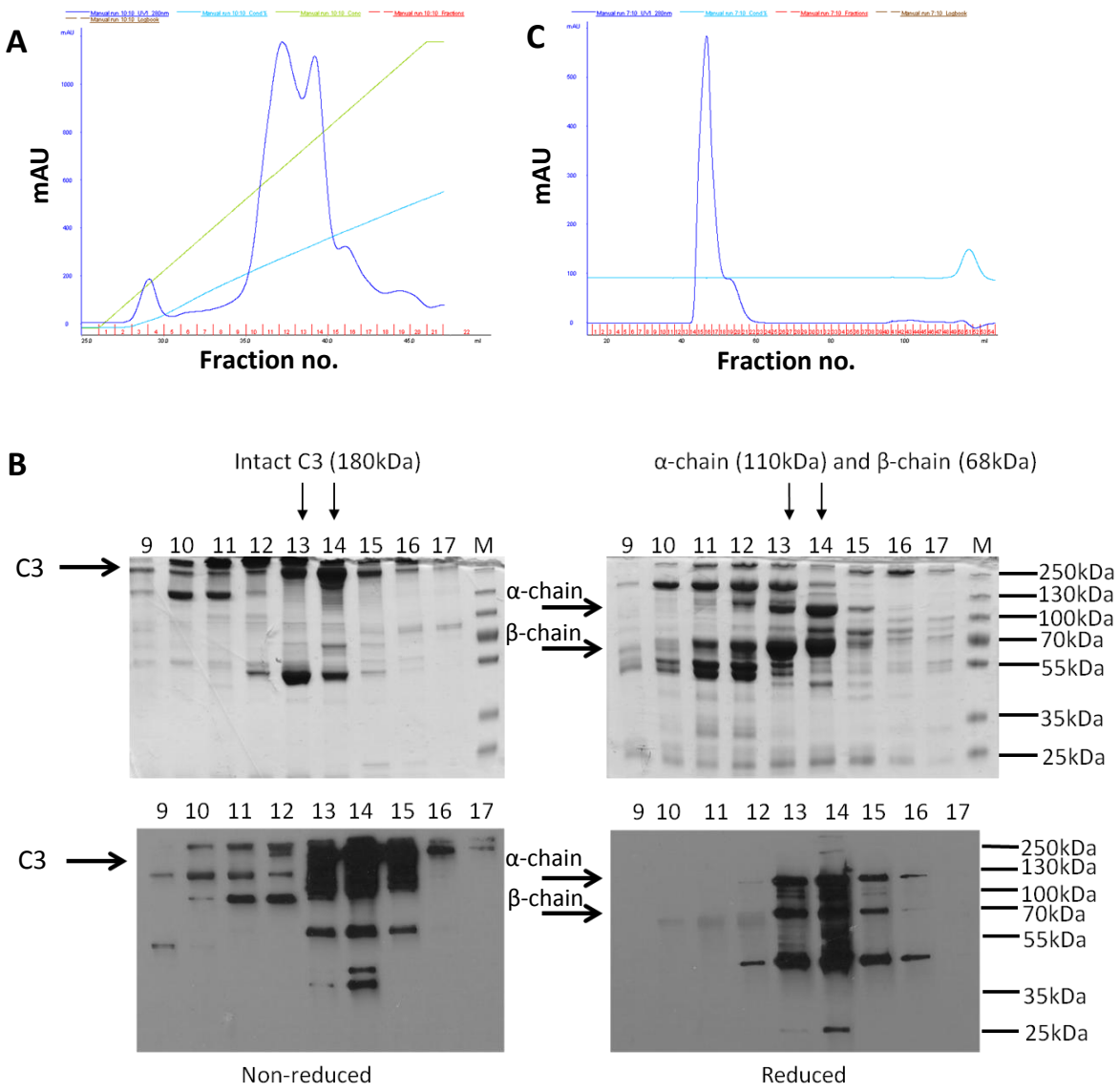


Figure 21: Purification of C3 from normal human serum

(A) The C3 containing fraction, obtained by precipitation of human serum, was applied to an anion-exchanger column and eluted with a linear salt gradient (% gradient = green line; % conductivity = light blue line). Several protein containing peaks were recorded. **(B)** The protein containing fractions 9-17 were analysed by reducing and non-reducing SDS-PAGE (upper left and right panel). The majority of C3 (encircled in white) was present in fractions 13 and 14. **(C)** Immunoblotting of the fractions with a rabbit anti-human C3 polyserum was carried out to confirm the identity of the bands (lower left and right panel). A number of proteolytic fragment of C3 were detected.

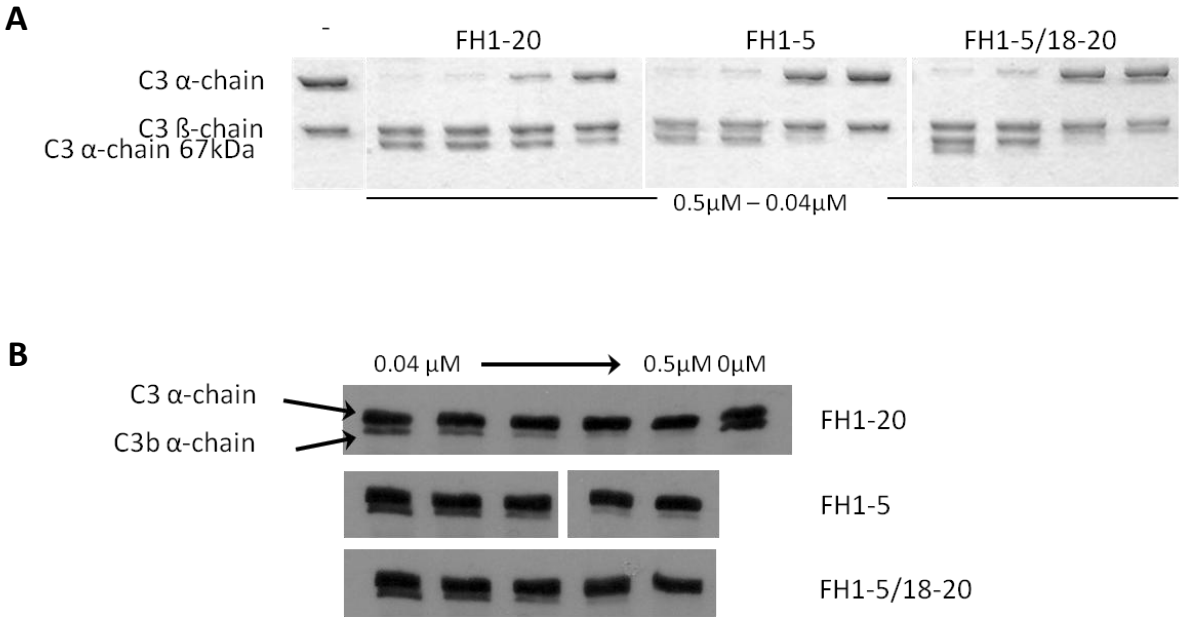


Figure 22: Factor I cofactor and anti-C3 convertase activity of FH1-5 and FH1-5/18-20

(A) Cofactor of FH1-5 and FH1-5/18-20 was demonstrated in a fluid-phase assay. In the absence of a cofactor (-) the C3 α-chain remains intact. Presence of a cofactor results in cleavage of the α-chain. Full length FH (positive control), FH1-5 and FH1-5/18-20 were included at concentrations ranging from 0.5-0.04 µM. Shown is one of four representative experiments. n = 4. **(B)** C3 convertase activity was also assessed in a fluid-phase assay. In the absence of a regulator (0 µM) spontaneous formation of the C3 convertase allows generation of C3b. The C3b α-chain is ~8 Da smaller than the C3 α-chain. In the presence of a regulator, formation of C3 convertases is inhibited and formation of C3b prevented, as indicated by decreasing amount of C3b α-chain with increasing concentration of regulator. Full length FH (positive control), FH1-5 and FH1-5/18-20 were included at concentrations ranging from 0.5-0.04 µM. n = 2.

3.2.8 Fluid-phase C3 convertase assay

FH prevents formation and accelerates decay of the AP convertase C3bBb by binding to C3b and displacing Bb. This function of the recombinant FH proteins were was assessed in a fluid-phase assay (Figure 22.B). The assay used relies on the spontaneous formation of C3(H_2O) which can, in the absence of a regulator, participate in the formation of C3 convertases and consequently cleave C3 to C3a and C3b. For the fluid-phase assay C3 was first mixed with full length FH, FH1-5 or FH1-5/18-20. FB and FD were then added to allow formation of the AP convertase from spontaneously hydrolysed C3. The samples were subjected to SDS-PAGE and immunoblotting to detect the relative amounts of C3 α-chain (~110 kDa) and C3 α'-chain (~102 kDa, C3b α-chain). All three proteins prevented formation of the AP C3 convertase in a concentration-dependent manner (Figure 22.B). The negative control shows mainly C3 α'-chain and only little C3 α-chain, indicating unhindered formation of the C3 convertase and cleavage of C3 into C3b and C3a in the absence of a regulator.

3.2.9 Solid-phase decay acceleration assay (SPR)

The ability of FH1-5 to accelerate decay of existing AP C3 convertases was assessed by SPR (Figure 23). When the opportunity for this experiment in collaboration with Dr D. Kavanagh (Newcastle University) arose, FH1-5/18-20 was not available in sufficient quantity to be included. The C3 convertases were formed by flowing FB and FD over C3b immobilised on a chip surface. Two negative control proteins were included – FH19-20 which lacks any decay acceleration function and a R83S mutant of FH1-4 (FH1-4 R83S) which was expected to show impaired decay acceleration function (negative control proteins prepared by Edwin Wong, Newcastle upon Tyne). Wild-type FH1-4, known to exert good decay acceleration function, was included as positive control (positive control generated by Edwin Wong, Newcastle upon Tyne). For each protein the chip was regenerated and new C3 convertase formed. The convertases were allowed to decay naturally before injection of the proteins (Figure 23). FH19-20 did not accelerate decay of the convertases while FH1-4 R83S showed a limited deviation from the dissociation profile for natural decay. Both FH1-5 and the positive control FH1-4 resulted in marked acceleration of convertase decay with FH1-5 showing better decay acceleration than FH1-4 as indicated by lower response units.

3.2.10 Haemolytic assay

The ability of FH1-5 and FH1-5/18-20 to protect erythrocytes from AP-mediated damage was assessed in haemolytic assays. Rabbit erythrocytes are particularly susceptible to AP-mediated lysis by human serum and sheep erythrocytes are less susceptible (Aminoff *et al*., 1976, Fearon and Austen, 1977). The reason for this difference is that human factor H has a higher affinity for C3b deposited on sheep erythrocytes than for C3b deposited on rabbit erythrocytes

because sheep erythrocytes display more GAGs on the surface. For the functional assessment of FH-5 and FH1-5/18-20 rabbit erythrocytes were hence used to assess fluid-phase regulation and sheep erythrocytes to test the cell-targeting domain of FH1-5/18-20. The cells were exposed to 1% human serum, as determined by a serum titration (Figure 24.A) in the presence of equimolar concentrations of full length FH (FH1-20), FH1-5, FH1-5/18-20 or BSA. BSA was included as negative control for protein buffering effect that may protect cells from haemolysis. As shown in Figure 24.B, addition of FH1-5/18-20 at 12.5 nM reduced lysis to ~60%, FH1-5 and FH1-20 reduce lysis to ~90% at this concentration. However this is similar to the BSA control. At 25 nM, FH1-5 and FH1-5/18-20 reduce lysis to less than 40% and FH1-20 reduced lysis ~60%. At higher concentrations all three proteins provide a comparable degree of fluid-phase protection. To test the cell surface-targeted FH1-5/18-20 sheep erythrocytes were treated exactly the same as the rabbit erythrocytes (Figure 24.C). Addition of 12.5 nM FH1-5/18-20 and FH1-20 reduced lysis to ~20%, while FH1-5 reduced lysis to ~70% which is comparable to the BSA control. At 25 nM FH1-5/18-20 and FH1-20 reduced lysis to ~18%, while FH1-5 reduced lysis to ~30%.

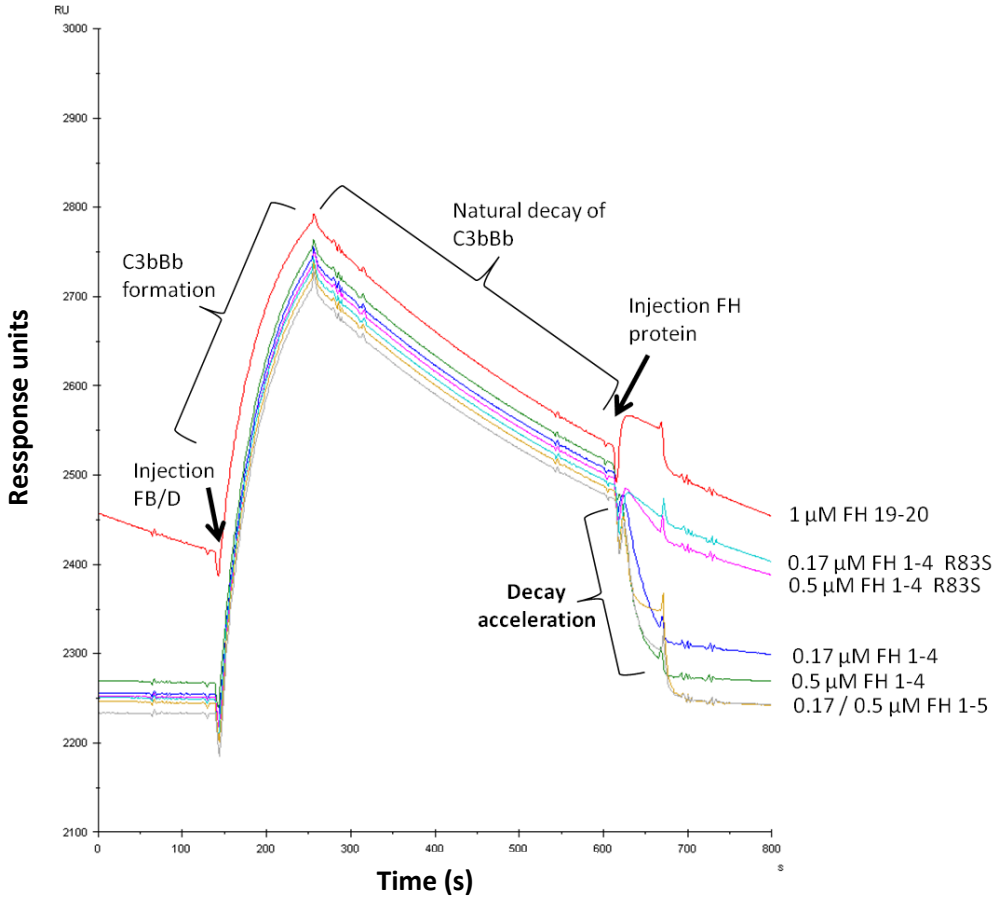


Figure 23: Decay acceleration activity of FH1-5 assessed by SPR

Decay acceleration activity of FH1-5 was assessed by surface Plasmon resonance. Alternative pathway C3 convertases were generated by flowing FD and FB across immobilised C3b (C3bBb assembly). The convertases were allowed to gradually decay (natural decay of C3bBb) before FH1-5 and the control proteins were injected in separate runs. Accelerated decay is evident from a steeper dissociation profile from the point of injection. FH SCRs 19-20 (FH 19-20) and a R83S mutant of FH SCRs 1-4 (R83S FH 1-4) were included as negative controls. The wild-type FH SCRs 1-4 (FH 1-4) were included as positive controls. Response units are shown on the y-axis, time in seconds on the x-axis. n = 1

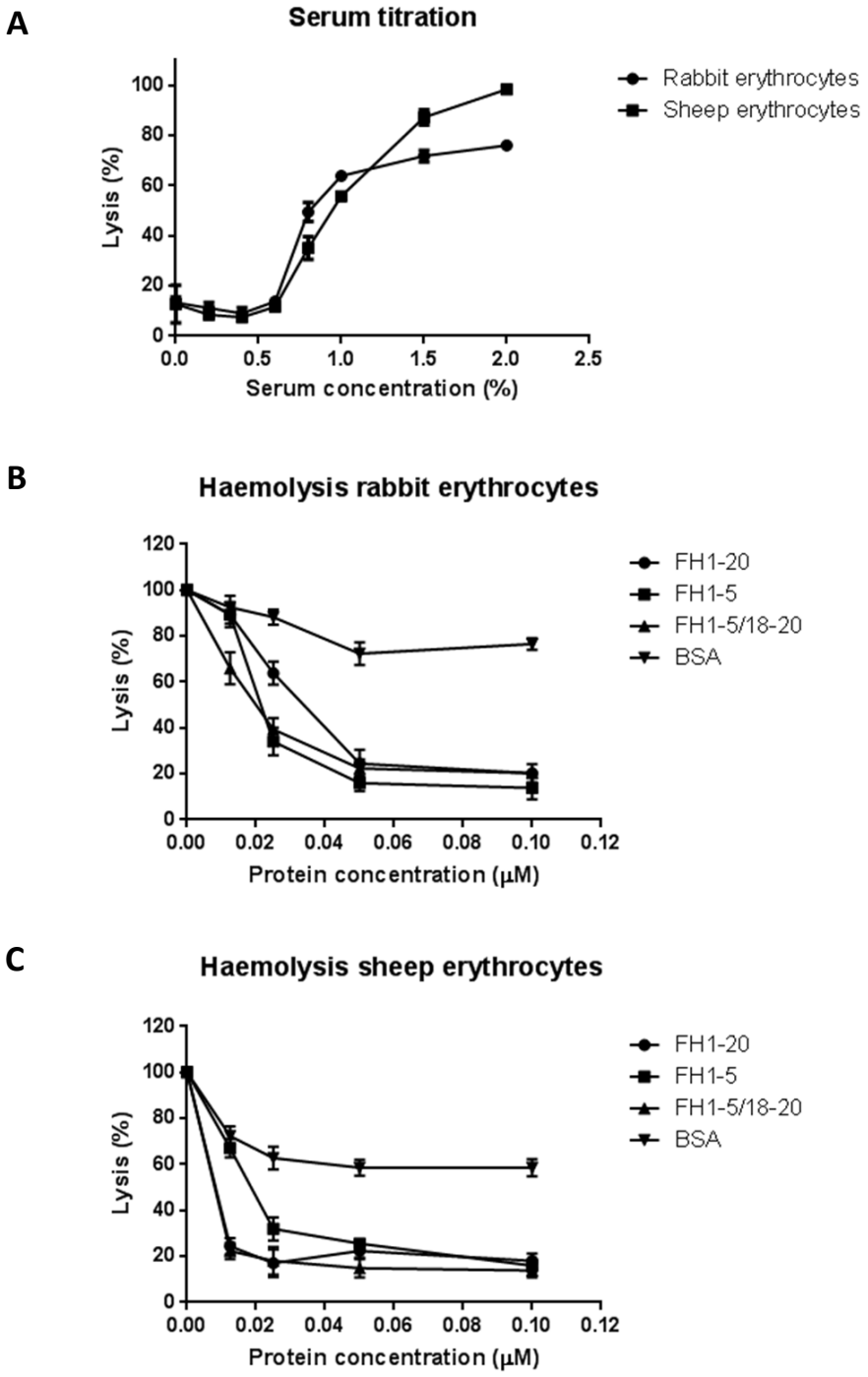


Figure 24: Haemolytic assays with rabbit and sheep erythrocytes

(A) Lysis of rabbit and sheep erythrocytes by human serum was titrated. Percentage lysis was calculated from a complete lysis control (= 100%). A serum concentration of 1% was used for testing protective function of the recombinant FH proteins. **(B)** Rabbit erythrocytes were exposed to human serum in the presence of FH1-20, FH1-5, FH1-5/18-20 or BSA at equimolar concentrations. **(C)** Sheep erythrocytes were exposed to human serum in the presence of FH1-20, FH1-5, FH1-5/18-20 or BSA at equimolar concentrations. Shown are average values of triplicate wells from one representative experiment of two.

3.3 Discussion

3.3.1 Expression of FH1-5 and FH1-5/18-20 in CHO cells

Complement FH is implicated in renal pathologies like aHUS and MPGN type II. The direct connection between FH deficiency or dysfunction and pathology has been clearly demonstrated in *Cfh* knockout mice (Pickering *et al*., 2002, Pickering *et al*., 2007). Recombinant FH proteins present an attractive alternative to current treatment options for these conditions. FH is a large glycoprotein with eight confirmed N-glycosylation sites and 40 disulphide bonds (Fenaille et al., 2007). Several attempts have been made to produce the full length protein. However, expression in insect cells, the mammalian cell line COS7 and plant cells failed to reach levels of therapeutic relevance (Sharma and Pangburn, 1994, Sanchez-Corral *et al*., 2002, Buttner-Mainik *et al*., 2011). The design of the proteins for this study, informed by current conformational models, reduced the 20 SCRs of full length FH to the SCRs required for regulation and cell binding. The resulting lower molecular weight (36 kDa and 54 kDa) was expected to address expression issues raised in previous studies and is also feasible for development of the two recombinant FH proteins, FH1-5 and FH1-5/18-20, as therapeutics.

The expression host was required to produce the relevant posttranslational modifications for secretion of biologically active protein. Although deglycosylation appears not to impact on the regulatory capacity of FH, the two disulphide bonds in each SCR are essential for structural integrity and stability (Jouvin *et al*., 1984, Kristensen and Tack, 1986). *E. coli* is a popular expression host for recombinant proteins due to low cost and high yields. Although most expression strains of *E. coli* are not able to form disulphide bonds, functional recombinant forms of MCP and a truncated version of CR1 have been successfully expressed in *E. coli*. (Fraser *et al*., 2002, Fremeaux-Bacchi *et al*., 2008). A disadvantage of expression in *E. coli* is that the harsh conditions required to retrieve of recombinant protein from inclusion bodies necessitates a refolding step in suitable buffers. Expression of recombinant proteins in yeasts such as *Pichia pastoris* offers the advantages of bacterial expression systems and the ability to synthesise all mammalian posttranslational modifications (Cregg *et al*., 2000). Several complement proteins have been expressed in *P. pastoris* (He *et al*., 1997, Pechtl *et al*., 2011, Herbert *et al*., 2012). Expression in *P. pastoris* can require complex optimisation of growth conditions or induction of expression and occasionally glycosylation may deviate from the native patterns with potential impact on function.

There are several mammalian cell lines that are suited for expression of secreted recombinant proteins (Wurm, 2004). CHO cells are the most prominent example and are currently used for the production of approximately 70% of all therapeutic proteins (Walsh and Jefferis, 2006).

Although not a low cost alternative to bacterial and yeast expression systems, CHO cells are very resilient and show high viability for extended periods of cultivation. Posttranslational modifications in CHO cells closely resemble those of native human proteins. For this study CHO cells were successfully used as expression host. This was demonstrated by ELISA and immunoblotting of the cell culture supernatant (Figure 17). Selection of the high expressing clones S21.5 (FH1-5) and P5 (FH1-5/18-20) significantly improved expression levels in comparison to the transfection pool (Figure 17.B). Yields for FH1-5 and FH1-5/18-20 were usually around 2 mg/L and 4 mg/L, respectively. Further rounds of transfection and selection of clones may have resulted in better expression levels. For the objectives of this study the established expression levels and protein yields were sufficient. Both cell lines allowed the purification of sufficient amounts for the *in vitro* functional testing and will provide sufficient amounts for use in animal studies. Genetically modified CHO cell strains and optimised expression protocols used in the industrial setting should allow large scale production of the proteins. The standard yield for the expression of recombinant protein in CHO cells is 50 mg/L in the industrial setting (Wurm, 2004).The histidine-tag incorporated in the constructs allowed IMAC purification. This worked well for both proteins under standard conditions (Figures 18 and 19). Addition of 40 mM imidazole effectively prevented binding of endogenous CHO cell proteins (Figures 18 and 19). Functional testing, discussed below, suggests that the proteins are functional. CHO cells are therefore a good expression host for FH1-5 and FH1-5/18-20.

3.3.2 FH1-5 and FH1-5/18-20 retain regulatory function comparable to native FH

The second aim of this chapter was to test the AP regulatory function of the proteins. FH fulfils three distinct functions in the regulation of the AP – C3b binding to prevent C3 convertase formation, FI cofactor activity and decay acceleration of the C3 convertase. The two principal C3b binding sites are located in SCR1-4 and SCR19-20, the latter also bearing the important C-terminal heparin binding site. Firstly, binding of FH1-5 and FH1-5/18-20 to C3b was assessed by ELISA. FH1-5 and FH1-5/18-20 both bound C3b with the anticipated differences in affinity (Figure 20.A). Full length FH, included as a positive control, showed a higher affinity than the two recombinant proteins, likely due to the presence of three C3b binding sites. FH1-5/18-20 showed a higher affinity than FH1-5 due to the presence of an additional C3b binding site in SCRs 19/20. This result allows two conclusions. Firstly it shows that SCR5, the hexa histidine-tag and SCR 18 provide a sufficient linker between the two binding sites to allow simultaneous engagement with C3b. While it is not possible to tell whether this simultaneous engagement is on the same or two neighbouring molecules, it is in accordance with the current model of FH binding to surface-bound C3b. The “folded-back” conformation of cell-surface associated FH is widely accepted and supported by recent structural characterisation of the central SCRs 10-15

(Aslam and Perkins, 2001, Okemefuna *et al*., 2008, Schmidt *et al*., 2010). Secondly, the findings imply that SCRs 6-17 may not be required for SCRs 1-5 and 18-20 to associate with C3b bound on a cell surface. An exact functional role of SCRs 8-17 has not been determined yet. A mere structural function for this large portion of FH is unlikely. In addition to C3b, C3d and heparin FH binds to CRP (via SCRs 7 and 20) and aggregates in the presence of high concentrations of zinc via SCR 7 (Okemefuna *et al*., 2008, Perkins *et al*., 1991, Perkins *et al*., 2010). It is therefore likely that one or more yet unidentified ligands exist that bind in this area of the protein. A third heparin binding site in SCR 13 is disputed after fragments comprising central SCRs of FH failed to bind to heparin. The ability of FH1-5 and FH1-5/18-20 to bind heparin was assessed by ELISA (Figure 20.B). As expected, FH1-5 did not bind to heparin and full length FH bound better than FH1-5/18-20, likely because of the presence of at least one additional heparin binding site. A heparin binding site in SCR 13 may only be functional in the context of the full length protein.

