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EXPERIMENTAL DESIGN OF A NOVEL TARGET TO ISOLATE HCV MONOCLONAL ANTIBODIES

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Abstract

Hepatitis C Virus currently affects up to 3% of the world's population. There is no effective vaccine yet available and the natural immune response to infection is largely inefficient. Progress has been made in isolating several broad-acting neutralizing antibodies that target the viral envelope protein E2. However, a dominant element of the epitopes targeted is an overlap with the highly conserved CD81 binding sites. Various E2 constructs were investigated as possible targets to be used in phage display panning of a combinatorial library of the phagemid vector pComb3H. HVR2 deletion showed optimal exposure of the CD81 binding sites and D535A disrupted known CD81 epitopes. A selection technique was designed to improve exposure of conserved sites on an E2 construct target molecule that disrupts CD81 epitopes while remaining conformationally correct. Optimisation of the screening methodology was used to assess the quality of enrichment of the library panning along with more efficient selection of specific clones. The approach adopted in this project isolated Fab clones specifically reactive to the protein target, one of which also showed preferential binding in acidic environments. Taken together, the information gathered on E2 and the implementation of the phage display method described will contribute to more effective ways of isolating novel antibodies.

Declaration

I hereby declare that this thesis is my own work and effort, and that it has not been submitted anywhere for any award. Where other sources have been used, they have been acknowledged.

Signature:

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Abbreviations

AP	**Alkaline Phosphatase**
APS	Ammonium Persulphate
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
CDR	Complementarity Determining Region
CD81	Cluster of Differentiation 81, tetraspanin
CLDN	Claudin
CHV	Canine Hepacivirus
CMV	Cytomegalo Virus
ddNTP	Dideoxy Triphosphates
DNA	Deoxyribose Nucleic Acid
E1	Envelope Glycoprotein 1
E2	Envelope Glycoprotein 2
ECL	Enhanced Chemiluminescence Solution
ELISA	Enzyme Linked Immunosorbent Assay
ER	Endoplasmic reticulum
FBS	Fetal Bovine Serum
Fc	Fragment Crystalisable region
fd	Filamentous phage
GNA	Galanthus Nivalis Agglutinin
GST	Glutathione S-Transferase
HAV	Hepatitis A Virus
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HGV	Hepatitis G Virus
HCV cc	Hepatitis C Virus cell culture
HCVpp	Hepatitis C Virus pseudoparticles
HDL	High Density Lipoprotein
HIV	Human Immunodeficiency Virus
HRP	Horse Radish Peroxidase
HVR	Hyper Variable Region
IFN	Interferon
Ig	Immunoglobulin
IgVR	Intergenotypic variable region
IVDU	Intravenous Drug Users
LB	Luria Bretani broth
LDL	Low Density Lipoprotein
MHC	Major Histocompatibility Complex
MLV	Murine Leukemia Virus
NS	Non Structural
OD	Optical density
OCLN	Occludin
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with 0.05% Tween
PCR	Polymerase Chain Reaction
PEG-IFN	Pegylated Interferon
PKR	Protein Kinase R
PNGase F	Peptide N-Glycosidase F
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis
sE2	Soluble E2
SR-BI	Scavenger receptor class B type I
TEMED	N,N,N',N'-tetramethylethylenediamine
TM	Transmembrane domain
UTR	Untranslated Region
VLDL	Very Low Density Lipoprotein
WB	Western Blot

1. Introduction

1.1. Hepatitis C Virus

There are at least nine different recognised hepatitis viruses: 8 human viruses designated as Hepatitis A-G and a Non-Primate Hepacivirus designated as NPHV. Despite the common nomenclature of the human viruses, they do not share common origins or phylogenies. The Hepatitis C Virus (HCV) belongs to the family *Flaviviridae*, which also contains the Dengue virus, Tick-Borne Encephalitis Virus (TBEV), and West Nile Virus (WNV). The term "flavi" comes from the Latin term for yellow. The prototypical member of the genus *Flavivirus,* the largest genus within the family *Flaviviridae,* is the Yellow fever virus. Many flaviviruses are insect or tick borne; HCV, however, is not. HCV was once the sole member of the genus *Hepacivirus,* until improved screening methods led to the discovery of more HCV-related viruses, expanding the genus. HCV is now the prototypical member of the genus *Hepacivirus* (Bukh 2011; Kapoor, Simmonds et al. 2011), which also contains the Hepatitis G Virus (HGV) (Simons, Pilot-Matias et al. 1995; Linnen, Wages et al. 1996) and the newly discovered Canine HepaciVirus [(CHV), recently reattributed as Non-Primate HepaciVirus (NPHV)] (Kapoor, Simmonds et al. 2011). Serological screening methods recently identified a novel equine hepacivirus that is very closely related to NPHV, shedding new light on the possible origins of the genus *Hepacivirus* and the historical host shifts by which the

hepaciviruses have crossed species barriers (Burbelo, Dubovi et al. 2012).

HCV is a human-tropic, single-stranded RNA virus comprising seven genotypic groups (1-7 with subtypes therein). Genotypes 1-3 are the most prevalent genotypes across the world, whereas genotypes 4-7 are restricted to smaller populations in Africa, the Middle East and East Asia (see Figure 1.2). The HCV genome is 9.6 kb, containing 10 genes encoding three structural proteins; core, E1, and E2; at least six non-structural proteins; NS2, NS3, NS4A, NS4B, NS5A, and NS5B; and one protein, p7, which is not yet confirmed as being either structural or non-structural (Figure 1.1). All HCV genotypes contain the same genes. Protein-sequence variation, however, can be as high as 30% among different genotypes (Okamoto, Kurai et al. 1992).

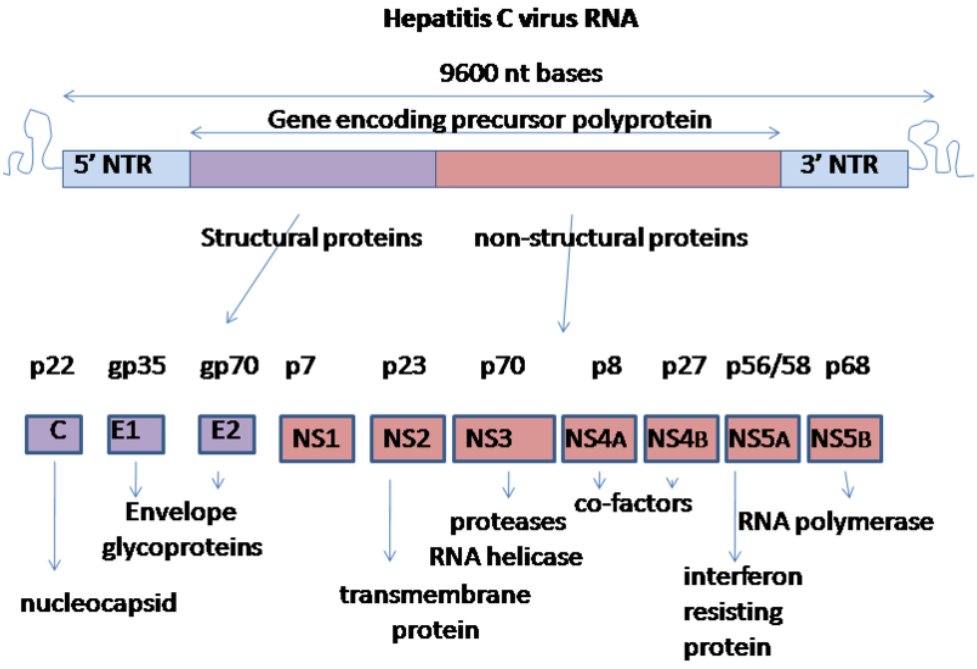

Figure 1.1 Hepatitis C Virus genome and gene products (Coln 2012).

All of the viruses in the family *Flaviviridae* are members of group-IV in the Baltimore classification system: they have a single-stranded, positive-sense RNA genome encoding a single polyprotein, which host and viral proteases cleave sequentially, producing functional proteins. The two HCV envelope proteins, E1 and E2, are homologous to the prM and E proteins in the TBEV and Dengue virus (Kuhns, Zhang et al. 2002; Zhang, Chipman et al. 2003). HCV has an error-prone RNA polymerase that, combined with a high rate of viral replication, produces a great amount of sequence variation within parts of the genome. HCV is phenotypically robust to genetic variation, resulting in genetically heterogeneous within-host populations known as quasispecies. Despite the high degree of genetic variability, HCV proteins contain many highly conserved regions, suggesting functional constraints. The E1 and E2 proteins, for example, contain highly conserved regions as well as highly variable regions. The variable regions may help the virus to evade host immune responses (see Section 1.6)(von Hahn, Yoon et al. 2007; Dowd 2009).

1.1.1. Epidemiology

Prior to being identified as a new virus in 1989 (Choo, Kuo et al. 1989), HCV was implicated as an icteric virus exclusive of the previously identified hepatitis A and B viruses. Researchers created a cDNA library using Non-A Non-B Hepatitis (NANBH)-infected sera and reconstructed a novel RNA genome distinct from the host genetic

material and similar to the genomes of the flaviviruses. The newly characterised HCV virus (Houghton 2009) accounted for up to 90% of the NANBH virus group, which currently affects 3% of the global population and almost 200 million chronically infected individuals worldwide (WHO 2011). The main routes of HCV transmission today are intravenous drug use in the developed world and unsafe medical procedures in the developing world (Maheshwari and Thuluvath 2010).

The source of infection is unidentified in up to 40% of HCV cases. Possible sources of HCV infection include needle-stick injuries and mother-to-child transmission during pregnancy or childbirth; infrequently, HCV can be transmitted sexually or through the sharing of personal items such as razors or toothbrushes (Prevention 2012). Widespread antischistosomal treatments carried out in Egypt from the 1960s through the 1980s inadvertently spread HCV through the use inadequately sterilised needles (Pybus, Drummond et al. 2003; Mezban and Wakil 2006). The HCV infection rate in Egypt climbed as high as 28% in 1991 (Saeed, al-Admawi et al. 1991); more than 10 years later in 2004, however, it had fallen to 10-13% (Mohamed 2004). The iatrogenic spread of HCV infection in Egypt resulted in the expansion of a previously rare variant, HCV genotype 4. Indeed, over the same period, iatrogenic transmission led to the expansion of HCV genotype 1b in Japan (Tanaka, Hanada et al. 2005) and HCV genotype 5b in South Africa (Tanaka, Kurbanov et al. 2006). Today, HCV genotypes 1-3 are

the most prevalent HCV variants worldwide; with genetically distinct populations dominating different regions (see Figure 1.2)

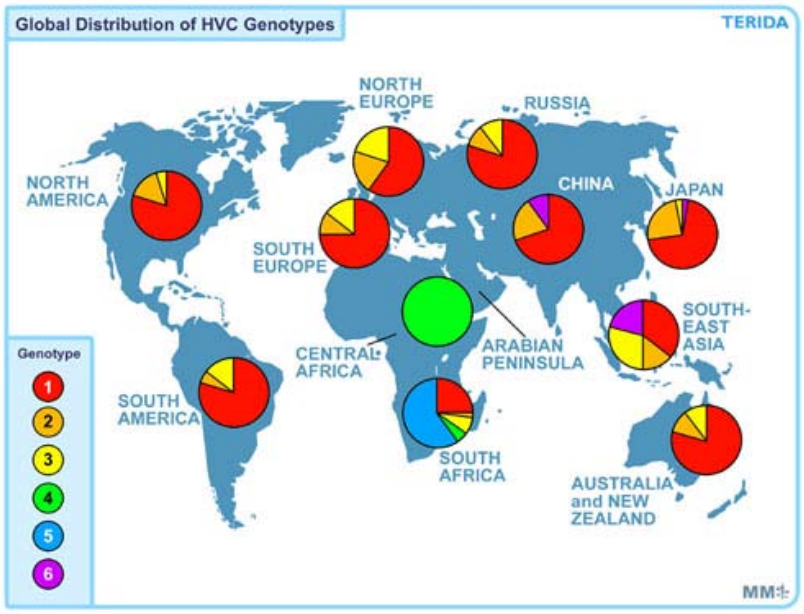

Figure 1.2 Global distribution of HCV genotypes.
The global distribution of HCV genotypes is not uniform. The genotype-specific challenges presented by different HCV variants make treatment difficult.

1.1.2. Diagnosis

HCV diagnosis comprises a number of different assay formats. A blood test for HCV developed in 1992 almost completely eliminated HCV transmission by blood transfusion (Wilkins, Malcolm et al. 2010). The most basic HCV diagnostic assay is the Enzyme Linked-Immunosorbent Assay (ELISA), which detects the presence of anti-HCV antibodies in blood serum. The ELISA is prone to false-positive results (AACC 2010), however, for individuals who have suffered an acute infection and subsequently cleared the virus, often sero-converting in the process, gaining HCV-specific antibodies. The antibodies persist in the sera of

seroconverted individuals, even if the virus is no longer present, resulting in false-positive ELISA results. An analysis of the ELISA signal-to-cut-off ratio can help determine if the presence of HCV antibodies is caused by an active infection or by a previously resolved exposure or an erroneous result (National Center for HIV/AIDS 2013). False-negative results can result from low antibody titres or low sensitivity or specificity of the diagnostic test. The Recombinant Immuno-Blot Assay (RIBA) is a more expensive and specific test that has a lower false-negative rate than the ELISA-based tests. Despite its higher specificity, the RIBA still relies on the presence of antibodies and therefore cannot detect infections in individuals who have not seroconverted, representing up to 7% of acute infections (Heller and Rehermann 2005), nor can it distinguish with any certainty between current or past infections.

The HCV Core-antigen test detects the presence of the viral Core protein in the serum (Lee, Peterson et al. 2001; Muerhoff, Jiang et al. 2002). The Core antigen can be detected within days of the initial infection, unlike HCV-specific antibodies, which often take several weeks or months to appear in the serum. The Core antigen test can be combined with antibody-based tests to provide a more detailed assessment of the stage of the HCV infection (Shah, Chang et al. 2003; Laperche, Elghouzzi et al. 2005).

Once an infection is confirmed, it is important to assess the viral genotype and the amount of virus present in the patient (viral load). The viral genotype can affect the effectiveness of different treatments. For example, interferon and Ribavirin therapy successfully clears ~55% of HCV genotype-1b infections, whereas it clears ~85% of HCV genotype-2 infections and HCV genotype-3 infections (Sy and Jamal 2006). If a patient is known to be infected by HCV genotype 1, the treatment can be amended to include the drug Telaprevir, which increases the HCV genotype-1 clearance rate to ~75% in treatment-naive individuals (Jacobson, McHutchinson et al. 2011).

To assess the treatment effectiveness and the clearance of infection, it is important to know the change in viral load over time. Assays such as the real-time Polymerase Chain Reaction (qPCR) can directly detect HCV RNAs and, more importantly, can be used to estimate the amount of virus present in a sample. Viral RNAs in a sample indicate actively replicating viruses and thus confirm an active or on-going infection. Measurements of the viral load taken before, during, and after a treatment can show the effectiveness of the treatment, allowing timely decisions to be made about whether to continue, extend, or amend the prescribed drug regimen, saving unnecessary expenditures on drugs, related healthcare, and treating side effects. If the viral load becomes undetectable in a patient for 6 months or more following the cessation of treatment, the individual is said to have achieved a Sustained

Virological Response (SVR), at which point the relapse rate is below 1% (Pearlman and Traub 2011).

1.1.3. Prognosis

HCV infections can be either acute or chronic. An acute infection may not manifest symptoms; however, failure to resolve an acute infection can lead to decades of chronic asymptomatic disease, progressing to cirrhosis, hepatocellular carcinoma (HCC), and extra-hepatic manifestations such as Sjörgen's disease (Haddad, Trinchet et al. 1992) and mixed cryoglobulinaemia (Dammacco, Sansonno et al. 1993). The viral genotype interacts with many other factors to influence likely clinical outcome of an HCV infection, making it impossible to predict the clinical outcomes of HCV infections with any certainty.

Less than a quarter of new HCV infections are estimated to resolve naturally (Nelson, Mathers et al. 2011), with the remaining infections becoming chronic. HCC develops in 1-3% of individuals sustaining a chronic HCV infection for more than 30 years (Goodgame, Shaheen et al. 2003), along with risk of liver failure and the need for a liver transplant (Wilkins, Malcolm et al. 2010; Rosen 2011). HCV infection increases the risk of HCC by promoting fibrosis, which can lead to cirrhosis. Once cirrhosis occurs, individuals have about a 1-4% chance annually of developing HCC (El-Serag 2002). With no HCV vaccine yet available, the costs associated with HCV are mostly related to the

treatment of infections and the secondary conditions resulting from infections.

1.1.4. Acute infection

In-depth study of the viral population dynamics and host immune responses during acute HCV infection is limited by the difficulties inherent in identifying patients during the acute phase of the infection. Early onsets of broad-acting and specific humoral and cellular immune responses are associated with the clearance of acute HCV infections (Lavillette, Morice et al. 2005; Pestka J. M., Ziesel et al. 2007). High viral serum titres generally appear a week or so after the initial infection, followed by a delay in the appearance of immune responses. Cellular responses appear approximately 1 month after the initial infection, whereas humoral responses appear as late as 2 months after the initial infection (Thimme, Oldach et al. 2001; Thimme, Bukh et al. 2002; Major, Dahari et al. 2004). HCV titers begin to decline 8-12 weeks after the initial infection, as the host antibodies start to become detectable (Figure 1.4) (Rehermann and Nascimbeni 2005). Many factors contribute to the delayed, and sometimes diminished, host response to HCV, with more yet to be identified. The delay in the HCV-specific immune response contributes to the high rate of HCV persistence after the acute-infection stage; the viral titre is very high and the viral population is very diverse by the time the host response becomes fully established, confounding the efficacy of the response.

Another characteristic impeding the resolution of HCV infections is the ability of HCV to replicate at different sites within the host. HCV can infect non-hepatic cells, raising the potential for viral reservoirs to persist after clinical clearance of the infection. Peripheral dendritic cells, granulocytes, B lymphocytes, and monocytes/macrophages are some of the cell types known to harbour HCV. Lymphocytes have been shown to be permissive to infection *in vivo* and *in vitro* (Lerat, Shimizu et al. 2000; Goutagny, Fatmi et al. 2003; Nowicki, Laskus et al. 2005; Radkowski, Gallegos-Orozco et al. 2005). Different HCV genotypes distribute non-randomly among hepatic and extra-hepatic compartments, supporting the hypothesis that HCV genetic diversity impacts on cell tropism (Kato 1998; Forton, Karayiannis et al. 2004; Roque-Afonso, Ducoulombier et al. 2005; Zehender, Maddalena et al. 2005). Replicative forms of HCV RNA have been found in the Peripheral Blood Mononuclear Cells (PBMCs) of patients whose liver or serum appears to be clear of the virus (Castillo, Rodriguez-Inigo et al. 2005), allowing the HCV infection to persist in subsets of PBMCs long after clinical resolution of the infection. Thus, non-hepatic cells can act as true HCV reservoirs, confounding efforts to prevent the resurgence of viral titres in chronically infected patients and in patients receiving liver transplants (Pham, MacParland et al. 2004).

Unabated, HCV replicates with a population doubling time of about 12 h (Dahari, Major et al. 2005), before slowing down significantly over

many weeks as host immune responses gain control of the viral titres (Figure 1.4). HCV can usually be detected in the blood by quantitative PCR about 1 week after the initial exposure (Group. 1999; Seeff 2002). The innate responses of hepatocytes slow the viral population-doubling time to about 7.5 days, reducing the rate of viral titre increase during the initial phase of the infection. Interferon I, plasmacytoid dendritic cells, and natural killer cells all act to slow the increase in viral titre during the first several weeks of the infection (Rehermann 2009). HCV, however, has multiple mechanisms to interfere with the signalling pathways of the innate-immune response (Polyak, Khabar et al. 2001; Ait-Goughoulte, Banerjee et al. 2010; Arnaud, Dabo et al. 2011). The best-known mechanism of HCV interference in the innate-immune response is the cleavage of host MAVS or TRIF by viral NS3-4A to counteract the host signalling of RIG-I or TLR3, respectively, following the intracellular detection of viral dsRNA (Li, Foy et al. 2005; Meylan, Curran et al. 2005). HCV proteins can also phosphorylate the interferon-induced Protein Kinase R (PKR), inactivating translation factor eIF2α, which normally facilitates the translation of antiviral Interferon Stimulated Gene (ISG) mRNAs (Garaigorta and Chisari 2009).

HCV has additional mechanisms to escape the adaptive-immune responses that occur later. HCV-specific T cells can be detected 5-9 weeks after the initial infection (Thimme, Oldach et al. 2001; Thimme, Bukh et al. 2002; Cox, Mosbruger et al. 2005); however, it takes at least

8 to 14 weeks for serum Alanine Transaminase (ALT) levels, an indicator of liver damage, to peak and up to 6 months for enhanced cross-reactivity antibodies and increased neutralisation titres to appear (Logvinoff, Major et al. 2004). The combined innate and adaptive immune responses bring down the viral titres in acute infections by several orders of magnitude after 10 to 15 weeks (Figure 1.3A). Viral interference mechanisms such as E2 suppression of NK cells (Rehermann and Nascimbeni 2005) along with escape mechanisms such as surface exposed highly variable regions of the envelope proteins allow viral titres to persist by preventing the host immune mechanisms from effectively clearing the virus. Patients who fail to clear HCV infections have been shown to have a weak or functionally impaired T-cell response (Thimme, Binder et al. 2012), possibly caused by regulatory T-cell suppression, lack of CD4+ T-cell cooperation, and viral escape. Persistent or chronic infection is a disease state in which the viral titre resurges periodically over many years, causing secondary conditions or remaining subclinical for decades. Consequently, prevention is much more effective in managing HCV infection than cure alone.

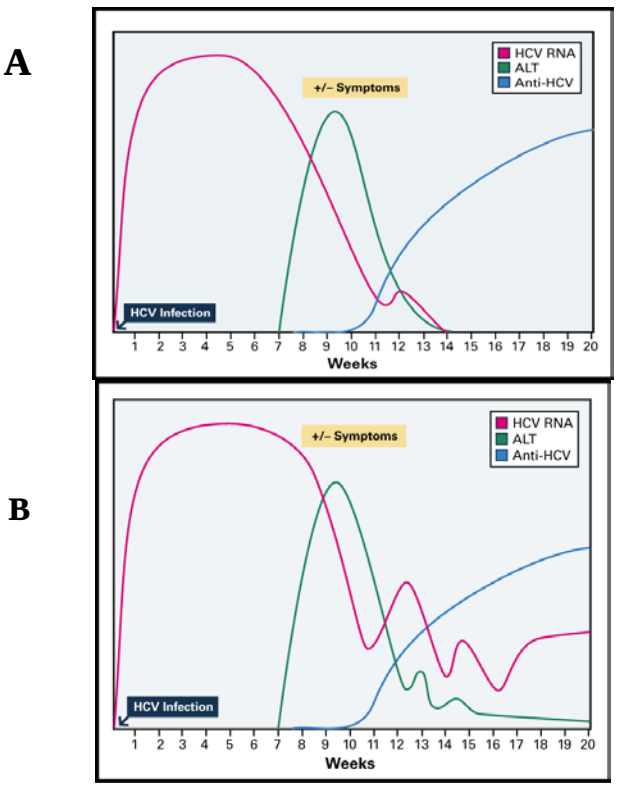

Figure 1.3 Viral kinetics in acute and chronic HCV infections
The initial 6-8 weeks following infection are often unnoticed, with many factors contributing to the development of an acute infection (**A**) or a persistent infection (**B**), determined by the presence of HCV RNA despite anti-HCV immune responses.

1.1.5. Treatment

The HCV genome is highly variable. There are, however, many highly conserved regions of the HCV genome, suggesting the potential for a universal HCV treatment. The large amount of genetic variation within and among HCV populations makes it difficult to manage and treat HCV infections. For example, patients infected by HCV genotype 2 or HCV genotype 3 are twice as likely as those infected by HCV genotype 1 to achieve SVR following pegylated interferon and Ribavirin therapy (Simmonds 2004; Zeuzem 2004). The current HCV treatment options are expensive and have varying efficacies against the different HCV genotypes. Recently, the standard treatment for HCV genotype-1

infection was changed to a three-drug regimen of pegylated interferon, Ribavirin, and Telaprevir. The addition of Telaprevir compensates for the genotype-specific resistance of HCV genotype 1 to pegylated interferon and Ribavirin alone.

1.1.5.1. Pegylated Interferon

The standard therapy for HCV infection over the past 10 years has been pegylated interferon and Ribavirin administered for a period of 24 or 48 weeks. Interferon is a natural antiviral cytokine which exhibits improved biological activity when pegylated. Pegylation, the addition of a PolyEthylene-Glycol (PEG) moiety to a protein, allows the protein to associate with two or three additional water molecules, making the compound function as though it is 5 to 10 times larger than other soluble proteins of the same mass (Kozlowski and Charles S. 2001). Interferon α has a mass of 19 kDa; the addition of a PEG moiety increases the actual mass to 31 kDa (Liang, Rehermann et al. 2000). The water shield around the pegylated compound helps protect the compound against enzymatic degradation, interactions with cell-surface proteins, renal clearance, and immunogenicity. The pegylated compound is thus more stable, especially across pH and temperature ranges (Monfardini, Schiavon et al. 1995). Clinically, pegylation increases the half life of the compound in the body about five fold, helping to increase and sustain serum concentrations, generating improved viral suppression, ultimately providing improved convenience

and better treatment outcomes for the patient (Kozlowski and Charles S. 2001) (Glue, Fang et al. 1999).

1.1.5.2. Ribavirin

Ribavirin is an antiviral drug commonly used against the major cause of lower respiratory tract infections; the *Paramyxovirus*: severe Respiratory Syncitial Virus (sRSV). Ribavirin, however, does not appear to have any direct effects on HCV. Moreover, when Ribavirin is administered alone to patients with HCV infections, only the adverse side effects of the drug occur (Brok, Gluud et al. 2006). Ribavirin benefits HCV therapy when it is combined with pegylated interferon, in which case it reduces HCV reproduction in a dose-dependant manner, improving liver histology and quality of life for the patient (Davis, Esteban-Mur et al. 1998; Chander, Sulkowski et al. 2002; Fried, Shiffman et al. 2002; Flamm and Chopra 2012). The mechanism of Ribavirin action is unknown, although multiple hypotheses have been proposed. Ribavirin is a nucleoside analogue hypothesised to inhibit Inosine-Monophosphate-DeHydrogenase, which causes a reduction in intracellular GTP concentration *in vitro*. The reduction in intracellular GTP concentration is thought to inhibit viral protein synthesis and replication (Streeter, Witkowski et al. 1973).

Ribavirin also has immunomodulatory and mutagenic effects (Crotty, Maag et al. 2000; Tam, Ramasamy et al. 2000). Indeed, Ribavirin was previously thought to act as a chain terminator, but studies using Polio

virus proved otherwise. Crotty (2000) found that the mutagenic activity of Ribavirin correlates directly with the antiviral activity, leading to the catastrophe hypothesis of Ribavirin action (Crotty, Maag et al. 2000). RNA viruses generally tolerate a great amount of genetic variability, allowing them to exist as quasispecies. There is an intrinsic limit, however, to the amount of genetic variability that a viral population can sustain and still remain infective (Eigen 1971; Eigen and Biebricher 1988). The loss of genetic fidelity eventually allows non-functional genetic sequences to accumulate within the viral genomes, and the virions lose the ability to successfully replicate and infect new host cells. The catastrophe hypothesis states that by acting as a mutagen, Ribavirin promotes the accumulation of deleterious mutations in the viral gene pool to the point where the viral particles can no longer successfully complete their replication cycle (Eigen and Biebricher 1988; Domingo and Holland 1994).

Ribavirin has also been associated with the induction of ISGs, which is correlated with the speed of the virological response to subsequent combination therapy (Feld, Nanda et al. 2007; Rotman, Noureddin et al. 2013). A high level of baseline ISG expression in the patient correlated with a slow virological response to combination therapy. Ribavirin pre-treatment increased the change in ISG expression level induced by combination therapy when the baseline ISG expression level was low, leading to a more rapid virological response. Ribavirin also induced interferon-inhibitory pathways more strongly when the

virological response was slow. Recently Ribavirin pre-treatment was shown to increase the induction of ISGs by pegylated interferon-combination therapy, although there was no associated change in the virological response (Rotman, Noureddin et al. 2013). Altogether, many more details about the mechanism of Ribavirin action remain to be elucidated.