The fluid-phase activation of the AP is effectively controlled FI and FH by cleavage of the C3b α-chain. Protection of $CfH^{-/-}$ $CfI^{-/-}$ mice from renal disease clearly emphasised this relationship (Rose *et al*., 2008). Cofactor activity of FH1-5 and FH1-5/18-20 was tested in a fluid-phase assay. Both proteins function as a cofactor to a similar degree as full length FH (Figure 22.A). Fusion of SCRs 18-20 downstream of FH1-5 appears not to impair cofactor function in SCRs 1-5. FH also controls the activation of the AP by preventing formation of AP C3 convertases and by accelerating decay of the AP C3 convertase. A fluid-phase assay was employed to test the ability of FH1-5 and FH1-5/18-20 to prevent formation of C3 convertases from spontaneously hydrolysed C3. Both proteins were able to inhibit the formation of C3 convertases in a concentration dependent manner (Figure 22.B). The level of inhibition is comparable to that of the native full length protein. The ability of FH1-5 to accelerate the decay of preformed C3 convertases was further assessed by SPR (Figure 23). For this C3b was immobilised on a chip and the C3 convertases generated by addition of FB and FD. FH1-5 showed higher decay acceleration activity in this experiment than FH1-4 (Figure 23). Both concentrations of FH1-5 reduced the RU reading to the same level, suggesting saturation of the system. This experiment was only performed once and should therefore be repeated in the future to confirm the findings.

Function of both proteins was further demonstrated in haemolytic assays. Protection of rabbit erythrocytes from AP-mediated lysis by FH1-5 and FH1-5/18-20 is due to fluid-phase regulation of AP activation and is comparable to full length FH (Figure 24.B). Compared to rabbit erythrocytes, FH has a higher affinity for C3b deposited on sheep erythrocytes (Aminoff *et al*., 1976, Fearon and Austen, 1977). FH1-5/18-20 and full length FH protected sheep erythrocytes

to a similar degree, whereas FH1-5 protected these cells to a lesser degree (Figure 24.C). There are several aspects of the haemolytic assays that should be addressed in future experiments. The relative protective effects of FH1-5 and FH1-5/18-20 compared native FH on rabbit and sheep erythrocytes would be more distinct if FH depleted serum was used in the assay. The use of a wider range of the recombinant proteins is also anticipated to accentuate the relative effects. C7/FH depleted serum could be included as an additional negative control to equimolar BSA to demonstrate the protective effect is mediated by control of the terminal pathway initiation. Similarly, it is of interest to ascertain AP-specific inhibition. This can be achieved by using FB/FH depleted serum.

Overall the functional analysis is comparable to the recent findings of Schmidt *et al.* and Hebecker *et al.,* who have both generated similar "mini-FH" proteins and found decay acceleration and cofactor activity of their respective constructs were comparable to native FH (Schmidt *et al*., 2012, Hebecker *et al*., 2012). Schmidt *et al.* included SCRs 1-4 and 19-20 and linked them with a sophisticated linker based on structural modelling; Hebecker *et al*. did not include an additional peptide linking SCRs 1-4 and 19-20. Schmidt *et al.* tested the ability of their construct to protect erythrocytes from PNH patients from AP-mediated lysis and found it provided better protection than native FH. The FH1-5/18-20 generated in the present work did not perform better in the sheep cell haemolytic assay that was performed. However, modification of the haemolytic assay as discussed above may reveal such effect.

3.3.3 Therapeutic potential of FH1-5 and FH1-5/18-20

The treatment options for aHUS and MPGN type II are limited. Successful treatment of aHUS patients with the therapeutic anti-C5 mAb Eculizumab is encouraging for the immediate patient outcome. However, Eculizumab suppresses the terminal pathway of complement and the many important roles of C5 fragments in mediating inflammatory processes. This results in an increased risk of infection. Any further adverse effects of long-term suppression of the complement system are not known. Ideally, treatment of aHUS should aim to restore AP regulation of the glomerular endothelium. FH1-5/18-20 is the ideal candidate to replace dysfunctional FH in this condition. Fakhouri *et al* showed that administration of human FH to *Cfh*$^{-/-}$ mice reverses the deposition of C3 metabolites in the glomerulus (Fakhouri *et al*., 2010). This supports the treatment of MPGN type II patients with plasma exchange and infusion but also implicates administration of recombinant FH may present as viable treatment alternative. Depending on the nature of the regulatory defect, FH1-5 or FH1-5/18-20 can find application in the treatment of MPGN type II.

FH1-5 and FH1-5/18-20 both display fluid-phase AP regulatory activity that is comparable to the full length protein in a number of assays. FH1-5/18-20 protects cells at the cell surface. The functional data generated in this study justifies future *in vivo* studies. The two reagents are useful tools to modulate disease development and progression in murine models of MPGN and aHUS (Pickering *et al*., 2002, Pickering *et al*., 2007). Application of FH1-5 and FH1-5/18-20 in these models will test their potential as therapeutics in these conditions. In $Cfh^{-/-}$ mice both proteins are expected to restore fluid-phase activation of the AP resulting in normal serum C3 levels and reduced GBM deposition of C3 metabolites. Without a cell binding domain FH1-5 may "downgrade" the MPGN type II like phenotype of the $Cfh^{-/-}$ mice to the aHUS like phenotype of the $Cfh^{-/-}$.Δ16-20 mice. Disease resolution and protection is therefore only expected for FH1-5/18-20, not FH1-5.

A newly identified ligand of FH, malondialdehyde (MDA), may be of interest for targeting FH to the site of tubular injury after ischaemia-reperfusion. MDA is a product of lipid peroxidation, a process that causes damage to the cell membrane through oxidative stress and is dramatically increased after renal ischaemia-reperfusion (Gueraud *et al*., 2010, Paller *et al*., 1984). Weisman *et al* characterised binding of FH to MDA on apoptotic and necrotic cells in the context of age-related macular degeneration AMD) (Weismann *et al*., 2011). They mapped the interactions sites of MDA with FH to SCRs 7 and 20. The binding site on SCR7 is of particular importance since the H402 variant of FH, associated with approximately 50% of AMD cases, shows markedly reduced binding to MDA (Thakkinstian *et al*., 2006, Weismann *et al*., 2011). A recombinant FH protein that contains SCRs 1-7 therefore presents a further alternative construct.

In conclusion, two recombinant FH proteins were successfully expressed in CHO cells and their ability to inhibit amplification of the AP of complement was demonstrated using several assays. In some conditions, e.g. anti-GBM nephritis, targeting of the site of complement activation/deposition is feasible. The GBM was identified as attractive target and the suitability of two mAbs to provide a targeting domain explored in the next chapter.

4 Characterisation of the candidate antibody mAb3

4.1 Introduction

In the previous chapter the generation of two recombinant FH proteins was discussed. Dysregulation or inappropriate activation of the AP of complement plays a role in pivotal role in aHUS and MPGN type II (see sections 1.2.3.2 and 1.2.4.5). The AP also plays an important role in renal ischaemia-reperfusion injury as well as immune-complex mediated nephritis by amplification of initial CP activation. In the context of these pathologies targeting of FH1-5 directly to the kidney may present an attractive alternative to the cell surface-targeted FH1-5/18-20. Targeting of a drug decreases dose requirements and possibly limits side-effects (Holers *et al*., 2013). A specific targeting mechanism would also provide a useful platform to deliver other / additional therapeutic molecules to the kidney. The GBM forms the interface between endothelial cells and podocytes, which together perform the separation of the filtrate from the blood. It is comprised of three layers with type IV collagen and laminin as the major constituent (Miner, 2012). The lamina rara externa and interna are covered in heparan sulphate. Several studies have illustrated the susceptibility of the GBM to complement-mediated damage in certain scenarios (Williams *et al*., 1984, Zipfel, 2006). In the human kidney, all AP regulators are expressed to varying degrees on different cell types (Ichida *et al*., 1994, Zipfel, 2006). The GBM lacks surface-bound regulators, but FH can be detected at the GBM in the normal human kidney, most likely associated with heparan sulphate, as shown by co-localisation of FH and type IV collagen (Licht *et al*., 2007). Deficiency of FH in mice causes deposition of C3 metabolites along the glomerular capillary walls (Pickering *et al*., 2002). In mice deficient in FH and FI C3 is only detected in the mesangium, demonstrating a fundamental role of FH for protection of the GBM from C3 deposition. The GBM may therefore present a suitable target for delivery of FH1-5.

I have designed scFv fusion proteins that will target FH1-5 to the glomerular basement membrane. Successful targeting of complement regulators to the site of complement activation or specific tissues and cell types has been demonstrated in several studies (Huang *et al*., 2008, Song *et al*., 2012, Spitzer *et al*., 2004). To target recombinant FH1-5 to the kidney I obtained the mAb3 hybridoma which secretes a mouse mAb (IgG1) with specificity to the NC1 domains of the α3-chain of type IV collagen (Johansson *et al*., 1993). The structure and assembly of type IV collagen is illustrated in Figure 25. For generation of mAb3 the mice were immunised with bovine NC1 domains but mAb3 cross-reacts with both murine and human NC1 domains. The NC1 domain of the α3-chain, also known as the "Goodpasture's antigen" is a well-characterised glomerular antigen (Borza and Hudson, 2003, Pedchenko *et al*., 2011). To exploit the binding properties of mAb3, the unknown sequence of the heavy and light chain

variable region had to be determined. It was also necessary to establish the *in vivo* availability of the antigen and specific binding to the kidney.

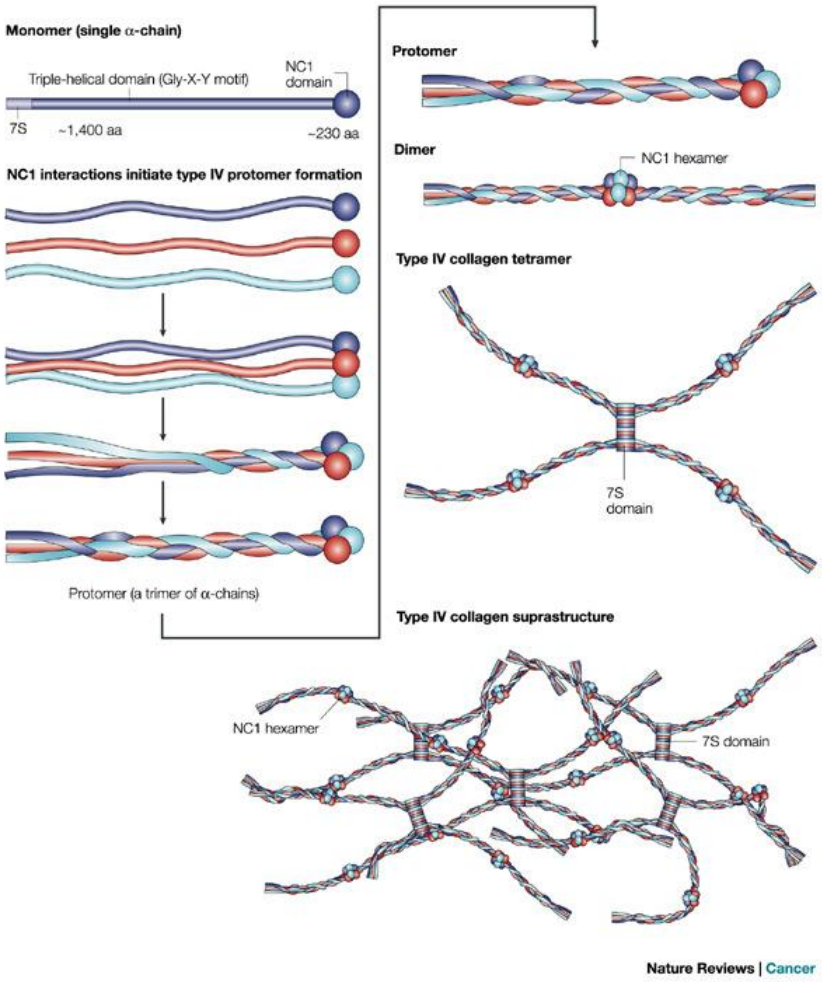


Figure 25: Structure of type IV collagen

Each individual α-chain of type IV collagen contains a 7S domain, a large triple helix and an NC1 domain. The NC1 domains of three α-chains initiate formation of a protomer, e.g. the α3,4,5 protomer. The NC1 trimers of two protomers dimerise and each dimer forms tetramers via the 7S domains. Many tetramers form the collagen IV meshwork. Diagram reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer; Kalluri, A.: Volume 3(6), 2003 (Kalluri, 2003).

The aims of this chapter were therefore:

1. Characterise the *in vitro* and *in vivo* binding properties of mAb3 (and 1G6)

2. Clone the correct mAb3 heavy and light chain variable regions

4.2 Results

4.2.1 Purification of mAb3

To allow characterisation of the binding properties of mAb3, the hybridoma was obtained (Johansson *et al*., 1993). The antibody purified from the hybridoma cell culture supernatant by affinity chromatography using a protein G column. The antibody was eluted from the column using a low pH buffer and 1 ml fractions collected (Figure 26.A). The fractions were analysed by SDS-PAGE to identify immunoglobulin containing fractions and to assess purity of the batch (Figure 26.B). The gel revealed a band of the expected size (~150 kDa) under non-reducing conditions and two bands of the expected size under reducing conditions. There was a double band at the expected molecular weight of the heavy chain. Elution of IgG in these fractions was further confirmed by immunoblotting with an anti-mouse IgG antibody (Figure 26.C).

4.2.2 Binding of mAb3 to bovine and murine type IV collagen NC1 domains

Bovine collagen type IV α3-chain NC1 domains were used to immunise the mice to generate mAb3 which was subsequently shown to cross-react with murine and human NC1 domains (Johansson *et al*., 1993). To verify that the IgG secreted by the hybridoma is mAb3, binding of the purified IgG to bovine and murine renal collagen type IV NC1 domains (Chondrex) was tested by sandwich ELISA. The antigen was coated to microtitre plates and binding of different concentrations of the purified IgG detected with an HRP-conjugated anti-mouse IgG antibody (Figure 27.A). To confirm specificity for the α3-chain, collagen type IV derived from the Engelbrecht-Holm-Schwarm (EHS) tumour which lacks the α3-chain, was included as negative control (Danielson *et al*., 1992). Binding of mAb3 to bovine NC1 domains and to a lesser degree to murine NC1 domains but not to EHS tumour collagen type IV was demonstrated. After significant difficulties were encountered during cloning of the mAb3 light chain variable region, as discussed later, an alternative mAb with similar specificity was sought. The alternative type IV collagen specific mAb was 1G6 (Zhang *et al*., 2008). Purified 1G6, an IgM, was not available for testing as the original cell line reportedly stopped secreting; a small amount of cell culture supernatants was provided by Dr M. Foster (Duke University, U.S.A.) for testing in the ELISA. The ELISA was carried out as for mAb3, the supernatant was serial-diluted across the plate, starting with undiluted supernatant (Figure 27.B). 1G6 bound to bovine and murine NC1 domains to a similar extent and showed some cross-reactivity with the control type IV collagen (EHS collagen type IV).

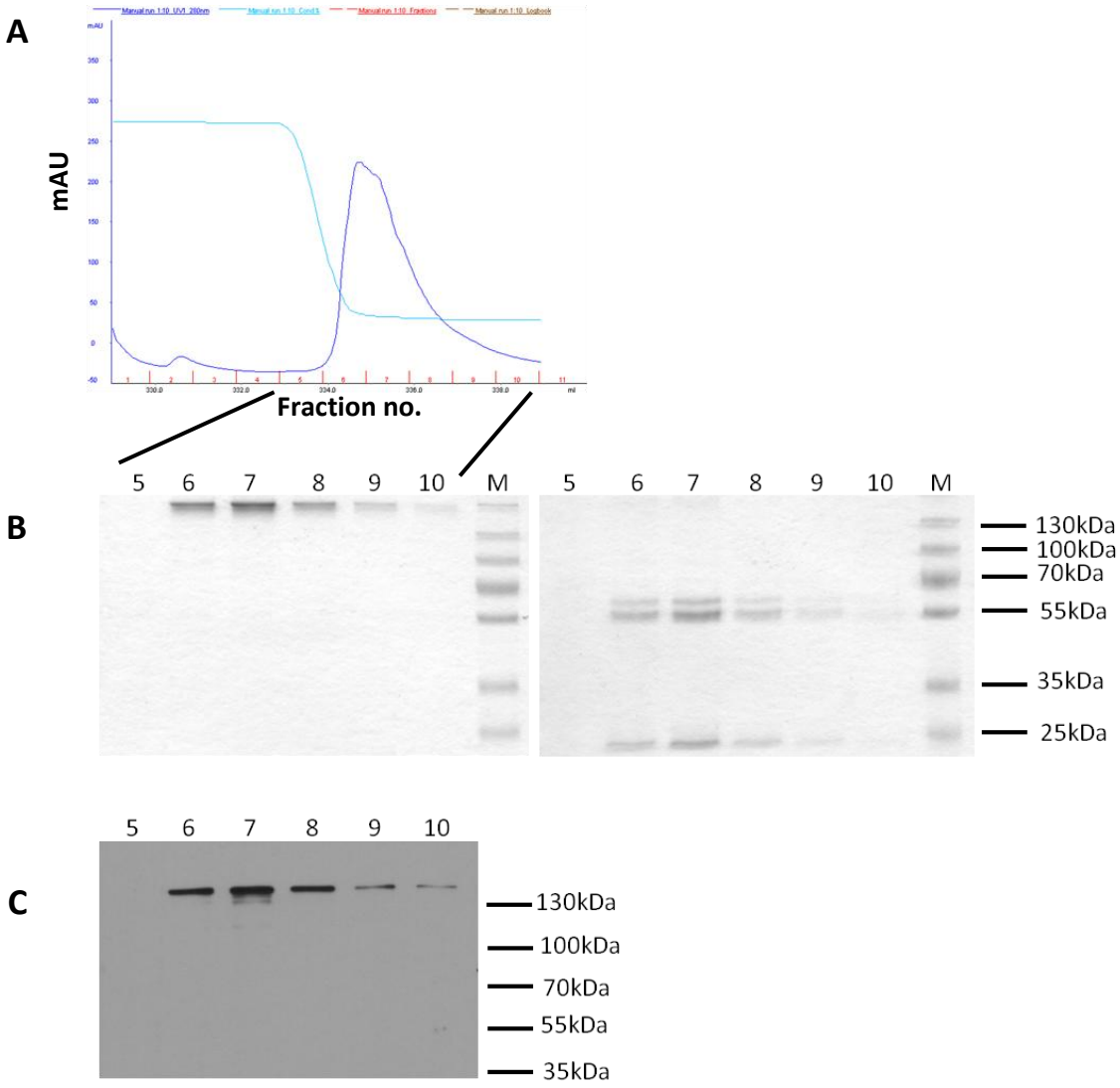


Figure 26: Purification and detection of mAb3:

(A) The chromatography trace shows elution of mAb3 from a protein G column with a low pH buffer. Absorbance units are shown on the y-axis and fraction numbers on the x-axis. **(B)** Protein containing fractions (5-10) were analysed by non-reducing and reducing SDS-PAGE and Coomassie blue staining. A single band of ~130 kDa was revealed under non-reducing conditions and three bands of ~25 kDa, ~55 kD and ~60 kDa were revealed under reducing conditions. **(C)** Immunoblotting of the fractions after non-reducing SDS-PAGE was carried out with a HRP-conjugated sheep anti-mouse Ig antibody. A single band of ~130 kDa was detected.

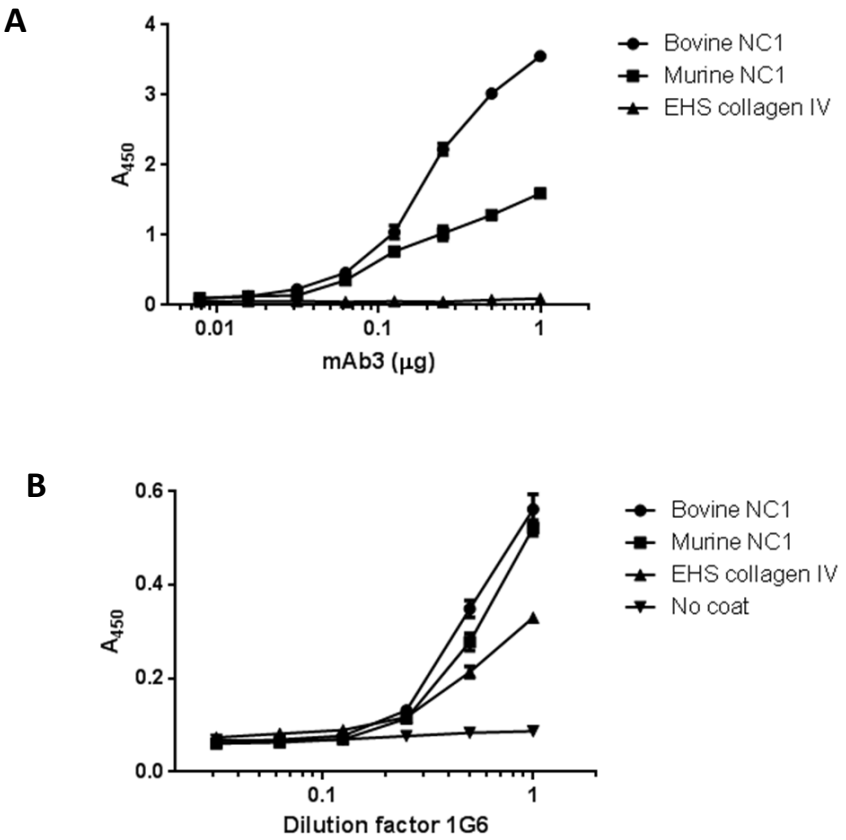


Figure 27: Binding of mAb3 and 1G6 to collagen type IV NC1 domains

(A) Binding of mAb3 to bovine and murine collagen type IV NC1 domains was tested by ELISA. Bovine and murine NC1 domains were coated microtitre plates and binding of mAb3 detected with a sheep anti-mouse Ig antibody. Type IV collagen from the Engelbreth-Holm-Schwarm (EHS) mouse tumour cell line, lacking the α3-chain, was included as negative control alongside uncoated wells (no coat). **(B)** For binding of 1G6 to bovine and murine NC1 domains the same experiment was carried out. Only cell culture supernatant was available for analysis, this was serial-diluted across the plate. Shown is one representative experiment of two.

4.2.3 *In vitro* and *in vivo* binding of mAb3 to mouse kidney

To further characterise mAb3, binding to normal mouse and human kidney sections was detected with a FITC-conjugated anti-mouse IgG antibody (Figure 28). In this *in vitro* experiment mAb3 stains the glomerular and tubular basement membranes. Binding of mAb3 to murine and human kidney sections has been shown previously (Johansson *et al*., 1993, Wei *et al*., 2006); however no study has demonstrated the *in vivo* binding properties of mAb3. To assess availability of the antigen *in vivo*, 20 µg of mAb3, 8D1 (positive control), isotype control (anti-Hen egg lysozyme) or PBS only were injected into mice via the tail vein. 8D1 is an isotype matched collagen type IV specific mAb3. Binding of 8D1 to murine glomerular basement membrane was previously shown *in vitro* and *in vivo* (Luo *et al*., 2010). The animals were sacrificed after 1 h and the kidneys, lungs, livers and spleens harvested. Binding of mAb3 was detected by staining cryosections of the kidneys and control organs with a FITC-conjugated anti-mouse IgG antibody (Figure 28). *In vivo*, mAb3 is only detected at the GBM as the tubular basement membrane is not accessible.

4.2.4 Cloning the mAb3 heavy and light chain variable regions

To exploit the binding properties of mAb3 to target FH1-5 to the GBM, the variable domains of the mAb3 heavy and light chains were cloned. For this mRNA was extracted from the hybridoma cells and heavy and light chain cDNA synthesised using degenerate primers that bind in the hinge region and kappa constant region, respectively (see methods). The highly variable 5' end of both chains only offers the leader sequence and framework 1 region for 5' end primer design (Figure 29). However, it is desirable to obtain the leader sequence for later alignment and expression. To allow for cloning of the variable domains including the leader sequence, 5' RACE was chosen for amplification. 5' RACE (rapid amplification of cDNA ends) is a PCR technique which is routinely used to amplify genes with unknown 5' (or 3') ends (Frohman *et al*., 1988).

4.2.4.1 Cloning of the heavy chain variable region by 5' RACE

To obtain the heavy chain variable region cDNA synthesised using an oligonucleotide priming in the hinge region, was used as a template for 5' RACE (Figure 29). The PCR product obtained was TA cloned and the DNA of several clones sequenced. The DNA sequence obtained was aligned with several sequences of functional heavy chain variable regions (Figure 30). There are no reports of contaminating IgG heavy chain mRNA transcripts and the sequence obtained matched that obtained previously with different primers (K.J. Marchbank), there was little doubt that the sequence obtained in several clones was that of the mAb3 heavy chain.