1.1.5.3. Telaprevir

Telaprevir improves the efficacy of combined Ribavirin and pegylated interferon therapy in patients infected by HCV genotype 1. In clinical trials, 72-74% of treatment-naive patients receiving Telaprevir alongside pegylated interferon and Ribavirin did not have detectable levels of HCV RNAs after 24 weeks of treatment, compared to only 55% undetectable viral RNA for those not receiving Telaprevir in the same drug regimen (Jacobson, McHutchinson et al. 2011; Sherman, Flamm et al. 2011). Telaprevir is currently being tested further in phase-IV trials. Telaprevir demonstrated efficacy against HCV genotype 1 in a study of patients who either previously failed to respond, or responded only partially, to treatment or relapsed following the cessation of pegylated interferon and Ribavirin therapy (Melnikova 2008; Zeuzem, Andreone et al. 2011). The study consisted of two treatment regimens: 48 weeks of pegylated interferon and Ribavirin alone and 48 weeks of pegylated interferon and Ribavirin with the addition of Telaprevir for the initial 12 weeks. Treatment with Telaprevir resulted in 6 times more positive virological responses in the previously null responders (31% versus 5%),

an increase of almost 3 fold for the previously partial responders (57% versus 15%) and more than 3 times more positive responses in the relapsers (86% versus 24%). Taken together, this data shows strong support for the use of Telaprevir in those for whom treatment has previously failed.

The mechanism of Telaprevir action is thought to involve the inhibition of the HCV NS3/NS4A protease, which is crucial for the viral life cycle. So far, the success of Telaprevir in treating HCV has been limited to HCV genotype 1. Telaprevir treatment outcomes are also influenced by host factors such as age (Sasaki, Matsui et al. 1997; Alter, Kruszon-Moran et al. 1999; Bellentani and Tribelli 2001), ethnicity (Howell, Jeffers et al. 2000; Jeffers, Cassidy et al. 2004; Nguyen, Whittemore et al. 2004), and genetic polymorphisms near the IL28B gene (Clark, Thompson et al. 2010; Clark, Thompson et al. 2011).

1.1.5.4. Drugs in development

Boceprevir is a protease inhibitor that has undergone phase-III clinical trials (Bacon, Gordon et al. 2011; Poordad, McCone et al. 2011). SVR rates in treatment-naive patients receiving pegylated interferon and Ribavirin for 28 or 48 weeks were improved by 28% when Boceprevir was added to the drug regimen (67-68% versus 40%) (Poordad, McCone et al. 2011). Boceprevir combined with pegylated interferon and Ribavirin is also being studied in previously treatment-naive patients co-infected by HCV genotype 1 and HIV. Interim results at 24 weeks

showed twice as many patients were HCV RNA negative when also receiving Boceprevir (70% versus 34%), thus strongly demonstrating the effectiveness of adding Boceprevir to the treatment regimen for such individuals (Franciscus 2012). In another study of partially responded or relapsed patients infected by HCV genotype 1 (Bacon, Gordon et al. 2011), those who received Boceprevir alongside pegylated interferon and Ribavirin had SVR rates 3 times higher than without the addition of Boceprevir (59% & 66% versus 21%).

Asunaprevir is a protease inhibitor currently in phase-II trials for the treatment of HCV (BMS 650032). HCV genotype-1 RNA levels dropped by more than three orders of magnitude in chronically infected, treatment-naive patients receiving Asunaprevir alone for 3 days (Pasquinelli, Eley et al. 2009; Pasquinelli 2012).

Daclatasvir is an NS5A inhibitor currently in phase-III trials (BMS790052). Interim results at week 12 from a phase-II trial among treatment-naive patients receiving pegylated interferon and Ribavirin with or without Daclatasvir (20 mg or 60 mg) showed that of the genotype 1 infected patients, almost 4 times as many patients receiving Daclatasvir had undetectable HCV RNA than those not receiving the drug as part of their treatment regimen (54% versus 14%). Genotype 4 infected patients however, showed either undetectable HCV RNA in 58% of those receiving a low dose (20mg) of Daclatasvir compared to all of those receiving a high dose (60mg) thus highlighting the importance

of dose as well as drug combination (Hezode, Hirschfield et al. AASLD 2011).

In one study, Daclatasvir and Asunaprevir are being tested in multiple combinations with other drugs and with one another in patients infected with HCV genotype 1 prior non-responders. Approximately 90% of the study participants have an unfavourable IL28B allele. After 24 weeks, all of the patients receiving Daclatasvir and Asunaprevir as a double therapy showed at least a four-orders-of-magnitude drop in HCV-RNA level, with 64% of the patients having no detectable HCV-RNA level at the end of the treatment and 36% who showed SVR at week 12, maintaining SVR through to week 24. All of the patients receiving a quadruple therapy of Daclatasvir, Asunaprevir, pegylated interferon, and Ribavirin achieved SVR at week 12, with 94% maintaining SVR through to week 24. High HCV-RNA levels eventually returned, however, in 55% of all the patients receiving the double therapy, whereas high HCV-RNA levels did not return in any of the patients receiving the quadruple therapy (Lok, Gardiner et al. 2012).

Sofosbuvir, a nucleoside analogue inhibitor of the NS5B RNA-dependant RNA polymerase, is a very promising anti-HCV drug being tested in multiple phase-III trials (GS-7977). At least two studies looked at the efficacy of Sofosbuvir administered alongside pegylated interferon and Ribavirin, and at least two more studies looked at Sofosbuvir administered alongside Ribavirin alone. As part of the

Proton study undertaken in 2011, treatment-naive patients infected with HCV genotype 1 received a combination of Sofosbuvir, Ribavirin, and pegylated interferon for 12 weeks, followed by Ribavirin and pegylated interferon alone for another 12 weeks. At the end of the study, 91% of the patients had achieved SVR (Lawitz, Gane et al. 2012). A similar study looked at treatment-naive patients infected by HCV genotype 2 or HCV genotype 3, receiving Sofosbuvir alongside Ribavirin and pegylated interferon for 24 weeks. All of the patients in the study achieved SVR after 24 weeks (Kowdley, Lawitz et al. 2012).

Drug monotherapies and combinations that do not include interferon are being investigated in the interest of reducing unwanted side effects while maintaining treatment efficacy. Sofosbuvir is a candidate for such studies, and some interim results have been published for its use with Ribavirin. In one study, 100% of treatment-naive patients infected with HCV genotype 2 or HCV genotype 3 who took Sofosbuvir alongside Ribavirin for 12 weeks achieved SVR without the blood abnormalities that can occur with other drug regimens (Franciscus 2012). In one arm of the ELECTRON study, prior non-responder patients infected by HCV genotype 1 were administered Sofosbuvir alongside Ribavirin, but not pegylated interferon, for 12 weeks. Unfortunately, eight of the nine patients who initially achieved SVR at 12 weeks relapsed within another 4 weeks (Gane, Stedman et al. 2012).

Altogether, the new drugs and treatment strategies currently in clinical trials hold much promise, although many difficulties and challenges remain. As the results of more trials are released in the coming months, a better outlook on which therapies hold the most promise will be possible.

1.2. HCV life cycle

Since 2005, cell culture based methods have allowed the study of the full life cycle of HCV (Lindenbach, Evans et al. 2005; Lindenbach, Meuleman et al. 2005). From this, the life cycle of HCV has been described in the following steps summarised in the Figure 1.2:

1) **Binding**: non-specific followed by specific interactions between viral coat and host proteins allow attachment of the virus into the host cell surface (discussed in more detail later in section 1.2 .

2) **Entry/Uncoating**: virion contents are released into the host cell cytosol.

3) **Translation**: HCV genome acts directly as an mRNA in its positive strand form, acting as a template to allow translation of a single long polyprotein initiated through an internal ribosomal entry site in the 5' untranslated region.

5) **Replication**: Cytoplasmic membrane-associated replication complexes form in a perinulcear membranous web. In these complexes, transcription of negative strand RNA intermediates are

catalysed to then generate positive strand progeny RNA molecules (Rice 2001; Egger, Wölk et al. 2002).

6) **Assembly**: intracellular membranes bud into the ER lumen to form cytoplasmic vesicles/particles containing genomic RNA and capsid proteins.

7) **Budding:** the newly formed particles migrate through the lipoprotein secretion pathway.

8) **Release**: the HCV particles are then transported to the plasma membrane, possible via an exosome or sorting body, before fusing with the plasma membrane to ultimately become an enveloped virion expressing viral and host coat proteins.

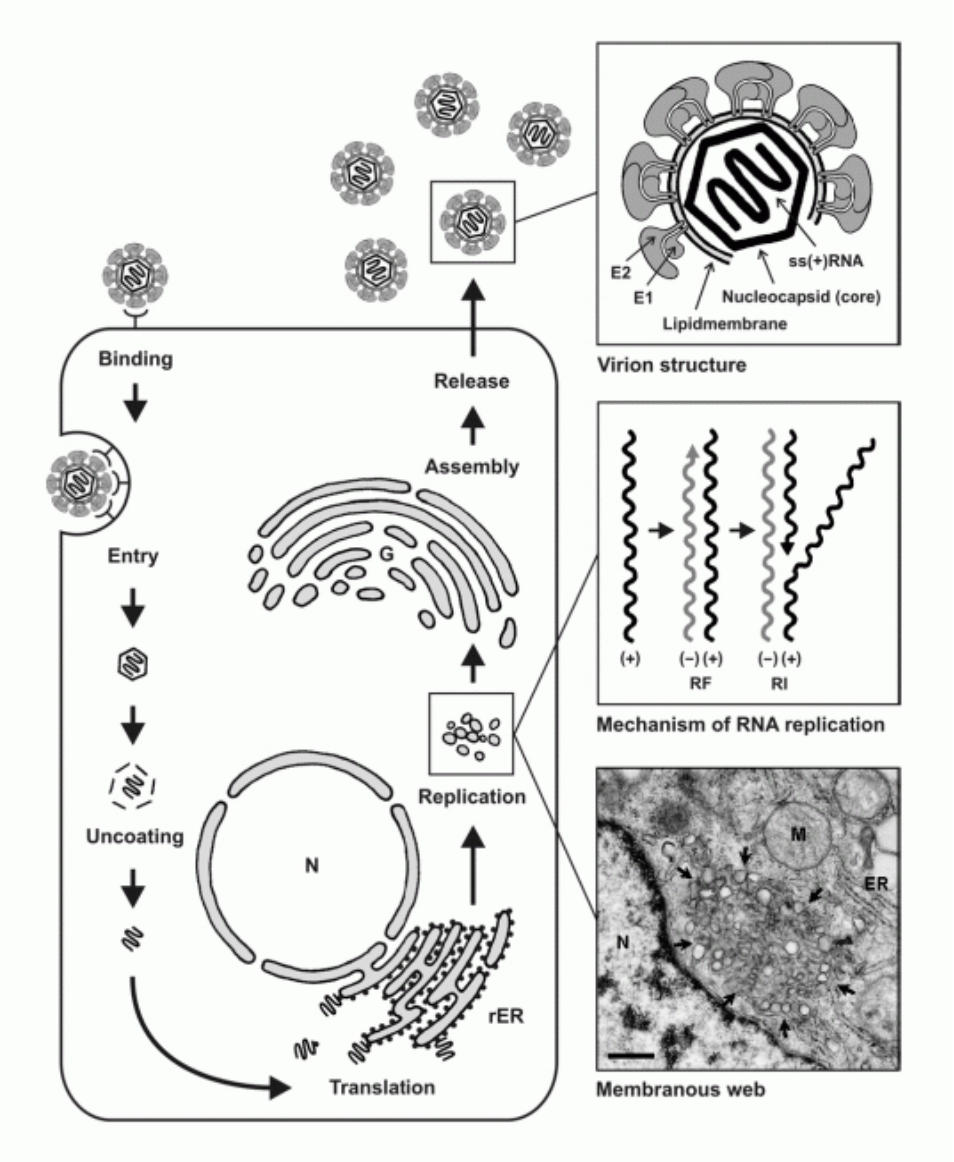

Figure 1.4 Stages of the HCV life cycle (Chevaliez and Pawlotsky 2006).

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1.3. Entry

HCV's two envelope proteins; E1 and E2, form a non covalent heterodimer involved in binding and entry with target cells (Lavie, Goffard et al. 2007; Vieyres, Thomas et al. 2010). Across the Flaviviridae family, the larger envelope protein of a pair such as for

Dengue virus and TBEV: the corresponding E(2) protein, is larger in size and appears to have a more dominant role over prM (E1) in entry (Smit, Moesker et al. 2011). Indeed E2 is thought to be immunodominant over E1 for HCV (Youn, Park et al. 2005). E2 of HCV has not been crystallised but a model has been proposed based on disulphide bridging and the related crystallised proteins prM and E (Krey, d'Alayer et al. 2010).

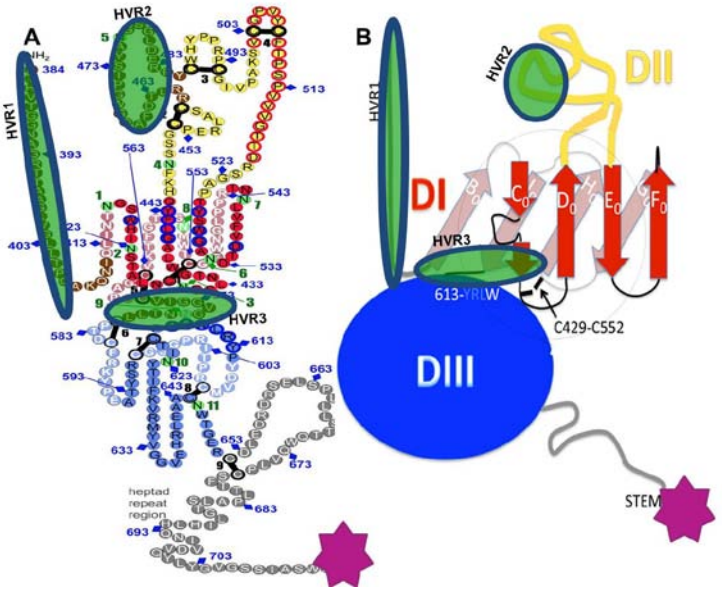

Figure 1.5 Schematic representation of the proposed structure of HCV E2 (Krey, d'Alayer et al. 2010).
A) The amino acid sequence model depicting the three domains as filled circles coloured: yellow (Domain II), red (Domain I), or blue (Domain III). Disulphide bonds are also marked as black links, green circles marked as "N" are the locations of the glycans and yellow filled circles of Domain II that have red rims mark the proposed fusion peptide. B) A more recent update of this model as a cartoon showing the general structure of the same 3 Domain colour coding (Keck, Xia et al. 2012). The three hypervariable sequence regions (HVR1, 2, & 3) are highlighted with blue rimmed green circles and the C terminus is marked with a purple star where the transmembrane domain would then begin.

Envelope proteins serve to identify and bind with specific target receptors on a host cell membrane so that a coordinated entry process can follow. Flaviviridae envelope proteins E2 (HCV) and E (Dengue) are

glycoproteins that possess a 3 domain structure containing important binding sites. E2 is proposed to comprise of 3 domains followed by a transmembrane domain anchoring the protein into the viral envelope. Across the E2 protein are binding sites for various host cell receptors, some identified, and others not yet located. Host receptors CD81 and SRB1 are two such E2 targets that have at least part of the binding sites characterized as being discontinuous across the E2 protein (Bartosch, Vitelli et al. 2003; Owsianka, Timms et al. 2006). The CD81 binding site has been characterized to involve up to four regions, though one is debated, all across the central first domain (Owsianka, Timms et al. 2006; Rothwangl, Manicassamy et al. 2008). SRB1 has also been shown to use conserved residues that are in close proximity to and also within a highly variable region that is located at the N terminus of the primary amino acid sequence of E2 (Bartosch, Verney et al. 2005). With more binding sites unknown, all of the E2 glycoprotein and E1 are still considered to be potentially involved in the process of binding and entry.

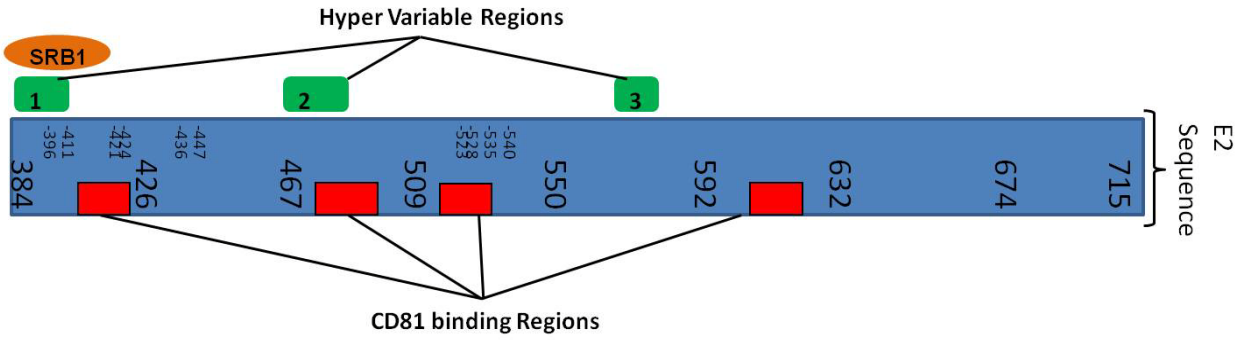

Figure 1.6 Linear sequence representation of E2
Starting from the N terminus residue 384 at the left hand side going to the C terminus at the right hand side. The Hyper-variable regions are depicted as

green boxes, CD81 binding sites as red boxes and the area containing SRB1 binding determinants marked with an orange box.

There are many details about the HCV entry process that have not yet been fully elucidated. Other flaviviruses such as Dengue virus (Modis, Ogata et al. 2004), WNV (Nybakken, Nelson et al. 2006), and TBEV (Rey, Heinz et al. 1995), however, with the aid of crystallized envelope proteins, have a fully characterized entry process and so are good references for likely mechanisms to be found in HCV. There are 9 main steps to flaviviral entry as reviewed by Smit (Smit, Moesker et al. 2011) outlined below:

1) **Circulation**: HCV virions are transported around the host in the bloodstream either as Lipo-Viro-Particles (LVP) or as empty nucleocapsid free sub-viral particles associated with apoB and complexed with antibodies to the envelope proteins of HCV. The LVPs consist of triglyceride rich lipoproteins (LDL and VLDL), associated with fully infectious virions (Bassedine, Sheridan et al. 2012)

2) **Adhesion:** When the viral particle first comes into proximity with a target cell there are weak interactions with the negatively charged glycosaminoglycans, such as heparin sulphate, that are abundantly expressed on cells. These low affinity attachments allow virus to concentrate at a cell surface.

3) **Binding:** Specific interactions can then occur between the envelope proteins of the virus and co-receptors found on the cell e.g. Laminin Receptor binding to Dengue Virus E protein (Thepparit and Smith 2004).

4) **Endocytosis:** Formation of a receptor complex triggers the formation of a clathrin coated vesicle by endocytosis to internalize the virus into the cell.

5) **Delivery:** The virus-containing endocytic vesicle migrates to deliver the virus to an early endosome.

6) **Endosome Maturation:** It must be noted that the subcellular compartment in which fusion occurs is most likely reliant on the pH-dependant membrane fusion properties of the virus in question. The early endosome carrying the virus can at this point mature into a late endosome.

7) **Envelope protein molecular changes:** Reaching a pH of 5-5.5 in the endosome triggers multiple effects in the viral envelope protein including dissociation, and energy releasing conformational changes that allow exposure of the fusion loop.

8) **Fusion loop insertion:** Fusion is initiated by the insertion of the fusion loop in the viral glycoprotein into the outer leaflet of the endosome membrane.

9) **Pore formation:** A fusion pore is formed which can then enlarge to allow-

10) **Nucleocapsid release:** The virion contents can now enter the cytosol of the target cell to initiate replication.

The entry process of HCV is thought to occur via a similar order of events to the other flaviviruses described, however some distinct differences have already been identified as HCV is functionally different. Firstly, unlike other flaviviruses HCV is transported through the circulation of the host by association with apolipoproteins. Once HCV reaches a target cell it can adhere to Low-Density-Lipoprotein-Receptors or the glycosaminoglycan chains of cell surface proteoglycans (Helle and Dubuisson 2007). Once the virus has reached a target cell, it is in closer proximity to cell surface expressed receptors with which specific interactions can occur. The first receptor to become involved with the HCV envelope proteins is Scavenger Receptor class B-1 (SRB1), followed by addition of the host tetraspanin receptor CD81 which both bind to E2 to form a receptor complex (Ziesel, Fofana et al. 2011). Two more receptors Occludin and Claudin-1 are also recruited. This is followed by the formation of a clathrin coated pit (Evans, von Hahn et al. 2007; Ploss, Evans et al. 2009). Entry determinants have so far been indentified for CD81 with particular focus on residues G530 and D535 (Drummer, Boo et al. 2006; Owsianka, Timms et al. 2006). Indeed the importance of residues 530 and 535 of E2 are highlighted by their critical role in the epitopes of several broad acting anti-E2 antibodies. Formation of a vesicle by endocytosis and a drop in pH to around 5 is finally followed by fusion of the host and viral membrane to release the

virus particle contents into the cytoplasm of the cell where replication then occurs (Blanchard, Belouzard et al. 2006).

Conformational changes are thought to occur in the envelope proteins of HCV in the entry process (Tscherne, Jones et al. 2006), in a similar fashion to that which occurs for HIV and Dengue virus (Smit, Moesker et al. 2011) (Sattentau and Moore 1991). The purpose of such alterations in conformation is to aid proximity of functional regions to their targets e.g., Dengue virus bending within the E protein to draw the viral and host membrane into contact for fusion. Conformational changes in envelope proteins can result in exposure of functionally important sites. An example of this is the interaction of HIV gp120 and host CD4 receptors, after which the binding site for CCR5 is unveiled from behind variable loops of viral gp120 to allow binding (Sattentau and Moore 1991).

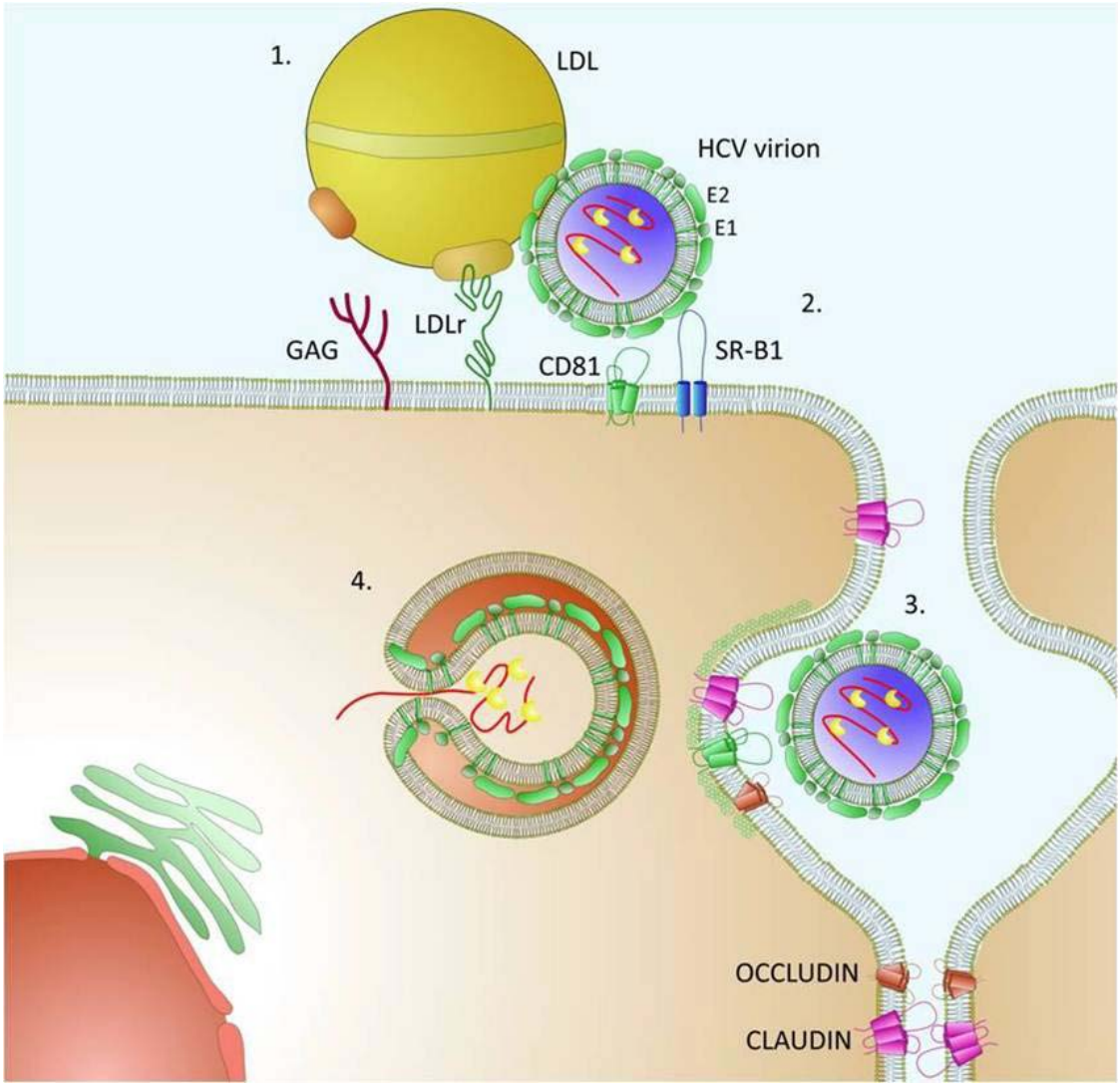

Figure 1.7 Cartoon depicting the steps involved in HCV entry. Beginning at stage 1 where virus is transported associated to LDL, adhesion to GAG and LDLR that allow stage 2 to occur where specific cell receptors interact with E2. Stage 3 is the recruitment of further cell surface receptors leading to formation of the endocytic vesicle resulting in stage 4 where virion contents are released into the host cell cytosol (Edwards, Tarr et al. 2012).

1.4. Immune Evasion

Viruses often conceal important binding sites on the envelope proteins to shield and protect them from antibody targeting. Solvent exposed regions of envelope proteins can possess important mechanisms of immune evasion such as glycan shielding (Helle, Vieyres et al. 2010)

(also reviewed in (Helle, Duverlie et al. 2011)) and sequence diversity (von Hahn, Yoon et al. 2007; Dowd 2009). To aid the concealment of central and functionally important regions of the envelope proteins, HCV is thought to use the glycan coat to physically obstruct anti viral access to precious regions. This mechanism is also adopted by other viruses like HIV and Influenza (Vigerust and Shepherd 2007). Initial work looking specifically at the function of the HCV E1E2 glycan coat has shown an improvement in immune response to E1 in the absence of glycosylation, thus supporting the notion that the glycan coat of HCV envelope proteins served as a shield (Fournillier, Wychowski et al. 2001; Liu, Chen et al. 2007). An additional mechanism in HCV immune escape is that the exposed parts of the envelope proteins consist of variable primary structure, resulting in variable secondary structure so that antibodies raised against these variable regions will be ineffective at clearing the mixed virus population that for HCV exists as a quasispecies.

The nature of an RNA polymerase means it is often error-prone, especially in the absence of proof-reading activity, and thus promotes sequence variation in the proteins it transcribes. In the E2 glycoprotein of HCV there have been 3 regions of hyper-variability identified that aid escape from immune targeting (see Figure 1.2). These regions designated Hyper-Variable Region 1, 2 (HVR1 & HVR2), and Intergenotypic Variable Region/IgVR (HVR3) reside in domain 1, domain 2, and between domain 2 and 3, respectively (Wiener, Brauer et

al. 1991; Kato, Oostuyama et al. 1992; McCaffrey, Boo et al. 2007). HVR1 is a strong inducer of antibodies thus drawing the focus of an antibody response away from more conserved regions (Zibert, Schreier et al. 1995). Though antibodies raised against HVR1 can be neutralizing, their limited breadth means they are mostly ineffective against a heterologous species (Zhou, Shimizu et al. 2000). Clinical effectiveness of antibodies is reliant on cross-neutralizing activity to allow targeting of the mixed viral population existing in patients. The antigenic variation displayed in the envelope proteins of the virus is a passive mechanism of immune escape. Another evasive mechanism exhibited by HCV is to spread the infection by cell-cell transmission through tight junctions thus avoiding circulating freely between cells where more antibodies roam (Timpe, Stamataki et al. 2008; Brimacombe, Grove et al. 2011). Consequently it has been shown that CD81 is not involved in cell to cell transmission of virus after an infection has been initiated highlighting that through this secondary route of transmission, virus evades extracellular exposure (Witteveldt, Evans et al. 2009; Jones, Catanese et al. 2010). This raises the importance of intracellular targeting of virus once an infection is already established to complement a given vaccine model.

1.5. Intracellular Targeting of HCV

The first immune phase following infection with HCV is dominated by innate responses. This immediate phase is considered the period of time up to 12 weeks post infection in which large amounts of type 1 interferon's are normally produced by hepatocytes and dendritic cells (Hiroishi, Ito et al. 2008). The production of interferon's in hepatocytes is initially induced as a consequence of detecting viral RNA. Briefly, pattern recognition receptors RIG-I and TLR 3 of hepatocytes recognise a polyuridine motif of the 3' untranslated region of the HCV genome in the cytoplasm (Saito, Owen et al. 2008), and double stranded RNA in endosomes (Kawai and Akira 2008) respectively. Via adaptor molecules a signalling cascade is initiated that uses IPS-1, TRIF and allows the phosphorylation of NFκB, IKKε & TNFR. These events lead to dimerisation of the transcription factor IRF3 which then translocates to the nucleus to activate transcription of interferon β in synergy with NFκB (thereby creating a positive feedback loop) (Rehermann 2009). HCV however has evolved mechanisms to obstruct this pathway as the viral proteins NS3/4A cleave TRIF and ISP-1 thereby blocking TLR3 and RIG-I signalling (Foy, Li et al. 2005).

Production of type 1 interferon's from neighbouring uninfected hepatocytes subsequently occurs in response to IFN β. Signalling via JAK/STAT pathways leads to production of PKR which inhibits translation of viral and host RNA, and production of IRF 7 which

induces IFN α, collectively creating an antiviral state and thus preventing infection from HCV (Pflugheber, Frederickson et al. 2002). Yet again HCV proteins have evolved mechanisms of interference. HCV core protein inhibits STAT1 phosphorylation thus inhibiting signalling that leads to IFN α production (Lin, Kim et al. 2006). Other cell types are also involved in the production of type 1 interferon's. Among dendritic cells there is a subset named plasmacytoid dendritic cells (pDC) which rapidly produce high amounts type 1 interferon's in response to viral infection. Recognition of viral components and nucleic acids in these cells occurs via pattern recognition receptors TLR 7 & 9 (Siegal, Kadowaki et al. 1999; Matsui, Connolly et al. 2009). Indeed the importance of pDCs and interferon production in viral infection is highlighted by a reduced frequency of pDCs and impaired interferon production by pDCs observed in the peripheral blood of chronic HCV patients (Kanto, Hayashi et al. 1999; Kanto, Inoue et al. 2004; Dolganiuc, Chang et al. 2006). Equally, an increased amount of conventional dendritic cells in acute infection is linked to clearance whereas loss in number of dendritic cells may increase risk of developing chronic infection (Wertheimer, Bakke et al. 2004). Responsiveness to pegylated interferon and Ribavirin treatment in HCV infection has been linked to expression of specific genes by gene profiling thus highlighting the importance of effective innate responses in clearing HCV infection (Chen, Borozan et al. 2005). Indeed DNA vaccines allow the induction of PKR to instigate cascades of interferon

inducible genes as little as two days post infection (Bigger, Brasky et al. 2001).

The production of cytokines following viral infection also allows cross talk between the innate and adaptive system as cytokines such as interferons contribute to priming of T cells. Cells presenting viral peptide antigens in their MHC I molecules such as hepatocytes, can interact with CD8+ T cells, stimulates them to produce IFN γ, stimulating neighbouring cells to inhibit HCV replication as part of an anti-viral state, or to lyse the infected cells via release of granzymes and perforins.

The T cell response has been considered critical for clearance of HCV infection (Lechner, Wong et al. 2000; Thimme, Oldach et al. 2001). Indeed depletion of CD4+ T (Grakoui, Shoukry et al. 2003) or CD8+ T cells (Shoukry, Grakoui et al. 2003) has been linked to viral persistence. The detection of fully functional virus specific CD4+ T cell responses that allow priming of CD8+ cells has also been linked to clearance of virus (Missale, Bertoni et al. 1996; Gerlach, Diepolder et al. 1999). More importantly, a temporal link has been made between the appearance of such T cell responses and the likelihood of achieving viral clearance (Cooper, Erickson et al. 1999; Lechner, Wong et al. 2000; Thimme, Oldach et al. 2001; Thimme, Bukh et al. 2002). Virus specific CD4+ T cells have been found up to 20 years after documented infection

(Takaki, Wiese et al. 2000), highlighting that it is not only B cell responses that are capable of being long lasting.

There has been mounting evidence that B cell responses also contribute to HCV clearance and protection. Characterisation of one particular cohort of patients exposed to the same HCV inoculum showed a direct correlation between rapid induction of neutralising antibodies and clearance during the acute infection (Lavillette, Morice et al. 2005; Pestka J. M., Ziesel et al. 2007).

Antibodies can also be used for intracellular targeting of virus. This has been successfully adopted for multiple viruses including the related West Nile Virus which has an entry pathway thought to reflect that of HCV (Irnai 1998; Zwick, Labrijn et al. 2001; Thompson 2009). Such antibodies target epitopes that are only exposed following conformational changes in the viral envelope proteins that occur in the endosome. The same approach has recently allowed identification of a peptide inhibitor of the HCV related Dengue Virus that also binds the envelope protein specifically following molecular changes as a result of internalization into the endosome (Schmidt , Yang et al. 2010). With proof that antibodies can be used to target virus intracellularly for various viruses including related viruses to HCV, it could be a promising avenue of research for HCV. However, the molecular differences between these viruses and their entry pathways will need to be considered before applying methods successful for Dengue and WNV to HCV. For example, Dengue virus infects cells via surface expressed Fc

receptors which are not present on hepatocytes. Also, repeated infection with Dengue allows enhancement of infection due to the involvement of Fc receptors as an entry factor. Such a mechanism has not been observed for HCV and so intracellular factors to be targeted for HCV infection will need to be specifically involved in HCV activity.

1.6. Kinetics of the antibody response

The dynamics of viral and host immune activity upon viral challenge can influence the progression and clearance of an infection. Normally the infection process consists of a burst of viral replication in the new host followed by a large production of specific antibodies that hopefully sequester the high viral titers. As the majority of HCV infections do not resolve, this scenario is not often the case. It has been observed that in acute infections of HCV, an early antibody response is mounted in some infected individuals that allows a more effective targeting of HCV in the host whilst the viral titers are low (Shimizu, Hijikata et al. 1994; Zibert, Schreier et al. 1995; Rosa, Campagnoli et al. 1996; Dowd 2009). Infections that become chronic can be characterized by a delayed antibody response (Ishii, Rosa et al. 1998; Pestka J. M., Ziesel et al. 2007). The consequence of neutralizing antibodies only being produced when the viral titer has become high is that there is also a greater diversity in the heterogeneous viral population thus making any antibodies produced less able to broadly target the quasispecies present. Given the challenges that face the mounting of an effective immune

response to HCV infection, treatment with potently cross neutralizing antibodies would be more likely to succeed if administered before exposure to virus rather than after infection was established (Meuleman, Bukh et al. 2011).

The administration of antibodies for managing viral infection is termed passive immunization and is used most commonly for the prevention of Hepatitis B, Tetanus, Varicella Zoster and Rabies following or preceding possible exposure (Keller and Stiehm 2000). The indication of protection from HCV infection using passive immunization was first observed retrospectively in 1987 following a double blind randomized study of the use of immune serum globulin between 1967 and 1970 (Conrad and Lemon 1987). This study revealed that prophylactic administration of immune serum globulin (ISG) allowed a significant drop in incidence of clinical hepatitis. When looking specifically at non-A, non-B hepatitis that is today known to consist of mostly Hepatitis C, there was still a significant difference between the test and placebo groups thus promoting the use of ISG prior to exposure. The same result was also demonstrated more recently following a retrospective study of the administration of human hepatitis B Immunoglobulin (Feray, Gigou et al. 1998). Research elsewhere has also demonstrated the ability of sera containing HCV-specific antibody to neutralize, prevent and clear infection *in vivo* (Choo, Kuo et al. 1994; Farci, Shimoda et al. 1996; Rosa, Campagnoli et al. 1996; Ishii, Rosa et al. 1998).

More recently, protection from HCV infection by passive immunotherapy has successfully been demonstrated in two animal models. Firstly, chimpanzees were given immunoglobulin preparations either containing or depleted of anti-HCV antibodies and then challenged with viral inoculum. Only the animal that was administered anti-HCV containing immunoglobulin demonstrated protection from infection by absence of detectable HCV RNA over the full 58 week test period following challenge (Yu, Bartosch et al. 2004). The two control animals however demonstrated infection, with seroconversion occurring at weeks 9 and 11 post challenge. Similarly, an infection study with passive immunotherapy in human liver-chimeric mice demonstrated protection in 55% (5/9) of mice that received HCV immunoglobulin compared to 0% in the control group (Law, Maruyama et al. 2008). While presence of HCV RNA was initially detected shortly after challenge, these animals demonstrated successful clearance of infection despite being an immune-compromised animal model, thus owing the successful outcome to the administration of HCV IgG. Taken together, the demonstration of complete protection in the chimpanzee and clearance in the immune-compromised mouse indicates potential for success with passive immunotherapy in humans to prevent re-infection following a liver transplant.

Active immunisation however, involves the administration of viral material to induce a response in the host. In 2005 a study distinctly showed the presence and involvement of induced neutralising sera in

the response to an accidental infection where the immune response, recovery and control of the HCV infection in humans was analysed in real time (Lavillette, Tarr et al. 2005). This study followed a nosocomial outbreak of HCV and thus was able to identify the normally hard to find spontaneous clearers along with tracing the immunological events for patients who did and didn't resolve their infection. A key finding from this study was that the HCV specific sera were able to neutralise/inhibit or facilitate *in vivo* HCV infection assays depending on whether the patient displayed an acute or unresolved infection by the end of the 6 month test period. Correlation of the neutralising sera with patients who spontaneously cleared their infection demonstrated the neutralising capability of human sera *in vitro* as well as indicating the *in vivo* potential. The other important indication from this study was the kinetics of the neutralising responses in relation to the outcome of the infection. In agreement with Pestka 2007 and Dowd 2009 (Pestka J. M., Ziesel et al. 2007; Dowd 2009), the appearance of an early neutralising response following infection strongly correlated with the clearance of the virus whereas patients showing a delayed antibody response were much more likely to remain chronically infected. So, the natural course of disease in humans promotes the need for neutralising antibodies immediately following (or prior to) infection to be able to effectively clear the virus.

Disease management can involve the use of human specific sera administered as passive immunotherapy as a preventative measure

against possible infection. Two studies have employed this rationale for HCV and shown the potential for use of human HCV specific sera in preventing infection (Davis, Nelson et al. 2005; Schiano, Charlton et al. 2006). Using purified antibody or polyclonal sera, these studies demonstrated the ability to reduce HCV RNA levels and induce high levels of serum antibodies following liver transplantation for chronically infected patients. Whilst the results weren't absolute, they do reveal further potential for the application of antibodies in controlling HCV infection outcome. The natural need for antibodies in clearing HCV infection can be seen in humans unable to mount normal antibody responses such as the condition hypogammaglobulinaemia in which there is a deficiency of all classes of immunoglobulins (Bjoro, Froland et al. 1994). In these individuals it has been shown that the incidence of chronic HCV infection following exposure is much greater than in healthy subjects and the disease progression much more severe. From this background of studies and data, efforts have been employed to search for and identify several broad acting potently neutralizing antibodies that have potential for use in preventative passive immunization.

1.7. Neutralising Antibodies and epitopes

Various methods and approaches have been employed in the search for neutralising HCV antibodies. The use of infected sera demonstrated the neutralising capacity of naturally induced antibodies in humans in the response to infection (Lavillette, Tarr et al. 2005). Examples of potent broadly neutralising antibodies are the mouse derived AP33 and human antibodies AR3A, A8 and 1:7 which are all capable of acting against at least 5 of 6 genotypic groups tested (Owsianka, Tarr et al. 2005; Johansson, Voisset et al. 2007; Law, Maruyama et al. 2008). The neutralising capacity of these antibodies has not been compared though they each have a varied activity profile, mostly showing the strongest neutralising potency against the world dominant genotypes 1, 2 and 3. All of these antibodies target the HCV envelope glycoprotein E2 with epitopes involving various discontinuous parts of the protein. This panel of antibodies shares a dependence on key residues for the binding of the important co-receptor for viral entry: CD81. The key involvement of CD81 in entry means the residues it binds to are thus highly conserved across all genotypes making antibodies that have these residues (G530 and D535) in their epitope more likely to be broad acting.

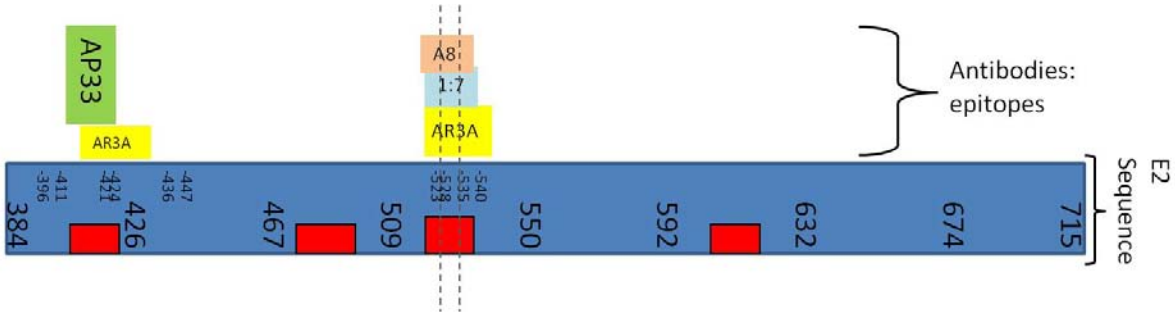

Figure 1.8 Linear representation of E2, marking the location of epitopes for neutralising antibodies 1:7, A8, AP33 and AR1A.
The dashed vertical lines depict the location of the highly conserved residues G530 and D535 crucial for CD81 binding.

CD81 is a key entry factor for HCV found on many cell types including human hepatocytes and some other cell types. During the entry process CD81 becomes part of the receptor complex with E2. The binding regions for the interaction between CD81 and E2 have been defined on both proteins. CD81 acts more precisely as a post attachment co-receptor and so its involvement precedes fusion and any conformational changes in E2 that might occur for it. The CD81 binding sites on the E2 protein are comprised of a collection of residues spread across the central domain I grouped into three main regions designated as residues 474-492, 522-551 and 612-619 being CD81 binding regions I, II and III respectively (Roccasecca, Ansuini et al. 2003; Tarr, Owsianka et al. 2006) (see Figure 1.3 & Figure 1.5). More recently however, it has been shown that it is only regions II and III that appear to be crucial for CD81 binding even though antibodies with epitopes involving the first region compete with CD81 (Owsianka, Tarr et al. 2005; Rothwangl, Manicassamy et al. 2008). Linked to its importance in entry, the CD81 binding regions have been the most studied highly conserved region of

E2 and have produced the predominant portion of the broad acting neutralising epitopes.

Current research has shown a predominance of broadly acting neutralising antibody to target CD81 binding regions. However this is not exclusive as there has been a most recent trend in research focusing on non-CD81 epitopes. Three groups have recently characterized novel broadly acting neutralising antibodies recognising novel epitopes (Sabo, Luca et al. 2011; Giang, Dorner et al. 2012; Keck, Xia et al. 2012). One study produced a set of these antibodies that did not block E1E2-CD81 interaction but could potently neutralise virus *in vitro* (Giang, Dorner et al. 2012). Another group produced an antibody that could block soluble E2 binding to CD81 and SRB1 in a cross reactive manner whilst not being mapped to any of the binding residues for these receptors (Sabo, Luca et al. 2011). The antibodies studied were derived from phage display biopanning and myeloma-splenocyte fusions and so these data support the search for further novel non-CD81 binding site cross-neutralising antibodies.

1.8. Hyper-variable regions

The conserved nature of the CD81 binding regions makes them ideal targets for antibody mediated neutralization. To help protect against this, the key CD81 binding residues are kept central to the E2 protein rather than being exposed on the most accessible surface reaches of the

upper domain II. According to the current proposed model of HCV E2, the most solvent exposed region of E2 is the second region of hypervariability (HVR2) representing residues 474-482 (Kato, Oostuyama et al. 1992). The first hypervariable region (HVR1), residues 384-410, is mapped to the start of the E2 sequence where it forms the beginning of Domain I (Wiener, Brauer et al. 1991) (see Figure 1.2). The third hypervariable region which is also known as Intergenotypic Variable Region (HVR3/IgVR) comprises residues 570-580 modelled to sit between domain I and III (McCaffrey, Boo et al. 2007). The location of these regions means they are easily accessible on the surface and thus are much more likely to be able to induce an antibody response than residues that are hidden in the core of the E2 protein.

Multiple viruses use the ploy of variable sequences to evade an effective antibody response or to draw away focus from important functional regions (Sattentau and Moore 1991; Wyatt, Sullivan et al. 1993; Gzyl, Bolesta et al. 2004). HVR1 on the HCV E2 protein lies close to and among highly conserved residues that are involved in the binding of entry receptor SRB1 (Bartosch, Verney et al. 2005). Another functional region identified on the surface of the E2 protein is the fusion motif that is suggested to be residues 502-520 (Albecka, Montserret et al. 2011) and so hypothetically, in the tertiary structure is thought to lie in very close proximity to the HVR2, collectively forming the uppermost tip of domain II of E2 (see Figure 1.5). HVR3/IgVR however has not been directly linked to any functional regions though neutralizing epitopes

have been identified in close proximity on either side of it in domain I and III. HVR3 also lies between the second and fourth putative CD81 binding sites found in Domain I and III respectively (Yagnik, Lahm et al. 2000; Roccasecca, Ansuini et al. 2003; Owsianka, Timms et al. 2006) (see Figure 1.3). Bridging domain I and III, IgVR also lies close to a loop of the domain I that holds one of the Antigenic Region-3 epitope containing areas (Law, Maruyama et al. 2008). On the other side of IgVR/HVR3 is a section of domain III where the highly conserved cross neutralising linear epitope for antibody ALP98 can be found (Owsianka, Clayton et al. 2001; Clayton, Owsianka et al. 2002). Whilst neutralizing epitopes can also be found within these hyper variable regions, they are mostly if not all unable to cross neutralize efficiently compromising the likelihood of raising an effective immune response *in vivo*.

One approach adopted in vaccine design is to remove/delete obstructive non-functional parts of an immunogen to increase the chances of mounting antibodies against crucial conserved regions. This approach has been tried out in HIV with various results. The use of recombinant HIV glycoproteins as a vaccine has shown some success in protecting against heterologous challenge in chimpanzees (Berman, Gregory et al. 1990; El-Amad, Murthy et al. 1995; Berman, Murthy et al. 1996). Removal of specific variable loops of the HIV envelope protein gp120 did not diminish virus function whilst also allowing an improvement in the induction of cell mediated responses, eliciting neutralising

antibodies, and improving exposure of neutralising epitopes/conserved regions (Wyatt, Sullivan et al. 1993; Barnett, Srivastava et al. 2001; Gzyl, Bolesta et al. 2004). Though HCV and HIV are of different virus groups and families, they share the same problem of having highly glycosylated envelope glycoproteins that house multiple highly variable sequence segments that occlude functional conserved regions (Wyatt, Sullivan et al. 1993; Prentoe, Jensen et al. 2011). So, the results for HIV provide supporting evidence to the possibility of there being a similar result in HCV. So far, variable region deleted HCV envelope glycoproteins haven't been tested for their function as immunogens in humans but their functional characteristics have begun to be looked at in recent years.

Within the structure of HCV E2, the HVR's are a candidate for removal to allow exposure of more central conserved regions such as the CD81 binding regions. Few groups have removed the HVR's in combination and singly to demonstrate that expression, secretion and antigenic profile are retained (Op De Beeck, Voisset et al. 2004; McCaffrey, Boo et al. 2007). Using a panel of cross domain conformational antibodies, wild type and triple deleted E2 (lacking HVR1, 2 & 3), showed minimal change in the global and core structure with a slight improvement of binding noted for some antibodies used. Functional ability was assessed to not be changed by HVR removal after demonstrating no loss or great change in the ability to bind to the key host receptor used in entry; CD81 (Roccasecca, Ansuini et al. 2003). The notion that deletion of HVR has

no or minimal detrimental effect on E2 structure or function has also been upheld elsewhere and thus has been an accepted conclusion (Op De Beeck, Voisset et al. 2004; Bankwitz, Steinmann et al. 2010).

The full function of E2 however is not limited to its binding to CD81 as at least three other receptors are also involved in the entry process. SRB1 is another E2 binding receptor for which deletion of only HVR1 has been tested. Testing of SRB-1 function has not been investigated as extensively as CD81 partly due to practical difficulties of producing soluble forms of SRB-1 that do not apply to CD81; which can readily be produced in a soluble form. The use of soluble SRB1 would allow more flexibility in the assays that can be used and also a quicker advancement of research into factors affecting SRB-1 and HCV E2 binding. Even so, without having tested any relationship with HVR2 or 3, only the relevance of HVR1 has thus been associated with SRB-1. Within HVR1 on E2 are highly conserved single residues; some of which have been identified as being involved in SRB1 binding (Bartosch, Verney et al. 2005). Initial research showed an inability of SRB-1 to bind E2 lacking HVR1 (Scarselli, Ansuini et al. 2002). More recently however, *in vitro* produced pseudo HCV virus lacking HVR1 from E2 was able to bind SRB-1 expressed on cultured cell lines and the deletion of HVR1 did not affect the ability of virus to infect cells (Bartosch, Verney et al. 2005; Voisset, Callens et al. 2005). In the absence of HVR1, the rate of entry of pseudo particle virus was however reduced implying a secondary manner in which HVR1 might affect entry (Bartosch, Verney et al.

2005). Whilst HVR1 is not thought to be essential for entry, it is influential in binding and the neutralising response in a genotype specific manner (Prentoe, Jensen et al. 2011) and so its effects on the rest of the protein still need further investigation.

In a similar fashion, it cannot be ruled out that the remaining HVR's of E2 could still exhibit direct or indirect effects on the protein or the entry process. Using genotype 2a virus cultured *in vitro*, HCV lacking HVR1 was seen to be able to infect normally, however a lack of HVR2 or 3 greatly reduced the virus' infectivity (McCaffrey, Gouklani et al. 2011). To try and understand this difference, a comparison has been made between initial cell entry that is dependent on CD81 not requiring any HVRs, and a cell to cell route via tight junctions that bypasses the need for CD81 (Timpe, Stamataki et al. 2008; Brimacombe, Grove et al. 2011). Data suggests cell to cell transmission of HCV is largely able to avoid antibody mediated neutralization that is otherwise effective before initial infection has occurred (Meuleman, Hesselgesser et al. 2008). So, whilst binding to CD81 or SRB-1 may not be greatly compromised by the removal of HVR's, binding to other receptors involved in entry could be.

1.9. Vaccine approaches

In the last 10 years there have been many clinical trials of preventative and therapeutic HCV vaccines. Therapeutic vaccines aim to

improve/induce immune activity of an infected host to enable clearing of an infection or reduction of viral load to a sustained virological response. Recombinant-E1E2, expressed in Chinese Hamster Ovary (CHO) cells in MF59 adjuvant, is a vaccine candidate from Chiron Corp, which has been developed as both a preventative and later as a therapeutic vaccine when administered in combination with existing drug regimens (Colombatto, Brunetto et al. 2009). In a recent phase 1b clinical trial, the therapeutic vaccine E1E2-MF59 was shown to have minimal adverse effects and second phase viral load decay was improved in those receiving the vaccine plus standard treatment with a viral median half life, measured from days 4-48, of 5.6 days compared to a half life of 10 days for those receiving standard treatment only. There were also concomitant increases in neutralising antibody titers, in subjects receiving standard treatment plus the vaccine compared to standard treatment alone (Colombatto, Brunetto et al. 2009; Frey, Houghton et al. 2010). Alvarez-Lajoncheres group used a DNA plasmid vaccine encoding HCV structural genes, and a recombinant core gene named (CIGB-230), administered therapeutically to patients who had previously not responded to drug therapy (Alvarez-Lajonchere, Shoukry et al. 2009). Neutralising antibody and T cell responses were present in the majority of patients along with an improvement in liver histology and fibrosis. Although viraemia persisted, the positive results are encouraging as a method to improve the host's ability/chances of clearing infection. Intercell produced a vaccine IC41 consisting of synthetic peptides encoding various highly conserved T cell epitopes

(Klade, Wedemeyer et al. 2008). Initially this vaccine model tested therapeutically showed promise at phase 2 trials by inducing T cell responses but without reductions in viral loads the trials did not go further. So, the vaccine schedule was optimized and then a significant drop in viral load was observed after only 4 months of treatment (Firbas, Boehm et al. 2010). Thus the vaccine IC41 provided proof of concept of using therapeutic vaccines specifically targeting T cell responses to contribute to the control of HCV infection.

There have also been multiple preventative vaccines tested with varying degrees of success. Stamatakis group also tested a recombinant HCV E1E2 vaccine for prophylactic use (Stamataki, Coates et al. 2007). They tested their vaccine in guinea pigs and saw substantial production of antibody titers that could neutralise pseudoparticle infectivity as well as heterologous strains of cell cultured HCV at low levels. Chiron/Novartis have also developed other prophylactic vaccine models based on ISCOMATRIX adjuvanted recombinant HCV proteins. In healthy humans a recombinant core protein vaccine induced anti-core antibodies and T-cell proliferative responses indicating good immunogenicity (Drane, Maraskovsky et al. 2009; Houghton 2011). Their other prophylactic vaccine model is a yeast derived fusion HCV polypeptide containing core and NS3-5 adjuvanted with ISCOMATRIX and was tested in chimpanzees showing vigorous multi-specific T-cell responses but no clearance was observed (Houghton 2011).

Neither the B nor T cell response to HCV infection has been proven to be solely and independently deterministic of viral clearance. So, to complement the focus on antibody mediated clearance, multiple vaccine candidates have been developed that focus on the T cell response. Folgoris group developed a prophylactic T-cell genetic vaccine comprising the DNA of genotype 1 HCV non-structural genes NS3-5B (Folgori, Capone et al. 2006). This vaccine was tested on chimpanzees, which are known to resolve HCV more easily than humans, and were given replication-defective adenoviral-vector vaccine injections, followed by application of a brief electrical field to the site of injection (electroporation) to encourage uptake of plasmid DNA into cells. All but 1 of the 5 vaccinated chimpanzees developed HCV specific CD4+ and CD8+ T-cell immune responses, and showed protection from acute infection from heterologous challenge. Whilst these findings are encouraging for the basis of priming T cell responses to aid clearance of acute infection in chimpanzees, they need to be supported in humans.