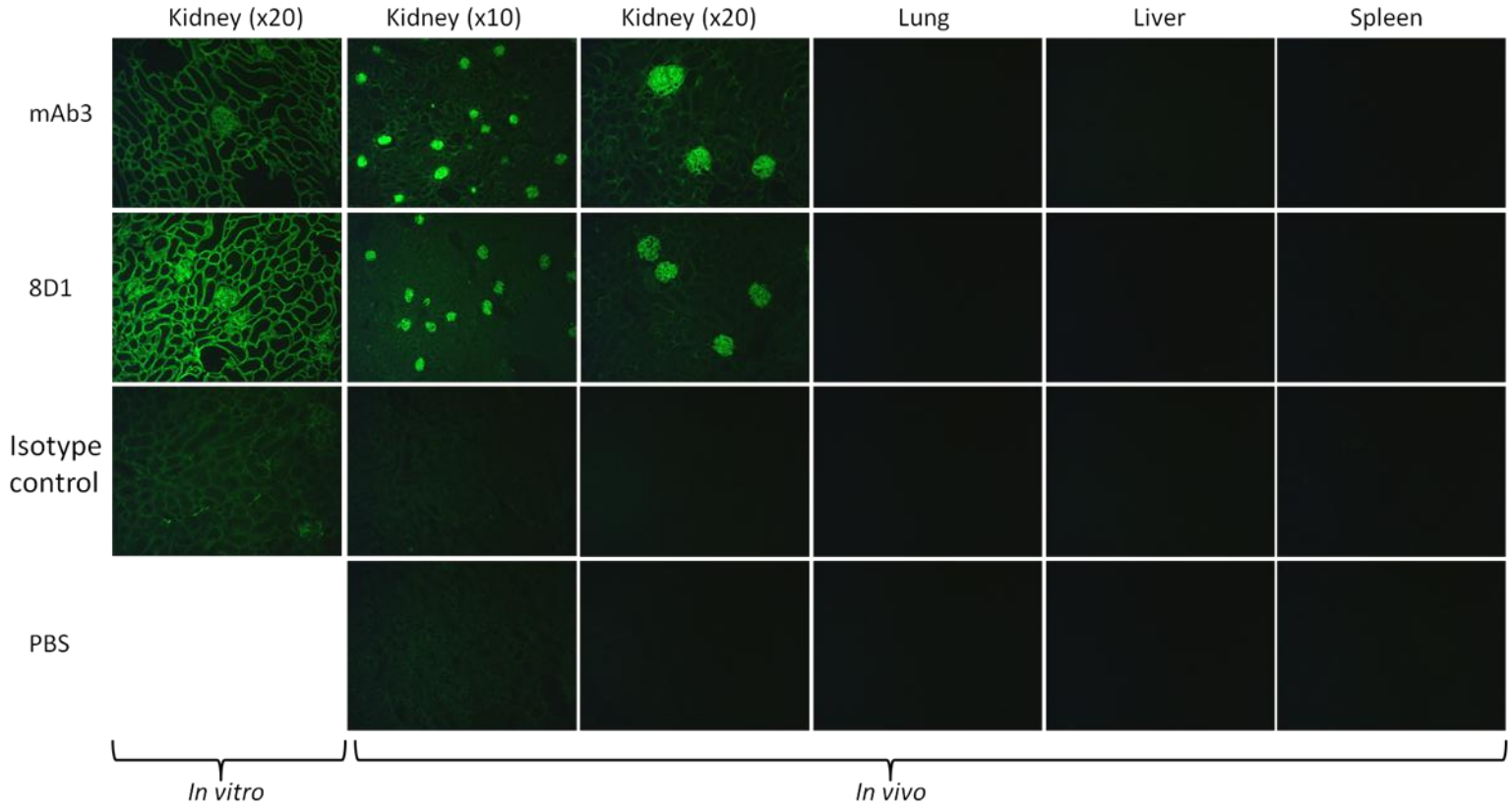


Figure 28: *In vitro* and *in vivo* binding of mAb3 to mouse kidney

To confirm the *in vitro* binding of mAb3 normal mouse kidney was stained with 3 µg/ml of mAb3, 8D1 (positive control) or an isotype control followed by a FITC-conjugated anti-mouse Ig secondary antibody (show is one representative experiment, n=3). To test *in vivo* binding 20 µg of each antibody in PBS were injected via the tail vein of normal Balb/c mice. The animals were sacrificed after 1h and cryosections of the kidney, lung, liver and spleen prepared and stained with a FITC-conjugated anti-mouse Ig antibody (n=2).

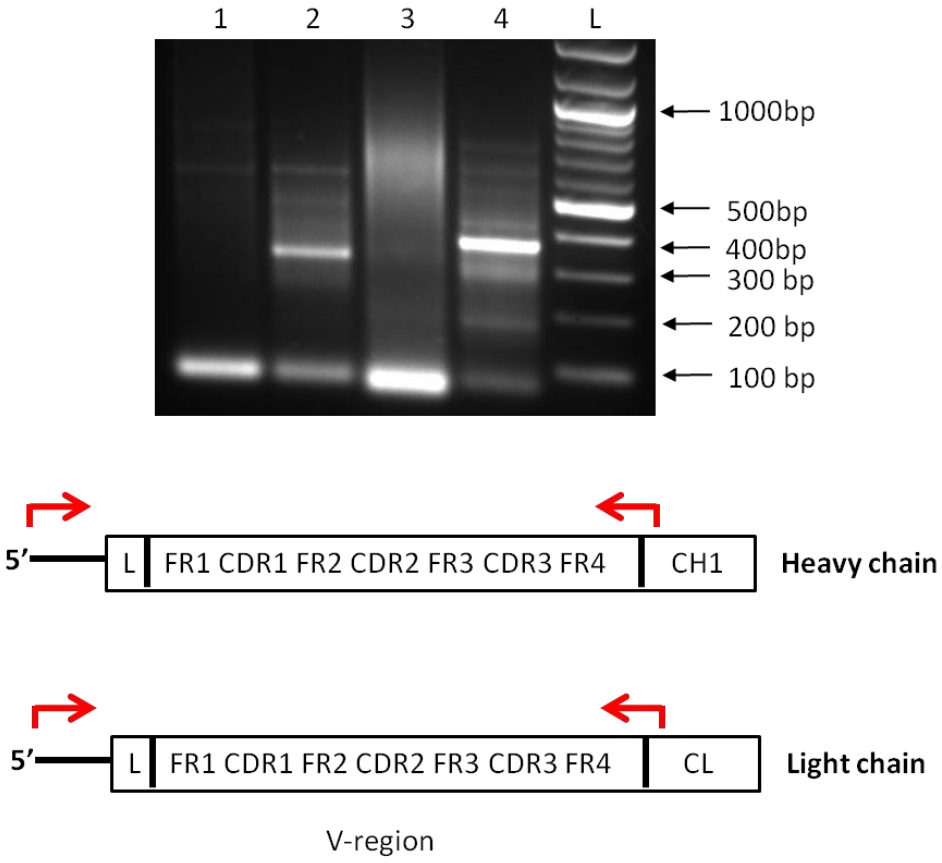


Figure 29: 5' RACE of mAb3 heavy and kappa light chain cDNA

5' RACE was used to amplify mAb3 heavy and light chain cDNA. The mock tailed (lane 1) or poly-C tailed (lane 2) heavy chain cDNA was amplified with the 5' anchor primer and MOCG12, priming in CH1 region. The mock tailed (lane 3) or poly-C tailed (lane 4) light chain cDNA was amplified with the 5' anchor primer and CKMosp, priming at the start of the light chain constant region.

	Leader sequence	FR1	CDR1	FR2
mAb3		QVQLQQSGAELVKPGASVKLSCKASGYTFT	EYTIH	WVKQRSGQGLEWIG
H5	MGWSWIFLFLLSVTAGVHS	QVHLQQSGAELVRPGTSVKVSCKASGYAFT	NYLIE	WVTQRPGQGLEWIG
H7	MAWVWTLLFLMAAAQSIQA	QIQLVQSGPELKKPGETVKISCKASGYTFT	HYPMH	WVKQAPGKSLKWMG
17-1A	MGWSRVFIFLLSVTAGVHS	QVQLQQSGAGLVRPGTSVKVSCKASGYAFT	NYLIE	WVKQRPGQGLEWIG

	CDR2	FR3	CDR3	FR4
mAb3	WFYPGSGSIKYNEKFKD	KATLTADKSSSTVYMELSRLTSEDSAVYFCAR	HEKGAYGNYFDY	WGQGTTLTVSS
H5	VINPGSDFTYYNEKFKG	RATLTADKSSSTAYMQLTSLTSDDSAVYFCAR	TIVTTDYFDY	WGQGTPLTVSS
H7	WINTKSGVPTYADDFKG	RFAFSLETSASTACLQITNLKNEDMATYFCVR	GGLYYDYFYGVDY	WGQGTSVTVSS
17-1A	VINPGSGGTNYNEKFKG	KATLTADKSSSTAYMQLSSLTSDDSAVYFCAR	DGPWFAY	WGQGTLVTVSA

Figure 30: Alignment of the mAb3 heavy chain sequence

The sequence of the mAb3 heavy chain, as obtained by 5' RACE, was aligned with the published heavy chain sequences of three other monoclonal antibodies: H5 (Hackett *et al*., 1998), H7 (Park *et al*., 2000) and 17-1A (Sun *et al*., 1987). Conserved residues in the leader sequence and framework region 1 were used to aid the alignment.

4.2.4.2 5' RACE of the light chain cDNA favours amplification of an endogenous transcript

Cloning of the light chain variable region had been attempted previously in the lab (K.J. Marchbank). However, expression difficulties and inability of the construct to bind its antigen prompted further analysis of the obtained sequence using the Basic Local Alignment Search tool (BLAST, National Centre for Biotechnology). The search returned a match with the sequence of an aberrant kappa light chain (JL0073). The mAb3 hybridoma was generated using SP2/0 cells as fusion partner, which is derived from the MOPC21 myeloma cell line (Carroll *et al*., 1988). This cell line produces an aberrant kappa light chain transcript which is often present in excess of the desired light chain and preferentially amplified (Carroll *et al*., 1988). A number of ways to eliminate the aberrant transcript have been reported in the literature.

The first approach to obtain the correct mAb3 kappa light chain entailed the standard 5' RACE that was successfully used for cloning of the heavy chain variable region. Anecdotal reports suggested that if a sufficient number of clones are analysed, a kappa light chain sequence different to the aberrant transcript can be obtained. 5' RACE was performed, the PCR product obtained TA cloned and the DNA of 25 clones sequenced. Of these clones, 22 contained the aberrant transcript, 2 reactions failed and 1 sequence obtained revealed only vector sequence (see Table 11). Due to the uncertainty of this method it was not pursued further.

Table 11: Sequencing results 5' RACE mAb3 kappa light chain

5' + 3' primer	No. of clones sequenced	Failed	Aberrant transcript	Other result (not immunoglobulin)
MKVP2+CKMOsp	4	1	3	0
MKVP11+CKMOsp	6	1	4	1
MKVP2+"Andy rev"	4	0	4	0
MKVP11+"Andy rev"	6	0	6	0
MKVP2+Light chain back *BamHI*	5	0	5	0
Total	**25**	**2**	**22**	**1**

The digestion of the aberrant transcript mRNA, hybridised with a specific antisense-oligonucleotide, by RNAse H, was attempted next (Ostermeier and Michel, 1996). However, the low working temperature of the enzyme appeared to allow non-specific annealing of the antisense-oligonucleotide resulting in degradation of all mRNA since there was no amplification of the treated mRNA (data not shown). This approach was also found not to work by a different member of the lab using a different template.

4.2.4.3 Leader specific primers and restriction digest to amplify the specific transcript

Next, a panel of degenerate primers, designed to prime in kappa light chain signal peptides, was tested. These primers (MKVP1, MKVP6, MKVP8, MKVP11) were designed to be different

from the signal peptide of the aberrant transcript (Delbat *et al*., 2001). A primer specific for the aberrant transcript (MKVP2) was included as control. The mAb3 light chain cDNA was first amplified by standard PCR using this panel of primers (Figure 31.A). Only MKVP2, the primer specific for the aberrant transcript, produced a band at the expected size. The PCR product was TA cloned and the DNA of several clones sequenced. As expected, all sequences obtained using MKVP2 matched the aberrant transcript. To test the primer panel on a different template, it was used to amplify the variable region of an irrelevant kappa light chain (Figure 31.D). MKPV1 and MKVP8 generated a product in addition to MKVP2. Sequencing of these products (carried out by Dr A. Knight) revealed that both MKVP1 and MKVP8 amplified a kappa light chain sequence different to the aberrant one. This demonstrated that the primer panel was working in discriminating against the aberrant transcript. The absence of amplification products for the mAb3 cDNA was either due to presence of only a relatively small amount of specific template in the synthesised cDNA or due the primers in the panel failing to bind the specific template DNA.

There was a possibility that a small amount of the correct light chain was present in the 5' RACE reaction. This was explored by using the 5' RACE reaction mixture as template for standard PCR with the same primer panel used above. As shown in Figure 31.B and 31.C, primer MKVP11 yielded a product with two different reverse primers. This product was TA cloned and sequencing again revealed the aberrant light chain sequence. The PCR product obtained with MKVP11 was further analysed by restriction digest. Juste *et al.* identified several restriction enzyme sites in the aberrant sequence that occur only at a low frequency in functional kappa light chains (Juste *et al*., 2006b). The MKVP2 and MKVP11 PCR products were subjected to restriction enzyme digest with Bci VI, one of the enzymes identified by Juste *et al*. (Figure 32.A). As shown, both PCR products are digested with Bci VI but show a different restriction pattern. This suggests a different sequence was amplified by MKVP11 but contradicts the sequencing result. To ensure the sequencing result was not due to a sample mix up, the MKVP11 PCR product was cloned three times and the DNA of several clones analysed. The sequencing result consistently showed the aberrant sequence.

The abundance of the endogenous transcript reportedly varies between SP2/0 derived hybridoma cell lines. Up to this point it was not possible to obtain the specific light chain sequence. This encouraged assessment of the endogenous transcript amount present in the mAb3 hybridoma in comparison with SP2/0 and NS0 cells which are both derived from the MOPC21 tumour. As shown in Figure 32.B the amplification levels of the mAb3 cDNA with MKVP2 greatly exceeded the levels obtained for the SP2/0 and NS0 cDNA.

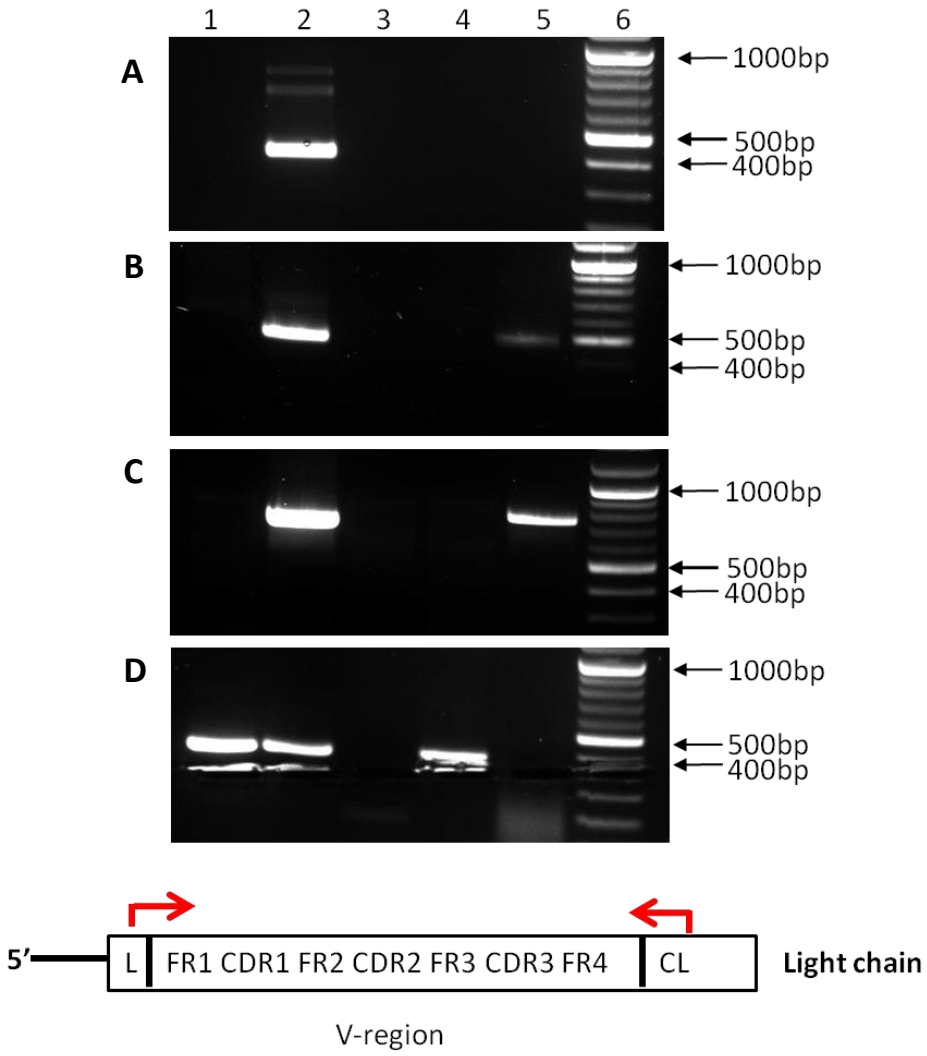


Figure 31: Amplification of mAb3 light chain cDNA leader sequence specific primers

A panel of leader sequence specific degenerate primers (1 = MKVP1, 2 = MKVP2, 3 = MKVP6, 4 = MKVP8, 5 = MKVP11) was employed to amplify the correct mAb3 kappa light chain. MKVP2 is a control primer designed to bind to the endogenous light chain leader sequence. Panel **A** shows amplification of mAb3 light chain cDNA with the primer panel and the "CKMOsp" reverse primer by standard PCR. Panel **B** shows nested amplification of a 5' RACE reaction (as shown in Figure 29) with the primer panel and "CKMOsp". The 5' reaction was amplified with an alternative reverse primer "Andy rev" as shown in panel **C**. Panel **D** shows amplification of control light chain cDNA, derived from a monoclonal antibody with irrelevant specificity, with the primer panel and CKMOsp by standard PCR. L = DNA ladder.

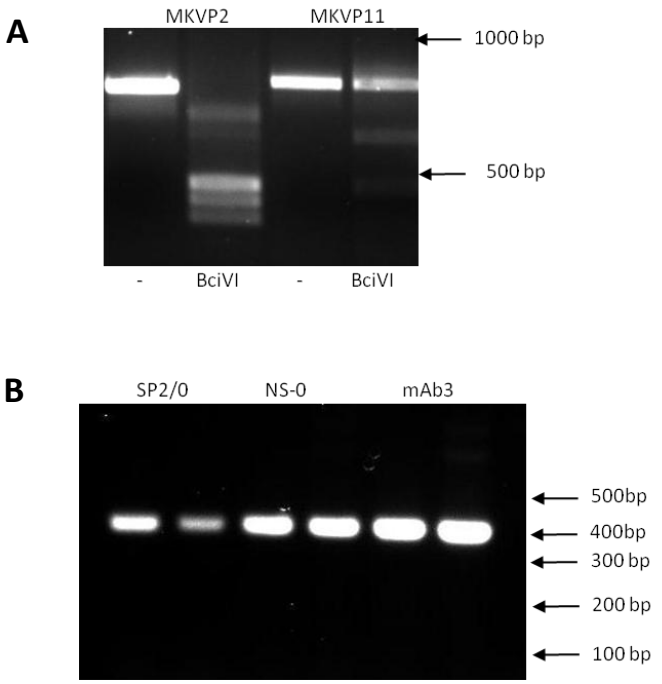


Figure 32: BciVI restriction digestion of the MKVP2 and MKVP11 PCR products

(A) The products obtained by amplification of the mAb3 light chain 5' RACE reaction with MKVP2 or MKVP11 were subjected to digestion with BciIV. A control reaction in absence of the enzyme was included (-). **(B)** SP2/0, NS-0 or mAb3 cDNA was amplified with MKVP2. Two amounts of template cDNA was used, with the left lane containing half the amount of template cDNA than the right lane (for SP2/0 the reverse is the case).

4.2.4.4 Combining FR1 specific primers and 5' RACE to amplify the specific transcript

Next, a degenerate primer for the framework region 1 was tested. This primer had previously been shown not to amplify the aberrant transcript (Delbat *et al*., 2001). Amplification with this primer yielded a clear single band of the correct size (Figure 33). The product was purified, TA cloned and plasmid DNA of several clones sequenced. All successfully sequenced clones (12 in total) revealed the same sequence, distinct from the aberrant light chain. Alignment of the obtained sequence (BLAST) confirmed a murine kappa light chain sequence. There was some variation in the framework region 1 due to the degenerate nature of the primer and no information about the signal peptide was obtained. To confirm the 5' end of the mAb3 kappa light chain, mAb3 specific primers were designed. One primer hybridised to the CDR3 region to allow for specific synthesis of the mAb3 kappa light chain cDNA. The specific cDNA was then amplified by 5' RACE using a mAb3 specific reverse primer hybridising to the CDR2 region and a single product was obtained (Figure 33). Sequencing of 8 clones revealed the same sequence for the leader peptide and confirmed the framework region 1.

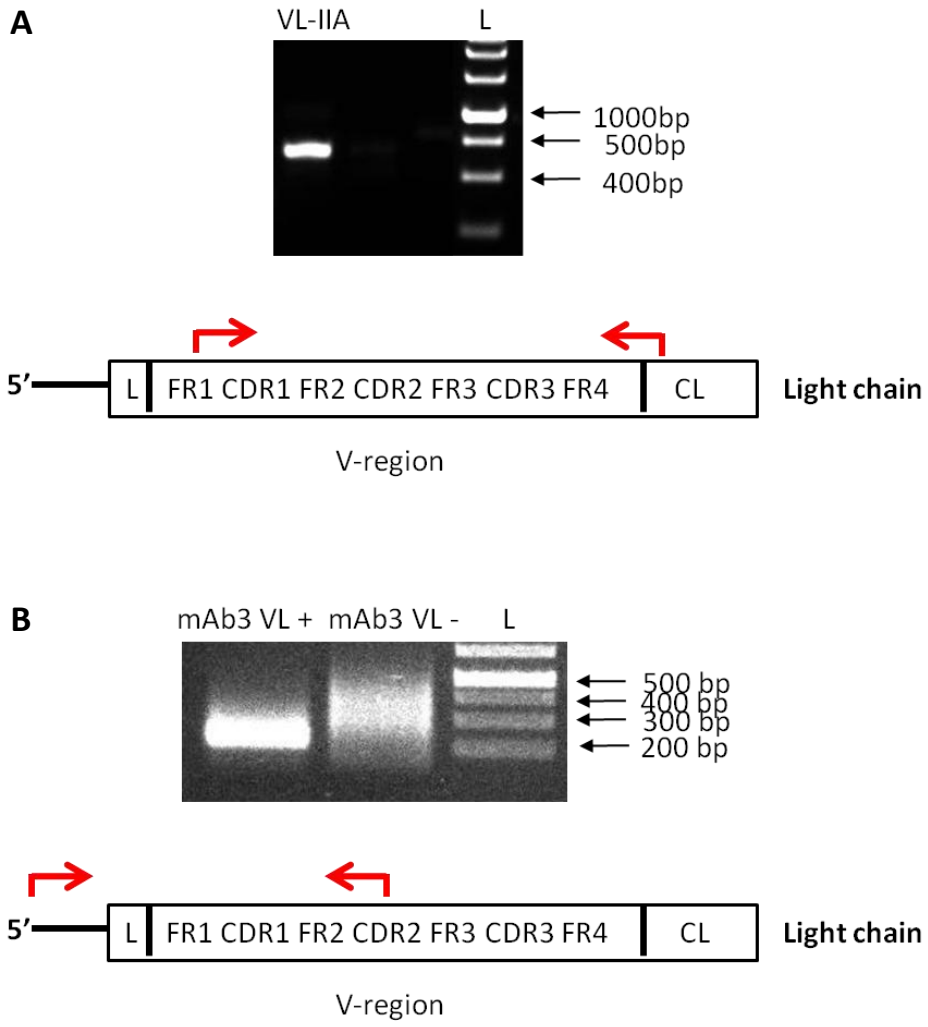


Figure 33: PCR of the light chain cDNA (FR1 primer) and 5' RACE (CDR2 primer)

(A) The mAb3 light chain cDNA was amplified with a FR1 specific degenerate primer (VL-IIA) by standard PCR using the "CKMOsp" reverse primer. **(B)** Specific mAb3 cDNA, synthesised with a CDR3 specific primer based on the sequence derived from the VL-IIA PCR product, was amplified by 5' RACE using the anchor primer and a CDR2 specific primer.

	Leader	FR1	CDR1	FR2
mAb3	MESQTQVFVY LLWLSGVDG	DVLMTQTQKFMSTSVGDRVSVTC	KASQNVGTNVA	WYQQKPGQSPKALIY
Aberrant	MDTLLLWWPGSTG	DIVLTGSPASLAVSLGGRATISV	ISYRASKSVSTSGYSYMH	WNQQKPGQPPRLLIY
H5	MESDTLLLWVLLLWVPGSTG	DIVLTQSPASLAVSLGQRATISC	RASESVSIRGAGLMH	WYQQKPGYPPKLLIY
H7		DVVMTQTPLTLSVTIGQPASISC	KSSQSLLDSDGETYLN	WLLQRPGQSPKRLIY
17-1A	MESQTLVFISILLWLYGADG	NIVMTQSPKSMSMSVGERVTLTC	KASQNVVTYVS	WYQQKPEQSPKLLIY

	CDR2	FR3	CDR3	FR4
mAb3	SASYRYS	GVPDRFTGSGSGTDFTLTISNVQSEDLAEYFC	QQYNSYPLT	FGGGTKLEIK
Aberrant	LVSNLES	GVPARFSGSGSGTDFTLNIHPVEEEDAATYYC	QHIRELTR	SEGGPSWK*
H5	AASNLES	GVPARFSGRGSGTDFTLNIHPVEEADAATYFC	QQSRRYPYT	FGGGTKLEIK
7	MVSKLDS	GVPDRFTGSGSGTDFFFTLKISRVEAEDLGVYYC	WQGTHFPT	FGGGTKLEIK
17-1A	GASNRYT	GVPDRFTGSGSATDFTLTISSVQAEDLADYHC	GQGYSYPYT	FGGGTKLEIK

Figure 34: Alignment of the mAb3 light chain sequence

The sequence of the mAb3 light chain, as obtained by PCR with a FR1 specific degenerate primer and 5' RACE, was aligned with the aberrant light chain sequence and published sequences of functional light chains of three other monoclonal antibodies: H5 (Hackett *et al*., 1998), H7 (Park *et al*., 2000) and 17-1A(Sun *et al*., 1987). Conserved residues in the leader sequence and framework region 1 were used to aid the alignment.

4.3 Discussion

Since the technology to establish B cell hybridomas to produce monoclonal antibodies (mAbs) was introduced, developments in the area of molecular cloning (e.g. rapid amplification of cDNA ends, phage display technology) have greatly advanced the engineering of antibodies (Kohler and Milstein, 1975, Nissim and Chernajovsky, 2008, Liu *et al*., 2008, Teillaud, 2012). It is now possible to produce small functional antibody molecules like single chain variable fragments (scFv) that only bear the antigen binding domains. These molecules are attractive molecules for use as therapeutics because of their small size (25kDa) which also make them suitable as fusion partner to other functional molecules. The majority of therapeutic scFvs under development find application in the treatment of cancer, mainly by targeting tumour cell surface antigens (Accardi and Di Bonito, 2010, Ahmad *et al*., 2012). Several studies have demonstrated the targeting of the AP regulators DAF and CD59 to a specific cell type by means of a scFv (Zhang *et al*., 2001, Spitzer *et al*., 2004, Song *et al*., 2012). This study aimed to characterise the binding properties of two candidate antibodies, mAb3 and 1G6, and to determine the sequence of the heavy and light chain variable regions of mAb3 to allow generation of type IV collagen specific FH1-5/scFv fusion proteins.