Vaccine delivery by infectious agents has been an approach employed successfully for combating infectious diseases (Paglia, Medina et al. 1998; Koesling, Lucas et al. 2001; Xie, He et al. 2007) and recently including HCV. Recently, a prophylactic prime boost strategy has been tested to induce both humoral and cellular responses in mice and macaques (Garrone, Fluckinger et al. 2011). The initial prime consisted of E1E2 containing recombinant adenovirus, following by a boost

consisting of virus-like particles pseudotyped with E2 and/or E1. Multi-specific T-cell responses and cross-neutralising antibodies were found in the test subjects. As with all data collected in non humans, further investigation would be needed in humans. Initial attempts at using attenuated *Salmonella typhimurium* containing an expression plasmid for delivery of HCV core protein as a prophylactic vaccine approach saw immune responses to the expressed protein but not the viral DNA (Lia, Ren et al. 2007). Optimisation of the vaccine involved the inclusion of the envelope protein E2 gene in a separate expression cassette of the plasmid to allow immune responses and antibodies in response to the translation of both core and E2 genes (Cao, Chen et al. 2011). Purified immunoglobulin collected from the infected mice used in the study, were able to neutralise the infectivity of HCV pseudoparticles of autologous and heterologous cell cultured virus *in vitro*. To address the potency of the viral vector to be used in a prophylactic vaccine, Colloca and his group assessed more than 1000 adenoviral vectors for use in humans as a vaccine delivery model according to immunogenicity and potency (Colloca, Barnes et al. 2012). From this selection pool, two vectors were tested as HCV vaccine models containing non structural proteins with the aims of priming the immune system in healthy human volunteers in a phase I trial (Barnes, Folgori et al. 2012). The vectors induced HCV-specific T cell responses targeting multiple proteins as well as different genotypes that were sustained for at least 1 year after vaccination. With these promising results, the ability to protect against HCV *in vivo* would be the next crucial challenge.

1.10. Glycoproteins as an Immunogen

Many vaccine models have been proposed for HCV including DNA vaccines, recombinant adenoviral vaccines and modified subunit vaccines. Each of these models show varying strengths for their potential in the human host (reviewed in (Torresi, Johnson et al. 2011)). Initial data that provided support for the use of modified envelope protein as part of a vaccine formulation was provided by Choos group in 1994 and Farcis group in 1996, who demonstrated that immunization of chimpanzees with a peptide vaccine based on the variable regions of envelope glycoprotein E2 could elicit neutralizing antibodies that prevented infection on subsequent challenge with autologous virus (Choo, Kuo et al. 1994; Farci, Shimoda et al. 1996). A multi-epitope immunogen based on the HVR1 region of E2 successfully demonstrated high titer antibody responses that could bind and inhibit HCV E1 and E2 of different genotypes (Torresi, Stock et al. 2007). Recently HVR1 has been shown to provide a secondary immune evasive function. Having already observed the ability to induce antibodies inherently limited in breadth, HVR1 also shields cross neutralising epitopes in the E2 glycoprotein (Bankwitz, Steinmann et al. 2010; Prentoe, Jensen et al. 2011). Importantly, it has also been recently published that deletion of HVR1 from E2 increases the susceptibility to neutralization (Bankwitz, Steinmann et al. 2010). The implication of this latest research is that E2 lacking HVR1 therefore has potential to induce greater antibody responses that will also be primed against more conserved neutralising

epitopes that might not be induced in the natural response to infection. The resulting antibody repertoire will thus have more chance of being able to potently cross neutralise and clear natural infection with HCV. With more research elaborating the role and influence of the HVR's to be done and more results from the various clinical trials of vaccine candidates to be released, vaccine research for HCV is far from finished. Indeed a model of HCV E2 lacking all three hypervariable regions has already been patented for use as an immunogen vaccine(Burton and Law 2010) .

1.11. Role of pH in entry

Envelope proteins like E1 and E2 of HCV are often the mediators of fusion between viral and endosome membranes. Various studies have provided evidence for specific regions of E1E2 having distinct roles in entry: such as receptor-co receptor binding and fusion (Pileri, Uematsu et al. 1998; Drummer, Wilson et al. 2002; Rothwangl, Manicassamy et al. 2008; Haid, Pietschmann et al. 2009). Production of functional HCV glycoproteins has been shown to be dependent on both proteins as they act as chaperones for each other along the processing pathway (Choukhi, Wychoski et al. 1998; Bartosch, Dubuisson et al. 2003; Brazzoli, Helenius et al. 2005). Fusion determinants have been identified on both E1 and E2 indicating a specific role for each of these envelope proteins within the fusion process (Drummer, Boo et al. 2007; Lavillette, Pecheur et al. 2007). The interdependence of E1 and E2 also

translates functionally as investigation of the relationship of E1 and E2 in fusion indicates that E2 is essential for fusion to occur (Haid, Pietschmann et al. 2009), with E1 acting as a companion protein (Yagnik, Lahm et al. 2000). Mutation of certain residues on E2 alters the kinetics of fusion with the effects differing according to whether residues are altered to a neutral or acidic amino acid (Haid, Pietschmann et al. 2009). This result supports two important notions. Firstly the importance highly conserved residues can have as the residues investigated by Haids group for their involvement in fusion are highly conserved and centrally located within the E2 protein just like the key residues of the many neutralising epitopes in CD81 binding sites have been so far (Rothwangl, Manicassamy et al. 2008; Haid, Pietschmann et al. 2009). The second notion is that the effect of the polarity of residues involved in fusion illustrates a role for pH/charge during or preceding fusion. Indeed it has been speculated that local hydrophobicity might induce rearrangements that have functional importance. So, just as receptor interaction can induce conformational effects in viral envelope proteins, alterations in pH/charge could also induce functionally important effects.

The entry process of HCV into the host cell involves the formation of a clathrin coated pit for endocytosis. It is the early endosome that HCV fuses with to release the contents of the virion into the host cell cytoplasm (Blanchard, Belouzard et al. 2006; Meertens, Bertaux et al. 2006). Fusion for HCV has been shown to be pH dependant (Tscherne,

Jones et al. 2006; Haid, Pietschmann et al. 2009). Using the *in vitro* pseudo particle model of HCV, pH change in the endosome was shown to be crucial for fusion as preventing the acidification with drugs such as bafilomycin consequently blocked infection/entry (Tscherne, Jones et al. 2006). In this setup the optimum pH for fusion has been observed to be pH 5.5 with a threshold of 6.3 (Lavillette, Bartosch et al. 2006). This finding is in line with other literature (Bartosch, Dubuisson et al. 2003; Hsu, Zhang et al. 2003; Lavillette, Morice et al. 2005) and pH dependant fusion events observed for related viruses in which pH 5-6 is also the optimum (Despres, Frenkiel et al. 1993; Corver, Ortiz et al. 2000; Mukhopadhyay, Kim et al. 2003; Lavillette, Bartosch et al. 2006). In enveloped viruses exposure to acidic pH results in inactivation of the virus due to induction of irreversible conformation changes in the envelope proteins. The structural changes imposed are normally necessary for the fusion of viral and endosomal membranes and so the timing of this conformational effect is thus important for the coordinated fusion process. Extracellular virions are resistant to low pH inactivation as HCV glycoproteins expressed on pseudo virions pre-incubated in acid pH before application to cell culture showed no irreversible changes or downstream effects in viral function (Meertens, Bertaux et al. 2006; Tscherne, Jones et al. 2006). From this, it is evident that more factors are involved for fusion.

1.12. E2 activation

Viral envelope protein activation has been observed for viruses other than HCV for example the related Pestivirus: Bovine Viral Diarrhoea Virus (BVDV), which requires an activation step such as disulphide shuffling to become acid sensitive (Krey, Thiel et al. 2005). Indeed it has been postulated that a prolonged incubation at 37^{o}C may be an activating step for HCV (Tscherne, Jones et al. 2006) as this is a necessary trigger that has been observed for the retrovirus ALV and Alphavirus SFV (Marsh and Bron 1997; Mothes, Boerger et al. 2000). Recently HCV neutralization has been shown to be affected by temperature and time-dependant binding (Sabo, Luca et al. 2012). The altered neutralising capabilities by antibodies following different binding incubations was seen to be a reflection of altered epitope exposure such that the incubation at 37^{o}C for a prolonged amount of time allowed “virus breathing” to occur in which differential exposure of epitopes can be seen. The concept of virus breathing is observed in other flaviviruses also in response to temperature incubations prior to infection (Lok, Kostyuchenko et al. 2008; Dowd, Jost et al. 2011). These data thus support that HCV E2 can be responsive to factors other than pH. Cholesterol and particle density are factors that have also been directly linked to HCV fusion (Lavillette, Bartosch et al. 2006; Haid, Pietschmann et al. 2009). Whether factors such as these would contribute as complimentary variables or as the missing sensitizing trigger for fusion pre-activation in HCV cannot yet be answered.

The full relationship between E2 conformation, pH and fusion is not yet fully elucidated as has been achieved for other viruses (Sattentau and Moore 1991; Modis, Ogata et al. 2004). Evidence for conformation changes in E2 in response to acid exposure has been found but no indication of the duration or any other conditions required was shown (Op De Beeck, Voisset et al. 2004; Keck, Li et al. 2005). E2 exposed to low pH prior to immunoprecipitation underwent drastic changes in global structure as the binding profile from a panel of conformation dependant antibodies was almost abolished. The association of E1E2 was also affected by the low pH condition as almost 75% appeared to have dissociated (Op De Beeck, Voisset et al. 2004). These data showed definite conformational changes in at least E2 due to treatment with acidic pH. In these experiments E1E2 was acidified for 20 minutes at 37 degrees Celsius and then neutralised to pH 7.5 from 5.5 before the immunoprecipitation indicating a permanent change occurred. Lavillette and Tschernes groups, both in 2006, supported this data by also showing temporary exposure of E1E2 to pH 5.5 with an incubation at 37^{o}C allowed the necessary activation for fusion to occur which was not observed at 4^{o}C (Lavillette, Bartosch et al. 2006; Tscherne, Jones et al. 2006). Tschernes study also showed extracellular HCV virion resistance to low pH despite the use of a 37^{o}C incubation. An important difference between their two main observations however can be noted in the duration of the warm incubation at low pH for and prior to fusion. The apparent resistance to low pH was noted following a 10 minute incubation at 4 ^{o}C as opposed to a full hour at 37 ^{o}C where fusion did

occur raising the question of whether the results would have been the same had both incubations lasted a full hour or if a 10 minute incubation at 37 ^{o}C would also have allowed fusion to occur (Evans, von Hahn et al. 2007). If HCV were to be similar to SFV and ALV, than the activation trigger for low pH sensitivity may not be observed in such a short period as 10 minutes, whereas over 20 minutes at 37^{o}C could allow it occur. Thus the time dependence observed in their study could be the duration of temperature incubation needed for activating HCV into a pH sensitive state.

In summary, entry of HCV into the host cell is a pathway of which fusion is just one step. Fusion itself requires multiple conditions/elements that haven't all been identified yet. Temperature, time, low pH and conformational changes in E2 are elements already investigated as a prerequisite for fusion of the virus and endosome membrane. More research on these factors could allow development of new antiviral strategies as has been applied elsewhere.

1.13. Targeting Fusion

Many viruses undergo conformational changes in their envelope proteins as part of the entry process (Sattentau and Moore 1991; Smit, Moesker et al. 2011). This can allow exposure of functional regions such as binding sites or fusion motifs. Fusion determinants and motifs have been identified on E1 and E2 (Drummer, Boo et al. 2007; Lavillette,

Pecheur et al. 2007), and there is evidence for a pH responsive conformational change in the HCV glycoproteins (Op De Beeck, Voisset et al. 2004; Keck, Li et al. 2005). The identification of new conserved and functionally important regions of E2 should be considered for containing new epitopes that can be targeted as part of an anti-viral strategy. When the envelope glycoprotein of HIV, gp120, binds to the host CD4 receptor, conformational changes are induced that allow exposure of the highly conserved binding site for CCR5 (Sattentau and Moore 1991). In a similar fashion, conformational changes in HCV E2 during the fusion process could allow exposure of conserved epitopes not normally accessible on the proteins surface. This concept has successfully provided a basis for anti-viral targeting where antibodies bind to the epitopes exposed after acidification and receptor binding induced conformational changes in the envelope proteins. These antibodies have successfully demonstrated the prevention of continuation to fusion in HIV (Zwick, Labrijn et al. 2001; Burton, Desrosiers et al. 2004) and the flavivirus West Nile virus (Thompson 2009). These scenarios are thus proof of principle for the use of antibodies targeting epitopes linked to the fusion process as an anti-viral strategy. So, in principle, antibody mediated inhibition of fusion for HCV could also be possible. Targeting epitopes that are involved in fusion or related conformational changes for HCV E2 could therefore provide an effective anti-viral strategy.

1.14. Antibody search methods

Edward Jenner first demonstrated the concept of vaccination by administering cowpox as a vaccine to prevent smallpox infection in a new individual. Today, advances in science and technology make it possible to produce and purify specific antibodies to use directly as passive immunotherapy. Immunologically competent mice can be induced to produce antibodies to HCV immunogens without succumbing to infection as murine hepatocytes are not permissive to HCV. Serum can then be collected and used experimentally to show the presence of antibodies from mice just as with sera samples from human infected individuals. This has been demonstrated by Lavillettes group in 2005. Following a nosocomial HCV outbreak they were able to track where they observed the prevention of infection in cell culture due to the neutralising properties of the antibodies present in the sera (Lavillette, Morice et al. 2005). This set up however presents a limited source of unpurified and uncharacterized antibody. In 1975 the technology to create immortalized antibody producing B cells called Hybridomas was developed by Kohler and Milstein who went on to receive a Nobel prize for it in 1984. In this set up spleen or blood derived from mouse B cells are extracted and treated with chemicals to encourage their “fusion” with myeloma cells to become immortal antibody producing cells/factories (see Figure 1.6A). With an eternal source of antibody it is then feasible to use binding assays to characterize the epitopes for such antibodies, test their neutralising

capacity and eventually clone the immunoglobulin genes using PCR technology to determine the genotype of these antibodies. Hybridoma technology has allowed the isolation and identification of murine antibodies H53, AP33 and ALP98: each of which are potently cross neutralising (Cocquerel, Meunier et al. 1998; Clayton, Owsianka et al. 2002; Owsianka, Tarr et al. 2005). Non-human antibodies however need to be humanized to reduce their immunogenicity when administered to humans and so beginning with human antibodies avoids this complication.

To overcome the need for species specific antibodies, adaptations have been developed to circumvent the need for human test subjects. Isolated murine immunoglobulin genes can be humanized by cloning so that the specific complementarity determining regions derived from mice immunoglobulin genes can be grafted into acceptor human antibody frameworks. This allows retention of the binding affinity and specificity. In the U.S nearly half of all Food and Drug Administration approved therapeutic monoclonal antibodies are humanized antibodies thus illustrating their safety and tolerance by humans (Marasco and Sui 2007). Otherwise, transgenic mice that have their immunoglobulin genes replaced by a human locus can be immunised to express human specific antibody and so extraction of their antibody producing cells will naturally produce human antibody. This approach has indeed been successfully adopted for generating human antibodies against Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) and rabies

(Coughlin, Lou et al. 2007; Sloan, Hanlon et al. 2007). Another technique is the use of human antibody producing B cells that can be derived from infected patients and chemically transformed to be immortalized allowing the potential for an infinite source of human antibody: similar in principle to the traditional hybridoma but with less technical difficulty and time required. Single B cells can be isolated using blood samples, from which antibody containing supernatants can be tested for reactivity against a given target. From this, the B cell can be cultured to be immortalized, or the immunoglobulin genes can be cloned by extraction of RNA and reverting to cDNA so that genes can be introduced into new expression vectors. This approach has been adopted for HIV and Influenza to yield potently neutralising human monoclonal antibodies (Simmons, Bernasconi et al. 2007; Scheid, Mocquet et al. 2009).

Biopanning methods have also been developed that allow screening of large numbers of antibodies at the same time to isolate highly specific antibodies of high affinity to a given target such as an envelope protein of a virus (applicable as library selection/screening as seen in Figure 1.9). A great advantage of biopanning is the ability to "screen"/check the binding of up to billions of different antibody variants at the same time and so this is a much more efficient set up for isolating specific antibodies. Several groups have used display technologies/biopanning to isolate broad acting and potently neutralising antibodies against the HCV E2 protein in the last 10 years thereby supporting its potential (Johansson, Voisset et al. 2007; Law, Maruyama et al. 2008; Perotti, Mancini et al. 2008; Giang, Dorner et al. 2012).

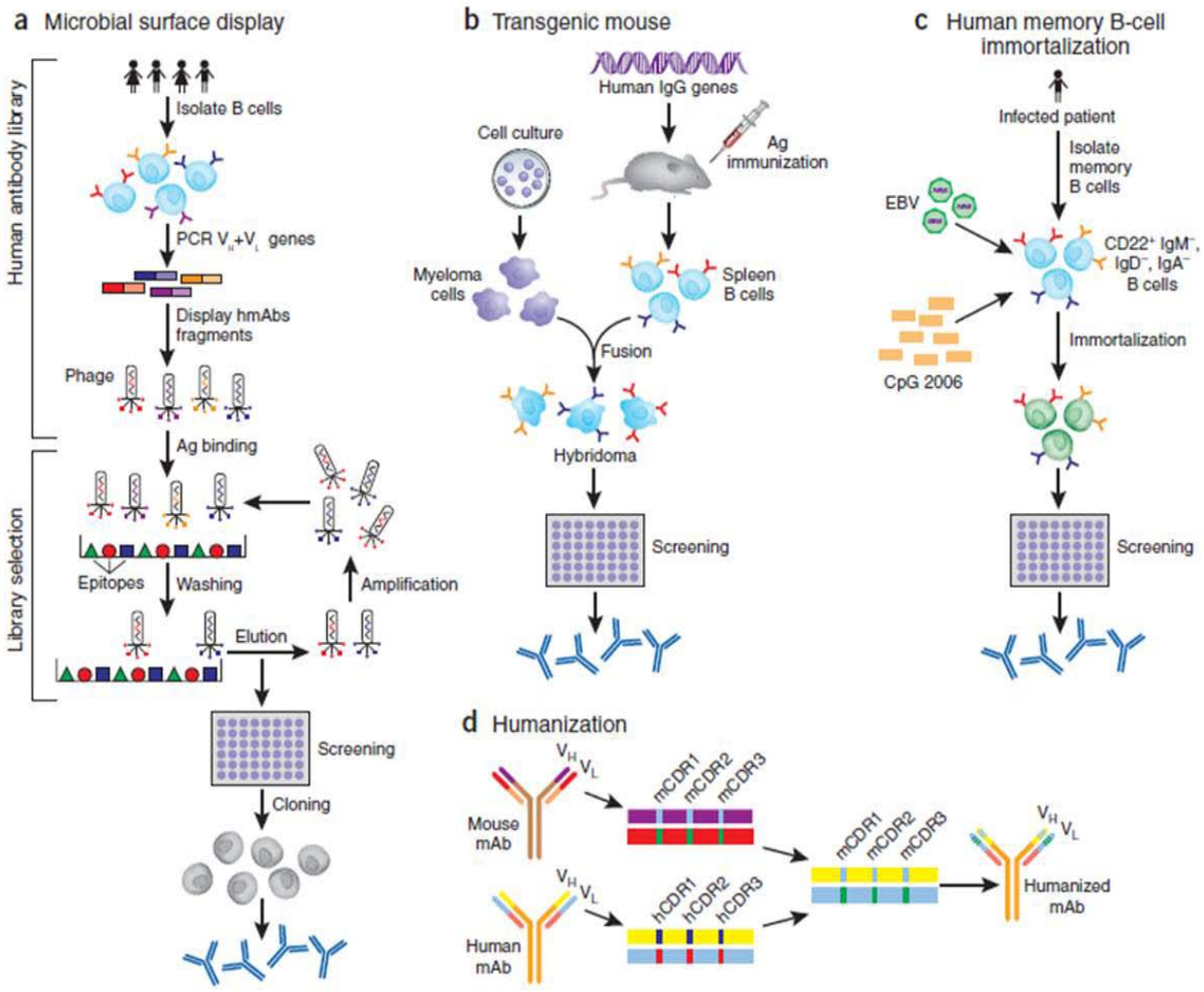

Figure 1.9 In vitro methods of generating monoclonal antibodies. A) Antibody producing B cells can be derived from immunologically naïve or mature individuals to generate a library for display platforms such as phage display. The library is used to screen for antibodies specific to a given antigen. B) Otherwise B cells can be collected from immunized mice (transgenic or otherwise) to form hybridomas to screen for production of specific antibodies. Likewise cells from a naïve or infected individual can be cultured to produce myeloma fusions to be screened just as hybridomas. C) Sera from infected individuals can be treated and screened to identify specific antibody producing B cells that can be further used to isolate antibodies. D) Non-human species specific antibody genes need humanizing by cloning (Marasco and Sui 2007).

1.15. Biopanning

Biopanning is the process of affinity selection of peptides based on their ability to bind to a specific target. The technology to create libraries of peptides to select from allowed an advance in efficient screening en masse in different formats. In 1989 cloning of antibody genes allowed cloning directly from lymphocytes such that combinatorial or single domain libraries of variable heavy and light chain genes could be constructed (Orlandi, Gusow et al. 1989). At this point libraries of millions of different antibody clones could be made. These antibody clones can then be expressed on the surface of bacteria as antigen based fragment (Fab) and probed with radiolabelled antigen (Huse, Sastry et al. 1989). This set up allowed Fab to be selected and identified according to their ability to bind to a given target such as a viral protein. Display technologies superseded the previously traditional hybridoma for production and selection of antibodies by coupling screening of genotype with phenotype. This then avoided the need to be able to produce a fully functional antibody which was time consuming, expensive and not guaranteed to work, before then needing to assess the characteristics of all antibodies that were successfully produced.

Possible platforms for the display of a given library of protein candidates can be; the surface of phage particles, complexed to ribosomes associated with mRNA, and cellular expression on microbial cells such as yeast (reviewed in (Hoogenboom 2005)). The different

platforms allow flexibility in the format of the proteins tested due to differences in presentation: phage particles for example display recombinant proteins as the protein displayed is fused with its own structural proteins. Microbial cell display however allows the use of native protein due to its expression on membrane surfaces. For screening and selection of antibody fragments, phage display is the most widespread as it is robust, simple to use and highly versatile. The selection process also holds high flexibility thus being advantageous over most other display platforms. A further advantage of using phage particles is that following biopanning, elution of the bound phage particle with its recombinant protein that contains the Fab can immediately infect new bacteria to begin a new cycle of expression. The first successful demonstration of this system to produce specific antibody was published in 1990 by McCaffertys group (McCafferty, Griffiths et al. 1990), and was quickly followed by more examples of the isolation of specific antibodies from different libraries (Barbas, Kang et al. 1991; Barbas, Björling et al. 1992; Barbas and Wagner 1995; Samuelsson, Yari et al. 1996; Tout and Lam 1997; Rader, Cheresh et al. 1998).

1.16. Phage Display

Since the first application of phage display in 1990, it has remained a popular method, having been further developed and adapted. Recently the phage display platform of biopanning has been used to develop a

disulphide stabilized variable fragment (dsFv) as an improvement on Fab (Jia, Yu et al. 2008). The phage platform allows multi- or mono-valent display, a large library size (10^{10} to 10^{11}), versatile scope and the ability to use the most formats e.g. scFv, Fab, dAbFab'$_2$ and even the recently developed dimerized scFv known as a diabody. Thus, phage has remained the main choice of platform for biopanning (reviewed in (Hoogenboom 2005)).

In the world of HCV antibodies, the majority of the prominent monoclonal broad acting neutralising antibodies have been derived by phage display biopanning in a Fab format (Johansson, Voisset et al. 2007; Law, Maruyama et al. 2008; Perotti, Mancini et al. 2008; Mancini, Diotti et al. 2009). Until recently these phage display derived antibodies all had a common link of having epitopes focused on the CD81 binding sites with residues 530 and 535. Recently however one of these groups has successfully extended the application of phage display to pull out novel non-CD81 neutralising antibodies by optimizing the selection/panning set up to favour epitopes in new areas of the E2 protein (Giang, Dorner et al. 2012). Like the other studies, a purified glycoprotein was still the target for the panning. However, the available binding space on the surface of the target was specifically shaped by epitope masking as has been demonstrated elsewhere for isolating novel antibodies of defined specificities (Ditzel 2002; Tsui, Tornetta et al. 2002). By capturing and masking the E1E2 target with antibodies of defined specificities, Giang and his group were able to ensure only

antibodies bound to the remaining “novel” space would be selected. Indeed novel antibodies were identified that did not compete with the previously identified and CD81 binding site residing epitopes that also demonstrated broad neutralising activity (Giang, Dorner et al. 2012). Whilst not blocking E1E2-CD81 interaction, the antibodies were potently neutralising at a pre and post attachment stage with the epitopes likely to reside in the third domain of E2, away from the identified binding sites for CD81 and SRB1. This recent data thus supports the use of the phage display platform for selection of novel cross neutralising epitopes on HCV E2. Indeed the groups that produced AR1-5 antibodies, 1:7, A8, L1 and the Fabs e137 and e20 all were derived using the phagemid vector set pComb3 (Barbas, Kang et al. 1991) of the M13 phage system.

1.17. M13

The most widely used phage vector for biopanning is the filamentous bacteriophage comprising fd, f1 and M13. These three phage species are so similar that they can be regarded as mutations of a single bacteriophage and so are often collectively referred to with the generic term M13 (Rasched and Oberer 1986), as will be done here. M13 belongs to the *Inoviridae* family of viruses and has a 6407 base pair DNA genome in a single stranded covalently bound circle that encodes 11 proteins (see Figure 1.7 & Table 1.1) (Day and Berlowitz 1977). Filamentous phage M13 is 7 nm wide and can be up to 2000 nm in

length. It has 11 different genes encoding proteins, each with a different role in the assembly and replication of the phage particle.

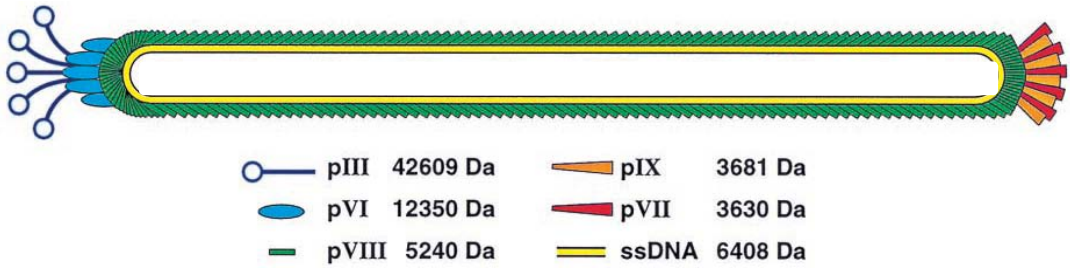

Figure 1.10 Schematic representation of the structure of the M13 phage particle structure
Depicted are the various capsid proteins and the circular DNA genome (adapted from (Gao, Mao et al. 1999)).

Table 1.1 Table listing the 11 genes of M13 phage and the function of the encoded protein.
(adapted from (Smith and Petrenko 1997)

M13 phage genes				
Symobol	Gene	Amino Acids	Molecular Weight (Da)	Function
	I	348	39,502	Assembly
	II	410	46,137	Replication
	III	406	42,522	Minor capsid protein
	IV	405	43,476	Assembly
	V	87	9,682	DNA binding
	VI	112	12,342	Minor capsid protein
	VII	33	3,599	Minor capsid protein
	VIII	50	5,235	Major capsid protein
	IX	32	3,650	Minor capsid protein
	X	111	12,672	Replication
	XI	108	12,424	Assembly

The phage particle consists of a long shaft comprised of a helical array of up to 2800 monomers of the major capsid/coat protein pVIII that encloses the circular single stranded DNA genome (see Figure 1.7) (Marvin 1998). The minor capsid proteins are clustered into two groups: one found at each end of the phage particle. Five copies of protein III and VI (pIII & pVI) are found at one end of the phage filament with the remaining capsid proteins pVII and pIX at the other (Grant and Webster 1984; Endemann and Model 1995). All other encoded proteins are involved in replication and assembly and so are the non structural accessory proteins such as pI and pIV which facilitate packaging and extrusion of the phage particle in co-operation with bacterial proteins. M13 phage infects male strains of *Escherichia coli* by pIII binding to the F' pilus, resulting in retraction of pilus allowing translocation of the phage genome into the bacterial cell cytoplasm (Henry and Pratt 1969; Stengele, Bross et al. 1990). Replication then begins with bacterial enzymes synthesizing the complementary strand of the phage genome to form a double stranded supercoiled replicative form (RF) to act as a template for transcription. Translated capsid proteins migrate and integrate into the cell membrane while further ssDNA is formed for conversion to more RF. The cycle of replication and translation continues until the concentration of pV and phage specific ssDNA has become equimolar so that all the new molecules of ssDNA are associated with a monomer of pV. At this point formation of a new phage particle can start with the help of accessory proteins like pI and pIV. Mature capsid proteins displace pV and accompany the ssDNA out

of the bacterial cell enclosing the genome as the phage filament forms from the cell membrane outwards. Once the end of the ssDNA molecule has been reached the progeny phage particle terminates and separates from the bacterial host cell to be released into medium. This replication cycle thus provides multiple advantageous elements for use of M13 as a vector for cloned DNA.

One such advantage conferred by the life cycle of the M13 bacteriophage is that the release of progeny phage is through the host's membrane rather than disrupting it and so M13 is non-lytic phage. The consequence of a non-lytic life cycle is simplification of the isolation process of purified phage and preparations from supernatants being relatively free from contaminating proteins (Russel and Model 1988). Also, the lysogenic infection cycle allows each infecting bacteriophage to produce up to 1000 progeny from a single cell cycle thus very high titers of phage can be recovered from small cultures: up to 10^{12} pfu/ml. The replicative process and structural aspects of the progeny phage are dictated by the ssDNA phenotypically as well as genotypically. The capsid proteins do not dictate the morphology of the phage particle but instead their association with the genome means that it is the genome itself that dictates the shape and size of the phage. Consequently, the length of the genome determines the length of the phage filament as the length is a variable factor reflected by insertions of cloned DNA experimentally (Zacher, Stock et al. 1980). Furthermore, the capsid genes can tolerate insertion of DNA to still correctly express the protein,

and most importantly insertion into the N terminus of pIII or pVIII (McConnell, Uveges et al. 1996; Smith and Petrenko 1997). These protein regions are hydrophilic, located on the exterior of the phage particle and so as a result of this, recombinant proteins are displayed at the extremity of the phage surface ideal for affinity selection (Minenkova, Ilyichev et al. 1993).

1.18. Phage library formats

Taken together, the features of the M13 bacteriophage life cycle have been used to display recombinant peptides fused to pIII (Bass, Greene et al. 1990; Cwirla, Peters et al. 1990; McCafferty, Griffiths et al. 1990; Barbas, Kang et al. 1991) or pVIII (Greenwood, Willis et al. 1991; Kang, Barbas et al. 1991) as a platform in affinity selection. The valency of pIII and pVIII on the bacteriophage particle confers restrictions on the application of M13 for affinity selection. A lower representation of pIII and thus any recombinant protein to be displayed allows affinity to be the property that is being selected for rather than avidity, which can be better selected with using the high valency representation of a recombinant protein VIII. So, with regards to biopanning in search for antibodies, gene III based libraries are the preferred choice over gene VIII peptide libraries that could be used for epitope mapping. Indeed the biopanning projects that produced the main HCV E2 neutralising antibodies mentioned previously all used gene III based libraries for their phage display (Johansson, Voisset et al. 2007; Law, Maruyama et

al. 2008; Mancini, Diotti et al. 2009). However, in using phage display libraries containing relatively large recombinant antibody proteins hundreds of amino acids in size, rather than libraries of the much smaller peptides around 8 amino acids, a physical restraint has been met as large DNA inserts into gIII result in reduced infectivity and in gene VIII packaging is disrupted (Kang, Barbas et al. 1991; Iannolo, Minenkova et al. 1995). This has led to library format adaptations; double gene or phagemid libraries (see Figure 1.8).

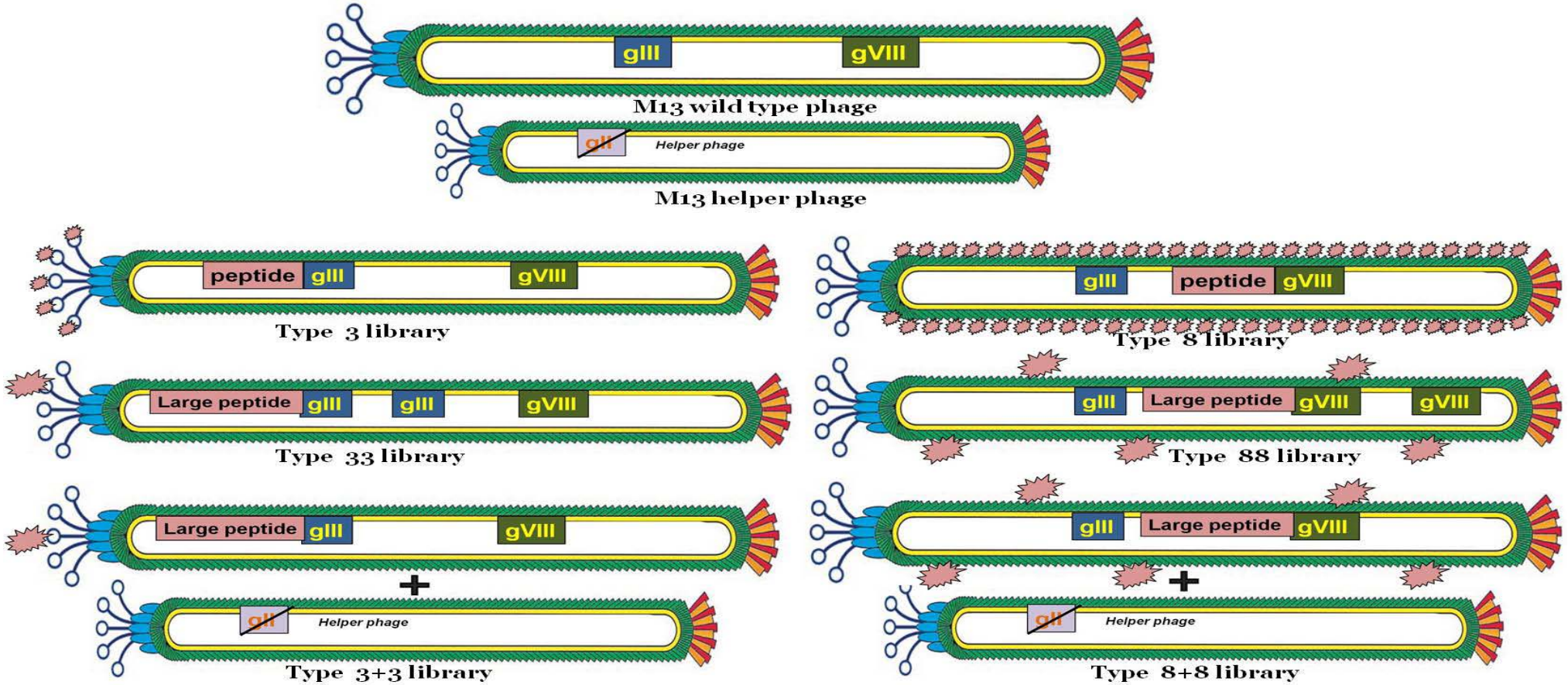

Figure 1.11 Classes of display vectors based in M13 (adapted from (Smith and Scott 1993) and (Gao, Mao et al. 1999). All phage vectors contain functional genes I and III-XI. Only the helper phage has a mutated gene II impairing its ability to replicate efficiently. The inner yellow circle represents the genome with relevant genes marked out in coloured boxes (blue: gene III, green: gene VIII, pink: fusion gene, grey: gene II) and pink shapes as the fusion peptides. All other symbols are illustrated in Table 1.1.

The double gene set up can be applied to gene III or gene VIII based libraries and are referred to as 33 or 88 respectively (see Figure 1.8). For these systems the expression plasmid encodes both the wild type phage gene 3 or 8, along with another recombinant gene to express the desired fusion protein 3 or 8. The advantage of there being a wild type gene alongside the recombinant version is to compensate for the loss of function observed when only the recombinant version is present. Having the two versions of the capsid gene present independently in the plasmid allows a degree of control over the induced expression of each depending on the promoter. The second approach to constructing an M13 library for large proteins is to use a second phage known as helper phage. This setup is also applicable to gene 3 or 8 libraries and is referred to as 3+3 or 8+8. In this setup the plasmid is known as a phagemid and encodes only one gene 3/8 that includes the protein gene to be displayed fused to the chosen phage capsid protein. To compensate for loss of function due to the large insert size, the fully functional wild type capsid gene is supplied via super infection with helper phage that is replicative impaired. The phagemid is thus unable to produce infective progeny in the absence of the helper phage, and the helper phage is unable to replicate efficiently resulting in preferential packaging of the phagemid genes. On the surface of the progeny phage particle will therefore be a mixture of wild type capsid proteins as well as the fusion protein. The advantage to the phagemid set up is that it is possible to culture, isolate and make bacterial stocks of the phage genome without producing fully infective phage. In 1991 a vector set for

3+3 phagemid called pComb was developed by Barbas' lab (Barbas, Kang et al. 1991). Indeed the main neutralising HCV antibodies derived from phage display were using the pComb phagemid vector for display of Fab fused to capsid protein III (Allander 2000; Law, Maruyama et al. 2008; Mancini, Diotti et al. 2009; Giang, Dorner et al. 2012).

1.19. Directed selection in biopanning

The high through put nature of biopanning is merely one of many advantages over *in vivo* methods for the isolation of antibodies. The choice of reagents as well as the conditions imposed can also shape the attributes of the antibodies derived from biopanning. The repertoire of the library to be used in biopanning can vary according to whether it was constructed from an immunologically naïve individual or not, along with whether it was created from bone marrow or peripheral blood mononuclear cells. These factors will determine the prevalence of antigen specific antibody clones that can be retrieved from the library. One study constructed phage display libraries from donors who had been vaccinated against a "plethora of different bio-threat agents" to specifically enhance production of antibodies against otherwise uncommon antigens (Wild, Maruyama et al. 2003; Bradbury, Sidhu et al. 2011). Antibodies able to recognise epitopes on proteins from multiple species have been isolated from a library derived from an immunologically naïve subject (Fellouse, Wiesmann et al. 2004; Liang, Wu et al. 2006). Conversely antibodies that distinguish between species

variants of antigens that differ by only one amino acid have also been derived from biopanning methodologies highlighting the functional flexibility (Ayriss, Woods et al. 2007). The affinity of antibodies can also be tailored by appropriate selection and screening protocols. Immunization of animals will typically produce antibodies via activation of B cells of limited affinity around 100 pM. The affinities of antibodies derived from *in vitro* display technologies however can reach as low as picomolar and femtomolar levels of antigen affinity (Boder, Midelfort et al. 2000; Hanes, Schaffitzel et al. 2000; Lee, Liang et al. 2004).

The conditions of the selection process can define the requisite antigen conformation thus also affecting the possible antibodies that can be derived. Conditions that can be used for this are alteration of pH, salt concentration, modulators and presence of competitors (Bradbury, Sidhu et al. 2011). Competitors have been used by Parsons and her group in 1996 to specifically deplete an antigen pool of haemoglobin using high affinity antibodies to the adult form so that only fetal haemoglobin remained for the selection of fetal haemoglobin specific antibodies (Parsons, Earnshaw et al. 1996). Similarly, Giangs group most recently used competitive inhibition of their HCV E1E2 antigen target to focus the selection towards antibodies targeting novel areas (Giang, Dorner et al. 2012; Keck, Xia et al. 2012). Site directed mutagenesis of the antigen target to be used as bait during the selection process has also allowed antibodies to be isolated that specifically do not have problematic residues in the epitope they target. Most recently

this was demonstrated by Keck who used HCV E2 protein as the target antigen protein in which the Aspartic acid at residue 535 had been mutated to Alanine thus abolishing many previously identified neutralising epitopes containing this residue (Keck, Xia et al. 2012).

The conformation of the chosen target antigen used in biopanning can also be modulated for a functional advantage. One study used small molecules to covalently lock a cell surface receptor specifically into the active/on form to use as the antigen target in biopanning thereby allowing selection of antibodies highly specific for the active/on form (Gao, Sidhu et al. 2009). The consequence for the antibodies selected was translated into the ability to probe cell surfaces for responses to various stimuli in the form of cell signalling. Likewise Watanabe was able to take advantage of limited conformational movement in the target antigen to confer a functional advantage in the antibodies that were isolated such that a specific dye fluorescence could be increased by up to 15,000 times upon antibody binding (Watanabe, Nakanishi et al. 2008).

1.20. Aims and approach

HCV is a medical burden of the modern world. Limitations in the ability to identify and treat infection create a great need for novel preventative measures and treatments. There is still great potential in the current methods that have generated broad acting potently neutralising monoclonal antibodies. Adaptations to the choice of antigen target and screening protocol were investigated to use in phage display biopanning with the aim of identifying a novel neutralising antibody against HCV E2. A broad acting neutralising antibody against HCV would be most valuable for use as passive immunotherapy in liver transplant patients, in which there is a high re-infection rate (Charlton 2003; Brown 2005). Pre and post-exposure prophylaxis would also benefit from effective antibodies as the current HCV drug regimen especially following liver transplant can be poorly tolerated (Crippin, McCashland et al. 2002; Schiano, Charlton et al. 2006). The main objectives of this project therefore were to;

1) Produce stable, monomeric and antigenically correct E2 protein of various selective conformers.

2) To assess the E2 form that could best allow selection of antibodies reactive to novel conserved epitopes in a biopanning format.

3) Investigate pH status of E2 as a pre-requisite condition for any conformational effects in E2.

4) Use the chosen E2 protein and defined conditions in phage display to isolate a novel specific neutralising antibody.

2. Materials and methods

2.1. Plasmids

A panel of 8 pMT plasmids containing a variety of codon optimized HCV isolate UKN2B2.8 glycoprotein constructs were kindly provided by Dr Thomas Krey and Dr Felix Rey (Institut Pasteur, Paris) (see Table 2.1). Each E1E2 construct was terminated at residue 715 thus lacking the E2 transmembrane domain allowing secretion into culture supernatant following production. E1 was retained in the construct to allow optimal production and folding of E2 as E1 and E2 are thought to act as chaperones to each other during protein synthesis. In addition, each construct contained a C-terminal one strep tag for purification.

In addition, a pMT construct corresponding to the HCV isolate UKN1A20.8 E2, truncated at residue 715 was provided by Dr Alex Tarr (University of Nottingham). Alanine replacement substitutions at positions G530 and D535 were made in this plasmid.

Table 2.1 Panel of E2 constructs derived from two genotype strains assessed as biopanning targets.

UKN 1A20.8	**UKN 2B2.8**	
Wild Type E1E2-715	Wild Type E1E2-715	ΔHVR1,2
G530A	ΔHVR1	ΔHVR1,3
D535A	ΔHVR2	ΔHVR2,3
G530A-D535A	ΔHVR3	ΔHVR1,2,3

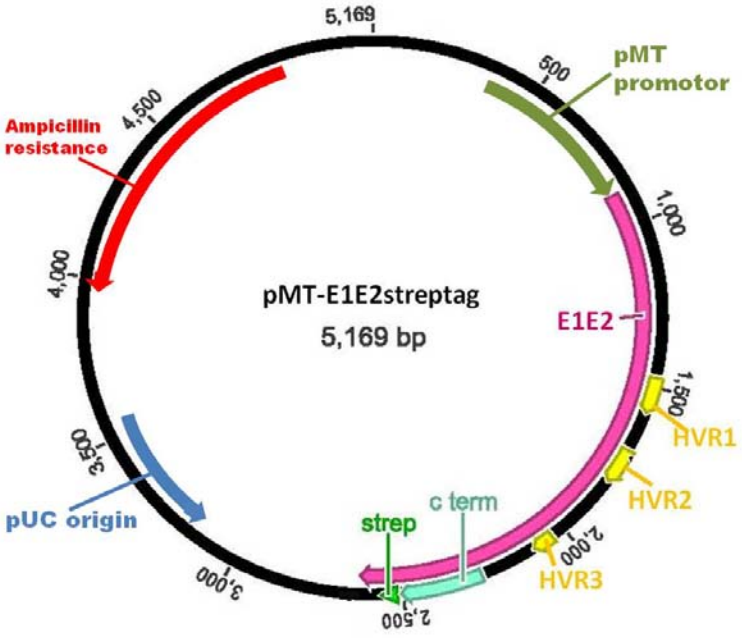

Figure 2.1Diagrammatic representation of the pMT plasmid format containing the E1E2-715 inserts of strain UKN1A20.8 or UKN 2B3.8. The location of the three hypervariable regions are illustrated along with the C terminal region and the strep tag used for affinity purification.

2.2. Site-Directed Mutagenesis

Residues 530 and 535 of HCV E2 are crucial for the binding of CD81 and pivotal for many neutralising epitopes previously identified. To allow selection of antibodies targeting non-CD81 region epitopes, these residues were mutated to alanine by site directed mutagenesis to disrupt any epitopes involving these residues. The commercial kit QuickChange site-directed mutagenesis (Strategene, The Netherlands) was used according to the manufacturer's protocol. The E2 clone of genotype 1A20.8 was thus used to create a G530A clone, a D535A clone as well as a G530D535AA clone in addition to the original wild type plasmid using the respective mutagenesis primer described in Table 2.3.

Table 2.2 Site directed mutagenesis primers used.
All primers used were sense strand reading.

Primer	Sequence (5'-3')	Nucleotide position[1]
1-D535As	GGGTGAGAATGAAACGGCTGTTTTCGTCCTGAACAA	1928-1963
2-G535As	GGCGCGCCTACCTACAGATGGGCTGAGAATGAAACGGACGTT	1908-1949
3-G530A-D535As	GGCGCGCCTACCTACAGATGGGCTGAGAATGAAACGGCTGTT	1908-1949

[1] primer position numbered according to H77 genome coordinates

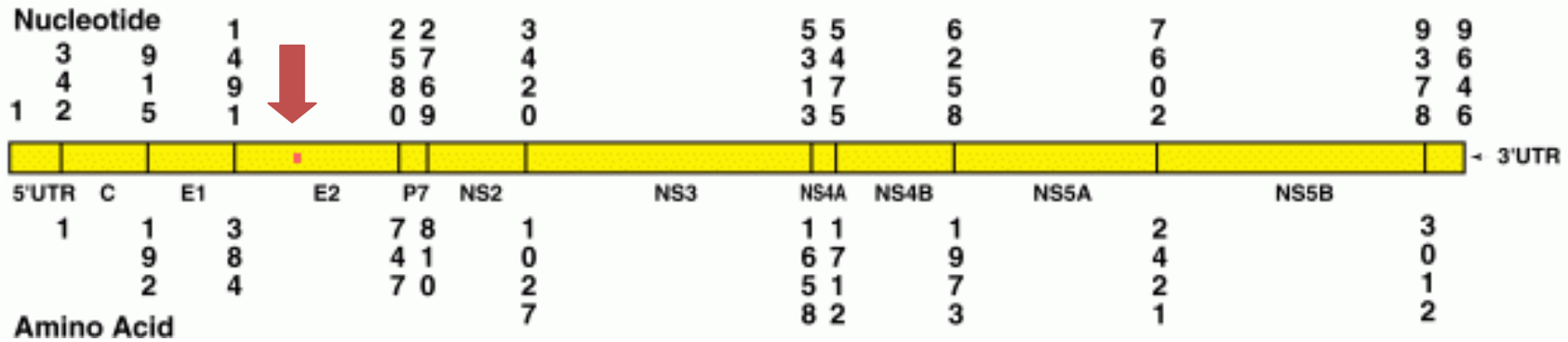

Figure 2.2 Schematic representation of primer locations (according to H77 coordinates) for primers presented in Table 2.3.
Marked by red arrow within the H77 genome framework.

Mutagenesis primers were synthesised by Eurofins MWG Operon* (Ebersberg, Germany) or DNA Technology A/S (Denmark). Cycling parameters for the mutagenesis reaction were 95^{o}C for 1 min, then 30 cycles of 95^{o}C for 1 min, followed by 55^{o}C for 1 minute, and 65^{o}C for 8.5 mins. The mutation reaction of G530A used both forward and reverse primers whilst mutation of D535A was successful using only one primer (*). The creation of G530-D535A used the G530A plasmid and further mutagenesis using the single primer for mutating D535A. Resulting mutant clones were sequenced across the entire E2 insert using dye terminator sequencing methods (Amersham Bioscience) to verify the correct mutation and no others had been incorporated into the clone.

2.3. S2 Cell culture

The various pMT soluble E2 constructs were expressed in Drosophila Schneider 2 cells. Cells were maintained in Insect Xpress media (Lonza) by incubation at 28 ^{o}C in a controlled environment of 5% CO_2 and 95% humidity (Johansson, Drakenberg et al. 2007).

Transfection was performed 24 hours following seeding 5x10^{6} cells in a volume of 2.5 mls of Insect free Xpress Media with glutamine (Lonza) in one well of a 6 well plate (Immulons). The transfection reaction consisted of Fugene 6 transfection reagent (Roche) at a ratio of 3:2 (Fugene µl : reporter DNA µg) according to manufacturer's instructions, 2 µg of reporter plasmid pMTE1E2, and 0.1 µg of reporter plasmid

pCoBlast conferring resistance to the antibiotic Blasticidin per transfection reaction. After 48hours Blasticidin (Invitrogen) was added to the cells at a concentration of 25 µg/ml and maintained for a period of 2 weeks to enable expansion of the Blasticidin resistant cell population. Following transfection, cells were maintained at all times at a concentration between $2x10^6$ and $1x10^7$ cells per ml of culture.

Expression of soluble E2 was induced by incubating cells with 500 µM $CuSO_4$ and continual shaking of cell cultures at ~105 rpm for a period of between 10 and 14 days. To recover expressed protein, culture supernatants were filtered through a 0.2 µm membrane and then subjected to affinity chromatography using Strep-Trap columns (GE Healthcare), according to manufacturer's instructions. Column fractions were assayed for total protein using the BCA assay (Thermo Scientific) and protein containing fractions were concentrated using 10 kDa size exclusion columns (Amicon) according to manufacturer's instructions. The quality, conformation and purity of the protein produced were then verified by ELISA, gel electrophoresis followed by protein staining and Western blot.

2.4. Protein quantification

Two methods were used for protein quantification: the BCA assay (Thermo Scientific) and nano-drop spectrophotometer analysis. The BCA assay was performed according to manufacturer's instructions, and

the test protein concentration in the sample was estimated by applying the resultant data to a regression equation produced from the values obtained for a set of protein standards. Regression analysis and estimations were performed using Excel (Microsoft Office 2007). To provide a second estimation of protein concentration, 2 µl of protein sample was quantified with a nano-drop spectrophotometer using the Beer-Lambert equation (absorbance value = extinction co-efficient x path length x analyte concentration).

2.5. Antibodies

Antibodies used in this study were human monoclonal antibodies A8, 1:7 (Johansson, Voisset et al. 2007) kindly provided by Mats Persson and the murine monoclonal antibodies AP33 and ALP98 provided by Arvind Patel (Owsianka, Tarr et al. 2005). The murine antibody H53 was kindly provided by Jean Dubuisson (Cocquerel, Meunier et al. 1998) and Mansun Law provided AR1A and AR3A (Law, Maruyama et al. 2008). Antibody specific to the strep-tag associated with the expressed E2, Anti-strep classic (IBA technology), was also used.

All antibodies and their properties are summarised in Table 2.3.

Table 2.3 Summary of all antibodies used in this study and information describing the epitopes.

E2 antibodies and epitopes						
Antibody	**Epitope**		**Neutra-lising**	**CD81 compe-tition**	**Broad acting**	**References**
	Residues	**Form**				
1:7	523, 529, 530, 535	Confor-mational	Yes	Yes	Yes	(Allander 2000; Johansson, Voisset et al. 2007)
A8	523, 529, 530, 535	Confor-mational	Yes	Yes	Yes	(Allander 2000; Johansson, Voisset et al. 2007)
ALP98	644-651	Linear	Yes	No	No	(Owsianka, Clayton et al. 2001; Clayton, Owsianka et al. 2002)
AP33	412-423	Linear	Yes	Yes	Yes	(Owsianka, Tarr et al. 2005)
AR1A	416-417, 485, 538, 540, 549	Confor-mational	No	No	No	(Law, Maruyama et al. 2008)
AR3A	424, 436-447, 523, 530, 535, 538, 540	Confor-mational	Yes	Yes	Yes	(Burton and Law 2010)
Anti-tag	Strep-tag	Confor-mational	-	-	-	IBA
H53	540-550	Confor-mational	No	No	No	(Cocquerel, Meunier et al. 1998)

2.6. SDS PAGE and Western Blot Analysis

To verify the size, stability and purity of the monomeric E2 proteins, ~5 µg were run on a sodium dodecyl sulphate polyacrylamide electrophoresis gel (SDS-PAGE; NuPAGE NOVEX Bis Tris mini 10 % Gel, Invitrogen) for 60 minutes at 200 volts. The gels were either stained or used for Western blotting as described below.

To visualise the E2 proteins and extrapolate size estimations relative to the marker ladder (Fermentas; Spectra Broad Range Protein ladder), Coomassie or silver staining was used. Silver staining was performed

using the reagents and protocols from Bio-Rad Silver Staining Kit. For the Coomassie Brilliant Blue Stain, the gel was shaken for 1 hour in 200 mls of Coomassie Brilliant Blue stain made up as; 450 mls distilled water, 450 mls methanol, 100 mls glacial acetic acid and 2.5 grams of Brilliant Blue R 250 (Sigma), all filtered. Coomassie stain was replaced with destainer made up as 45% glacial acetic acid, 45 % distilled water and 10 % methanol and left for 1-2 hours changing destainer 3-5 times as necessary.

For Western blot analysis, proteins present in PAGE gels were transferred on to a nitrocellulose membrane at 200 volts for 60 minutes in a Semi-dry conductor transfer cell (Bio-Rad). Following transfer, membranes were blocked on slow rock for 1 hour in 50 ml of 5% milk-PBS Tween at room temperature. After 3 washes with 0.05% PBS-Tween, the membrane was probed with AP33 supernatant diluted in PBST 1/400 for 1 hour at room temperature. After 3 washes, 1/1000 anti-mouse alkaline phosphatase (A2429, Sigma Aldrich), was added diluted in 0.05% PBS-Tween and rocked for 1 hour at room temperature. The membrane was then washed thoroughly with PBS-Tween and soaked in BCIP/NBT solution (Sigma FAST) for colour development on the membrane.

2.7. Enzyme Linked Immuno-Sorbent Assays

2.7.1. GNA capture ELISA

Functional testing of the purified soluble E2 was achieved using a GNA capture enzyme immunoassay. Wells of a 96-well plate (Immulons) were coated with 100 ng/well of *Galanthus nivalis antigen* (GNA; Sigma), overnight at +4°C. Unbound GNA was removed and non specific binding sites were blocked for 2 hour with 200-300 µl of 5% milk-PBS Tween at room temperature. The plate was then washed 3 times with PBS-Tween. Purified E2 sample was diluted 1:100 and 50 µl was added to each well of the plate, left at room temperature for 2 hours followed again by three washes in 0.05% PBS-Tween. After washing, 50µl of 1 µg/ml either A8, 1:7 and AP33 monoclonal antibody diluted in 0.05% PBSTween was added to the wells for 1 hour at room temperature, then washed as before. Lastly, 50 µl of anti-species immunoglobulin G (IgG)-Alkaline Phosphatase (Sigma) used at 1:5000 diluted in 0.05% PBS was added to the wells for 1 hours before 5 washes and addition of 100 µl of Blue-Phos microwell phosphalyse substrate system (KPL) and reading the plate with the micro-plate spectrophotometer at 620 nm (Molecular Devices V max plate reader). Protein samples that resulted in an OD reading of at least twice the negative control were considered positive. Depicted in Figure 2.2A.

2.7.2. CD81 binding assay

The genotype 2B panel of soluble E2 constructs were tested to verify if they maintained the ability to bind CD81 protein. An ELISA 96 well plate (Immulons) was coated with 100 µl per well of 5 µg/ml soluble E2 protein diluted in carbonate bicarbonate buffer (Sigma) and left overnight at 4^{o}C in a humidified box. The assay was continued as described in section 2.7.1 except the second incubation was with purified soluble CD81 LEL protein fused to a GST tag that had been added to the ELISA plate at 50 µl of 10 µg/ml diluted in PBSTween. The plate was then covered and left at room temperature for 2 hours. All other steps were the same. Depicted in Figure 2.3B.