4.3.1 Binding characteristics of mAb3 and 1G6

The *in vitro* binding of mAb3 and 1G6 to bovine, murine and, in the case of mAb3, human type IV collagen has been described previously (Johansson *et al*., 1993, Zhang *et al*., 2008). Whilst mAb3 specifically bound bovine and murine NC1 domains but not the EHS control type IV collagen, 1G6 showed some cross-reactivity with the control collagen (Figure 27). 1G6 may therefore be less suited than mAb3 to deliver FH1-5 specifically to the kidney. No published data describes the *in vivo* binding properties of mAb3. The antigen for Goodpasture's disease is located within the NC1 domain hexamer of collagen type IV, which are cross-linked by sulphilimine bonds (Vanacore *et al*., 2009, Pedchenko *et al*., 2011). In a recent study, Luo *et al* investigated the suitability of mice as model for passive transfer of autoimmunity by immunisation with patient antibodies (Luo *et al*., 2010). For this they compared cryptic antigen (Goodpasture's (GP) auto-antibodies) with accessible antigen (Alport allo-antibodies). *In vitro*, no binding of GP auto-antibodies to native NC1 hexamers was detected whilst reactivity to dissociated hexamer subunits or acid-urea treated tissue was comparable to the Alport allo-antibodies which reacted under both conditions. This study encouraged the question, whether the antigen for mAb3 would be available in an *in vivo* setting. The reported binding pattern of mAb3 to the glomerulus was confirmed by staining normal mouse kidney cryosections with mAb3. Type IV collagen of the glomerular and tubular basement membrane was detected by mAb3 (Figure 28). To establish *in vivo* binding of mAb3, C57BL/6 mice were injected with 20 µg

of mAb3, the control antibodies 8D1 and anti-HEL or PBS only. Cryosections of the harvested kidneys were stained with the same FITC-conjugated secondary antibody as used for the *in vitro* sections. As expected, binding of mAb was only detected at the GBM, since the basement membrane of intact tubuli is not exposed to mAb3 (Figure 28). This confirms that the NC1 domains of type IV collagen, in C57BL/6 mice, are not disguised by additional sulphilimine cross-links. The data also shows, that mAb3 can penetrate the glomerular endothelium and at least partially the GBM.

The reactivity of mAb3 with collagen IV in tissues other than kidney was also determined. Collagen IV is a general constituent of basement membranes and the six chains (α1-α6) appear in three different protomers. In addition to GBM and renal tubule the α3.α4.α5(IV) protomer is found in the eye and in alveolar, testicular, cochlear and placental basement membranes. It was shown that mAb3 weakly binds NC1 domains derived from bovine lens, but not human placental NC1 domains (Johansson *et al*., 1993). Binding affinities of mAb3 appear to differ between tissues, in accordance with variation of relative quantities of α3.α4.α5(IV) and interestingly the degree of cross-linkage of NC1 dimers (Langeveld *et al*., 1988, Pullig, 1992, Khoshnoodi *et al*., 2008). Lungs, livers and spleens were collected from the mice and no binding of mAb3 or control antibodies was detected (data not shown).

4.3.2 Overcoming an overexpressed endogenous kappa light chain

The cloning of the mAb3 hybridoma variable regions presented two challenges. The highly variable 5' end of both chains only offers the leader sequence and framework 1 region for 5' end primer design. However, it is desirable to obtain the leader sequence for later alignment and expression. To allow for cloning of the variable domains including the leader sequence, 5' RACE was chosen for amplification. The heavy chain was successfully amplified with this method (Figure 29). Alignment with several functional heavy chain variable regions sequences suggested that a heavy chain (Figure 30) was cloned. The mAb3 hybridoma was generated using SP2/0 cells as fusion partner, which is derived from the MOPC21 myeloma cell line (Johansson *et al*., 1993). This cell line produces an aberrant kappa light chain transcript which is often present in excess of the specific light chain (Carroll *et al*., 1988). When attempting to clone the specific kappa chain the majority of clones obtained will contain the aberrant transcript but not the specific kappa chain. A number of approaches have been developed to evade the aberrant transcript (Michel, 1996, Delbat *et al*., 2001, Yuan *et al*., 2004, Juste *et al*., 2006a). We have combined several of these methods in an attempt to achieve successful amplification of the mAb3 kappa light chain.

The digest of the aberrant transcript mRNA, hybridised with a specific antisense-oligonucleotide, by RNAse H, presented a simple but seemingly unspecific solution to the

problem (Michel, 1996). The low working temperature of the enzyme appeared to allow non-specific annealing of the antisense-oligonucleotide resulting in degradation of all mRNA (data not shown).

Delbat *et al.* deemed the use of 5' RACE as disadvantageous and resorted to a panel of highly degenerate leader sequence primers used against a primer specific for the aberrant transcript (Delbat *et al*., 2001). This allowed identification of a primer that does not amplify the aberrant transcript based on the amount of amplification obtained. This panel of four highly degenerate kappa leader sequence primers (MKVP1, MKVP6, MKVP8, MKVP11) and a primer known to amplify the aberrant transcript (MKVP2) were used to amplify the mAb3 light chain variable regions. Only MKVP2 yielded a product (Figure 31.A). This may be because neither of the other primers recognises the specific transcript or could reflect the relative abundance of the aberrant transcript. A nested PCR of the 5'RACE product using the degenerate primer panel and two different reverse primers (Figure 31.C and 31.D) again yielded good amplification with MKVP2, but also MKVP11 generated a single product. Based on the amplification levels, these results suggest that MKVP11 has specifically amplified the desired transcript. However, sequencing revealed that the sequence of the amplified DNA is that of the aberrant light chain.

To further test this, the PCR products were digested with *BciVI* (Figure 32.A). The restriction sites on the aberrant transcript were mapped and the frequency of the identified restriction sites in functional full length kappa light chains determined (Juste *et al*., 2006a). Three restriction sites with a frequency of less than 20% were identified, with *BciVI* occurring in only 4.19% of sequences. Restriction digest with these enzymes could thus be utilised to digest usual PCR or 5'RACE products to separate both transcripts or to exclude amplification of the aberrant transcript. Both the MKVP2 product and the MKVP11 product were digested by *BciVI* but showed restriction fragments of different sizes. This again indicated that the sequence of the MKVP11 amplified DNA has a difference sequence than the aberrant transcript. Cloning of the wrong PCR product presented a possible explanation for the results obtained. However, repeats of the same procedure yielded the same sequencing result. There is no plausible explanation for the somewhat contradictory findings obtained with the leader sequence primers and the *BciVI* digests.

Delbat *et al.* used further primers, hybridising to the framework region 1, to confirm the results obtained with the first primer panel. One of the primers identified to amplify only the specific kappa light chain (VII-A) was used to amplify the mAb3 light chain cDNA. The single band that was obtained with this primer (Figure 33) was TA cloned and the DNA of several clones sequenced. Analysis of the sequences obtained revealed the same kappa light chain sequence in all clones, very distinct from the aberrant kappa light chain sequence (Figure 34). The degenerate nature of the VII-A and fact that it hybridises in the FR1 region did not allow

cloning of the leader sequence and resulted in some variation in the FR1 region between the DNA clones obtained. To confirm the FR1 region and to obtain the leader sequence gene-specific primers hybridising in the CDR3 and CDR2 region were designed for synthesis of specific cDNA and subsequent 5' RACE (Figure 33.B). The completed mAb3 light chain sequence was aligned to several other functional kappa light chain sequences (Figure 34).

In this project several methods were adopted to retrieve the type IV collagen specific mAb3 kappa light chain sequence. Ultimately PCR with highly degenerate FR1 region primers followed by 5' RACE with gene specific primers allowed cloning of the correct light chain, including the signal sequence. The findings suggest that combination of these published methods presents a feasible, relatively simple approach. The degenerate primers panels can be used to identify a light chain sequence different from the endogenous light chain (Delbat *et al*., 2001). The obtained sequence, which may lack part of the 5' sequence, can then be used to design gene-specific primers for use in 5' RACE to obtain the entire sequence.

The difficulties encountered whilst cloning the mAb3 light chain variable regions prompt discussion of the approach chosen to generate an antibody fragment specific for type IV collagen. In principle there are two ways to generate a scFv. If a hybridoma that secretes an antibody of the desired specificity is available, the variable regions can be cloned directly from the cell line. The potential disadvantages of this approach are, as demonstrated in this study, the presence of contaminating endogenous transcripts that may be preferentially amplified. Depending on the position of the antigen binding site the folding of the resulting scFv may be incorrect and result in less or no binding of the antigen. Although there are several examples of functional scFv that were cloned from a parent antibody, the majority of scFvs reported in the literature were generated by phage display technology. A general or pre-selected scFv phage display library allows simultaneous selection of several candidate scFvs based on affinity of the folded protein. Later folding issues and loss of affinity can largely be prevented. Fusion of the scFv to a fluorescent tag, like GFP, could greatly facilitate the further functional analysis. For this project, it may well be worth pursuing this approach as humanised libraries can be used that may have a lower risk of immunogenicity in comparison to the murine scFvs generated.

Despite the problems encountered, the sequence of a kappa light chain was cloned from mAb3 cDNA and the alternative mAb 1G6 was established as possible alternative. The next challenge was to assemble the variable regions of mAb3 and 1G6 in a format suitable for fusion to FH1-5 to utilise the binding properties of the parent antibodies.

5 Generation of type IV collagen specific FH1-5/scFv proteins

5.1 Introduction

This chapter will combine the reagents generated in chapter 3 and the sequence information obtained in chapter 4 to generate a FH1-5 molecule that is targeted to the glomerulus by means of a collagen type IV specific antibody fragment. In chapter 3 the FH1-5 protein was expressed, purified and its complement regulatory function established in *in vitro* assays. Chapter 4 dealt with the characterisation of the binding properties of the monoclonal antibodies, 1G6 and mAb3. The sequence of the 1G6 variable regions was known, for mAb3 the variable regions were cloned and the sequence information obtained. The scFv format was chosen to exploit the binding properties of these two antibodies.

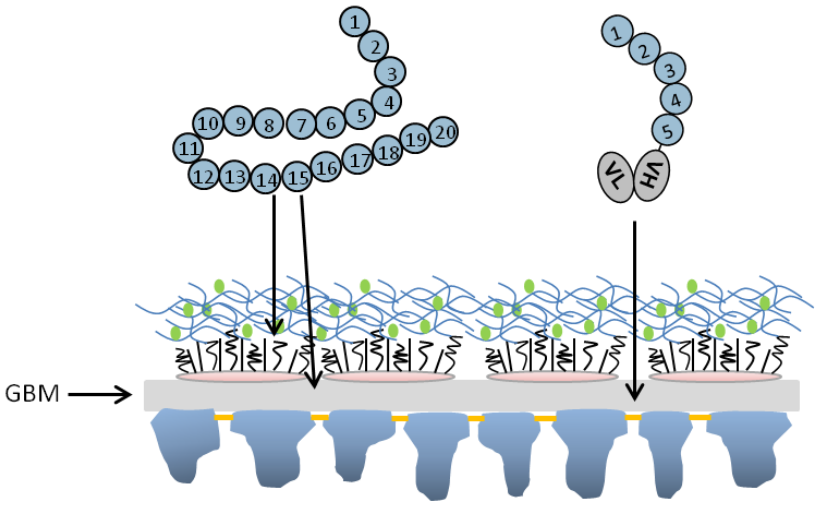


Figure 35: GBM-targeted FH1-5/scFv fusion proteins

Fusion of FH1-5 to a scFv antibody fragment specific for the NC1 domains of the type IV collagen α3-chain allows targeting of the regulator to the relatively unprotected GBM. FH1-5 can exert decay accelerating and FI cofactor activity locally at the GBM.

The site of complement activation and the resulting tissue damage is often restricted to a particular organ in complement-mediated conditions. Targeting of a complement inhibitor enables concentration of the therapeutic molecule at the site of complement activation and prevents systemic inhibition of complement and its diverse functions.

The aims of this chapter were therefore to:

1. Generate FH1-5/1G6 scFv and FH1-5/mAb3 scFv DNA constructs
2. Express and purify both fusion proteins
3. Characterise the binding properties of the two functional domains of the fusion protein

5.2 Results

5.2.1 Generation of the FH1-5/1G6 scFv expression vector

The sequence for the 1G6 variable regions was published and the scFv was therefore synthesised (see Appendix B) by MWG (Zhang *et al*., 2008, Sackey, 2008) The synthesised gene was provided in a plasmid (CR2.1 1G6 scFv) and the scFv DNA could thus be obtained by transformation of competent cells with the plasmid DNA, followed by plasmid preparation and restriction digests or by amplification of the scFv by PCR. The latter option was chosen since it allowed introduction of alternative restriction sites for downstream cloning of the fragment.

The cloning strategy for this construct is depicted in Figure 36. In brief, the two DNA fragments for the fusion protein were amplified by standard PCR from the according plasmid DNA. The primers were designed to introduce a *SalI* site at the 5' end and a histidine tag (His_6) and *KpnI* site at the 3' end of FH1-5 and a 5' *KpnI* site and 3' stop codon and *NheI* site for the 1G6 scFv. The two fragments (shown in Figure 37.A) were purified and ligated using a T4 ligase. The ligation product was amplified by PCR using the 5' primers for FH1-5 and the 3' primer for the scFv (see Figure 37.A). The resulting fragment was digested with *SalI* and *NheI* and the gel-purified insert ligated with the *SalI*/*NheI* digested pDEF N2.30. Colonies resulting from the transformation of competent cells with this ligation reaction were screened by colony PCR using primers flanking the multiple cloning site of pDEF (Figure 37.B).

Of these screened colonies, two clones were chosen to transfect CHO cells clones A7 and A8). Untransfected cells were eliminated from the transfection pool by antibiotic selection (hygromycin B). The resistant cells were allowed to grow to confluence and supernatant analysed by ELISA and immunoblotting (Figure 38.A). The A8 supernatant was analysed by sandwich ELISA. In neat supernatant a very low level of OX24 captured FH1-5/1G6 scFv was detected with a polyclonal anti-FH antiserum. Detection levels of A8 were increased by concentration of the protein in supernatant using spin-columns or nickel-NTA resin. Wild-type CHO cell culture supernatant was used as negative control. Culture supernatant of a histidine-tagged FH protein (Ba1) was included as positive control for the nickel-NTS resin enrichment. No protein was detected by immunoblotting and intracellular retention of the protein excluded by including cell lysates (Figure 38.B). Since the nickel-NTA resin allowed enrichment of the protein from the supernatant, purification of a small batch of protein was attempted using a nickel-charged chelating column (Figure 38. C). The collected fractions were analysed by SDS-PAGE followed by immunoblotting (Figure 38.D).

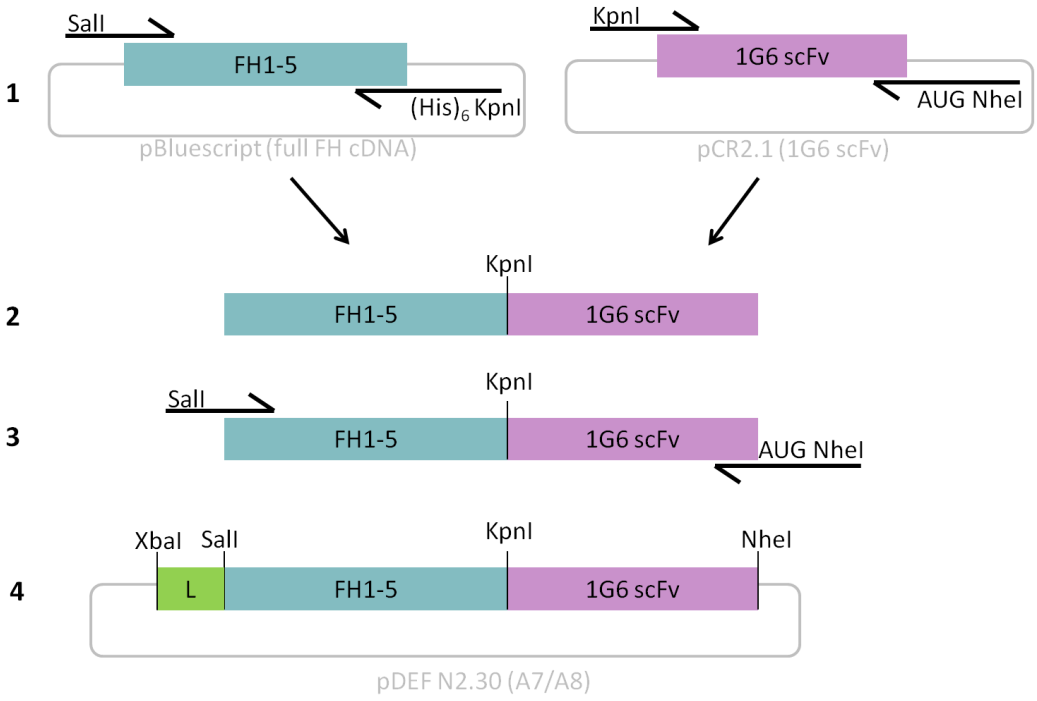


Figure 36: Cloning strategy FH1-5/1G6 scFv

(1) SCRs 1-5 were amplified from pBluescript containing the full length FH cDNA. The primers were designed to introduce a *SalI* restriction site at the 5' and a histidine-tag and *KpnI* site at the 3' end. The synthesised 1G6 scFv gene was amplified from PCR2.1 with primers introducing a 5' *KpnI* site and a stop codon and *NheI* site at the 3' end. **(2)** Both PCR products were *KpnI* digested and ligated. **(3, 4)** The ligated DNA fragments were amplified by PCR with the external primers and cloned into pDEF N2.30.

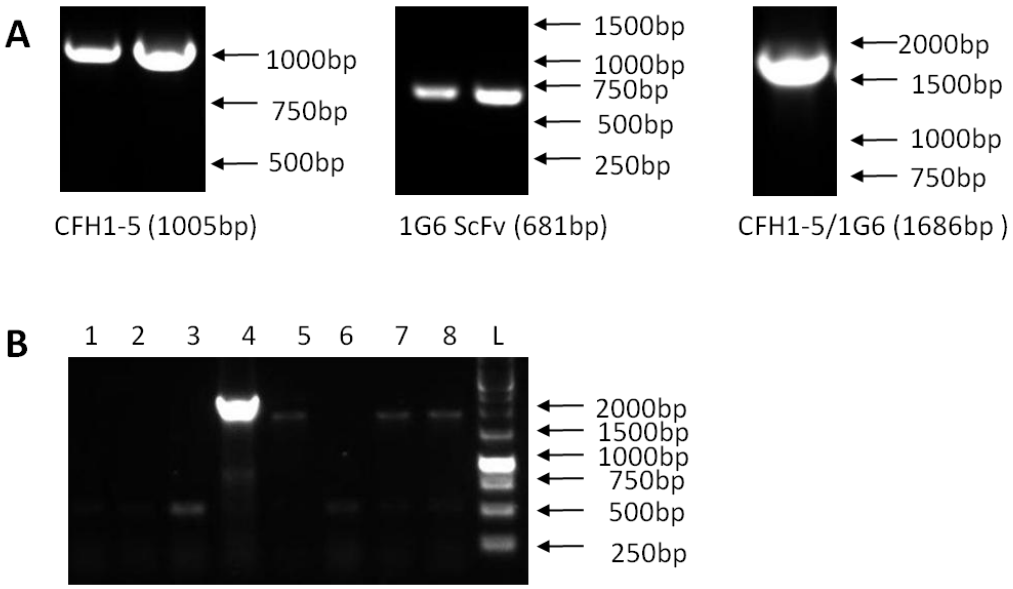


Figure 37: PCR assembly and cloning of FH1-5/1G6 into pDEF N2.30

(A) The PCR fragments for the FH1-5/1G6 scFv fusion construct were amplified individually by standard PCR and appropriate restriction sites introduced. Two different concentrations of template DNA were used and a band of approximately 1000 bp obtained for FH1-5 and a band of approximately 650 bp was obtained for the 1G6 scFv fragment. After ligation of the *KpnI* digested PCR fragments, the ligated DNA was amplified using the flanking primers. The resulting PCR product (approximately 1686) was purified and digested with *SalI* and *NheI* and ligated with the *SalI*/*NheI* digested pDEF N2.30 mammalian expression vector. **(B)** *E. coli* DH5alpha competent cells were transformed with the ligation mixture and colonies screened by colony PCR using primers flanking the pDEF multiple cloning site.

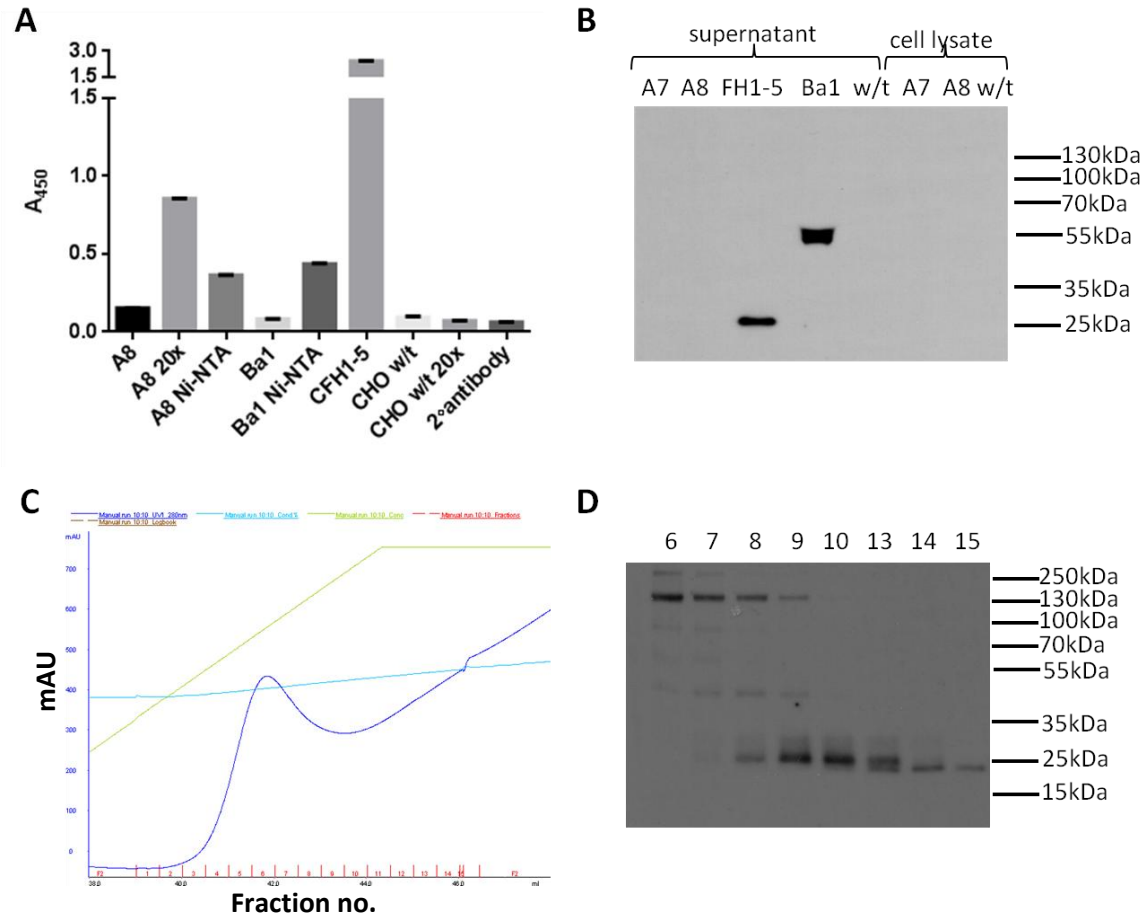


Figure 38: Detection of FH1-5/1G6 scFv expression in transfection pools

The expression levels of CHO cells (transfection pool) transfected with either FH1-5/1G6 scFv DNA clone A7 or A8 were assessed by ELISA and / or immunoblotting. **(A)** To assess A8 expression by ELISA, plates were coated with OX24, and bound FH proteins in neat A8 supernatant (A8), 20-fold concentrated A8 supernatant (A8 20x) or A8 protein enriched with Ni-NTA resin (A8 Ni-NTA) detected using a polyclonal anti-human FH antiserum. Supernatant containing a histidine-tagged control protein was used as positive control (Ba1; Ba1 Ni-NTA) for the Ni-NTA resin concentration. Neat FH1-5 supernatant was included as a positive control. Supernatant from wild-type CHO cells was used as negative control, either neat (CHO w/t) or 20-fold concentrated (CHO w/t 20x). **(B)** Neat supernatants (A7, A8, FH1-5 or Ba1) or cell lysates (A7, A8) were analysed by SDS-PAGE followed by immunoblotting using the same detection antibody as for the ELISA. **(C)** 75ml of A8 supernatant from the transfection pool was purified using a nickel-coated metal chelating column and eluted with a imidazole gradient (0-0.5M). **(D)** The fractions were analysed by SDS-PAGE and immunoblotting as for (C).

The main elution peak contained primarily non-specifically bound proteins; the later fractions (7-9) showed a faint band of approximately the expected size (~52 kDa under non-reducing conditions). More prominent bands in fractions 8-15 were two bands of ~25 kDa in size. These correspond to FH1-5 fragments. The low expression level prompted further analysis of the sequence. An additional leader sequence was identified upstream of the *SalI* site used to clone FH1-5 into N2.30. Since this could have contributed to low expression of the protein, the insert was removed from N2.30 and ligated with S21 as outlined in Figure 39. The correct DNA sequence of clones 3 and 4 was confirmed by sequencing. These two clones were subsequently named F3 and F4 and taken forward for transfection.

5.2.2 Generation of the FH1-5/mAb3 scFv expression vector

The initial strategy for generation of the FH1-5/mAb3 scFv expression vector is outlined in Figure 40. The VH and VL fragments were amplified by standard PCR (Figure 41.A). A 5' *KpnI* site and a 3' overhang for the $(G_4S)_3$ linker (adapted from (Kontermann and Dübel, 2010) were introduced in the VH fragment and a 5' linker overhang and 3' stop codon and *NheI* site in the VL fragment. The scFv was assembled by extension overlap PCR, in which the compatible linker overhangs serve as primers to link the two fragments but the favoured product is produced by the VH 5' primer and the VL 3' primer (Figure 41.A). The FH1-5 fragment and mAb3 scFv were, as for the FH1-5/1G6 scFv above, assembled by restriction digest followed by ligation and PCR with the flanking 5' and 3' primers. The resulting DNA fragment (Figure 41.B) was cloned into S21 using the *SalI* and *NheI* restriction sites and the DNA of several clones analysed by sequencing. The sequencing results consistently showed extensive mismatching in the region of the $(G_4S)_3$ linker (example shown in Figure 42). Due to time constraints, optimisation of this approach with regards to annealing temperature and template concentration was not feasible. Instead, the mAb3 scFv was synthesised (Eurofins DNA Operon, Munich) and the cloning strategy adapted accordingly (Figure 43.A1 and 43.A2). The DNA sequence of the mAb3 scFv is shown in Appendix B. To propagate the plasmid containing the synthesised mAb3 scFv gene, competent cells were transformed and the plasmid DNA (pCR2.1 mAb3 scFv) purified. The pCR2.1 mAb3 scFv plasmid DNA was amplified, digested with *KpnI* and *NheI*, purified and ligated with the *KpnI*/*NheI* digested, purified F3 plasmid (FH1-5 1G6 scFv) (Figure 43.B). Competent cells were transformed with the ligation mixture and resulting colonies screened by colony PCR (Figure 43.C). PCR of clones 1, 13 and 15 yielded a band of the expected size for FH1-5 and the mAb3 scFv. These clones were taken forward for sequence analysis. All three clones contained the correct sequence; clone 13 was taken forward for transfection of mammalian cells.