2.7.3. GNA capture titrations

Titrations of antibody or soluble E2 protein were performed in GNA (Galanthus Nivalis Antigen, Sigma) capture ELISA to assess the effect of HVR removal on epitope exposure. This assay was as described in section 2.7.1 and either the primary antibody or the E2 was titrated. E2 titration consisted of concentrations starting at a sub-saturating 5 µg/ml and titrated 3-5 fold for >5 dilutions. After washing the plate as described before 1-2 µg/ml of test antibody was applied diluted in PBSTween for 1 hour. When the antibody was titrated, E2 was applied at 1 µg/ml and the antibody was titrated from ~5µg/ml in 3-5 fold for >5 dilutions. All other details remained the same.

2.7.4. Point mutant binding comparison

Point mutated soluble strain 1A20.8 E2 of residues G530 and D535 to alanine residues were compared in a set point ELISA. Briefly, 96 well ELISA plates (Immulons) were coated with 2 µg/ml of soluble E2 diluted in carbonate bicarbonate buffer (Sigma), covered and left over night at 4^{o}C. All other steps were carried out as described in section 2.7.1. Depicted in Figure 2.3C.

2.7.5. Antigenic testing of pH effect

To assess the temporal relevance of pH related effects in E2, the stage at which acidification of E2 occurred in the ELISA was compared. In one set up, 10 µl of soluble E2 protein was diluted in 90 µl of PBSTween of pH 7.5. Twenty µl of 0.1 M sodium acetate buffer (Sigma) was added to reduce the pH to 5.5 and this solution was incubated at 37^{o}C for 20 minutes. Following this, the pH was raised back to 7.5 by addition of 20 µl of 0.1 M HEPES buffer (Sigma) before diluting the E2 in carbonate bicarbonate buffer to an appropriate volume and concentration for coating the ELISA plate. For this experimental set up, the primary antibody incubation was thus using antibody diluted in PBSTween of pH 7.5 and continued accordingly. Alternatively the acidification occurred with the primary antibody incubation in which the binding event occurred in the presence of the acidic pH. The rest of the assay continued as described in section 2.7.1, as depicted in Figure 2.3C.

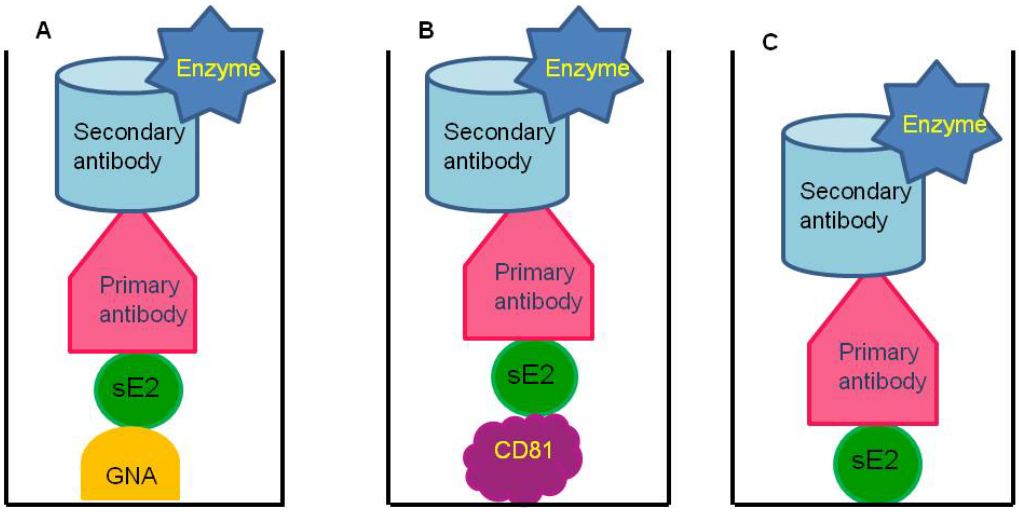

Figure 2.3 Diagrammatic representation of the components of the various ELISA set ups.
A) GNA capture ELISA which was used for E2 and antibody titrations against HVR deleted E2 in section 4.2.2, B) CD81 capture ELISA to test set point relative binding of HVR deleted E2 panel to CD81 in section 4.2.1, and C) E2 coated ELISA as used for comparing point mutated and wild type E2 binding to antibodies, with or without acid treatment: in sections 4.2.2, 4.2.3, 5.2 respectively.

2.8. Statistical analysis

The statistical significance of the effect of transfection reagent to reporter DNA ratio in section 3.2.2, E2-antibody binding relative to wild type in section 4.2.2 and CD81 binding by HVR deleted 2B2.8 E2 protein in section 4.2.1 was assessed by one way ANOVA analysis comparison of means with $p<0.05$. In section 4.2.2 EC50 values were derived from a curve regression analysis of sigmoidal dose type curves. Binding threshold (Ct) was determined as the x axis value (µg/ml of titrated protein) at which the binding curve y axis value (OD) met twice the negative control signal value (OD). Linear regression analysis of the Ct values against the EC50 values used a two tailed Spearman rank test where alpha is 0.05. Significance of the Ct values across the panel of

HVR deleted mutants was derived with one way ANOVA test comparing means with $p<0.05$. Analysis of the effect of sera in culture media on protein production in section 3.2.4 was performed with a t test for paired 2 sample means. One way ANOVA was performed in Section 5.2 assessing the effect of acidification of E2 on epitope exposure. All tests were performed with Graph pad Prism: version 5 software (Licensed by University of Nottingham).

2.9. Phage display

2.9.1. Affinity selection

The recombinant phage library screened in this project was created and previously used successfully with a different protocol to generate broad acting neutralising antibodies (Allander 2000). The biopanning process consisted of a cycle of input of purified phage in solution to a selection platform, enrichment of the phage and concentration to a purified solution (see Figure 2.4). The affinity selection allowed recognition of specific binding clones to be taken forward. Briefly, two 1.5 ml tubes containing 10 µl of streptavidin coated paramagnetic beads (DYNAL) and 500µl of PBS-0.05% Tween 20 5% milk were rotated for 1 hour to allow thorough mixing of the beads and the blocking solution. One of the tubes was then placed on a magnetic rack to wash the beads three times with PBS to remove the blocking solution. In this tube 400 µl of 1% BSA solution and 100 µl of phage solution was added to the beads and incubated at 37^{o}C for 20 mins. The free solution was then removed

from the beads and mixed with 100 ng of soluble E2 protein (D535A mutant of genotype 1A 20.8) and 10 µl of 5% BSA with 0.05% Tween 20, incubated on a rotation wheel for 2 hours at room temperature. Two test groups were designed for the affinity selection; one in which the addition of the 1% BSA was of a pH 5.5 solution to maintain acidic pH throughout the selection, and the second group in which E2 was acidified in a volume of <100 µl for 20 mins at 37^{o}C prior to dilution in the phage mix at pH 7.5.

Specific binders were then recovered as follows. The second blocked tube of streptavidin coated magnetic beads was washed 3 times with PBS-0.05% Tween 20 carefully and the phage-E2 mix added. The mixture was then incubated at 37 ^{o}C for 20 minutes and re-suspended twice to ensure thorough mixing. After placing the tube on a magnetic rack, the supernatant was carefully removed and the beads washed with 500 µl of PBS-0.05% Tween 20 5-10 times followed by a wash with PBS only then sterile water only. Bound phage were eluted from the beads by addition of 500 µl of elution buffer (0.1 M HCl-glycine, pH 2.2, with 1 mg BSA/ml), re-suspension and incubation for 10 minutes. The tube was then placed on a magnetic rack and the supernatant removed, taken into a new tube and neutralised by addition of 50 µl of 2 M Tris-base. This final solution constituted the eluted phage mix (eluate).

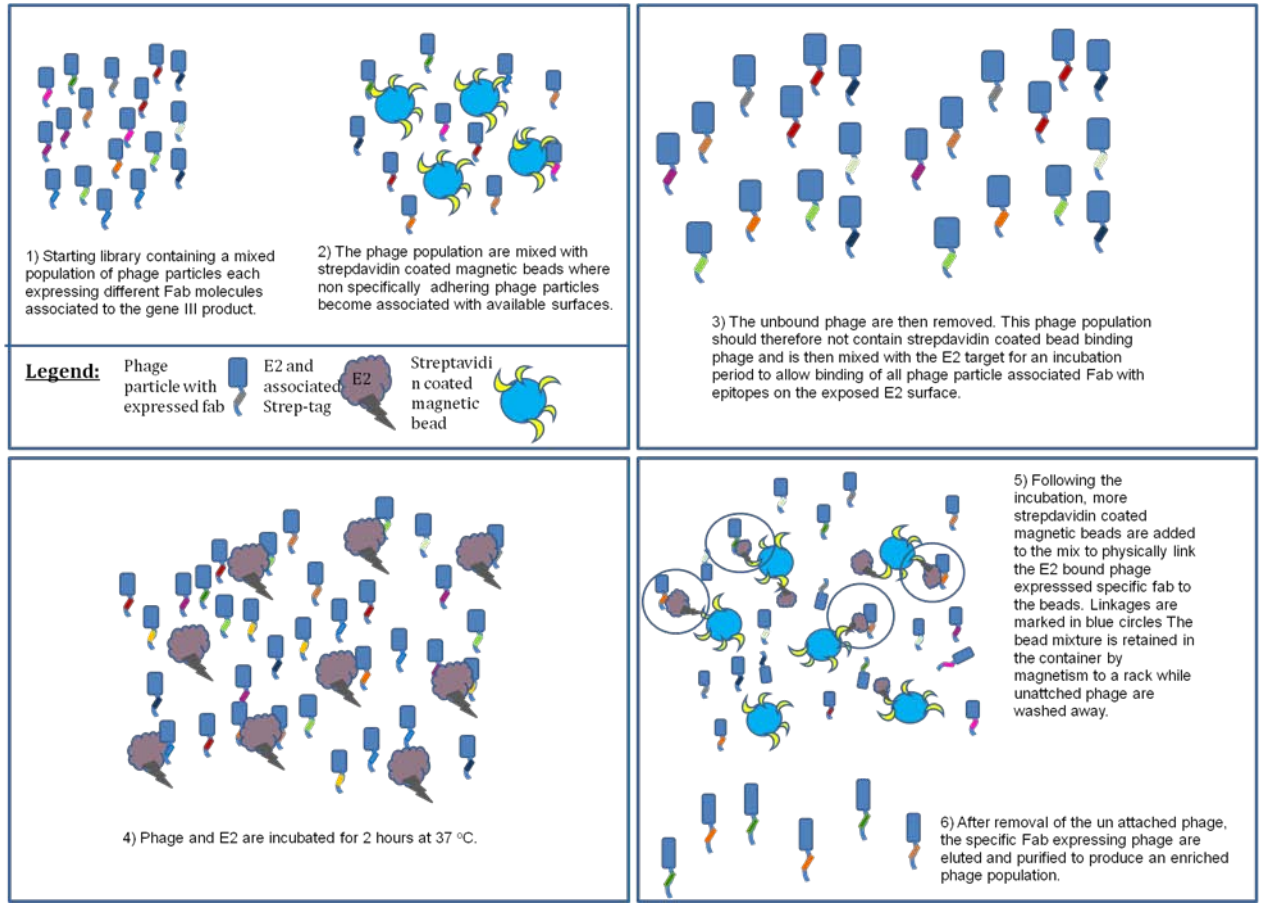

Figure 2.4 Schematic representation of enrichment of phage during panning.
Enrichment of the phage population here involves increasing the specificity while decreasing the diversity of the expressed fab present.

2.9.2. Library expansion

Following affinity selection, the eluted phage mix/enriched library were expanded to minimize loss of rare target-binders during subsequent rounds. Amplification of the library was therefore carried out in the XL 1 Blue bacteria, initiating a new round of panning with a 2 ml culture of ~1.65 x 10^8 bacteria/ml. The absorbance of this culture was read at 600 nm and the concentration of bacteria estimated with the equation 1.0 OD_{600nm}=3.3x10^8 bacteria/ml. The bacterial culture was infected with 100 µl of eluted phage and left to infect for 15 minutes at room temperature in a 50 ml conical tube. Following this, 6 mls of warm SB media supplemented with 1.6 µl of 100 mg/ml ampicillin were added to the culture and shaken at 250 rpm at 37^{o}C for 1 hour, after which a further 2.4 µl of ampicillin were added and another hour incubation followed. Bacteria were then transferred to a sterile 500 ml culture flask containing 91 ml of SB media supplemented with 100 µg/ml ampicillin in which bacteria were then super-infected with VCS M13 helper phage at a MOI of 30 before growing for a further 1.5-2 hours shaking at 37^{o}C. Finally, 50 µg/ml kanamycin were added and the culture grown overnight (16 hours) at 37 ^{o}C.

The following morning, bacteria were cleared from this culture by centrifugation at 4 000 x g for 15 minutes and the phage subsequently recovered from the supernatant by two rounds of precipitation as described in section 2.9.3. The concentration of phage expanded in the

library was then determined by titration as described in section 2.9.4 and ~$1x10^{12}$ phage subsequently used as the input to further rounds of affinity selection.

2.9.3. Phage precipitation

To isolate phage from bacterial cultures, PEG-precipitation was used. Phage containing culture supernatants were recovered to remove bacteria and other debris by centrifugation at 4 000 x g for 15 minutes (Thermo: Heraeus Cryofuge). Phage were precipitated from the supernatant by addition of $1/5^{th}$ volume of PEG (20 % [w/v] polyethylene glycol-8000, 2.5 M sodium chloride) for 1 hour at 4 ^{o}C and collected by centrifugation at 15 000 x g for 15 minutes (Sorval). Phage pellets were then re-suspended in 0.5-2 ml sterile PBS before a second precipitation with $1/5^{th}$ volume of PEG, for 1 hour on ice. Phage were subsequently collected by centrifugation at 10 000 x g for 20 minutes (MSE Micro Centaur) and re-suspended in a final volume of 0.2-2 mls 1% BSA or sterile PBS (where phage precipitation began with smaller culture volumes of ~35 ml, the final re-suspension occurred at the smallest volume of 0.2 mls).

To remove any remaining bacterial debris the solution was separated by centrifugation at 10 000 x g for 5 minutes at 4 ^{o}C and the supernatant collected. The concentration of phage within the expanded/enriched library was determined by titration as described in section 2.9.4, and

$1x10^{12}$ phage subsequently used as the input to further rounds of affinity selection.

2.9.4. Estimation of phage titer

To estimate the concentration of phage particles in the preparations, phage solutions were diluted in sterile PBS and 10 µl added to 90 µl of mid log phase XL 1 Blue bacteria culture, left to infect at room temperature for 15 minutes before spreading onto agar plates containing 100 µg/ml of ampicillin an grown overnight at 37 ^{o}C. The titer was then calculated as the number of colonies multiplied by 100, then divided by the dilution factor expressed as phage particles (cfu) per ml.

2.9.5. Isolation of phage clones

Individual bacterial colonies were obtained by streaking bacteria on LB agar plates containing 100 µg/ml ampicillin and grown overnight at 37^{o}C. Single colonies were then transferred to 3 ml of SB media containing 100 µg/ml ampicillin and grown at 37 ^{o}C overnight again (16 hours). Bacteria were recovered from this culture by centrifugation at 10 000 x g for 3 minutes and either stored at -80^{o}C in media containing 30% glycerol to make a glycerol stock, or the phagemid DNA was purified using a miniprep kit (Qiagen). Expansions from the glycerol stock of the bacterial culture were then performed as described in section 2.9.2 followed by PEG precipitation. Purified phage were then

reactivity tested in an ELISA format as described in section 2.9.6. Purified DNA was also used for sequence analysis and restriction enzyme digests.

2.9.6. Phage displayed peptide ELISA

To determine whether phage clones enriched from the library directed specific binding to the selecting protein, reactivity was assessed by enzyme-linked immunosorbent assay (ELISA) in which the optimum concentration for these components was determined empirically (data not shown). Optimal sensitivity of this immunoassay was achieved by coating the ELISA plate with target molecule used in affinity selection at a concentration ~100µg/ml diluted in carbonate bicarbonate buffer and loading 50 µl per well for overnight binding/incubation at 4^{o}C. The unbound well contents were then shaken out and replaced with 300 µl of PBS-0.05% Tween 20 5% milk, covered and incubated for 4 hours at room temperature. Following blocking, wells were washed 3 times with PBS-0.05% Tween 20 and approximately 1x10^{11} phage particles were added to the wells, and then allowed to bind for 1 hour. The wells were washed again with PBS0.05% Tween 20 and bound phage were subsequently detected by incubation with either anti-fd antibody (Sigma) or anti pIII antibody (Sigma), both diluted 1:1000 in PBST. The assay continued with appropriate secondary antibody and substrate as described in section 2.7.1 and the absorbance was measured as described.

2.9.7. PCR screen of phagemid clones

To be able to discriminate phagemid clones containing the correct sized gene inserts a PCR screen was designed using the primers described in Table 2.4 and illustrated in Figure 2.5.

Table 2.4. Specifications of the primers used for PCR screen and sequencing of phagemid clones.
Combinations of primers were used to specifically amplify gene inserts individually or the whole segment from the light up to and including the heavy chain.

Primer	Target	Direction	Sequence
SEQGb	~50 nucleotides downstream of V region: Heavy	antisense	GTCGTTGACCAGGCAGCCCAG
SEQLb	~50 nucleotides downstream of V region: Lambda	antisense	GAAGTCACTTATGAGACACAC
SEQKb	~50 nucleotides downstream of V region: Kappa	antisense	ATAGAAGTTGTTCAGCAGGCA
Ompseq	Omp leader: Light	sense	AAGACAGCTATCGCGATTGCA
Pelseq	Pel leader: Heavy	sense	ACCTATTGCCTACGGCAGCCG

Figure 2.5 Schematic representation of the linear coding region of the pComb3H phagemid gene inserts and associated amplification primers.
Omp and Pel leader sequences flag the beginning of each gene insert allowing specific primers to amplify all inserts where these sequences are present. Likewise, primers were designed that amplified from the 3' constant region, also distinguishing lambda from kappa light chains.

Amplification reactions were set up in 12.5 µl volumes containing 2.5 pmol each of a combination of a sense and an anti-sense primer (see

Table 2.5) for a product ~600 bp, representing the individual Fab chain genes or a ~1200 bp product, containing both chain genes. The reaction also included 1 mM of dNTPs, 0.3125 U of HotStarTaq DNA polymerase (Qiagen), and 1x PCR Buffer. Two µl of overnight culture diluted 1:100 was used as reaction template. The PCR-cycling parameters were 15 minutes at 95 ^{o}C followed by 40 cycles of 94 ^{o}C for 45 seconds, 61 ^{o}C for 45 seconds, 72 ^{o}C for 3 minutes, subsequently followed by a terminal step of 72 ^{o}C for 10 mins.

3. S2 Protein expression

3.1. Expression systems

Biopanning is a method for isolating favourable ligands such as specific antibodies based on chosen characteristics. The characteristics being targeted are imposed by the chosen target protein used to isolate the ligand/antibodies. As such, the primary aim of this project was to generate a panel of possible target proteins to use for the enrichment of antibody libraries. In this project, HCV entry is the biological process that is being targeted and for this reason, conditions imposed on the chosen viral protein E2 during entry are to be considered. To perfectly mimic the behaviour of the protein *in vivo*, the functional characteristics of the potential target protein to be used in biopanning need to be assessed and verified. The expression system in which a protein is produced i.e. mammalian, bacterial or insect cell lines can impose different constraints on the final state and activity of the purified protein. Bacteria can be used to express high levels of protein but due to a lack of complex post translational modifications that occur in eukaryotic cell lines, the biological activity of the protein produced and correct folding can be problematic (Baneyx and Mujacic 2004). Given that during infection with HCV, the replication and assembly of progeny virus occurs in human cells via mammalian enzymatic pathways and using human cell specific molecular interactions, the viral proteins are thus produced with eukaryotic glycosylation and

chaperones. For this reason alone a eukaryotic expression system for viral proteins *in vitro* is preferable. However, whilst mammalian cell lines are excellent for the production of membrane anchored and secreted proteins, the cell lines are largely inflexible as they are labour intensive, need a carefully controlled environment, show low expression levels, and require expensive media and time as it can take months to develop a stable cell line (Wurm 2004; Cha, Shin et al. 2005).

Filamentous fungi and yeast cell lines can also be used to express recombinant proteins at high levels. However, membrane-anchored or secreted proteins like HCV E2 are produced at low levels compared to intracellular proteins (Gerngross 2004). Insect cell lines are also a prominent eukaryotic platform for protein expression as they can express virtually all types of heterologous recombinant protein (Douris, Swevers et al. 2006). Initial research demonstrated that the protein in question in this project- HCV E2, produced in insect cell lines using baculovirus or in yeast cell lines, did not reflect the functional characteristics that are known to occur *in vivo* (Rosa, Campagnoli et al. 1996). However, the comparative E2 expressed by the mammalian cell line from the same study was able to retain functional characteristics. This observation was most likely due to different capabilities of the cell lines to glycosylate the proteins appropriately.

In 2004, a new set of vectors for insect cell lines became available for producing recombinant proteins thus eliminating the need for the previous cumbersome method that required Baculovirus infection of the cells (Farrel, Swevers et al. 2005). An inherent problem with using a virus to transform a cell line by infection is an increased amount of proteolysis. The recovery of protein produced also requires cell lysis and therefore the maintenance of cell lines and continuity of protein production does not occur. With the pMT series of vectors for insect cell protein expression by Invitrogen (Hyung-Bae, Sun-Hong et al. 2002; Invitrogen 2012), there is full integrity of post translational modifications, a stable physiological environment and a high yield of secreted recombinant protein from serum-free media. The only limitations to insect cell protein expression systems lies in the type of post translational modifications and like all non-mammalian cell lines, correct folding. S2 cells, however, have been developed to maintain the ability to process complex glycans and thus correct formation of proteins that are suboptimal in other insect cell lines (Kim, Shin et al. 2005). The insect cell line Drosophila Schneider 2 (S2) is well-adapted to post translational modifications such as N-glycosylation as it has folding machinery that promotes correct disulphide bonding in the ER. This makes it especially suitable for the expression of multi-domain glycoproteins such as HCV E2 and Fab molecules (Harrison and Jarvis 2006). Indeed multiple groups have used S2 cells successfully for the production of Fab and HCV E2 ((Kim, Shin et al. 2005; Johansson, Drakenberg et al. 2007; Backovic, Johnasson et al. 2009; Krey, d'Alayer

et al. 2010) also reviewed in (Douris, Swevers et al. 2006)) and the protocols developed by these groups have therefore been adopted and assessed in this project.

The first aim of this project was to use the Drosophila S2 cell expression system outlined by Johanssons group (Johansson, Drakenberg et al. 2007), to produce variations of the chosen target protein HCV E2 as soluble monomeric stable protein. However, before being used in phage biopanning, it was important to validate the antigenicity and general characteristics of the expressed protein.

3.2. Results

3.2.1. Transient Protein Production

The first aim of this project was to verify that the glycoproteins produced in the S2 cell culture system were correctly folded. For this, transient test inductions were performed. A period of 24-48 hours for test protein expression was used in this project following the protocol outlined in a 2007 paper by Johansson and his group (Johansson, Drakenberg et al. 2007). The test inductions performed in this project showed positive signals that were clearly distinguishable from the negative producing cell strains and controls and as such the cell strains that were expanded were confidently deemed to be producing high levels of protein that were verified to be correctly folded according to ELISA experiments with conformation sensitive antibodies.

3.2.2. Transfection Reagent Ratio Effect

To optimise the transfection process and consequently the protein yields, a variety of transfection conditions were assessed. The product instructions of the transfection reagent Fugene 6 suggest two transfection reagent: reporter DNA ratios, which are 3:2 and 6:1. Multiple transfections were performed with the constructs 1A20.8-G530A and 1A20.8-D535A. The comparative variable between the various transfections was the amount of Fugene 6 reagent used in the reaction noted as the ratio of reagent relative to the 2 µg of reporter

plasmid DNA used. Forty eight hours post-transfection the supernatants were harvested and the amount of E2 protein produced was compared by ELISA using the monoclonal antibody AP33 (see Figure 3.1).

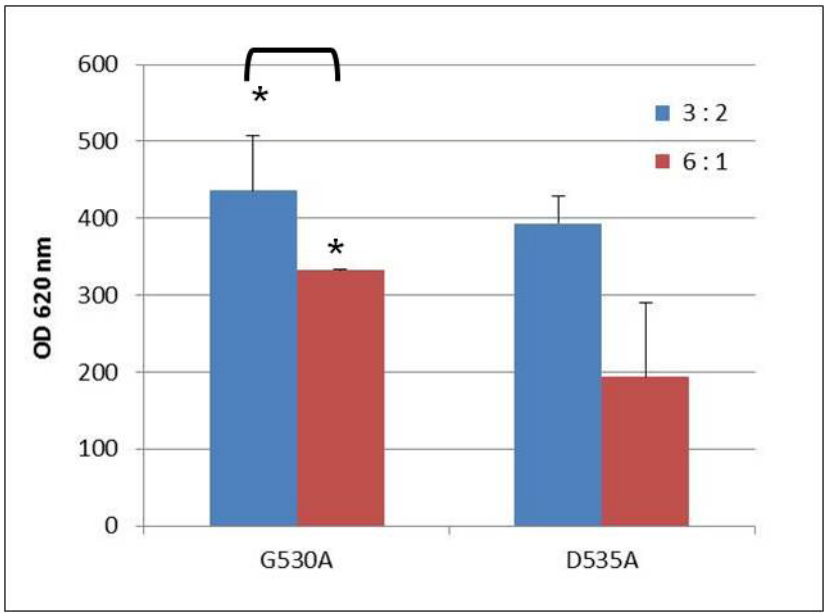

Figure 3.1 Effect of the ratio of transfection reagent to reporter plasmid on protein production.
After 2 days of selection and 24 hour test induction with 500 µM copper sulphate solution. Tested via GNA coated ELISA detecting E2 protein with AP33 antibody followed by alkaline phosphatase conjugated anti mouse antibody and substrate BCIP reading optical density at 620 nm. Asterisks indicate significant difference *$p<0.05$ using a one way analysis of variance comparison of means.

A Fugene to sample ratio of 3:2 produced more protein than the 6:1 ratio, although this was only statistically significant for one of the E2 constructs (G530A). Therefore, the suggested range of transfection ratios were deemed not to affect protein production to a great extent and the most economical ratio of 3:1 also provided in the product instructions was kept for all further transfections.

3.2.3. S2 cell Protein Expression

Eleven different E2 constructs were transfected into S2 cells and the relative amount of protein produced was determined by BCA assay and nanodrop spectrophotometry. Protein yields varied from 0.93 mg/l-5.73 mg/l (see Table 3.1). This variation did not appear to fit any pattern relating to the constructs in question as the highest expressers were observed for both genotype groups regardless of the modification of each construct.

Table 3.1 Average yield of purified E2 for each construct produced of each genotypic strain.

Protein was quantified with a nanodrop spectrophotometer and BCA assay. Protein yield is expressed as amount of pure protein collected per litre of culture supernatant. E2 of strain UKN1A20.8 contained different mutational replacement of residues to alanine whilst the UKN2B2.8 strain of E2 constructs contained various combinations of deletions of the hypervariable regions HVR1, HVR2 and HVR3 (Δ1,2, and 3 respectively).

E2 construct: UKN1A20.8	Amount of protein (mg/l)	E2 construct: UKN2B2.8	Amount of protein (mg/l)
		Wild type	3.03
Wild type	1.67	**Δ1**	5.73
G530A	5.00	**Δ2**	1.89
D535A	1.50	**Δ3**	1.69
G530A-D535A	2.40	**Δ1,2**	0.93
		Δ1,3	5.07
		Δ1,2,3	1.60

3.2.4. Sera in S2 culture media

To evaluate the impact of culturing the cells in serum free-media, two culture methods were compared. S2 cells, at a density of $5x10^6$ cells /ml were split into two pools. One pool was cultured using the standard serum free media whilst the second pool was grown supplemented with 10% fetal bovine serum. Cell density was recorded regularly with each passage occuring when cells reached a density of ~$5x10^6$ cells/ml.

Table 3.2 Average growth of cells per day of culture expressed as cell density X 106 / ml of culture /day of culture.