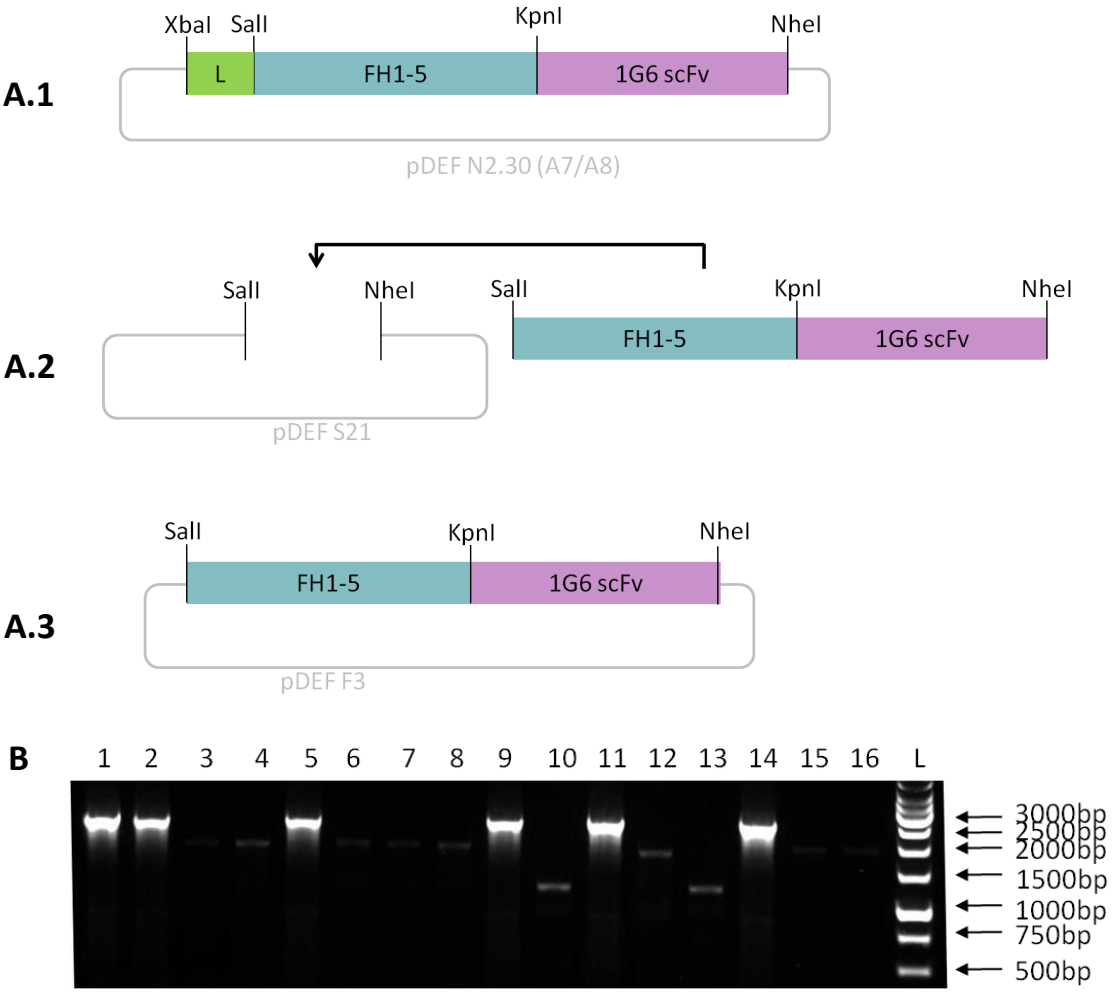


Figure 39: Transfer of FH1-5/1G6 scFv from N2.30 (A7) to pDEF S21

(A.1) To transfer the FH1-5/1G6 insert from the A7 pDEF N2.30 vector to pDEF S21 (which lacks the CD33 signal peptide) A7 was digested with *SalI* and *NheI* (A.2) and ligated with the *SalI*/*NheI* digested pDEF S21 to form pDEF F3 (A.3). **(B)** Competent cells were transformed with the ligation mixture and resulting colonies screened by colony PCR using primers flanking the pDEF multiple cloning site.

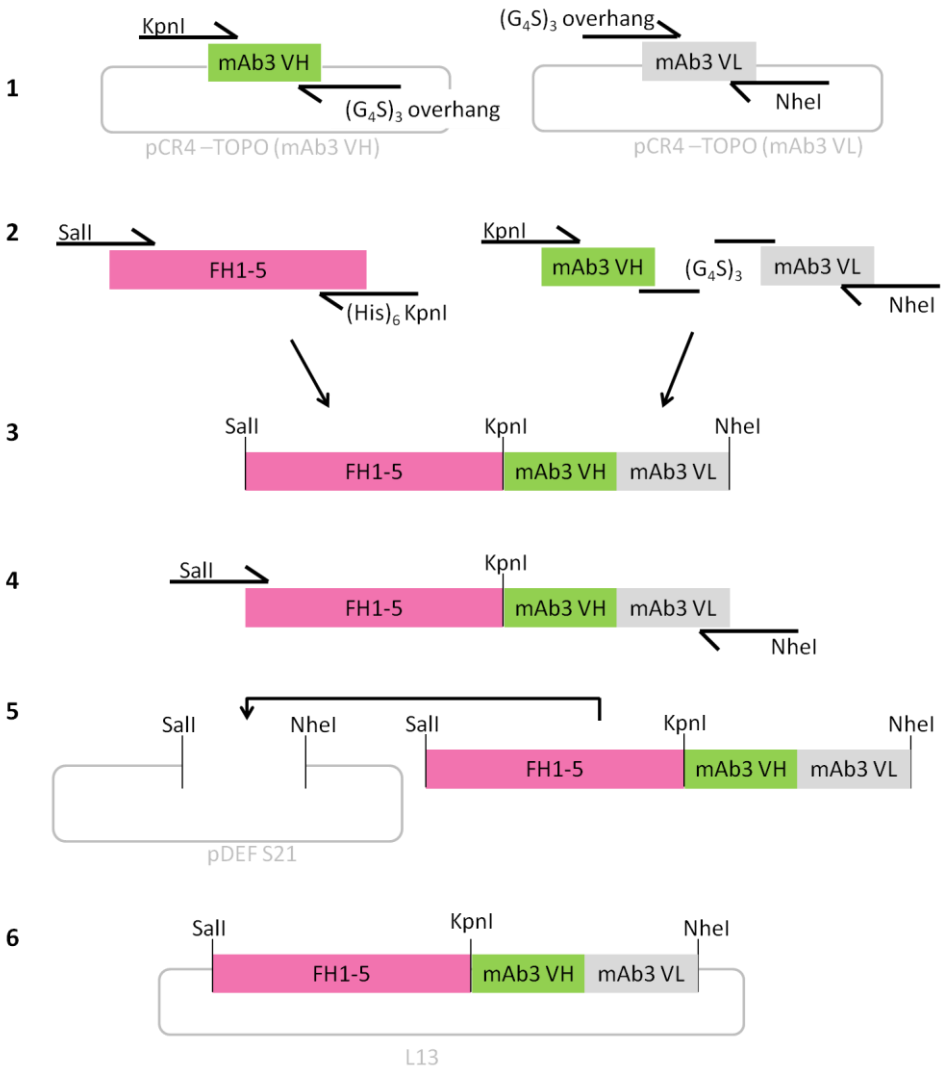


Figure 40: Cloning strategy FH1-5/mAb3 scFv

To assemble the FH1-5/mAb3 scFv fusion protein, the three individual genes were amplified by PCR to introduce restriction sites and overhangs for downstream cloning. **(1)** The mAb3 VH and VL were amplified from the PCR4-TOPO clones with confirmed sequence. A 5' *KpnI* site and 3' overhang for the glycine serine linker were introduced in the VH and a 5' partly complementary linker overhang and 3' stop codon and *NheI* site in the VL. **(2)** The FH1-5 DNA was amplified by PCR and a *SalI* site and histidine-tag / *KpnI* site introduced at the 5' and 3' ends, respectively. The VH and VL DNA was fused by overlap extension PCR. The partly complementary linker served to prime the two fragments and external primers were used for amplification. **(3)** The amplified FH1-5 DNA and the product of the overlap extension PCR were purified, *KpnI* digested and ligated. **(4)** The ligation mixture was then used as a template to amplify ligated DNA fragments. **(5)** The resulting PCR product was digested with *SalI* and *NheI* and ligated with the *SalI*/*NheI* digested pDEF S21 vector to obtain the final expression vector **(6)**.

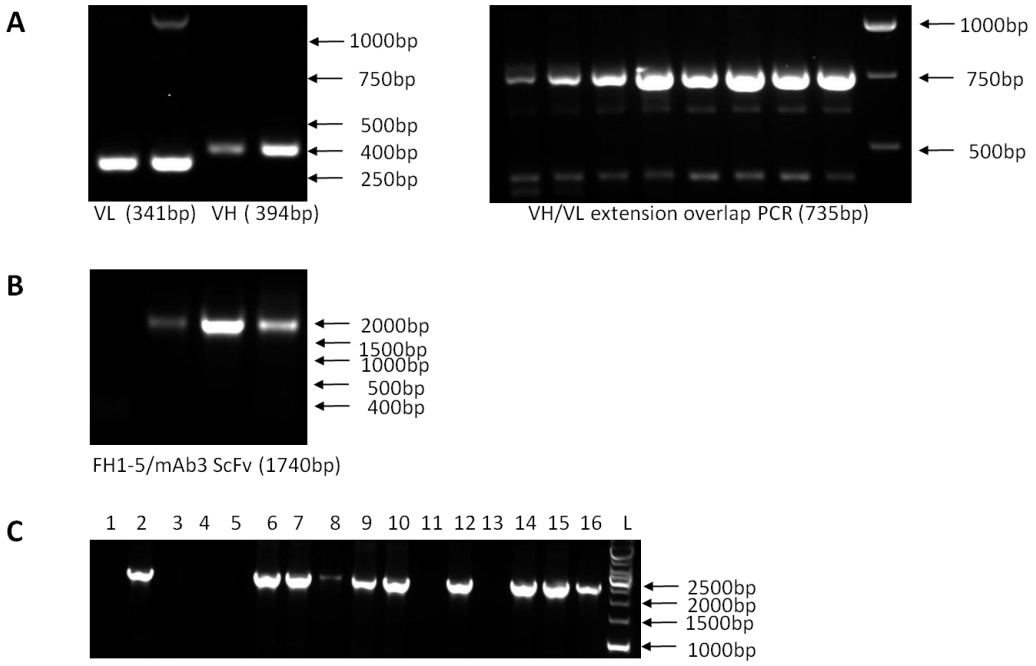


Figure 41: Assembly of the FH1-5/mAb3 scFv by PCR

(A) The mAb3 VH (394 bp) and VL (341 bp) were first amplified individually by PCR using two different amounts of template DNA each. The scFv DNA (735 bp) was then assembled by overlap extension PCR using a range of different concentrations. **(B)** PCR was then used to amplify the ligation reaction of FH1-5 and the mAb3 scFv using three different DNA ratios. The product obtained was then *SalI*/*NheI* digested and ligated with the *SalI*/*NheI* digested pDEF S21. **(C)** Colonies resulting from transformation of competent cells with the ligation mixture were analysed for the presence of insert by colony PCR using primers flanking the pDEF multiple cloning site.

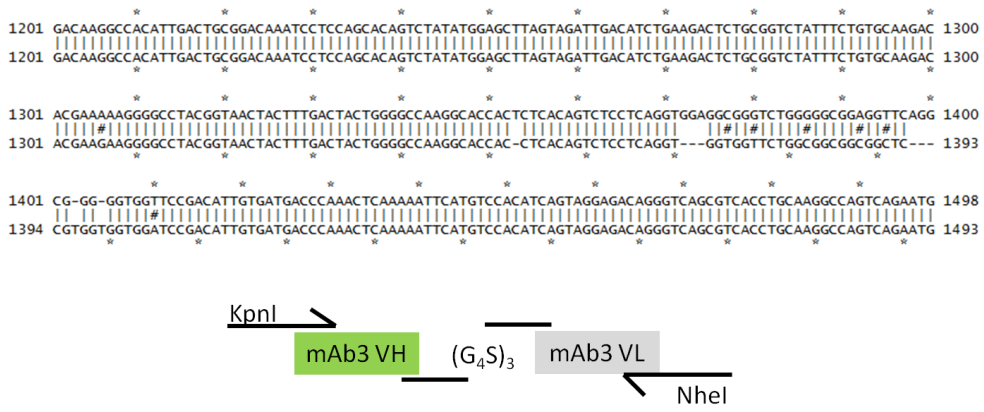


Figure 42: Mismatched linker after the mAb3 scFv overlap extension PCR

Sequencing of all clones generated using overlap extension PCR for assembly of the mAb3 scFv showed extensive mismatching and deletions in the linker region. The sequence shown is representative of 10 clones that were sequenced from two independent cloning experiments.

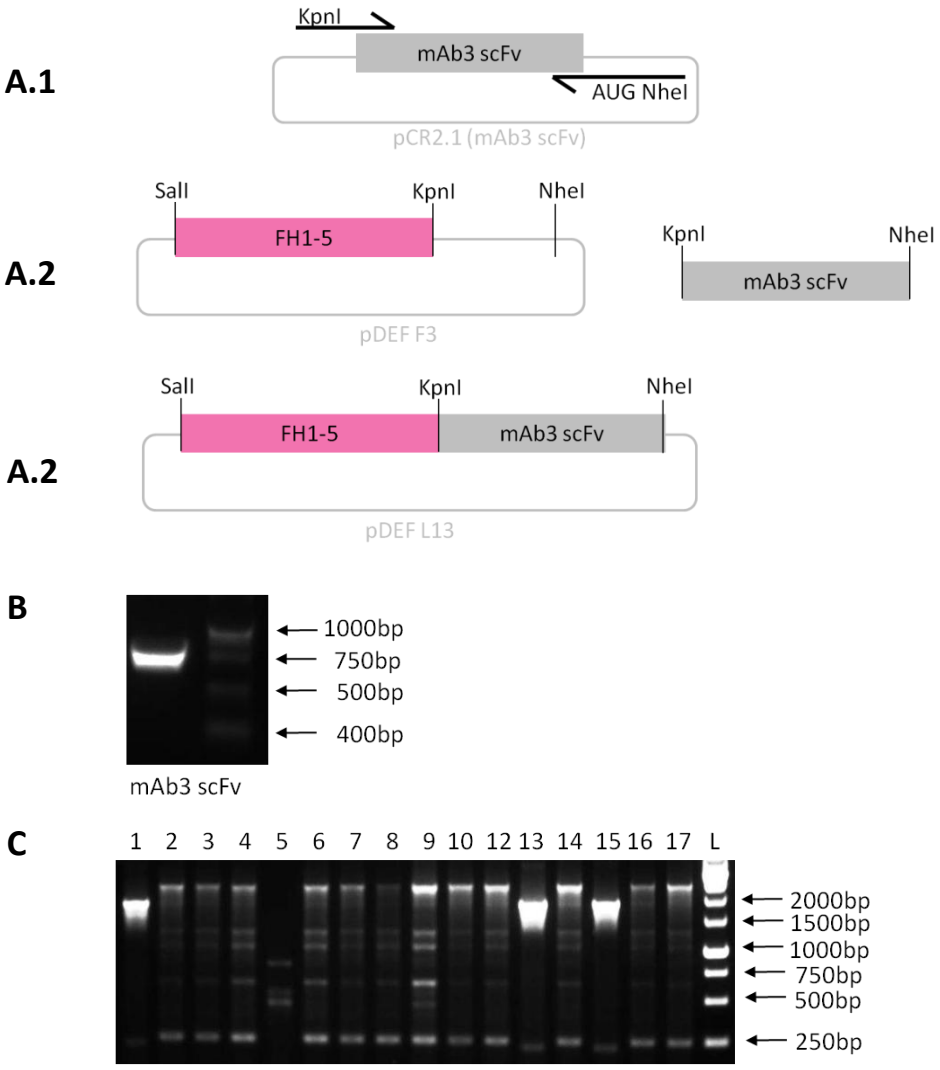


Figure 43: Cloning of FH1-5/mAb3 scFv vector from synthesised mAb3 scFv

(A.1) The mAb3 scFv was amplified from the synthesised gene in pCR2.1 by PCR using primers that introduce a *KpnI* site and stop codon / *NheI* site at the 5' and 3' ends, respectively. **(A.2)** The fragment generated was ligated with the *KpnI*/*NheI* restricted pDEF F3 (Fh1-5/1G6 scFv) resulting in FH1-5/mAb3 scFv ("L"). **(B)** Amplification of the synthesised mAb3 scFv from PCR2.1. **(C)** Colony PCR of the "L" transformed *E. coli* colonies.

5.2.3 Expression of the FH1-5/scFv fusion proteins in CHO cells

CHO cells were chosen as expression host for both FH1-5/scFv constructs. These cells provided satisfactory protein yield for the FH1-5 and FH1-5/18-20 constructs. Wild-type CHO cells were transfected with F3, F4 (1G6 scFv) or L13 (mAb3 scFv) plasmid DNA using the jetPRIME transfection reagent (as described in the methods sections). After selection with hygromycin, individual colonies were isolated with cloning discs and protein expression tested by immunoblotting which served to test integrity of the proteins at the same time (Figure 44). For the L13 transfections five clones were isolated, of which all but L13.1 showed expression of the fusion protein as indicated by detection of a band of the correct size (~62 kDa) by immunoblotting with a polyclonal anti-human FH antiserum (Figure 44.A). The protein runs lower under non-reducing conditions due to the presence of 12 disulphide bonds, ten in FH1-5 and two in the scFv part. The expression levels of the clones analysed were similar, with L13.2 showing slightly higher expression. For the F3 and F4 transfections, four and five clones were isolated, respectively. For all clones, except F3.2 and F4.1 a band of the correct size was detected. The predicted molecular weight of the FH1-5/1G6 scFv is ~62 kDa. Of the expressing clones, F3.3 and F4.2 showed the highest levels of expression. However, all clones showed two bands in addition to the band of the presumed intact protein. These lower molecular weight bands were likely the FH1-5 fragment of the fusion protein with different degrees of reduction. Analysis of the proteolytic cleavage sites in the protein sequence of FH1-5/1G6 scFv using online tools (Appendix C) did not reveal a unique site near the linker region of the fusion protein (PeptideCutter, ExPASy; (Walker *et al*., 2005). The temperature dependence of the cleavage of was tested by storage of fresh, sterile-filtered supernatant at 37 °C, 4 °C or -20 °C for 24 hours. Supernatants of F3.3 and L13.2 were included and the samples analysed by non-reducing SDS-PAGE followed by immunoblotting with OX24 and the polyclonal anti-human *CFH* antiserum (Figure 44.B and 44.C). The results indicate that storage at different temperatures does not affect the fragmentation of F3.3 or L13.2.

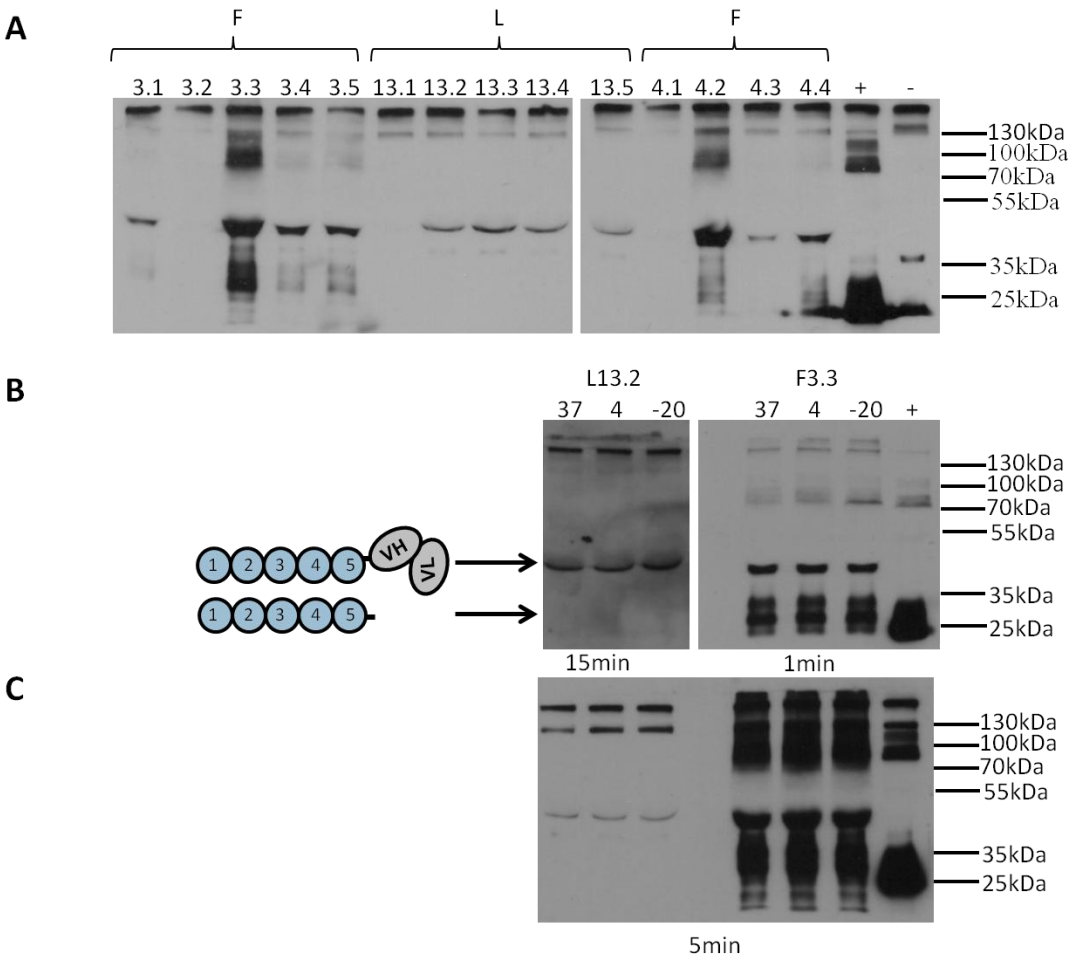


Figure 44: Expression screen and effect of temperature on stability

(A) Cell culture supernatant of five F3 clones (F3.1, F3.2, F3.3, F3.4, F3.5), four F4 clones (F4.1, F4.2, F4.3, F4.4) and five L13 clones (L13.1, L.13.2, L13.3, L13.4, L13.5) was analysed by SDS-PAGE followed by immunoblotting. Recombinant proteins were detected with a polyclonal goat anti-human FH antiserum, followed by a HRP-conjugated donkey anti-goat Ig antibody. FH1-5 was included as positive control (+), wild-type CHO cell supernatant as negative control (-).
(B) Stability of FH1-5/1G6 scFv at different storage temperatures (37, 4 or -20 °C) was assessed SDS-PAGE and immunoblotting. Proteins were detected with OX24 followed by a HRP-conjugated sheep anti-mouse Ig antibody. **(C)** Alternatively membranes were probed with a polyclonal anti-human FH antiserum as in (A).

5.2.4 Purification of the FH1-5/scFv fusion proteins

For purification of the FH1-5/scFv fusion proteins a hexa histidine-tag was included between the FH SCRs and the scFv. First the same conditions (40 mM imidazole in the binding buffer) were used as for purification of FH1-5 and FH1-5/18-20. However, no or very little protein was eluted under these conditions and for the fragmented FH1-5/1G6 scFv the FH1-5 fragment bound stronger as suggested by the later elution in Figure 35. Binding and elution of FH1-5/mAb3 scFv was therefore tested using a range of imidazole concentrations (Figure 45). The chromatography traces show decreasing elution peak size with increasing concentration of imidazole. The highest amount of eluted FH1-5/mAb3 scFv is eluted in the presence of 10 mM imidazole as detected by immunoblotting (Figure 45.E). This concentration of imidazole was therefore used to purify the fusion proteins from the cell culture supernatant (Figures 46 and 47). For the purification of FH1-5/1G6 shown in Figure 46 1 L of cell culture supernatant was applied to the column. The bound protein eluted in a main peak followed by a small peak. Immunoblotting of the collected fractions after non-reducing SDS-PAGE revealed several bands (Figure 46.B). Fractions 7-9 contain three main bands. The band at ~45 kDa corresponds to the intact FH1-5/1G6 scFv and the band at over 55 kDa corresponds to migration distance of reduced FH1-5/1G6 but may, like the band at ~100 kDa, be aggregated or multimerised protein. Additional lower molecular weight bands appear in fractions 10-12. These bands at ~25 kDa and ~30 kDa correspond to the size of FH1-5 fragments separated from the scFv. The number of bands shown under non-reducing conditions is reduced to two clear bands under reducing conditions as shown in Figure 46.C. The band at ~ 36 KDa corresponds to FH1-5 and the band of approximately 62 kDa corresponds to the intact FH1-5/1G6 fusion protein. The FH1-5/mAb3 fusion protein was purified and analysed in the same way (Figure 47). For the purification shown 500 ml of cell culture supernatant were applied to the column in the presence of 10 mM imidazole and bound protein eluted in a single peak (Figure 47.A). The Coomassie blue stained SDS-PAGE gel shows a band of the correct size (below 55 kDa under reducing conditions, 62 kDa under non-reducing conditions) in lanes 9 and 10 in addition to several contaminating protein bands (Figure 47.B). The identity of these bands was confirmed by immunoblotting; a single band of the correct size was detected in fractions 7-11 (Figure 47.C). Both fusion proteins were concentrated and buffer exchanged using concentrator spin-columns with a 10 kDa molecular weight cut-off. However, no or very little protein was detected in the concentrate by SDS-PAGE followed by Coomassie blue staining or immunoblotting. This suggests that the protein may have precipitated or strongly interacted with the spin-column filter.

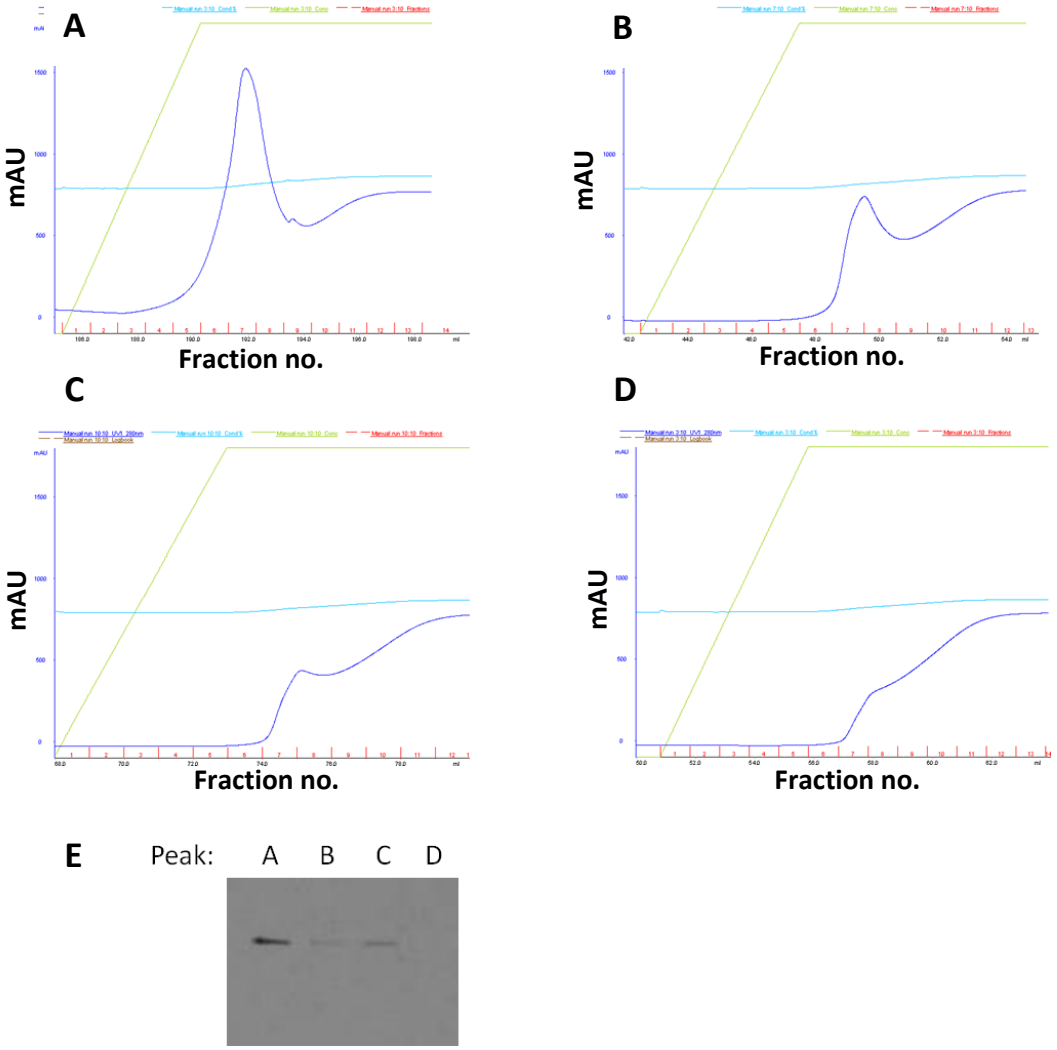


Figure 45: Optimisation histidine tag purification L13.2

(A-D) Binding of L13.2 to the nickel-coated chelating column was tested in the presence of 10, 20, 30 or 40 mM imidazole (A-D). Protein bound to the column was eluted with an imidazole gradient (0-0.5 M, green line) and 12 1 ml fractions collected (x-axis). Absorbance of the eluted protein is given in mAU (y-axis). **(E)** The peak fraction of each elution was analysed by immunoblotting after SDS-PAGE under non-reducing conditions (anti- human FH polyclonal antiserum). The amount of eluted protein decreases the higher the imidizaole concentration in the binding buffer.

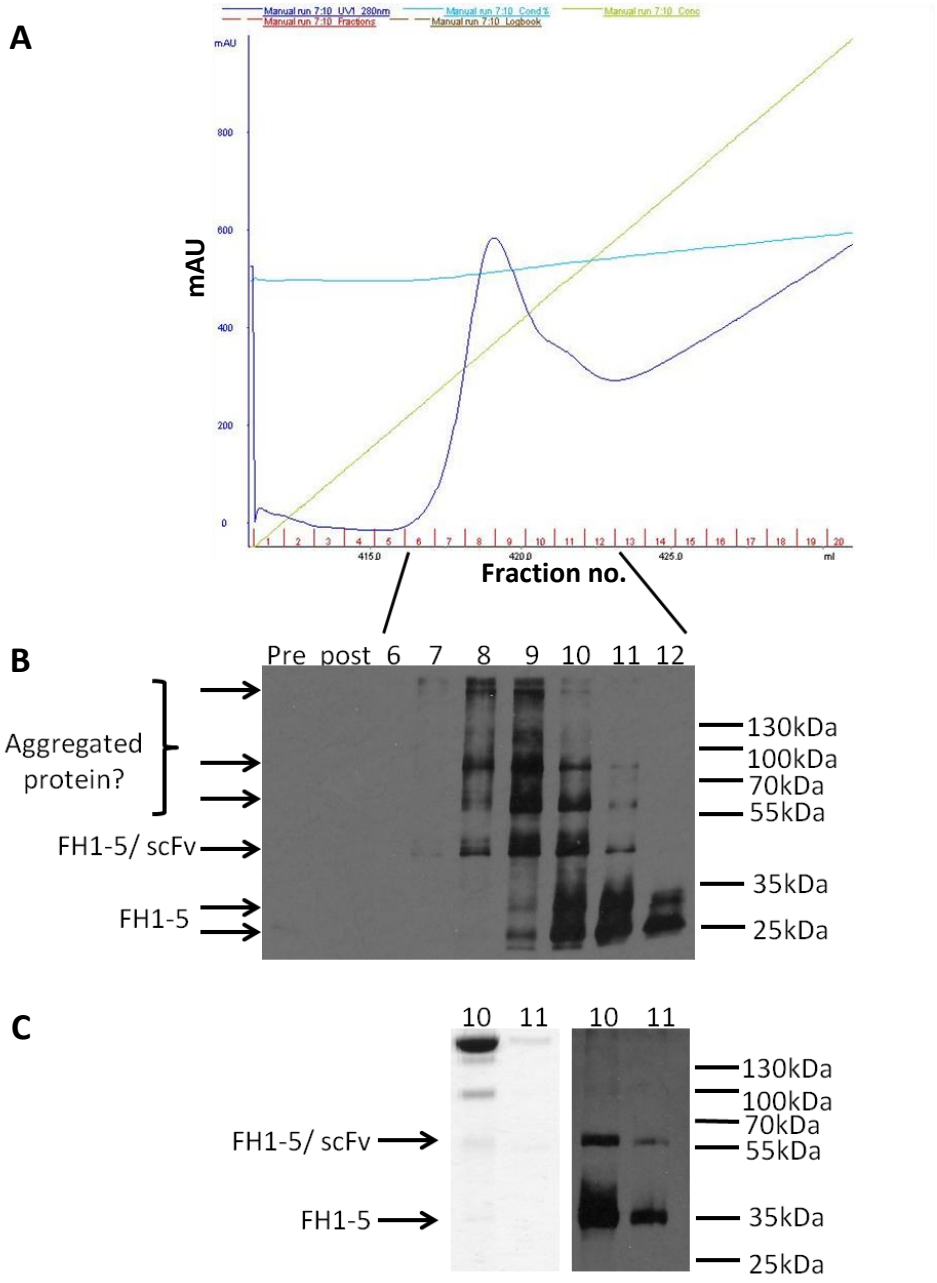


Figure 46: Histidine tag purification of FH1-5/1G6 scFv

(A) FH1-5/1G6 scFv was purified from CHO cell culture supernatant using IMAC. Protein bound to the column in the presence of 10 mM imidazole was eluted with a imidazole gradient (0-0.5 M, green line) and 20 1 ml fractions collected (x-axis). Absorbance of the eluted protein is given in mAU (y-axis). **(B)** Fractions 6-12 and pre and post column samples were analysed by immunoblotting after SDS-PAGE under non-reducing conditions (anti- human FH polyclonal antiserum). Identified bands are indicated by arrows. **(C)** Fractions 10 and 11 were analysed by SDS-PAGE under reducing conditions, followed by Coomassie blue staining or immunoblotting (anti-human FH polyclonal antiserum). Identified bands are indicated by arrows.
The data shown is representative of several independent experiments.

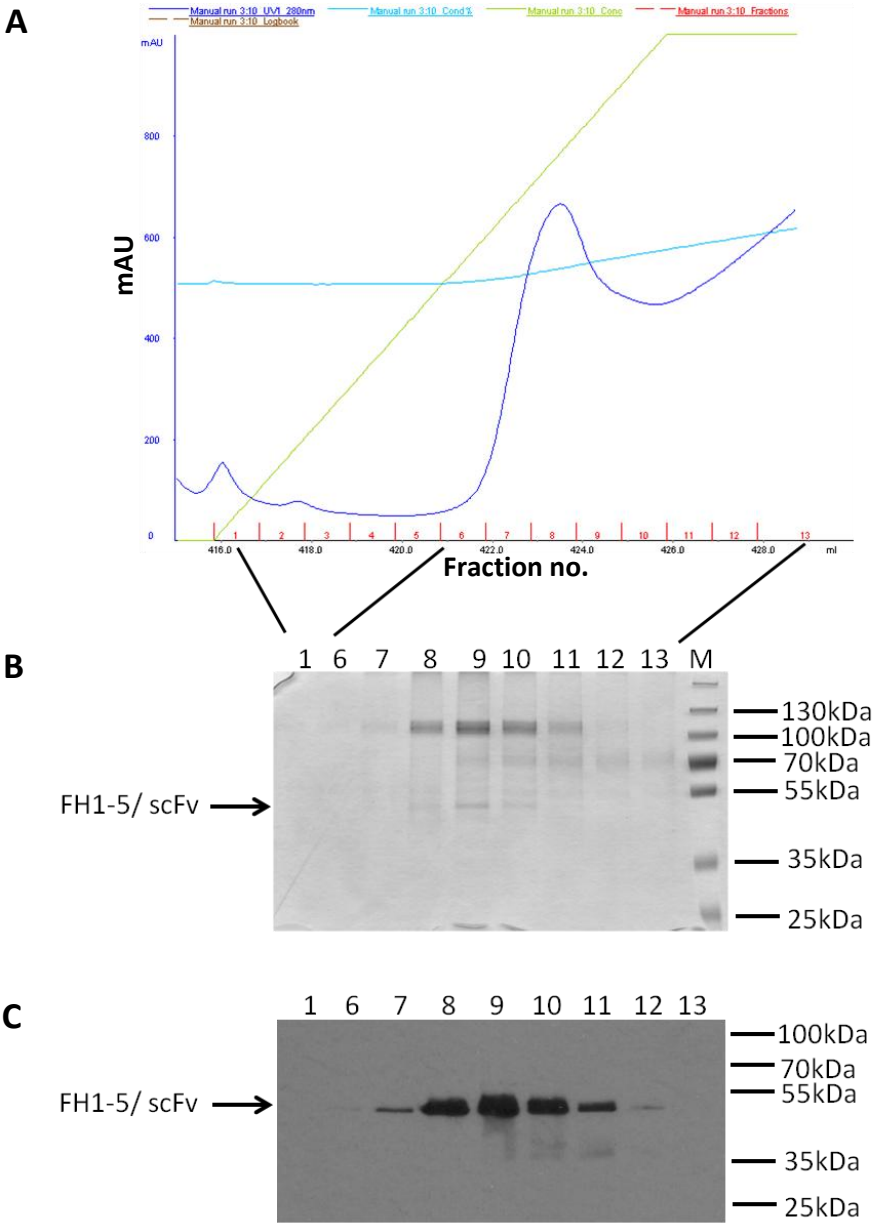


Figure 47: Histidine tag purification of FH1-5/mAb3 scFv

(A) FH1-5/mAb3 scFv was purified from CHO cell culture supernatant using IMAC. Protein bound to the column in the presence of 10 mM imidazole was eluted with a imidazole gradient (0-0.5 M, green line) and 13 1 ml fractions collected (x-axis). Absorbance of the eluted protein is given in mAU (y-axis). **(B)** Fractions 1 and 6-13 were analysed by SDS-PAGE under non-reducing conditions followed by Coomassie blue staining. The band for FH1-5 / mAb3 scFv is indicated by an arrow. **(C)** The fractions were also analysed by immunoblotting (anti- human FH polyclonal antiserum). Identified bands are indicated by an arrow.
The data shown is representative of several independent experiments.

The histidine-tag purification did not work as well as for FH1-5 and FH1-5/18-20. The low concentration of imidazole in the binding buffer allowed binding and co-elution of non-specific proteins along the relatively small amount of fusion protein. An alternative affinity method was therefore used in an attempt to improve purity. OX24 is a mAb against FH and binds in SCR 5. As described in the methods section, an OX24 column was prepared by coupling of the mAb to the NHS-activated sepharose matrix of a 1 ml column (GE Healthcare). For the purifications shown in Figure 48.A (FH1-5/1G6 scFv) and 48.B (FH1-5/mAb3 scFv) 500ml of CHO cell culture supernatant was applied to the column and the protein eluted with a low pH glycine buffer (pH 2.5). Elution is expected to occur when the conductivity drops, i.e. when the PBS used to wash the column after application of the supernatant is replaced with the elution buffer. For FH1-5/1G6 scFv an elution peak of approximately 800 mAU was recorded (Figure 48.A). The four collected fractions were analysed by SDS-PAGE followed by immunoblotting (Figure 48.A). A main band of ~36 kDa was detected. This indicates almost complete fragmentation of the fusion protein, possibly due to the low pH of the elution buffer. For FH1-5/mAb3 scFv no elution peak was observed and the fractions therefore not further analysed (Figure 48.B). Purification of the fusion proteins was further attempted using protein L columns. Protein L is a bacterial protein that binds most human and some murine kappa light chain subtypes of all antibody subclasses. It is suitable for purification of scFvs and importantly does not bind bovine immunoglobulins. 200 ml of cell culture supernatant was applied to the protein L columns for each fusion protein and eluted with a low pH buffer as for the OX24 column. As shown in Figure 48.C no peak was recorded for FH1-5/1G6 scFv and a peak of ~20 mAU was recorded for FH1-5/mAb3 scFv (Figure 48.D).

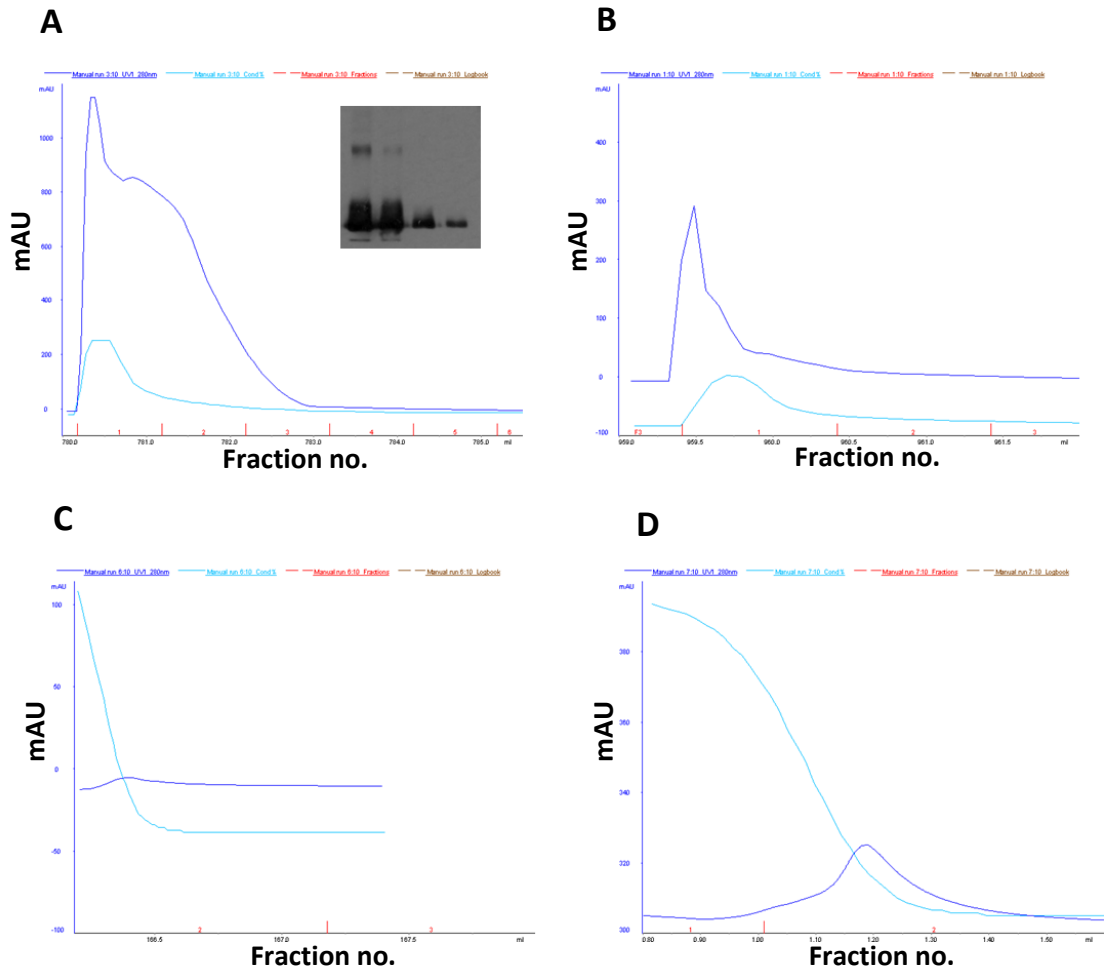


Figure 48: OX24 and protein L purification of FH1-5/1G6 scFv and FH1-5/mAb3 scFv

(A) FH1-5/1G6 scFv CHO cell culture supernatant was applied to an OX24 column and bound protein eluted using a low pH buffer (conductivity shown as light blue line). Fraction 1 and 2 were analysed by reducing SDS-PAGE followed by immunoblotting (anti-human FH polyclonal antiserum). **(B)** CHO cell culture supernatant was applied to an OX24 column and eluted with a low pH buffer. Due to the low absorbance reading the fractions were not further analysed. **(C)** / **(D)** FH1-5/1G6 scFv supernatant and FH1-5/mAb3 scFv supernatant was applied to a protein L column and elution performed with a low pH buffer (conductivity shown as light blue line). Due to the low absorbance reading the fractions were not further analysed.
Shown are representative elution profiles from several experiments.

5.2.5 Binding of FH1-5/1G6 scFv and FH1-5/ mAb3 scFv to C3b and type IV collagen

No intact, pure protein was obtained for functional analysis of the FH1-5/scFv fusion proteins due to low expression levels and difficulties purifying the protein from the cell culture supernatant. The ligand binding was therefore carried out by sandwich ELISA using cell culture supernatant. To assess the antigen-binding function of the scFvs, type IV collagen NC1 domains isolated from bovine and murine renal GBM (Chondrex Inc.) were used as model antigen. Collagen type IV from the murine Engelbreth-Holm-Swarm tumour cell line lacks the α3-chain and was included as negative control. The NC1 domains and control collagen were coated on microtitre plates and binding of the fusion proteins contained in the cell culture supernatant was detected in the same way as binding to C3b. FH1-5/mAb3 scFv showed no binding to bovine, murine or EHS collagen (Figure 49.A, B and C). FH1-5/1G6 scFv bound to bovine and murine NC1 domains and showed some reactivity with the EHS collagen type IV (Figure 49.A, B and C). No non-specific binding was detected for the wild-type CHO cell supernatant, BSA or the irrelevant fusion protein (C3d-Fc). Binding of FH1-5/1G6 scFv and FH1-5/mAb3 scFv to C3b was also tested. C3b was coated on 96-well microtitre plates and incubated with the cell culture supernatants which were serial-diluted across the plate. Wild-type CHO cell culture supernatant was included to control for non-specific binding of endogenous CHO cell proteins or bovine proteins derived from the FCS. In addition BSA and C3d-Fc were included as controls for non-specific binding. Binding of the protein to C3b was detected with a polyclonal anti-human FH antiserum. As shown in Figure 49.D binding of FH1-5/1G6 scFv to C3b was detected, the signal for FH1-5/mAb3 scFv was only marginally above the background. This probably reflects on the expression levels of this construct. FH1-5/mAb3 scFv was therefore transiently expressed in HEK293t cells and as shown in Figure 50.A expression levels improved. Using this supernatant it was possible to demonstrate C3b binding (Figure 50.B), however binding to bovine and murine NC1 domains was still low (Figure 50.C, D).

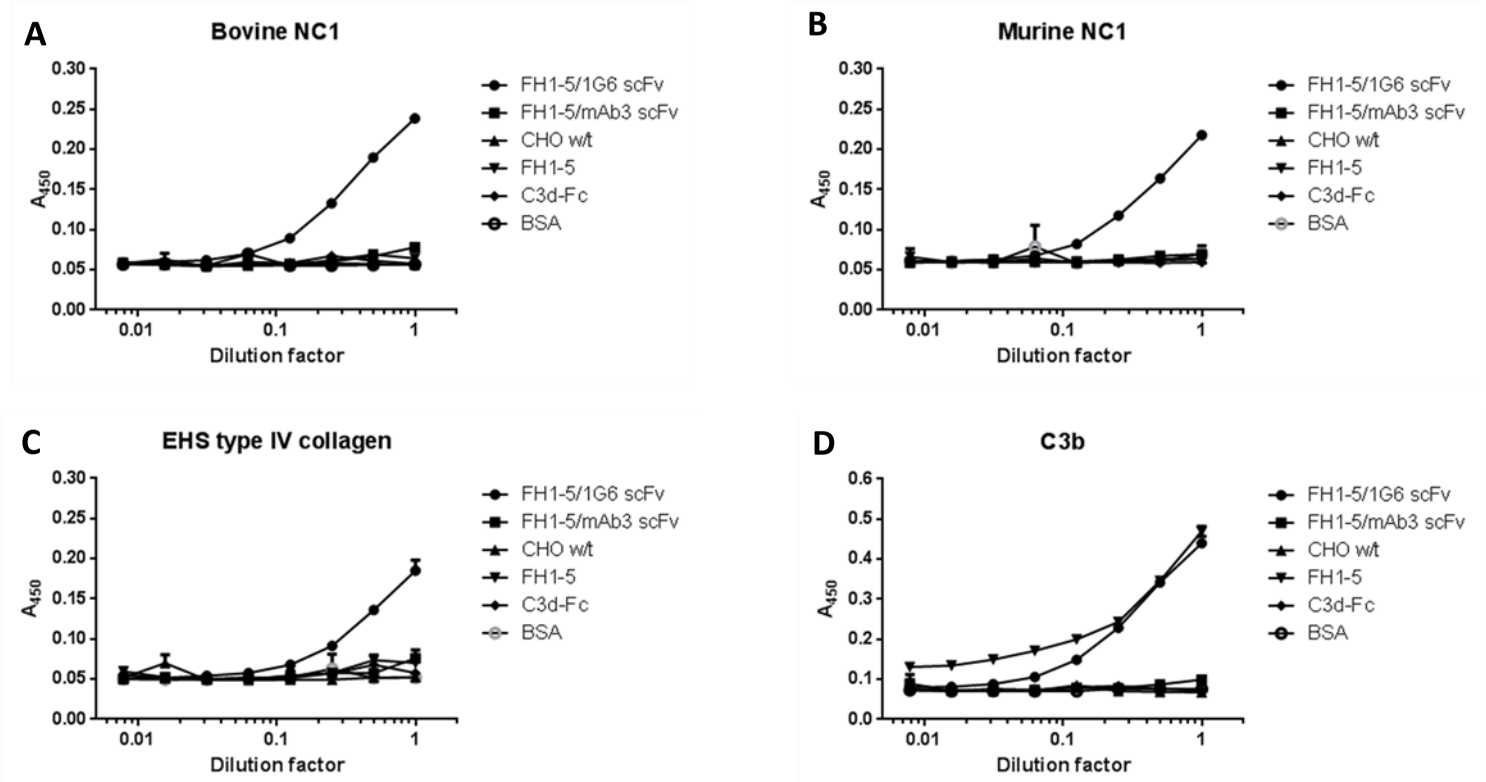


Figure 49: C3b and NC1 domain binding of FH1-5/1G6 scFv and FH1-5/mAb3 scFv

(A/B/C) Binding of FH1-5/1G6 scFv and FH1-5/mAb3 scFV, present in cell culture supernatants, to bovine and murine NC1 domains or EHS collagen coated on microtitre plates was detected with a goat anti-human FH polyclonal antiserum followed by an HRP-conjugated donkey anti-goat Ig antibody. Wild-type CHO cell culture supernatant, BSA and an irrelevant fusion protein (C3d-Fc) were included as negative controls. **(D)** Binding of both fusion proteins to C3b, coated to microtitre plates, was detected in the same way as binding to the NC1 domains. Wild-type CHO cell culture supernatant, BSA and an irrelevant fusion protein (C3d-Fc) were included as negative controls. Supernatant from the FH1-5 expressing CHO cell line was included as positive control. Samples were applied in triplicates, shown is one representative experiment of two.