Growth in serum-free media (SFM) or with addition of 10% foetal bovine serum over several passages was compared. This process was repeated multiple times with passages occurring when cell cultures reached a density of ~$5x10^6$ cells/ml, and cells were seeded at ~$1x10^6$ cells/ml. Averages are recorded here. Two way ANOVA with Bonferroni post test showed no significances with alpha as 0.05.

Passage	Cell growth (cells/(day x10^6/ml))			
	Serum Free Media		Media + 10% FBS	
	average	St. Dev.	average	St. Dev.
1	0.499	0.053	0.712	0.096
2	0.361	0.081	0.384	0.054
3	0.688	0.068	0.408	0.065

Table 3.2 shows that initially, cells receiving serum-containing medium grew more rapidly as the cell growth was almost 1.5 times greater for serum-treated cells compared to standard serum-free medium cells. By the time the third passage occured however, this trend had reversed as cell growth became almost twice as great for the serum free cells as for those receiving serum. Statistcial analyses of the data using a one way ANOVA test showed no significant differences among the trends observed. So, whilst using FBS-containing medium for S2 cell culture

may initially improve cell growth, any increase in cell proliferation was short-lived.

The impact of protein production on S2 serum-treated cells was subsequently assessed. Cell strains transfected with E2 constructs of genotype 1A or 2B were cultured in the presence or absence of 10% FBS throughout the selection period. Following selection, media were replaced with serum-free media only and a test induction was performed. The amount of protein produced was assessed by ELISA using the monoclonal antibody AP33 (see Figure 3.2). The amount of protein produced in the presence or absence of serum was similar, and paired t-tests for two sample means confirmed that there was no significant difference in protein yield between the two culture conditions.

Table 3.3 Numerical data and statistical analysis of the comparison between protein expressions from S2 cells receiving serum-containing or serum-free medium.

Assay format is GNA coated ELISA detecting expressed E2 with anti-strep tag antibody and substrate BCIP read at OD 620 nm. The statistical analysis was performed using a T test for paired two sample means

Protein production (OD 620 nm)				
	E2 strain			
	1A		2B	
	fbs	sfm	fbs	sfm
Average	1.62	1.78	0.54	0.75
St dev.	0.15	0.15	0.03	0.07
p value	0.0319		0.0227	

3.2.5. Purified E2 Protein Panel

Having defined optimal transfection and expression conditions, the S2 expression system was utilised to produce a variety of target E2 proteins for subsequent use in antibody biopanning experiments. The two E2 constructs chosen as possible target candidates for biopanning were the hypervariable deleted E2 in the genotype 2B2.8, and E2 of genotype 1A20.8 with mutagenesis of residues 530 and 535 (see Table 2.1). The E2 constructs were transfected into S2 cells, stable lines were established and then protein expression was induced with copper sulphate. Following 10-16 days of induction, supernatants were harvested, and run through affinity chromatography columns for purification and the amount of E2 protein yielded was analysed by Western blot and Coomassie gel staining (see Figure 3.3 & Figure 3.4). The purification of expressed soluble E2 successfully yielded monomeric, highly pure E2 protein. This panel of soluble E2 proteins were shown to be within a size range of 40-50 kDa with slight variations due to varying states of glycosylation. Deletion of the hypervariable regions contributed a maximum of about 8 kDa in line with the amount of genetic sequence excised from the protein coding gene. Inter-assay variation of the estimated size of E2 indicated sizes between 40 kDa and 50 kDa. An estimated size of ~50kDa for S2 expressed soluble E2 has been shown elsewhere (Krey, d'Alayer et al. 2010) and so this is a satisfactory E2 protein. Correct fold of the E2 produced was confirmed with conformation-sensitive antibodies 1:7, A8 and the antibody AP33

that has conformational determinants, in ELISA format for each batch of protein produced.

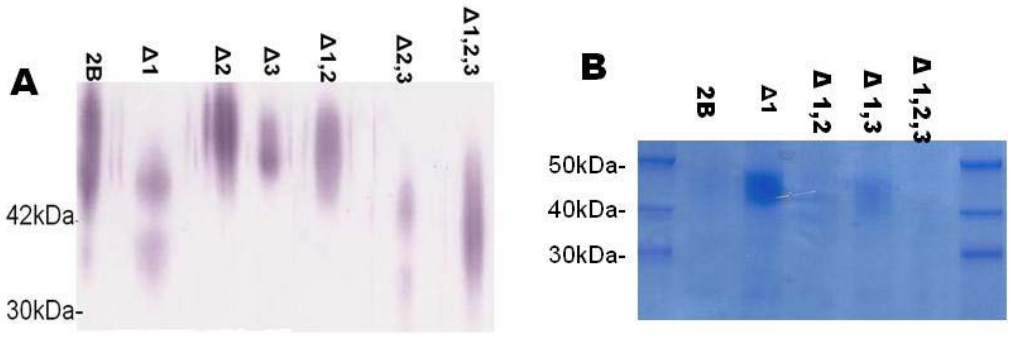

Figure 3.2 Western blot (A) and Coomassie staining (B) of the deletion mutant E2 protein panel and the wild type 2B2.8 isolate.
A) An antibody specific to the C terminus streptavidin tag located on each E2 was used to probe the blot followed by species specific antibody conjugated with Alkaline Phosphatase for which the substrate was BCIP/NBT. **B)** 2-5 µg of purified protein was run on an SDS-PAGE gel followed by protein staining with Instant Blue Coomassie stain. The panel of E2 proteins are wild type sequence as found in the clinical isolate, with variants containing deletions of the hypervariable regions. The expected size of E2 is ~50 kDa, deletion depending.

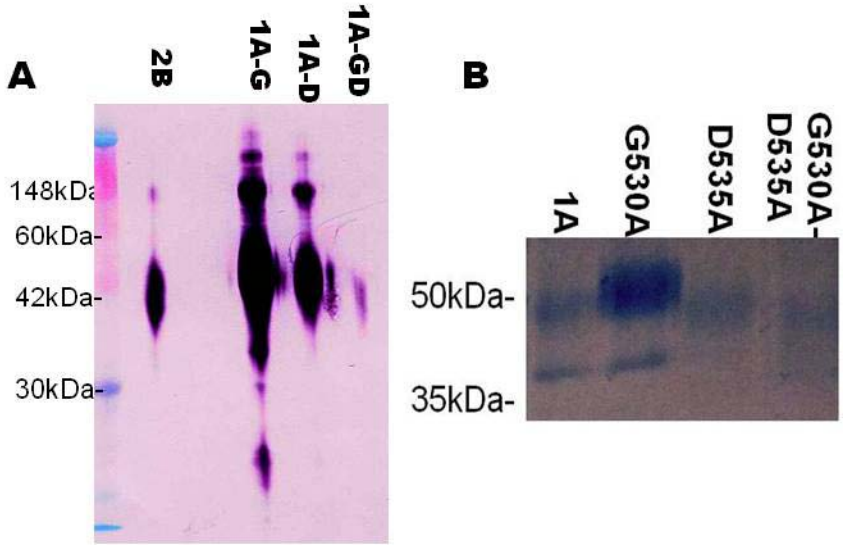

Figure 3.3 Panel of genotype 1A20.8 soluble E2 proteins in western blot (A) and Coomassie staining (B).
A) An antibody specific to the C terminus streptavidin tag located on each E2 was used to probe the blot followed by species specific antibody conjugated with Alkaline Phosphatase for which the substrate was BCIP/NBT. Surrogate Wild type protein is of strain 2B, with other proteins of strain 1A. **B)** 2-5 µg of purified protein was run on an SDS-PAGE gel followed by protein staining with Instant Blue Coomassie stain. The panel of E2 proteins are wild type sequence as found in the clinical isolate, with variants containing alanine replacement mutagenesis of residues G530 and D535.

Smaller sized variants are visible for some of the E2 constructs (see Figure 3.4 & Figure 3.3). These variants were not consistently detected using reducing or non reducing SDS-PAGE and nor were any other variant sizes observed that would otherwise imply degradation of the protein preparations. Therefore, the size variants might possibly be due to variation in the glycosylation of the E2. Overall, the soluble protein produced can be considered homogeneous, and stably produced. It also proved technically difficult to demonstrate all proteins consistently on Coomassie staining and Western blots, possibly due to the low sensitivity of coomassie staining and the relatively low concentration of some of the protein samples. Assessment of the protein panel according to fold/conformation was therefore next addressed and is discussed in section 4.

3.2.6. Deglycosylated purified E2

In order to assess the degree and nature of glycosylation of the S2 expressed soluble E2 protein in this project, glycosidase digestion experiments were performed (see Figure 3.5). Reduced and denatured wild type E2 showed a single band close to 50 kDa and thus the protein was stable and of the expected size. The single band shows that the protein was stable and not degraded. When the same protein preparation was also digested with pyrogenase F (NEB) to remove high mannose and complex N-linked glycans, two bands sized approximately ~35 and ~40 kDa were produced (indicated by red arrows in the middle lane of Figure 3.5A) thus a loss of up to 15 kDa was observed when N-linked glycan groups were removed. The remaining core E2 mass reflects E2 expressed in mammalian cells supporting that this protein is properly produced (Slater-Handshy, Droll et al. 2004).

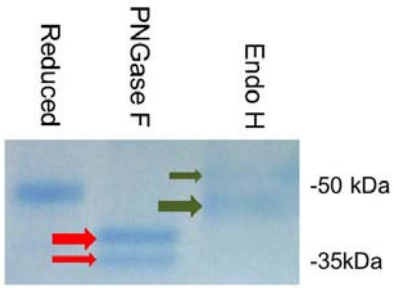

Figure 3.4 SDS-PAGE analysis of glycan status of reduced and denatured S2 expressed E2 protein.
Wild type genotype 1A20.8 soluble E2 monomeric protein reduced and denatured (left hand lane) or further treatment by either: PNGase F (middle lane-red arrows), or Endo H (right hand lane- green arrows).

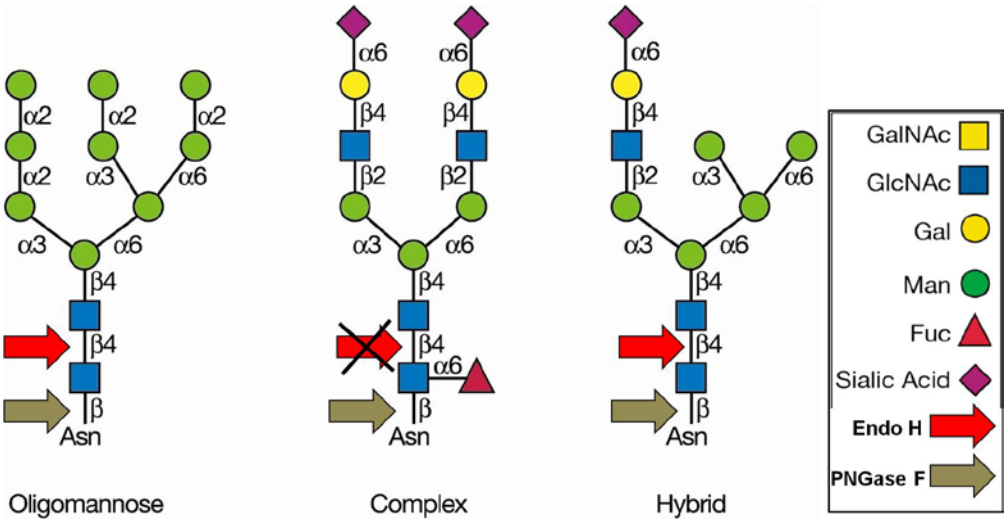

Figure 3.5 Illustration of the differential digestion of PNGase F and Endo H.

PNGase F is able to cleave between asparagines moieties and proximal N-acetylglucosamines providing there is no α1,3 fucosylation. EndoH however cleaves between two N-acetylglucosamine moieties in the absence of any fucosylation.

Glycosidases are enzymes that can cleave glycan moieties from proteins of which endoglycosidase H and pyrogenase F are two examples. Both enzymes cleave asparagine associated N-acetylglucosamine (GlcNAc) linkages but act at different points with varying activity. Figure 3.5 shows that pyrogenase F targets the link between the asparagine residue and the proximal N-acetylglucosamine, except in the case of the latter being α1-3 fucosylated. Endoglycosidase H however cleaves between two N-acetylglucosamines except when either of these moieties has been fucosylated. Human, insect and plant N-linked glycans most commonly contain α1-6 rather than α1-3 groups and so pyrogenase can be used to cleave a predominant portion of complex oligosaccharides (Varki, Cummings et al. 1999). The presence of 2 bands of similar sizes in Figure 3.4 following pyrogenase F treatment indicates two states of glycan digestion: almost complete and complete. Optimisation of the

pyrogenase treatment using more enzyme or a longer incubation period may be sufficient to complete digestion leaving a single band of only one protein species at ~35 kDa. The right hand lane of Figure 3.4 shows the size of the soluble E2 protein following digestion by Endoglycosidase H (NEB) with the bands marked by green arrows. Endoglycosidase H removes high mannose and hybrid oligosaccharides but cannot cleave fucosylated N-acetylgucosamines that therefore depicts complex oligosaccharides. Figure 3.5B & C show the different points at which endoglycosidase H and pyrogenase F cleave the main glycan groups. The use of endoglycosidase H and pyrogenase F thus distinguishes complex N-linked glycans from the other subtypes excluding the much less common α1-3 fucosylated oligosaccharides which pyrogenase F cannot process. According to Figure 3.4, the mass specifically attributable to non complex N-linked glycans could be interpreted to be around 5 kDa. However, when analysing a pool of protein molecules it is not possible to distinguish between differentially glycosylated molecules or to be able to ascertain if specific glycans have been modified to the same extent on each molecule. The two bands in the endoglycosidase H treated lane are of size ~50 kDa and ~45 kDa showing some undigested protein along with digested protein in which only 5 kDa of glycan mass was cleaved. Taken together, these data show only partial digestion by endoglycosidase H, which could be improved by optimising the digestion protocol.

Figure 3.3 shows the panel of genotype 1A E2 proteins, some with alanine replacements. Some of these proteins showed a fraction of

smaller sized variants indicating differential processing. Given the amount of post translational modifications required for E2 and the limited scope for this in the S2 cells the E2 was produced in, the size variation is likely a product of varying states of glycosylation. The double mutant (G530AD535A) the D535A E2 protein showed the least amount of this size variant, which was not consistently observed. Also, the wild type 1A E2 protein which did show a size variant in Figure 3.3 showed normal core mass following glycosidase analysis. Therefore the G530A, D535A & G530AD535Asoluble E2 produced were taken to have a stable core though glycosidase analysis on all E2 samples would have been preferable for a more confident evaluation.

Taken together, the E2 produced in this project is stable, highly pure, with a core mass of expected size and a glycan coat of ~15 kDa. The missing 10 kDa of glycan shield from the soluble E2 produced in S2 cells is likely due to limited post translational modifications that are otherwise present in mammalian cells. Glycan deletion has been shown to affect glycoprotein function whilst aiding epitope exposure (reviewed in (Helle and Dubuisson 2007)). Therefore, by having a thinner glycan coat that still retains the complex glycans, immunogenicity can be favoured hopefully with minimal effect on function. The production of stable, pure protein with a correct core mass and antigenicity thus supports that the E2 protein here closely reflects the viral protein as produced *in vivo*.

3.3. Discussion

A study in Cryotechnology by Santos and her group (Santos, Jorge et al. 2007) tested the expression profile of S2 cells using the same protocol on a different reporter protein to allow fluorescent analysis of protein expression. S2 cells were observed to start producing detectable levels of the reporter protein as little as 5 hours post induction when induced with 500 µM $CuSO_4$, showing up to 90% of cells present exhibiting expression by day five post induction (Johansson, Drakenberg et al. 2007). As an indicator of the expression pattern of S2 cells cultured using the same conditions, these data support that production of protein in S2 cells should be detectable by 24 hours and consequently that any production below detection level by this point could not be deemed an indicator of efficient expression. Given the clear separation between cell strains correctly producing E2 protein and those that did not, the cell strains expanded in this project were confidently deemed to be exhibiting efficient production of high levels of protein as reported elsewhere (Johansson, Drakenberg et al. 2007; Gilmartin, Lamp et al. 2012).

In 3.2.2 the transfection ratio of the Fugene 6 reagent and reporter DNA was tested and the choice of ratio adopted was deemed to have no great effect on protein production. However, given that the ratios tested and the proteins produced in this project were of satisfactory amounts and within the ranges described by others using the same and similar

systems (reviewed in (Douris, Swevers et al. 2006)), this does not discount the fact that greater differences could be observed using different proteins or constructs thereof.

Cell culture based techniques can be replicated exactly and yet have varying output of the culture assays. Multiple groups have used S2 cells for expression of HCV E2 (Johansson, Drakenberg et al. 2007; Backovic, Johnasson et al. 2009; Gilmartin, Lamp et al. 2012) or other proteins (reviewed in (Douris, Swevers et al. 2006)) with various reported outputs of protein from 0.13 mg/litre of supernatant to over 40 mg/litre (Gårdsvoll, Werner et al. 2004; Lim, Kim et al. 2004; Douris, Swevers et al. 2006; Johansson, Drakenberg et al. 2007; Gilmartin, Lamp et al. 2012). Krey observed in a panel of E2 constructs that even different batches of the same cell strain had outputs that varied by up to 20% (Krey, d'Alayer et al. 2010). Taken together, the variations in protein production observed here are in range of those observed by other groups. Thus, the S2 expression system here corresponds to its use by other groups. Variation in protein expression consequently cannot be considered an indicator of any hindrance to expression due to any of the particular constructs.

The use of sera in media is a condition that has its benefits and disadvantages for different cell lines and purposes. Serum in cell culture media is generally considered to improve the survival of cell lines, growth and to increase protein expression. However, the inclusion of

animal-derived components in cell culture allows a potential risk of contamination by mycoplasma and prions (Kallel, Jouini et al. 2002). Mycoplasma contamination is very costly for loss of work, time and reagents along with sometimes being very hard to identify and then eliminate. The European Medicine Evaluation Agency (EMEA) and the Food and Drug Administration (FDA, USA) have consequently encouraged the elimination of the use of substances of animal origin (Castle and Robertson 1999; Batista, Moraes et al. 2009). The consequence of this is the development of cell culture methods that are serum free. For S2 cell culture, protein expression has been shown to be highly efficient without the use of serum (Johansson, Drakenberg et al. 2007; Gilmartin, Lamp et al. 2012) though some groups still choose to use serum for an improved yield (Backovic, Johnasson et al. 2009). However, the presence of serum in the media not only affects the activity of the cells, but can also hinder the process of protein purification from supernatant by affinity chromatography. So, the data collected provides further evidence for there being no distinct advantage to the use of sera in S2 cell culture and protein production.

A variety of studies have shown the influence of glycosylation on envelope proteins regarding fold and function (Fournillier, Wychowski et al. 2001; Slater-Handshy, Droll et al. 2004; Liu, Chen et al. 2007). One factor that can affect the glycan processing of viral proteins is the expression system used to produce them (Baneyx and Mujacic 2004; Gerngross 2004). As previously noted, different expression systems

have different capabilities for post translational modifications, and heavily glycosylated proteins like E1 and E2 can thus be very much affected by the system they are produced in. So far the soluble E2 expressed in S2 cells here and by Krey in 2010 (Krey, d'Alayer et al. 2010), differs from the majority literature describing E2 produced in mammalian cells and derived from cell lysates (Dubuisson, Hsu et al. 1994; Fournillier, Wychowski et al. 2001; Op de Beeck, Cocquerel et al. 2001; Slater-Handshy, Droll et al. 2004; Liu, Chen et al. 2007) according to one factor. This factor is the difference in size since E2 purified from mammalian cells has been shown to have a mass of ~60 kDa compared to ~50 kDa for S2 expressed E2. The 2004 paper by Slater-Handshy (Slater-Handshy, Droll et al. 2004) shows expressed E2 protein derived from mammalian cell lines with an observable inter assay variability ranging from ~55 kDa to ~66kDa for the same E2 construct. However, even at the smallest possible size of 55 kDa for the E2 protein truncated at residue 715, this is still larger than the soluble E2 protein also truncated at residue 715 from Kreys work in 2010, and this project which is closer to 50 kDa (see Figure 3.4). Inter-assay variability for determining the size of the soluble E2 within this project has given soluble E2 sizes from 45 to 55 kDa. The tag for affinity purification of the E2 protein used by Slater-Handshy(Slater-Handshy, Droll et al. 2004) is the c-myc/His tag from the pcDNA3.1 vector (Invitrogen) and is detailed as adding 3 kDa to the recombinant protein size according to the manual. This project and Kreys (Krey, d'Alayer et al. 2010) used the pMT vector for expression in the S2 cells with the

given tag replaced with the Strep tag II (IBA) which will add a maximum of 1 kDa to a recombinant protein. This makes the difference of affinity tag insufficient to account for the size difference of E2 produced from S2 cells and mammalian cells. Therefore, the main contributing factor for size differences observed in E2 produced here and elsewhere lies in the ability to produce the different post translational modifications of the chosen expression system.

Slater-Handshys group (Slater-Handshy, Droll et al. 2004) demonstrated that the removal of all glycans from E2 caused a loss of around 20 kDa of mass by treatment with tunicamycin (Slater-Handshy, Droll et al. 2004). The mass of their fully glycosylated E2 protein was around 60 kDa, and the mass following complete glycan digestion became ~35 kDa as seen here. Thus a core mass of unglycosylated E2 being ~35 kDa is not significantly affected by the expression system and is correct in either mammalian cell lines or S2 cells. So the differences in mass observed in glycosylated E2 are confidently assigned to the glycan coat as the glycans produced in each cell line differs along with the actual mass of those glycans. Analysis of the glycosylation status of E2 expressed in mammalian cells showed a difference in size of 5-10 kDa between complete glycan digestion and non complex N-linked glycan digestion with endoglycosidase (Slater-Handshy, Droll et al. 2004). The difference in size following the two digestions is therefore attributable to complex N-linked glycans and other glycans such as O-linked glycans. Similarly, a ~10 kDa size

difference was observed here between digestion with pyrogenase F and endoglycosidase, thus reflective of mass contributed by complex N-linked glycans. The size difference contributed by non-complex N-linked glycans on E2 protein expressed in mammalian cells is up to 20 kDa (Slater-Handshy, Droll et al. 2004). In S2 cells, the core mass of E2 matches that of mammalian expressed protein and the mass of non complex N-linked glycans is no more than ~5kDa. Therefore, whilst inter and intra-molecular differences in glycosylation limit the scope of these data and the consequential interpretation, this project clearly shows that in S2 cells, a much smaller portion of the glycan coat on expressed E2 is made up of non complex N-linked glycans than in mammalian cell-expressed E2.

Current literature has shown that mammalian cell-expressed E2 protein collected from medium was resistant to digestion with endoglycosidase H (Slater-Handshy, Droll et al. 2004). The same protein collected from cell lysates however was still susceptible to the same digestion indicating that late stages of protein production induce endoglycosidase resistance. The E2 protein produced here was collected from culture supernatants and thus could also have potentially incorporated resistance to endoglycosidase. Figure 3.5A shows two bands following endoglycosidase H digestion: the first band at the same size as undigested protein, possibly due to resistance to endoglycosidase and the second band 5 kDa smaller. Taken together, it is possible that the S2

expressed E2 protein produced here exhibited partial resistance to endoglycosidase digestion as observed elsewhere.

4.Characterisation of HCV E2 proteins: identifying possible targets for enrichment of antibody libraries

4.1. Preface

In this project, a range of E2 target proteins were evaluated for use in biopanning against a phage antibody display library in order to isolate antibodies targeting novel epitopes. All of the constructs expressed good yields of E2 protein which, when analysed by PAGE and western blot, were shown to be predominantly monomeric and stable. These proteins were then subject to antigenic characterisation using a range of antibodies recognising linear and conformational epitopes, to identify which of these constructs would be most useful in the subsequent biopanning experiments.

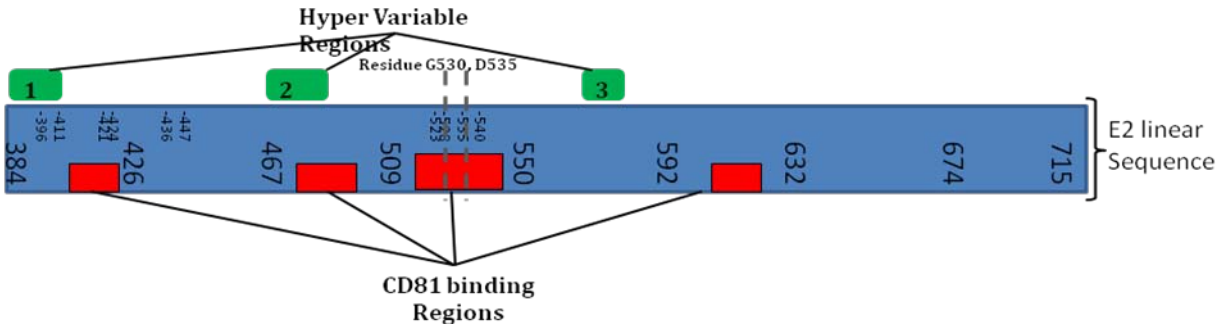

Figure 4.1 Schematic representation of the linear format of E2 marking the various deletions/mutations made to create a panel of immunogen candidates, HVR's and CD81binding sites:
The three hypervariable regions are marked with green boxes, the two key CD81 binding residues G530 and D535 are marked by dashed lines and CD81 sites depicted with red boxes.

4.2. Results

4.2.1. HVR's collectively shield CD81 binding sites

Before testing hyper variable deleted E2 as a possible biopanning target, the ability to bind CD81 protein was tested as an indicator of conformation. CD81 protein interacts with E2 over 4 contact regions forming a conformation dependant interaction and this interaction is essential for cell mediated entry.

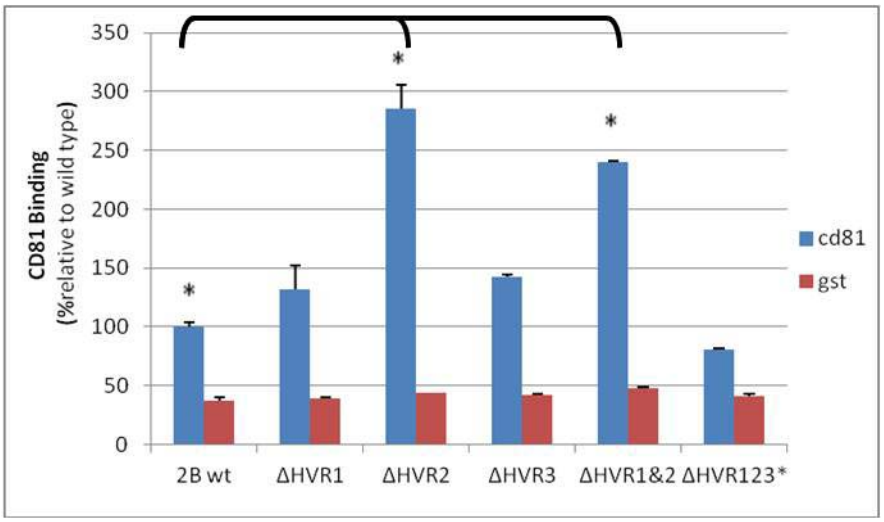

Figure 4.2 GST tagged soluble human CD81 binding to E2.
Strain 2B2.8 wild type E2 and variants were tested in an ELISA with 5 µg/ml of E2 probed with 10 µg/ml of CD81-GST or GST alone followed by detection with mouse anti-human CD81 antibody conjugated with horse radish peroxidise (1/5000) secondary antibody and BCIP/NBT substrate. Statistical analysis using one-way analysis of variance (ANOVA) comparison of means: all bars relative to 2B Wild Type at 95% confidence limit (*, $p<0.05$).

A simple ELISA assay was used to compare the effect of all combinations of variable region deletions in E2. Figure 4.2 shows the relative binding of soluble E2 with CD81 protein fused to a GST tag. Equal amounts of all E2 proteins were used so that the signal is comparable between the different E2 constructs. All the E2 protein

constructs tested bound the CD81 protein. Differences in overall binding affinity were evident and of these, the increased CD81 binding (relative to the wild type) observed for the ΔHVR2 and the ΔHVR1&2 constructs reached statistical significance. Only one E2 construct showed reduced CD81 binding (ΔHVR1, 2&3), but this reduction was not significant. It should be noted that this construct also contained a deletion of the c-terminal region of the ectodomain, from c-terminal residues 645-715, which might have affected binding.