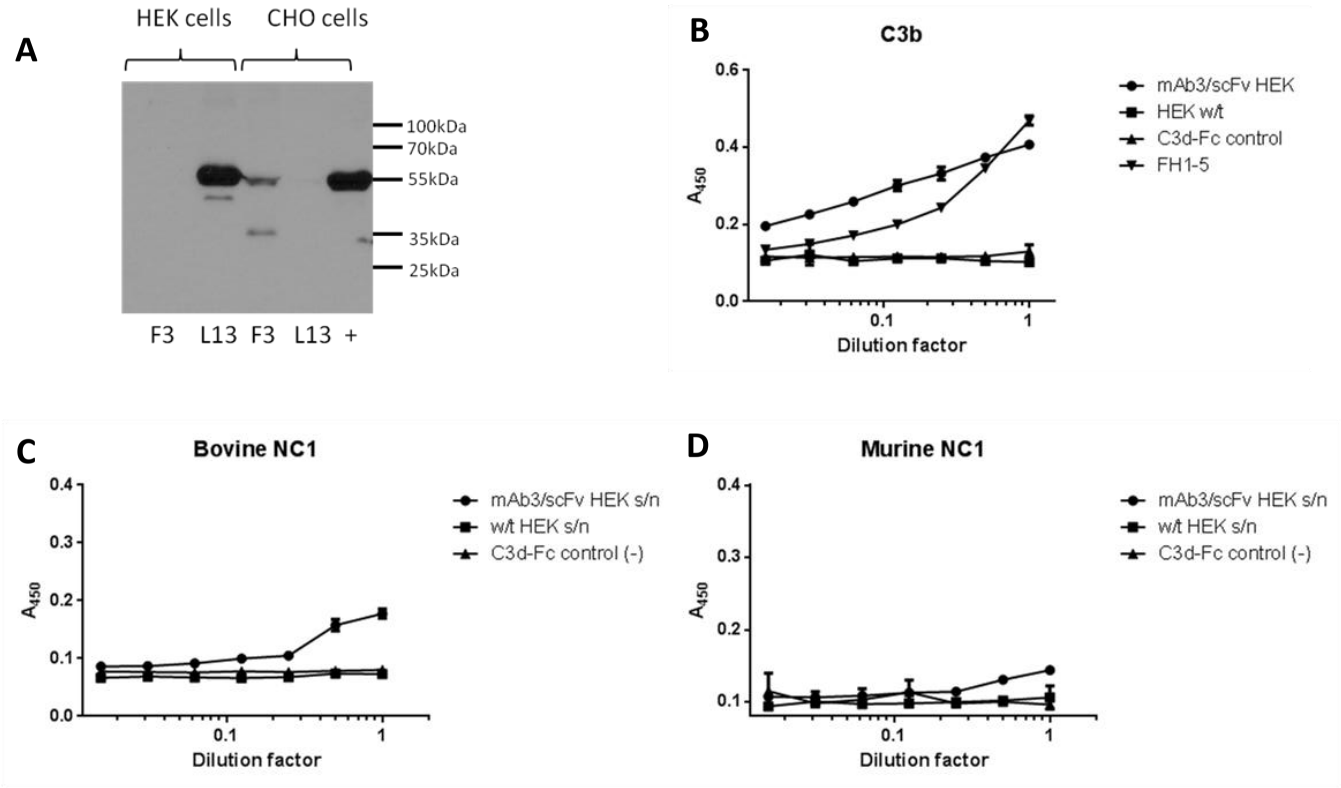


Figure 50: Ligand binding of FH1-5/mAb3 scFv transiently expressed in HEK293t cells

(A) HEK 293t cells were transfected with the FH1-5/mAb3 scFv and FH1-5/1G6 scFv DNA constructs and supernatants analysed immunoblotting after 48 h. FH was as described above. Supernatants of the stable transfected CHO cell lines were included for comparison. FH1-5/18-20 was included as positive control. **(B)** Binding of FH1-5/mAb3 scFv to C3b was detected as described above. Wild-type HEK293t cell culture supernatant and an irrelevant fusion protein (C3d-Fc) were included as negative controls, FH1-5 as positive control (n=1). **(C/D)** Binding of FH1-5/mAb3 scFv to bovine and murine NC1 domains was detected as described above. Controls included as for C3b binding. All samples were applied in triplicates, shown is one representative experiment of two.

5.3 Discussion

5.3.1 Generation of FH1-5/scFv fusion proteins

Two FH1-5/scFv fusion proteins were generated. The 1G6 scFv was synthesised directly and the mAb3 scFv was synthesised after several attempts to assemble the scFv by overlap extension PCR failed (Figures 41 and 42). The scFv were joined downstream of FH1-5 by PCR assembly (Figures 37 and 43). Expression of FH1-5 and FH1-5/18-20 in CHO cells was successful; this expression host was therefore also used for the fusion proteins. In the cell-surface targeted FH1-5/18-20 construct the histidine tag is located between SCRs 5 and 18. Purification of this protein in presence of 40 mM imidazole to reduce non-specific binding worked very well. For both FH1-5/scFv construct the internal position of the histidine tag is not feasible. Both fusion proteins do not tolerate imidazole concentrations higher than 10 mM for binding which results in significant binding of non-specific proteins (Figure 45). This problem is easily addressed by placing the histidine-tag to at the N-terminus of the protein. The "linking" histidine tag can either remain or be replaced with an alternative linker. Attempts to purify the fusion proteins by other affinity methods (OX24, L-protein) were not successful. Further, small amounts that were purified were lost on concentrator spin columns. This may be due to adsorption of the protein to the filter membrane. Loss of some protein due to such non-specific interaction is not unusual, however may be detrimental when dealing with a small amount of protein.

In addition, fragmentation of the FH1-5/1G6 scFv is a great concern. Analysis of proteolytic cleavage sites in the sequence with online tools returned no unique cleavage site in the area of concern (between histidine tag and start of the heavy chain variable region). The fragmentation could be addressed by grafting of the CDRs from the 1G6 scFv into the mAb3 scFv framework. To address expression levels it would be necessary to repeat the transfection of CHO cells and screen a larger number of clones to identify higher expressing clones. The expression host may be the limiting factor for these constructs, although similar constructs have previously been successfully produced in CHO cells. Alternative expression hosts are human embryonic kidney (HEK) cells and SP2/0 cells. As discussed in the general introduction, expression in a yeast strain like *P. pastoris* may present an alternative to increase protein yields. For this the sequence of both genes would have to be codon-optimised and transferred into a suitable expression vector. Once expression problems are solved the constructs should be characterised functionally.

5.3.2 Functional analysis of the FH1-5/scFv fusion proteins

Low expression levels and purification difficulties limited the functional analysis of the constructs. Cell culture supernatants were therefore used to assess binding to C3b and collagen type IV NC1 domains by sandwich ELISA. Both proteins bind to C3b (Figure 49.D and 50.B), differences in binding likely reflect on the slightly higher expression of the FH1-5/1G6 scFv expressed in CHO cells. This is also the construct that undergoes fragmentation. It is therefore not possible to say to what extent the observed binding is due to intact FH1-5/1G6 scFv or the fragmented FH1-5.

Binding of the constructs to bovine or murine collagen IV NC1 domains was tested by sandwich ELISA (Figure 49.A, B and C). The binding pattern of FH1-5/1G6 scFv to murine and bovine NC1 domains and the control collagen are similar to that of the parent antibody (Figure 27.B). This suggests that the 1G6 scFv has retained its ability to bind to the α3-chain NC1 domains of type IV collagen. This construct is very unstable and due to its broader binding pattern is not worth pursuing further. No significant binding of the FH1-5/mAb3 scFv to NC1 domains was detected (Figure 49.A, B). Although transient expression in HEK293t cells improved the expression levels for this construct no antigen binding was detected (Figure 50.C, D). This suggests that the scFv has either lost the ability to bind the antigen or that the conditions used did not allow detection of binding. An early ELISA showed binding of this construct to bovine NC1 domains, however this was not replicated in several repeat experiments. The bovine and murine NC1 domains used contain a mixture of α3, α4 and α5 NC1 domains. If mAb3 bound more specifically to the α3 than 1G6, there would be a lower antigen concentration coated for mAb3 in each ELISA than for 1G6. This, combined with lower expression levels may provide a possible explanation for the failure to detect antigen binding of this construct. It may be useful to produce recombinant NC1 domains. This would allow production of the specific chain and would make economic sense.

The next step would be to detect glomerular binding to human, murine and bovine kidney sections. For this study monomeric NC1 domains derived from bovine or murine GBM extract were used as model antigen in the sandwich ELISA (Figure 49.C and 49.D). In the GBM the NC1 domains are present in hexamers and cross-linked with sulphilimine bonds. Binding of the mAb3 and 1G6 scFv to the antigen in its native confirmation should be confirmed. After establishing *in vitro* binding of the constructs, the *in vivo* binding should be established as for the parent antibody mAb3 (Figure 28). Purified 1G6 was not available for testing in this study and may not be available in the future. *In vivo* binding of 1G6 was therefore not established. If 1G6 is a multimeric IgM, its ability to penetrate and bind to the GBM *in vivo* may be restricted by its size.

5.3.3 When is targeting of FH1-5 to the GBM useful?

The more obvious case for targeting of an AP regulator to GBM is to treat the glomerulonephritides with direct involvement of the GBM. In anti-GBM nephritis the GBM is the site of complement activation and targeting of FH1-5 would effectively regulate the AP and could ameliorate damage. In MPGN type II C3 metabolites, resulting from insufficient fluid-phase regulation of the AP, deposit along the GBM (Smith et al., 2011). Targeting of FH1-5 to this site may be beneficial and aid clearance of C3 fragments, but will not restore normal C3 levels in the serum. FH1-5 (for patients with C3NeF) and FH1-5/18-20 (for patients with FH deficiency) would achieve exactly this and therefore present more attractive options for treatment of MPGN type II than the GBM targeted constructs. As discussed earlier, aHUS patients with mutations in the C-terminal have sufficient fluid-phase control of the AP and complement attack is mainly directed against the glomerular endothelium (Pickering et al., 2007, de Cordoba and de Jorge, 2008). Arguably a therapeutic AP regulator should therefore be able to interact with the endothelial cell surface (e.g. FH1-5/18-20). The vicinity of the GBM may be sufficient to provide adequate "local regulation" of the AP by type IV collagen-anchored FH1-5.

The case for targeting FH1-5 to the GBM in the context of renal ischaemia-reperfusion injury is less clear-cut. The contribution of the AP to renal ischaemia-reperfusion injury has been demonstrated in several studies using animals deficient in complement activators and regulators (Lien *et al*., 2003, Vieyra and Heeger, 2010). The primary site of injury after renal ischaemia-reperfusion is the tubular epithelialium. The endothelial surface layer and glycocalyx contribute to the permselectivity of the filtration barrier (Friden *et al*., 2011, Salmon *et al*., 2012). Mild ischaemia-reperfusion injury causes remarkable changes in the glycocalyx and possibly the GBM that result in severe proteinuria even before cellular changes become evident (Andersson *et al*., 2007). The tubular epithelium itself can activate the AP and FH is an important regulator for this cell type (Renner *et al*., 2010, Renner *et al*., 2011). While the epithelial cells are rendered vulnerable as a result of the hypoxic conditions, the proteinuria resulting from the altered filtration barrier allows leakage of serum proteins, including albumin and complement components (Eddy and Giachelli, 1995). The protein load impairs binding of FH to the TEC surface and the conditions are favourable for complement activation (Buelli *et al*., 2009).

FH has a clear role for protection of renal endothelium and epithelium and administration of additional FH may be beneficial during renal reperfusion. Systemic inhibition of complement by administration of soluble CR1, an anti-C5 mAb or an anti-C5aR antagonist improved the outcome of renal allografts in mice and rats (Pratt *et al*., 1996, de Vries *et al*., 2003a, de Vries

et al., 2003b, Lewis *et al*., 2008). For reasons discussed previously targeted approaches have several advantages. In an attempt to block local complement activation cell or tissue targeted approaches were developed. These include the sLex linked TP10 (TP20) targeted to endothelial cells and Mirococept, targeted to epithelial and endothelial cells via a fatty acid tail (Rittershaus *et al*., 1999, Pratt *et al*., 2003, Picard *et al*., 2000, Patel *et al*., 2006). FH1-5, targeted to the GBM (or FH1-5/18-20) may exert similar protective effect as the agent generated by Pushpakumar and colleagues. They used lipid vesicles to target the vaccinia virus control protein (VCP) to the renal endothelium and demonstrated markedly reduced renal injury following reperfusion (Pushpakumar et al., 2011).

Although the FH1-5/scFv fusion proteins generated in this project remain in the early stages of development, they have potential to find application in a range of AP-mediated renal pathologies.

6 General discussion

6.1 Summary

The alternative pathway of complement activation is a potent and effective first line of defence against invading pathogens. A number of cell-surface and fluid-phase regulatory proteins are in place to tightly control the AP and protect the host cells from AP-mediated damage. FH is, alongside FI, the principal fluid-phase regulator of the AP and has a particular role in protecting the glomerular endothelium, the GBM and tubular epithelial cells from AP-mediated damage. These roles have been demonstrated in *Cfh*$^{-/-}$ mice and mice that express FH that lacks the C-terminal SCRs 16-20 (*Cfh*$^{-/-}$.Δ16-20) which develop renal pathology that resembles human MPGN type II and aHUS, respectively (Pickering *et al*, 2002 and 2007). In other conditions, including other types of nephritis and ischaemia-reperfusion injury, regulation of the AP can be overwhelmed and complement activation ensues. Therapeutic intervention in these conditions with regards to the involvement of the complement system is currently mainly limited to the anti-C5 mAb Eculizumab. The complement system offers several therapeutic targets and several promising agents are undergoing testing in preclinical and clinical development (Wagner and Frank, 2010, Ricklin and Lambris, 2007a). C3 and C5 are popular targets but blocking their function is associated with adverse effects such as immunosuppression and effects on other functions of complement, e.g. the clearance of immune-complexes. Over the past decade complement has emerged a mediator of several physiological processes that is finely balanced. Long-term inhibition of individual components may therefore entail further adverse effects than those known to date (Holers et al., 2013). Regulator based therapeutics like Mirococept (membrane-targeted soluble CR1) and TT30 (human CR2-FH fusion protein) are promising agents currently in clinical trials (Wagner and Frank, 2010). The use of recombinant complement regulators, especially targeted ones, has shown promising results in several models of complement-mediated disease states (Holers et al., 2013).

The objective of this project was to generate and functionally assess recombinant FH proteins, targeted to the cell-surface and to type IV collagen in the GBM, as potential therapeutics to target uncontrolled AP activation in the kidney. Four such proteins were generated:

- FH1-5 and FH1-5/18-20 were expressed in CHO cells and the *in vitro* functional analysis completed. Both constructs retained the capacity to regulate the AP in the fluid-phase. FH1-5/18-20 regulates the AP in the fluid-phase and on a cell-surface.

- To target FH1-5 to the glomerulus, the variable regions of the monoclonal antibody mAb3 were cloned after successful elimination of a contaminating endogenous light chain transcript.

- Two type IV collagen specific FH1-5/scFv fusion proteins were produced. One based on the published sequence of mAb 1G6 and the other based on the mAb3 derived variable regions. Binding of FH1-5/1G6 scFv to C3b and bovine and murine NC1 domains was demonstrated by ELISA. No significant antigen binding was established for FH1-5/mAb3 scFv and some binding to C3b was demonstrated.

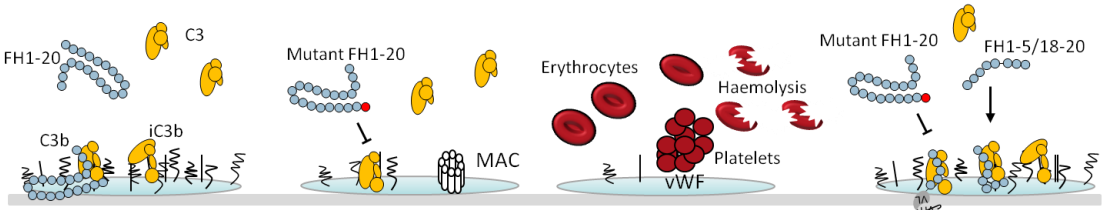
6.2 Production of recombinant FH proteins

Biological therapeutics have emerged as a very successful class of drugs. Monoclonal antibodies comprise the majority of approved agents, however the development of bi-functional or bi-specific fusion proteins takes this class of drugs to a new level. The possibilities of such multi-functional proteins seem endless; however practical hurdles limit their realisation. Initial attempts to produce full length FH did not yield therapeutically relevant quantities (Buttner-Mainik *et al*., 2011). Recently, Schmidt *et al* achieved the expression of large amounts of recombinant full length FH in *P. pastoris (Schmidt et al.,* 2011*)*. The *CFH* gene was optimised for the preferred codon-usage of *P. pastoris* but also with respect to repeated or rare codons in *CFH* that may have resulted in low yields in previous attempts (Schmidt *et al*., 2011). The binding properties of the type IV collagen specific mAb3 (Figures 27 and 28) were to be exploited as targeting mechanism of FH1-5 to the GBM. The strategy for this entailed cloning of the mAb3 variable regions and construction of a FH1-5/scFv fusion construct. MOP-C derived hybridoma fusion partner cell lines like SP2/0 produce, occasionally vast amounts, of an endogenous aberrant kappa light chain (Carroll *et al*., 1988). The endogenous light chain mRNA can greatly impact on the success of V-region cloning from hybridoma cell lines that were generated on a MOP-C background. The mAb3 hybridoma was generated with SP2/0 as fusion partner and excessive expression of the aberrant transcript prevented cloning by standard 5' RACE. A number of approaches to discriminate against the aberrant transcript are described in the literature. These range from simple anti-sense nucleotide directed RNAse H mediated cleavage of the aberrant transcript mRNA to much more complex ribozyme mediated elimination of the mRNA in the hybridoma cells.

6.3 Recombinant FH proteins for AP-mediated renal pathologies

In the present work novel recombinant FH proteins were generated and are proposed as new treatment options for a range of AP-mediated renal pathologies. Since no *in vivo* work was carried out with these proteins, theoretical application of the agents generated (FH1-5, FH1-5/18-20, FH1-5/scFv) in the treatment of aHUS, MPGN type II, anti-GBM nephritis and renal ischaemia-reperfusion injury will be discussed (Figures 51 and 52).

A) Recombinant FH proteins suitable for treatment of aHUS

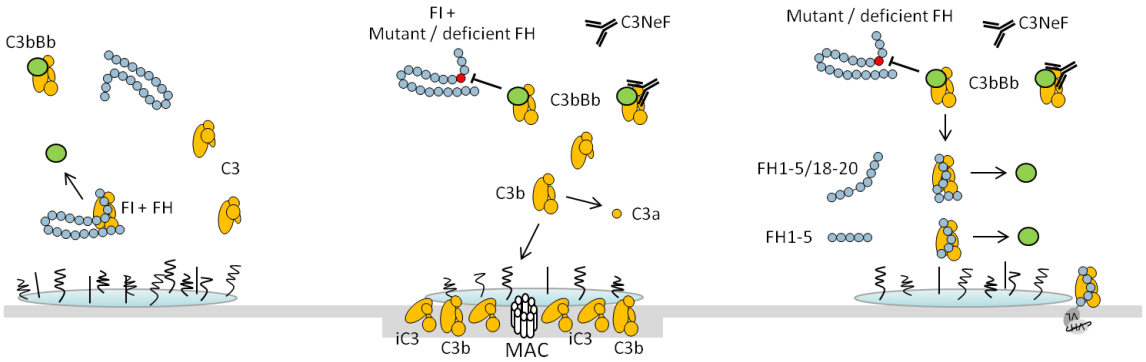


Normal state

Cell-binding dysfunction of FH in aHUS

Application of recombinant FH proteins

B) Recombinant FH proteins suitable for treatment of MPGN type II



Normal state

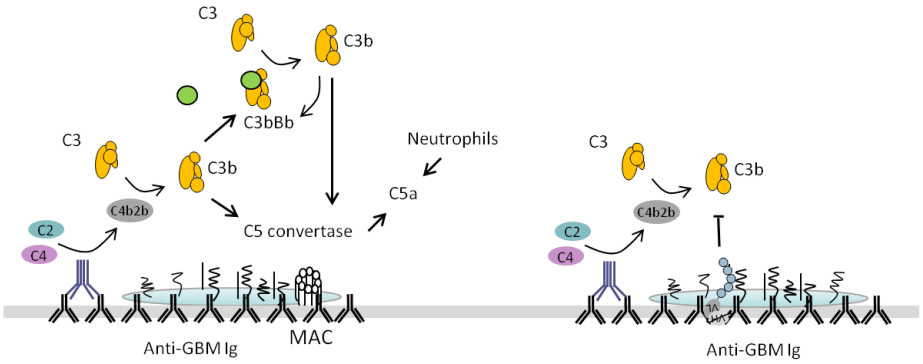
Fluid-phase dysregulation of the AP in MPGN type II

Application of recombinant FH proteins

Figure 51: Application of recombinant FH proteins in aHUS and MPGN type II

(A) In the normal state FH protects the glomerular endothelium from complement attack by binding and inactivating deposited C3b. If the cell-binding domain of FH is rendered dysfunctional by mutation or autoantibodies, C3b is not activated. Complement activation ensues and the endothelial cells are ultimately damaged by formation of the MAC. The damaged cells can bind vWF which initiates the formation of thrombi, resulting in haemolysis and renal dysfunction. Recombinant FH 1-5/18-20 would effectively replace dysfunctional FH and protect the renal endothelium. GBM targeted FH1-5/scFv may protect the endothelium by controlling complement activation locally.
(B) FH is essential for fluid-phase regulation of the AP and exerts this role with FI. If FH cannot effectively control the AP because of mutation of the regulatory domain or the presence of C3NeF, C3 is activated and its metabolites are deposited along the GBM, resulting in MPGN type II. Recombinant FH1-5/18-20 and FH1-5 would restore fluid-phase regulation of the AP. GBM-targeted FH1-5/scFv would aid the clearance of deposited C3 metabolites.

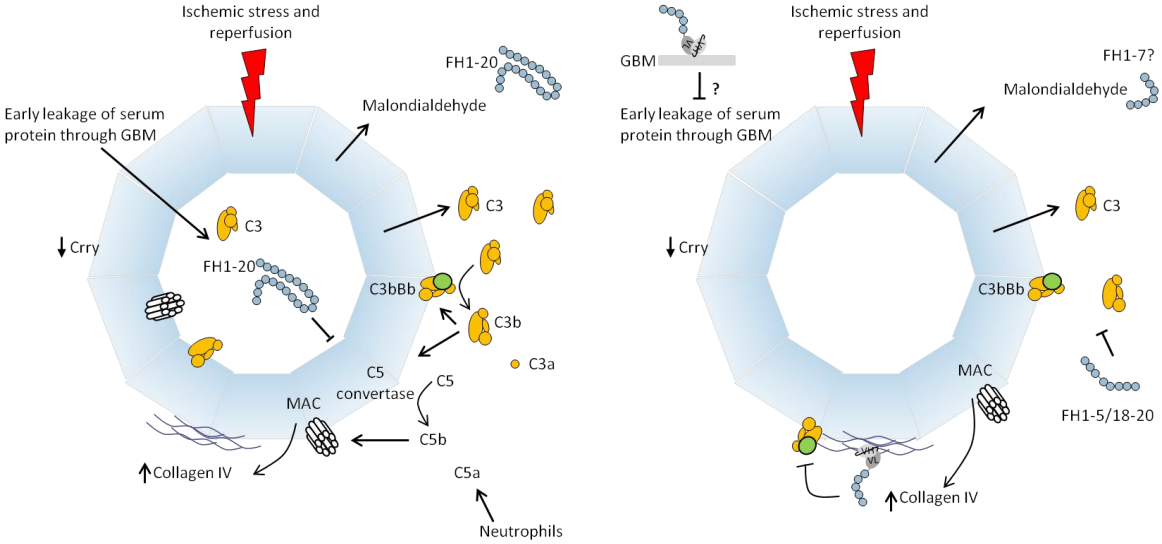
A) Recombinant FH proteins suitable for treatment of anti-GBM nephritis



Complement activation in anti-GBM nephritis

Application of FH1-5/scFv in anti-GBM nephritis

B) Recombinant FH proteins with potential application in renal ischaemia-reperfusion injury



Effects of complement activation in renal ischaemia-reperfusion injury

Possible therapeutic application of recombinant FH proteins

Figure 52: Recombinant FH proteins in anti-GBM nephritis and ischaemia-reperfusion

(A) In the pathogenesis of anti-GBM nephritis with complement involvement the AP is activated secondary to the CP which is initiated by anti-GBM Ig. GBM targeted FH1-5/scFv would control local AP activation and stop the inflammatory response.
(B) Ischemic stress renders tubular epithelial cells (TECs) susceptible complement mediated damage upon reperfusion and induces C3 secretion by TECs which contributes to complement activation and attraction of inflammatory cells. MAC insertion triggers upregulation of type IV collagen secretion. Early changes at the GBM permeability allows leakage of serum proteins, including complement. The protein load further encourages complement activation by interfering with effective binding of FH. Malondialdehyde (MDA), a marker of hypoxic stress, binds FH via SCR 7. GBM-targeted FH1-5/scFv may ameliorate reperfusion injury by moderation of GBM changes at the early stage or could alternatively bind to exposed collagen IV. A recombinant FH protein (SCRs 1-7) may be targeted to the site of hypoxic stress upon reperfusion via its interaction with MDA. FH1-5/18-20 may also be beneficial in controlling complement activation in this scenario.

The important role of FH for protection of the glomerular endothelium from AP-mediated damage has been clearly demonstrated in *Cfh*$^{-/-}$.Δ16-20 mice (Pickering *et al*., 2007). Without a functional cell binding domain mice spontaneously develop aHUS-like renal disease. The pathology in *Cfh*$^{-/-}$.Δ16-20 mice, compared to MPGN type II-like disease in *Cfh*$^{-/-}$ mice, depends more on the terminal pathway (de Jorge *et al*., 2011). This explains the successful treatment of aHUS patients with the anti-C5 mAb Eculizumab (Mache *et al*., 2009). The current treatment options include plasma infusion or exchange, haemodialysis and transplantation (Taylor *et al*., 2010). In the present study FH1-5/18-20 and FH1-5/scFv were generated and are proposed as new treatment options for aHUS. FH1-5/18-20 could be used as replacement therapy for the partially dysfunctional native FH as it bears the essential functional domains of FH. Its ability to protect from AP-mediated lysis by binding C3b on a cell surface was demonstrated in sheep erythrocytes (Figure 49.C).

FH1-5/scFv would localise at the GBM and may provide protection by controlling AP activation in the vicinity of the endothelial cells. Importantly, both agents may offer a reduced level of immunosuppression. Use of FH1-5/18-20 in an individual with circulating FH sufficient fluid-phase regulatory activity may cause some degree of immunosuppression. The extent will depend on the dosage necessary to achieve protection in the glomerulus. The advantage of FH1-5/scFv over FH1-5/18-20 is therefore the GBM targeting, which would address the problem of systemic inhibition. Whether FH1-5/scFv at the GBM is in sufficient proximity to protect the endothelial cells has be determined *in vivo*. MPGN type II is, like aHUS associated with dysfunctional regulation or over-activation of the AP. It is the deficit in fluid-phase regulation that drives uncontrolled C3 turnover and results in deposition of C3 metabolites along the GBM (Figure 51.B). The complement system, the AP in particular, is therefore a valid target. The therapeutic benefit of FH replacement has been demonstrated in *Cfh*$^{-/-}$ mice and there are reports that Eculizumab is effective in treating some MPGN type II patients (Fakhouri *et al*., 2010, Bomback *et al*., 2012, Herlitz *et al*., 2012). Compstatin/POT4 appears to be a suitable agent to correct the over-activation of C3. However, systemic inhibition is likely associated with immunosuppression and other effects on functions of the complement system. FH1-5/18-20 and FH1-5 are proposed as new treatment options for the treatment of MPGN type II (Figure 51.B).

Based on the *in vitro* functional data generated in the present work, both proteins would effectively regulate the fluid-phase metabolism of C3. FH1-5/18-20 would be suited for patients with mutations in the regulatory domain of FH (SCRs 1-4). Administration of FH1-5 to these patients would likely “downgrade” pathology of MPGN type II to aHUS, as fluid-phase regulation may be restored but still ineffective at the endothelial cell surface. FH1-5 may be

suited to treat patients with normal circulating levels of functional FH in which C3 metabolism is driven by the presence of C3NeF that stabilises the C3 convertase or gain of function mutations in C3 or FB. Restoration of serum C3 levels is the long-term aim for treatment of MPGN type II; long-term administration of FH1-5/scFv or similarly TT30 may not be beneficial. However, it might be useful to apply this protein as short-term treatment to aid clearance of the GBM from deposited C3.

The situation in anti-GBM nephritis is very different to MPGN type II. Here the GBM is the site of complement activation and deposition and makes the ideal target for drug-targeting. Although deposition of Ig against a glomerular antigen initially triggers activation of the CP, the cascade is dramatically amplified by the AP (Figure 52.A). FH1-5, anchored in the GBM via an anti-collagen IV moiety, would likely intercept AP activation and reduce pathology. The AP also contributed to tubular injury in renal ischaemia-reperfusion injury and selective inhibition of the AP with an anti-FB mAb protects the tubular epithelium from AP-mediated damage (Thurman *et al*., 2003, Thurman *et al*., 2005, Thurman *et al*., 2006b). The pathology in renal ischaemia-reperfusion injury is complex (Figure 52.A illustrates some of the roles of complement). Importantly, TECs contribute to injury by secretion of C3 and can readily activate the AP on their cell surface. MAC deposition results in increased production of type IV collagen whereas changes of endothelial glycocalyx and GBM alter permeability early on after reperfusion and allow leakage of serum proteins. The leaked proteins include complement proteins and contribute to further complement activation whilst preventing FH from binding to the TECs. Several attempts have been made to address the activation of complement therapeutically. Pratt *et al.* have demonstrated that treatment of rat kidneys with a cell-surface targeted soluble CR1 effectively reduced ischaemia-reperfusion injury after transplantation (Pratt *et al*., 1996, Pratt *et al*., 2003). Mirococept (APT070) binds to glomerular and tubular cells and provides protection at all sites targeted by complement activation. Figure 52.B illustrates how FH1-5/18-20 and FH1-5/scFv could be applied to modify AP activation during renal ischaemia-reperfusion injury.

6.4 Conclusion and future work

Given more time several aspect of this project could be continued and improved and alternative approaches to generating a type IV collagen specific antibody fragment explored.

6.4.1 FH1-5 and FH1-5/18-20

The experiments presented in this work imply great potential for FH1-5/18-20 as therapeutic for different AP-mediated renal pathologies. There are several areas of improvement for the haemolytic assay which should be addressed. The relative protective effects of FH1-5 and FH1-5/18-20 compared native FH on rabbit and sheep erythrocytes would be more distinct if FH depleted serum was used in the assay. The use of a wider range of the recombinant proteins is also anticipated to accentuate the relative effects. C7/FH depleted serum could be included as an additional negative control to equimolar BSA to demonstrate the protective effect is mediated by control of the terminal pathway initiation. Similarly, it is of interest to ascertain AP-specific inhibition. This can be achieved by using FB/FH depleted serum. It would be useful to confirm the decay acceleration of immobilised C3b (SPR) by FH1-5 and to include FH1-5/18-20 in repeat experiments. The findings of the heparin ELISA could be confirmed by detecting binding of FH1-5/18-20 to human umbilical vein endothelial cells (HUVECs) or to CHO cells (compared to a GAG deficient CHO cell line) by flow cytometry or immunocytochemistry. Importantly the expression levels of FH1-5 and FH1-5/18-20 allow production of sufficient protein to study the effect of the protein *in vivo*. Here the first objective is to test the effect of FH1-5 and FH1-5/18-20 on serum C3 levels and glomerular C3 deposition in $Cfh^{-/-}$ mice.

6.4.2 FH1-5/scFv fusion proteins

Although the FH1-5/1G6 fusion protein replicates the binding properties of the parent antibody 1G6, the binding is not exclusive to the α3 NC1 domains. This makes therapeutic application questionable. It may be worth to use the construct on normal human and mouse kidney sections to check how the ELISA result translates into tissue distribution.

The FH1-5/mAb3 scFv fusion protein showed low expression levels which made functional analysis difficult. The first step to address this problem would be to test an alternative expression host like HEK 293t cells which are known to give good expression levels in transient expression. This may provide an opportunity to assess binding to C3b and NC1 domains by ELISA. No human type IV collagen α3 chain NC1 domains were available for use in this study and the bovine and murine NC1 domains are expensive. It would therefore also be necessary to produce these proteins recombinantly. An affinity column prepared by coupling of mAb3 to the matrix would allow production of highly pure antigen.

If further attempts to detect binding fail it may be necessary to explore other antibody formats such as single-domain antibody or “minibody”. A further possibility to obtain an antibody fragment of the desired specificity is phage display. Synthetic and naturally derived human scFv and single domain antibody libraries are commercially available that could be employed to identify strong binders.

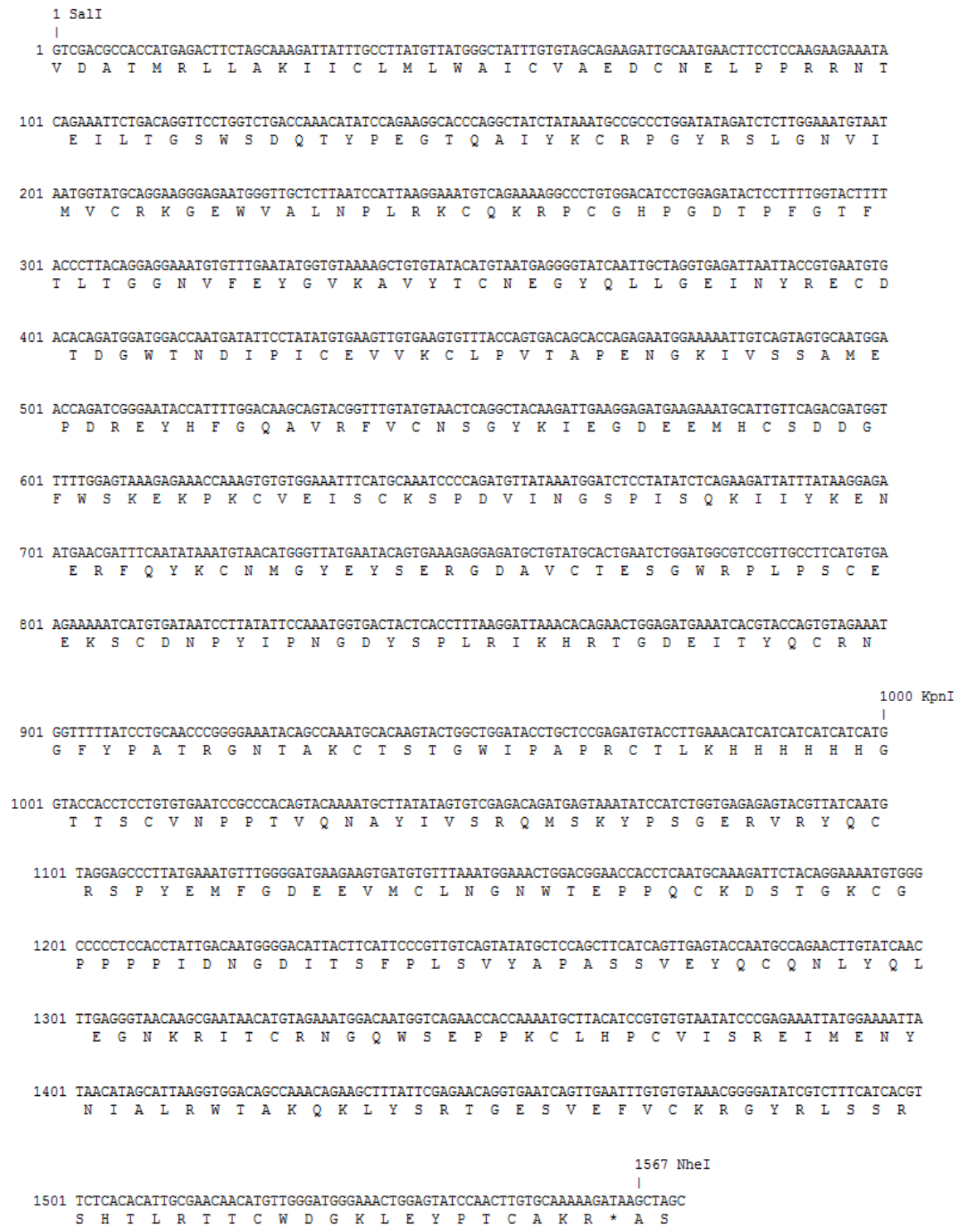
7 Appendix

7.1 Appendix A

FH1-5 (S21) DNA and amino acid sequence

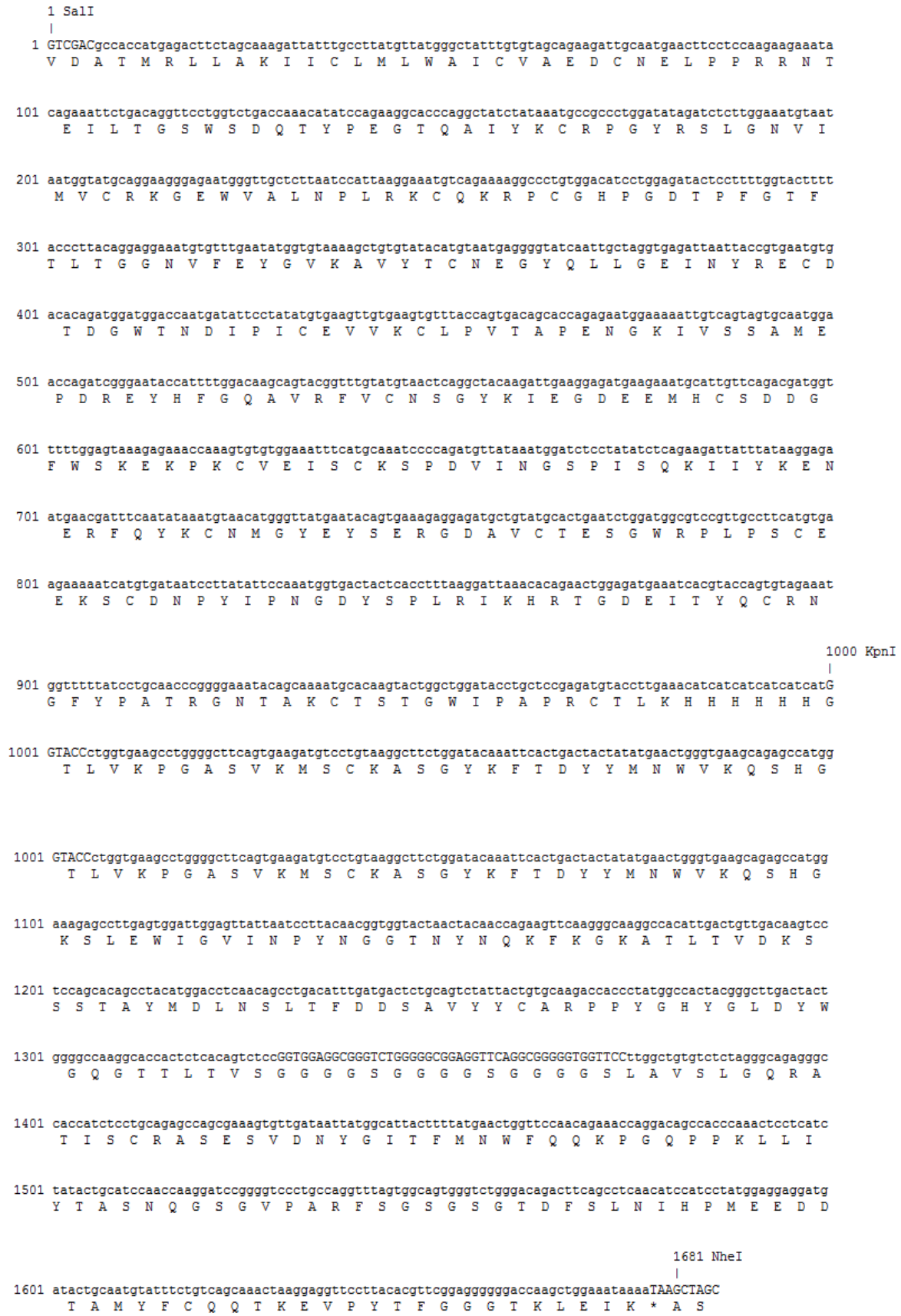
```
    1 SalI
    |
  1 GTCGACgccaccatgagacttctagcaaagattatttgccttatgttatgggctatttgtgtagcagaagattgcaatgaacttcctccaagaagaaata
    V  D  A  T  M  R  L  L  A  K  I  I  C  L  M  L  W  A  I  C  V  A  E  D  C  N  E  L  P  P  R  R  N  T

101 cagaaattctgacaggttcctggtctgaccaaacatatccagaaggcacccaggctatctataaatgccgccctggatatagatctcttggaaatgtaat
      E  I  L  T  G  S  W  S  D  Q  T  Y  P  E  G  T  Q  A  I  Y  K  C  R  P  G  Y  R  S  L  G  N  V  I

201 aatggtatgcaggaagggagaatgggttgctcttaatccattaaggaaatgtcagaaaaggccctgtggacatcctggagatactccttttggtacttttt
     M  V  C  R  K  G  E  W  V  A  L  N  P  L  R  K  C  Q  K  R  P  C  G  H  P  G  D  T  P  F  G  T  F

301 acccttacaggaggaaatgtgtttgaatatggtgtaaaagctgtgtatacatgtaatgaggggtatcaattgctaggtgagattaattaccgtgaatgtg
    T  L  T  G  G  N  V  F  E  Y  G  V  K  A  V  Y  T  C  N  E  G  Y  Q  L  L  G  E  I  N  Y  R  E  C  D

401 acacagatggatggaccaatgatattcctatatgtgaagttgtgaagtgtttaccagtgacagcaccagagaatggaaaaattgtcagtagtgcaatgga
      T  D  G  W  T  N  D  I  P  I  C  E  V  V  K  C  L  P  V  T  A  P  E  N  G  K  I  V  S  S  A  M  E

501 accagatcgggaataccattttggacaagcagtacggtttgtatgtaactcaggctacaagattgaaggagatgaagaaatgcattgttcagacgatggt
     P  D  R  E  Y  H  F  G  Q  A  V  R  F  V  C  N  S  G  Y  K  I  E  G  D  E  E  M  H  C  S  D  D  G

601 ttttggagtaaagagaaaccaaagtgtgtggaaatttcatgcaaatccccagatgttataaatggatctcctatatctcagaagattatttataaggaga
    F  W  S  K  E  K  P  K  C  V  E  I  S  C  K  S  P  D  V  I  N  G  S  P  I  S  Q  K  I  I  Y  K  E  N

701 atgaacgatttcaatataaatgtaacatgggttatgaatacagtgaaagaggagatgctgtatgcactgaatctggatggcgtccgttgccttcatgtga
      E  R  F  Q  Y  K  C  N  M  G  Y  E  Y  S  E  R  G  D  A  V  C  T  E  S  G  W  R  P  L  P  S  C  E

801 agaaaaatcatgtgataatccttatattccaaatggtgactactcacctttaaggattaaacacagaactggagatgaaatcacgtaccagtgtagaaat
     E  K  S  C  D  N  P  Y  I  P  N  G  D  Y  S  P  L  R  I  K  H  R  T  G  D  E  I  T  Y  Q  C  R  N

901 ggtttttatcctgcaacccggggaaatacagcaaaatgcacaagtactggctggatacctgctccgagatgtaccttgaaacatcatcatcatcatcatT
    G  F  Y  P  A  T  R  G  N  T  A  K  C  T  S  T  G  W  I  P  A  P  R  C  T  L  K  H  H  H  H  H  H  *

      1003 NheI
      |
1001 AAGCTAGC
      A  S
```

FH1-5/18-20 (P5) DNA and amino acid sequence

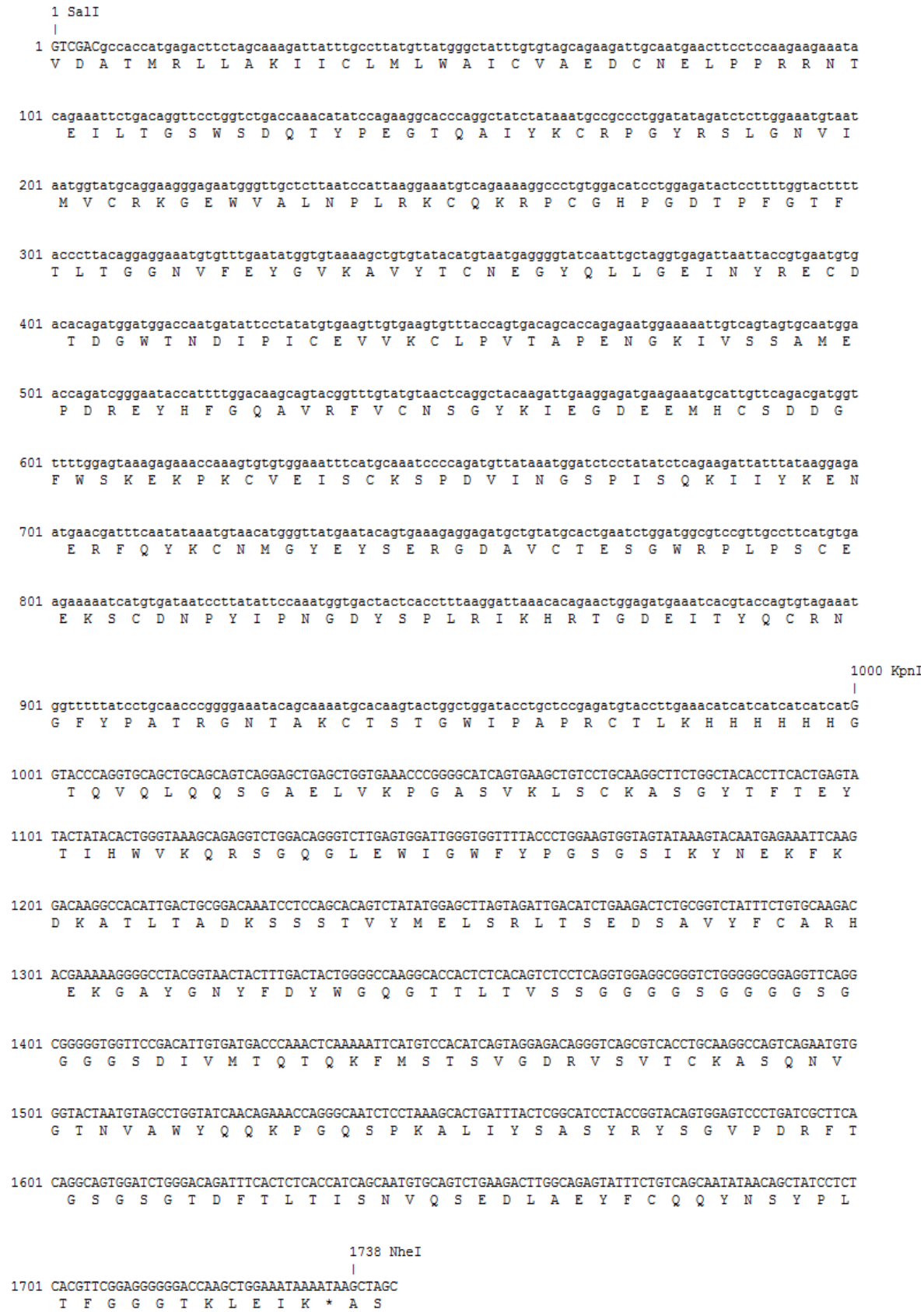


7.2 Appendix B

FH1-5/1G6 scFv DNA and amino acid sequence



FH1-5/mAb3 scFv DNA and amino acid sequence



7.3 Appendix C

These enzymes cleave the sequence:

Name of enzyme	No. of cleavages	Positions of cleavage sites
Arg-C proteinase	23	2 27 28 53 57 67 78 83 127 166 175 232 246 257 281 285 295 303 319 419 462 468 509
Asp-N endopeptidase	25	19 38 89 129 131 136 164 186 193 194 213 247 267 275 287 351 393 402 409 410 427 473 517 528 529
Asp-N endopeptidase + N-terminal Glu	57	18 19 22 30 38 43 69 89 104 115 122 127 129 131 136 141 152 162 164 166 184 186 187 188 193 194 200 206 213 228 230 241 244 247 252 262 263 267 275 287 288 351 366 393 402 409 410 427 470 473 517 526 527 528 529 540 552
BNPS-Skatole	11	13 37 71 134 198 256 314 357 368 430 483
CNBr	12	1 11 64 162 190 239 341 355 402 481 526 533
Chymotrypsin-high specificity (C-term to [FYW], not before P)	58	13 37 50 56 71 93 96 104 106 112 118 126 134 168 170 176 182 197 198 227 233 235 241 243 256 271 277 292 298 314 348 350 353 354 357 368 375 381 385 401 409 415 416 422 425 429 430 476 480 483 484 497 510 519 534 535 544 546
Chymotrypsin-low specificity (C-term to [FYWML], not before P)	106	1 3 4 10 11 12 13 33 37 50 56 59 64 71 74 77 93 96 98 104 106 112 118 120 121 126 134 162 168 169 170 176 182 190 191 197 198 227 233 235 239 241 243 256 271 277 280 284 292 298 314 322 324 325 326 327 328 329 332 341 348 350 353 354 355 357 362 366 368 375 381 385 391 401 402 404 407 409 415 416 422 424 425 427 429 430 436 455 459 476 480 481 483 484 494 495 497 510 519 521 526 534 535 544 546 552
Clostripain	23	2 27 28 53 57 67 78 83 127 166 175 232 246 257 281 285 295 303 319 419 462 468 509
Formic acid	25	20 39 90 130 132 137 165 187 194 195 214 248 268 276 288 352 394 403 410 411 428 474 518 529 530
Glutamyl endopeptidase	32	19 23 31 44 70 105 116 123 128 142 153 163 167 185 188 189 201 207 229 231 242 245 253 263 264 289 367 471 527 528 541 553
Hydroxylamine	5	154 217 274 296 376
Iodosobenzoic acid	11	13 37 71 134 198 256 314 357 368 430 483
LysC	35	6 51 68 79 82 109 145 156 183 200 202 204 211 224 228 236 265 283 308 323 334 340 344 349 359 364 384 386 388 395 487 493 540 551 555
LysN	35	5 50 67 78 81 108 144 155 182 199 201 203 210 223 227 235 264 282 307 322 333 339 343 348 358 363 383 385 387 394 486 492 539 550 554
NTCB (2-nitro-5-thiocyanobenzoic acid)	26	8 15 20 51 65 79 84 113 128 140 145 177 191 204 209 236 250 261 266 293 308 319 342 416 466 535
Pepsin (pH1.3)	137	3 3 9 10 11 12 12 13 32 33 36 37 42 49 50 56 58 71 73 76 92 95 96 97 98 103 104 105 106 112 117 118 119 120 120 121 125 126 133 134 167 169 170 176 181 182 196 197 197 198 227 233 235 240 241 242 243 255 270 276 279 291 292 299 313 322 332 347 348 349 350 352 353 353 354 356 357 365 367 368 374 380 381 384 385 391 400 401 403 404 406 407 408 409 414 415 415 416 424 425 427 428 429 429 430 435 436 454 455 458 459 475 476 479 480 482 483 483 484 494 494 496 497 510 518 519 520 521 533 534 534 535 543 545 546 551 552
Pepsin (pH>2)	67	3 3 9 10 11 12 32 33 58 73 76 92 95 96 97 98 103 104 119 120 120 121 169 170 176 196 197 233 279 322 332 349 350 365 384 385 391 403 404 406 407 408 409 427 435 436 454 455 458 459 479 480 483 484 494 494 510 518 519 520 521 534 535 545 546 551 552
Proline-endopeptidase	8	54 84 88 203 258 335 488 525

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Proteinase K	206	3 4 5 7 8 10 12 13 14 15 17 18 24 30 32 33 34 37 41 42 46 48 49 50 56 59 62 63 65 71 72 73 74 77 91 93 95 96 97 98 99 103 104 106 108 110 111 112 113 118 120 121 124 126 131 134 135 138 140 143 144 147 149 150 151 157 158 161 168 170 173 174 176 177 182 184 197 198 206 208 215 216 221 225 226 227 233 235 241 243 249 250 252 256 259 271 272 277 280 282 286 290 291 292 298 299 301 302 306 307 310 312 314 315 317 321 322 331 332 333 337 339 345 348 350 351 353 354 357 358 366 368 369 371 372 375 379 381 385 389 390 391 392 393 399 400 401 404 407 408 409 413 414 415 416 418 422 425 427 429 430 434 435 436 437 438 455 456 457 459 463 464 465 469 473 476 478 479 480 483 484 494 495 496 497 498 499 506 508 510 517 519 521 523 531 532 534 535 539 542 544 545 546 550 552 554
Staphylococcal peptidase I	29	19 23 31 44 70 105 116 123 128 142 153 163 167 185 188 201 207 229 231 242 245 253 263 289 367 471 527 541 553
Thermolysin	115	2 3 4 6 7 9 10 11 13 14 16 17 32 47 48 58 61 62 63 64 71 72 73 76 92 95 97 102 103 107 109 110 119 120 139 143 148 156 157 160 161 169 172 173 175 176 183 196 205 215 220 224 225 232 238 249 279 281 297 300 306 321 331 332 336 338 340 344 349 354 357 365 368 370 371 384 388 390 392 399 401 406 408 412 413 417 426 435 437 454 455 456 458 462 464 468 472 477 479 480 483 493 494 495 498 507 509 520 522 525 531 532 534 545 551
Trypsin	50	2 6 27 28 51 57 68 78 79 82 109 127 145 156 166 175 183 200 204 211 224 228 232 236 246 265 281 283 285 295 303 308 319 323 340 344 349 359 364 384 386 388 395 462 468 493 509 540 551 555

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Publications

Oral presentations:

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Publications:

I was involved in setting up the haemolytic assay in this publication:

KAVANAGH, D., PAPPWORTH, I. Y., ANDERSON, H., HAYES, C. M., MOORE, I., HUNZE, E. M., BENNACEUR, K., ROVERSI, P., LEA, S., STRAIN, L., WARD, R., PLANT, N., NAILESCU, C., GOODSHIP, T. H. & MARCHBANK, K. J. Factor I autoantibodies in patients with atypical hemolytic uremic syndrome: disease-associated or an epiphenomenon? *Clin J Am Soc Nephrol,* 2012, 7:417-26.

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