4.2.2. E2 antigenic profile is HVR modulated

It is believed that the removal of specific variable loops in the HIV envelope protein gp120 better exposes conserved regions thought to be involved in receptor binding and neutralising antibody recognition (Wyatt, Sullivan et al. 1993; Gzyl, Bolesta et al. 2004). Without the crystallised structure of E2, conformational effects of specific deletions in the E2 protein cannot be determined. In order to clarify how the HVR's impact the structure and antigenicity of E2, we probed the various E2 constructs with a panel of well-characterised antibodies. Preliminary binding assays indicated that for some of the antibodies and constructs, binding saturation would not be possible (not shown). This precluded the use of the standard Scatchard analysis of binding affinities. Therefore, in order to be able to compare across constructs and antibodies, we utilised a method that compares binding threshold

values (Ct), i.e. the lowest antibody concentration that provides a positive signal in ELISA (McKeating, Zhang et al. 2004).

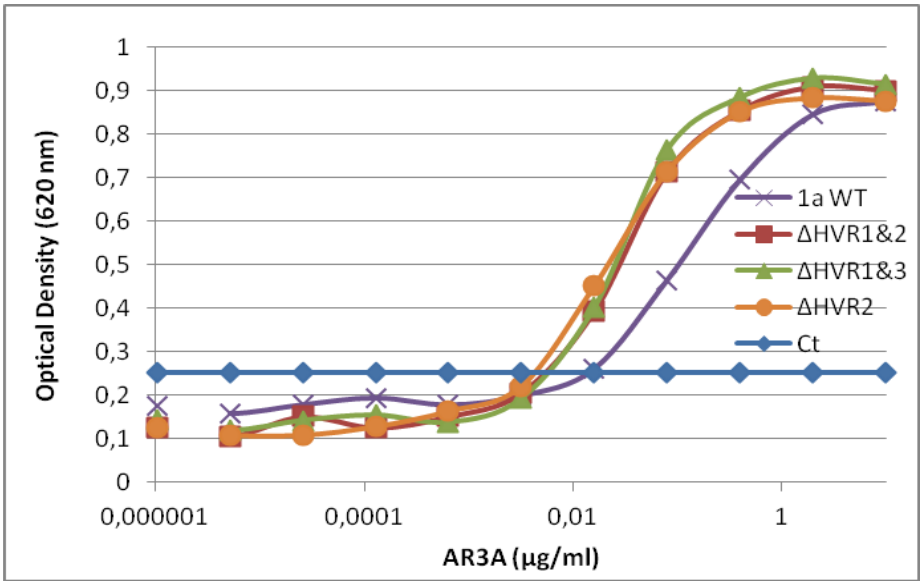

Figure 4.3 Titration curve for AR3A against E2 variants.
Full binding curves were achieved with AR3A antibody titrated from 10 µg/ml in 5 fold dilutions on a 96 well ELISA plate coated with 1 µg/ml of genotype 2B2.8 E2 variants. Bound AR3A was detected with goat anti human alkaline phosphatase secondary antibody (1/500). A BCIP/NBT substrate was applied and OD was read at 620 nm. Ct values averaged at 0.2516 with SD 0.0363.

To validate this approach, we compared the Ct value with the estimated EC50 value, for a sub-analysis using protein-antibody pairs that were able to reach saturation binding. An example of the full titration curve for antibody AR3A against a variety of proteins is shown in Figure 4.3. In a similar assay by McKeating and Zhang, the point of the curve which meets twice the amount of signal achieved by the negative control was used as the threshold for positive signal (McKeating, Zhang et al. 2004). To validate that this choice of comparison point among different data sets reflected the relationship given by EC50 comparison, a correlation of the EC50 versus the threshold value (Ct) according to McKeating and Zhang's method was analysed.

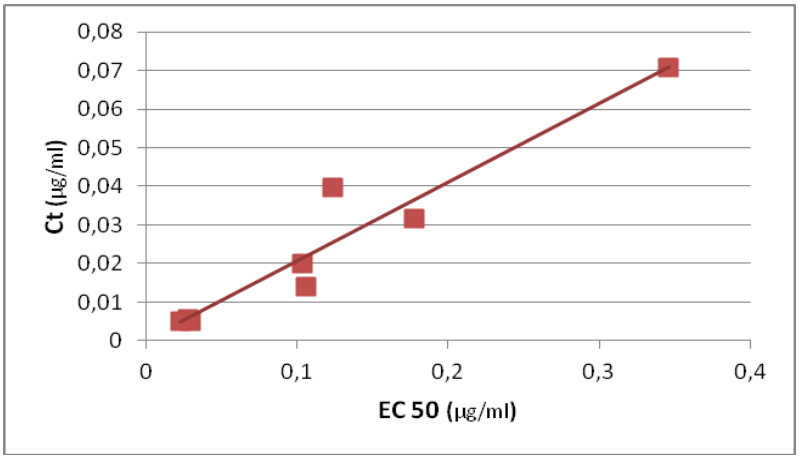

Figure 4.4 EC50s of binding curves with AR3A and a panel of E2 variants plotted against binding threshold values derived from the same data.
Statistical analysis of this correlation was performed with Spearman Rank test with significance level of 0.05 and the relationship was shown to be significant with a two tailed p value of 0.0046. This choice of threshold value has previously been used by McKeating and Zhang 2004 (McKeating, Zhang et al. 2004).

In the analyses of AR3A binding to a range of E2 constructs, there was a correlation between the Ct and EC50 (see Figure 4.4) and this correlation was significant in a Spearman rank test ($p<0.01$). Having validated that the Ct represents a good surrogate for affinity, we utilised this approach to assess the binding of various antibodies to each of the E2 constructs. The amount of antibody and E2 protein available also posed another restriction on how to use the antibodies and purified E2 at hand. A titration ELISA typically involves a set amount of target protein bound to a plate via a connector molecule such as GNA, followed by the application of varying concentrations of antibody from a very high dose to a very dilute dose, which allows a full binding curve to be attained. In this set up, only one step of the full assay is varied, this being the concentration of the primary antibody. With all other

components of the assay remaining constant, comparative interpretation of the data is possible.

Restricted availability hampered the amount of some of the antibodies that could be used in the assays. Therefore, to reduce the amount of antibody required, two different assay formats were compared: one whereby the E2 was captured and the antibody titrated, and the other capturing E2 dilutions and detecting this with a fixed amount of anti body. A hierarchy of E2 protein binding profiles was maintained in each titration. Calculation of the binding threshold in each data set was also maintained with a high correlation coefficient of 0.9479 between the two sets of data supporting that the titration approach does not affect the hierarchy of binding among a given set of proteins (See Table 4.1). Thus with limited amounts of antibody, titrating the E2 protein would save the amount of antibody required. Therefore, where antibody was lacking, the format for which E2 was titrated was used in replacement. However, whilst both formats could be used to reflect binding differences between different proteins by each antibody, quantitative interpretation was consequently restricted to within each antibody-format data set and not across the different formats.

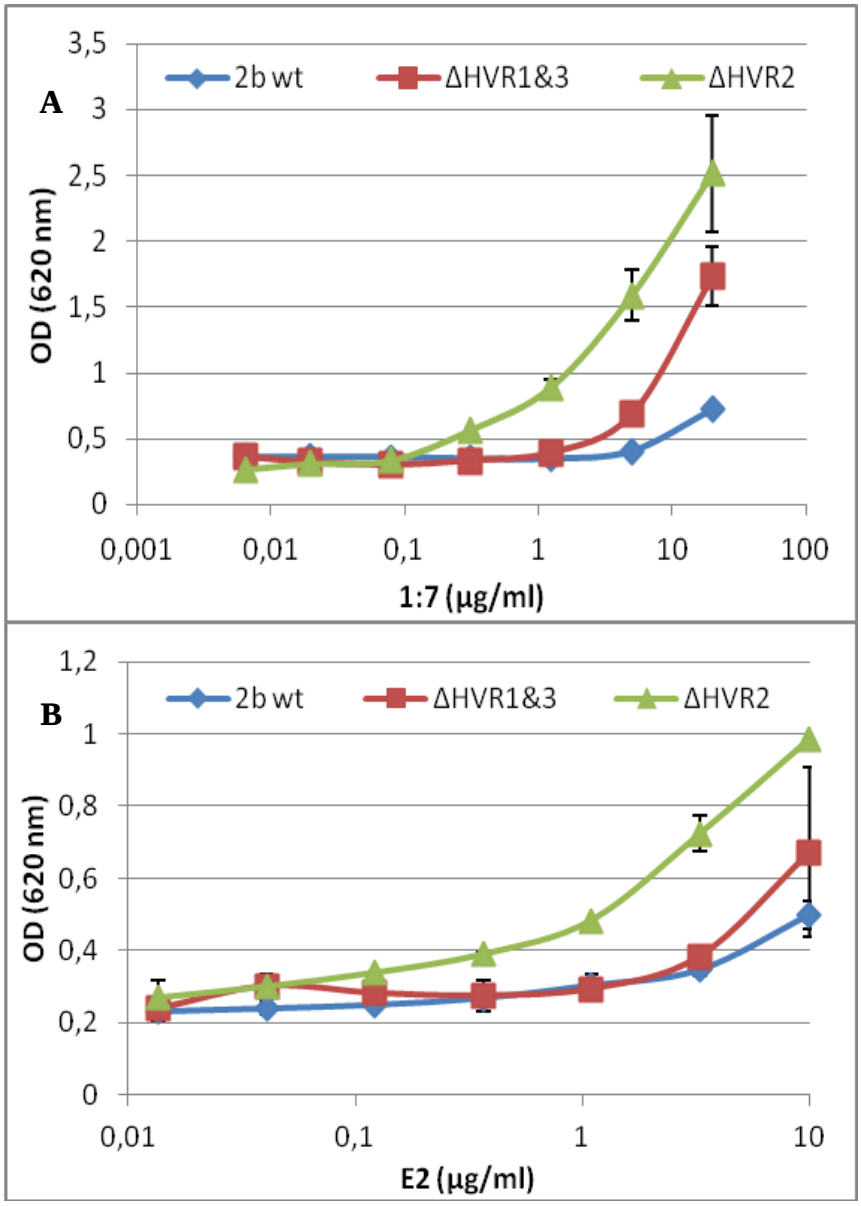

Figure 4.5 Titrations of E2 test protein or primary antibody were performed in ELISA to check the relationship of binding for each set up.

The non-titrated protein was used at 1 µg/ml whilst the titrated protein was diluted. The primary antibody here is 1:7 with E2 genotype 2B2.8 wild type, ΔHVR1&3 and ΔHVR2. 1:7 was titrated from 20 µg/ml in 5 fold dilutions (**A**), whereas E2 was titrated from 10 µg/ml in 3 fold dilutions (**B**).

Table 4.1 Analysis of threshold values derived from ELISA titration comparison.

Statistical analysis of the correlation between the threshold values obtained according to each titration set up was performed with a Pearson correlation test. Where E2 was titrated, 1:7 was applied at 1μg/ml and where 1:7 was titrated, E2 was applied at 1μg/ml. The titrated protein is thus the variable from which the threshold value is derived.

Threshold values for binding (μg/ml)		
E2 construct	**E2**	**1:7**
2B Wild Type	6.0843	8.1439
ΔHVR1&3	3.0693	1.4698
ΔHVR2	0.5653	0.2318
Pearson Correlation coefficient	0.9479	

Having established the optimal assay to be used, binding curves of neutralising antibodies AP33, A8 and AR3A were attained against the panel of purified soluble E2 constructs. These three antibodies represent epitopes located across the central domain I of the proposed structure of E2 (Owsianka, Tarr et al. 2005; Johansson, Voisset et al. 2007; Law, Maruyama et al. 2008). AP33 recognises a linear epitope located within a CD81 binding region within DI (Owsianka, Clayton et al. 2001; Owsianka, Tarr et al. 2005). The involvement of the residues in this area however has been disputed as having any direct involvement with CD81 binding (Rothwangl, Manicassamy et al. 2008) and so AP33's competitive inhibition of CD81 binding (Owsianka, Clayton et al. 2001) is possibly due to steric hindrance. A8 and AR3A recognise conformation-sensitive epitopes, which overlap with the CD81 binding

domain, and include residues 530-535 within DI (Johansson, Voisset et al. 2007; Law, Maruyama et al. 2008). The Ct values of each E2 construct for the three antibodies have been plotted as a % relative to the wild type E2 protein of genotype 2B2.8 (see Figure 4.6). A higher Ct value, indicates a larger amount of titrated protein (AR3A was titrated whereas AP33 and A8 had E2 titrated) required to achieve a binding signal equal to twice the negative control. Unlike the CD81 binding, access/exposure of each epitope varied widely over the panel of E2 constructs. Constructs containing the HVR2 deletion demonstrated significantly improved binding to the CD81 binding site antibodies, as did the construct containing the triple deletion. These data did not provide a consistent reflection of that seen for the CD81 binding, however together they do indicate that the removal of HVR2 results in the increased exposure of the CD81 binding site, at least in the context of soluble E2.

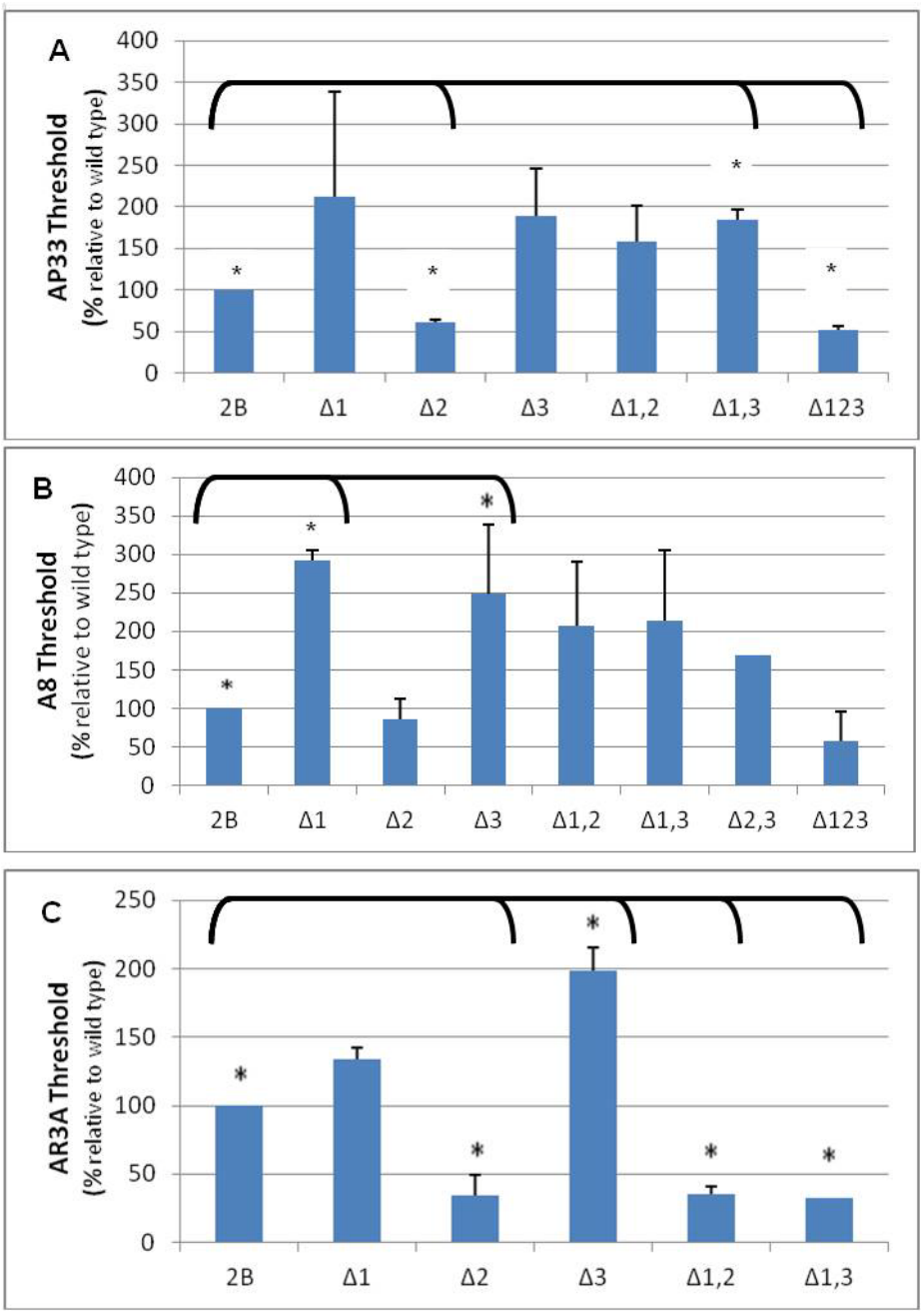

Figure 4.6 Comparative binding of titrated genotype 2B2.8 E2 variants with 1 µg/ml of AP33 and A8 antibody, and 1 µg/ml of E2 for titration of AR3A antibody:

AP33 (A), A8 (B) and AR3A (C), detected with goat anti human/mouse alkaline phosphatase (1/5000) and BCIP/NBT substrate. Statistical analysis using one way analysis of variance (ANOVA) of multiple comparisons: all bars relative to 2B Wild Type at 95% confidence limit (*, $p<0.05$).

The remaining deletions showed a variable effect on epitope exposure. However, the removal of HVR1 or 3 showed the strongest hindrance to the ability of antibodies to bind their epitopes. AP33 has an epitope most focused on an area near HVR1. The most detrimental effect on AR3A binding to its epitope was the removal of HVR3 alone. HVR3 links two E2 domains that contain CD81 binding sites which are thought to also encompass at least part of the AR3A epitope. Site directed mutagenesis has identified specific residues within DI that are thought to be important for AR3A binding. The epitope for AR3A involves residues from multiple CD81 binding sites and thus changes in AR3A binding should be most reflective of the global form of E2.

The only HVR that overlaps with the CD81 binding region is HVR2. Rather than hindering CD81 interaction with E2, the removal of HVR2 allowed an improvement in binding by CD81 protein and the various antibodies examined here. Previous studies have shown that HVR1 and 2 can modulate CD81 binding, although this relationship was in a strain specific manner (Roccasecca, Ansuini et al. 2003). There is also no current evidence that the antibodies used in this study recognise epitopes that encompass HVR2. HVR2 is located close to the putative fusion peptide proposed to lie at the uppermost region of Domain II according to Kreys model (Krey, d'Alayer et al. 2010). Other viruses including related flaviviruses have hydrophobic fusion peptides that are hidden from the aqueous solvent-accessible surface on the native proteins. These peptides become exposed following conformational

changes as a result of the binding process (Maeda, Kawasaki et al. 1981; Sattentau and Moore 1991; Smit, Moesker et al. 2011). Given the data shown here that HVR2 is most important for mediating access to conserved regions, should a fusion related conformational change in E2 occur during entry, it would be of interest to see how HVR2 is affected or potentially involved.

In practice, it has recently been shown that even with HVR2 only removed, HCV virus loses its ability to infect new cells (McCaffrey, Gouklani et al. 2011). Using pseudo particle virion and cell-cultured virus, the removal of any of the HVR's abolished the ability to infect new cells. This may be a reflection of the HVR's involvement in entry at some point downstream of binding. The loss of functionality in an immunogen, may not necessarily impact on the ability to induce antibodies to relevant epitopes, however it indicates a change in the form of the protein that may equally cause a loss of local or global structural resemblance, beyond the scope of the analyses performed here, to the native form. Therefore, in the aim of designing an immunologically relevant E2 immunogen that allows optimal access to conserved regions for identifying non-CD81 epitopes, a different approach than HVR deletion might be more successful. Also, the over-exposure of the CD81 binding sites (which dominate the known neutralising epitopes on E2 so far) in the HVR-deleted E2 means that as an antigen target in the biopanning process, the HVR deleted E2 as target would encourage rather than separate identification of epitopes

and antibodies equally afflicted with dependence on the CD81 binding sites.

4.2.3. G530 and D535 in CD81 epitopes

CD81 binding sites have previously provided the focus of conserved neutralising epitopes. The main cross neutralising epitopes on E2 identified so far have commonly been linked to residues G530 and D535 (Johansson, Voisset et al. 2007; Law, Maruyama et al. 2008; Perotti, Mancini et al. 2008; Mancini, Diotti et al. 2009). Evidence of cross-neutralising non -CD81 binding site associated antibodies has been shown (Grollo, Torresi et al. 2006; Torresi, Fischer et al. 2007). More recently, different methodologies have isolated and characterised novel cross neutralising antibodies that do not involve CD81 residues; although one of these antibodies may target an epitope involving the first CD81 binding region and competes with CD81 binding possibly by steric hindrance (Sabo, Luca et al. 2011; Giang, Dorner et al. 2012). What these data confirm is the reality of finding novel non CD81/G530-D535 neutralising epitopes. As a result, the second approach adopted for design of an optimal biopanning immunogen target was to specifically mutate the residues G530 and D535 of the E2 protein.

The first step in assessing point mutated E2 was to verify the effect on E2 conformation using the antibody H53 known to be broad-acting, conformation-sensitive and does not compete with CD81 (Cocquerel, Meunier et al. 1998). This antibody does not recognise E2 of genotype 2

and so the low signal observed in Figure 4.7 against the wild type 2B E2 confirms this and thus reflects background noise. Equally, the point mutations were performed in genotype 1A20.8 E2 which H53 does recognise.

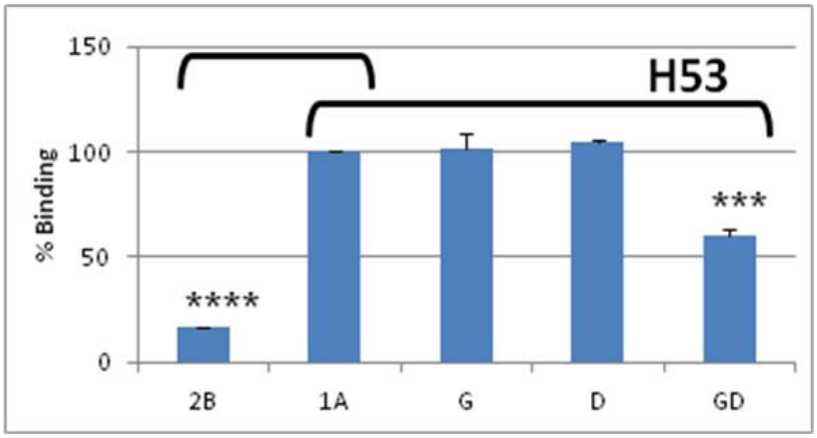

Figure 4.7 Comparison of binding by non CD81 conformational mouse antibody H53 to E2 of genotype 2b and 1a in which CD81 binding residues G530 and D535 have been point mutated to alanine individually and together (G, D and GD respectively).

GNA capture ELISA with 1 µg/ml of soluble E2, probed with H53 supernatant 1:500 and detected with species specific alkaline phosphatase antibody with BCIP substrate. Data plotted relative to wild type 1A E2 protein. One way ANOVA was performed for each E2 construct relative to 1A wild type with Bonferroni post test with alpha as 0.05 (***=p<0.001, ****=p<0.0001).

H53 demonstrated a consistent binding profile against the panel of 1A E2 constructs with no discernible difference among the single point mutations. The double mutation of G530 and D535 together did however cause a drop in binding, which indicates that changing both residues results in a loss of conformation. Therefore, of the choice between the three point-mutated versions of the E2 analysed, either of the single mutations would be preferable as a choice of target for biopanning as it retains native conformation.

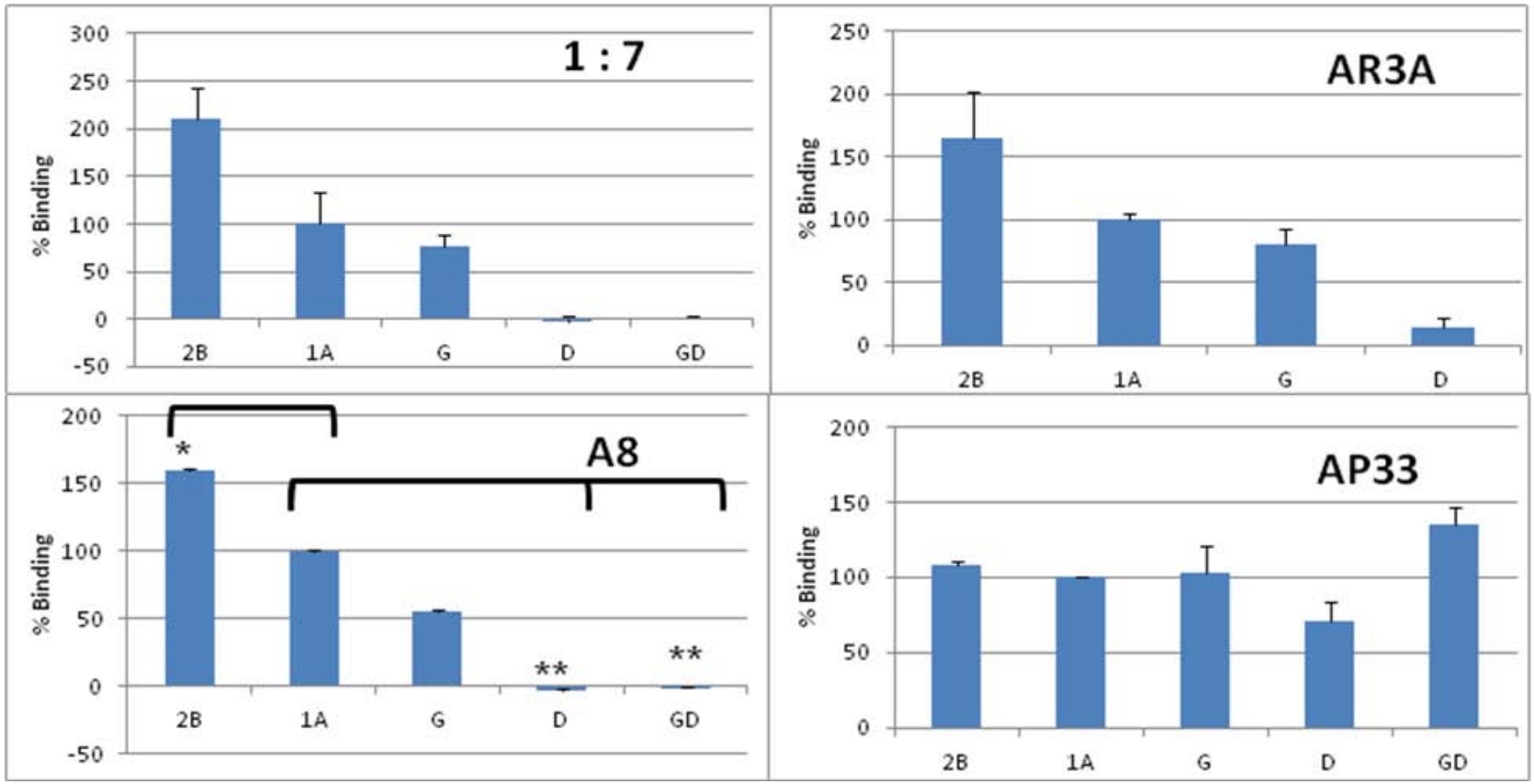

Figure 4.8 Comparison of binding by CD81 competing antibodies to E2 of genotype 2b and 1a in which the key CD81 residues G530 and D535 had been point mutated singly or together to alanine.

GNA capture ELISA with 1 µg/ml of soluble E2, probed with primary antibody at 1 µg/ml and detected with species specific alkaline phosphatase antibody with BCIP substrate. Data plotted relative to wild type 1A E2 protein. One way ANOVA relative to 1A binding was performed for each E2 construct with Bonferroni post test at alpha as 0.05 (*=p<0.05, **=p<0.01, ***=p<0.0001, ****=p<0.00001).

The dependence on residues G530 and D535 by known CD81 binding site epitopes was assessed in order to determine which E2 construct could abolish the known epitopes without compromising E2 conformation. In a GNA-coated ELISA, the relative binding of each E2 protein tested here was plotted against the wild type 1A. For all antibodies the more influential residue for maintaining the epitope was consistently D535 (see Figure 4.8). Binding to the epitopes for A8, 1:7 and AR3A was almost completely abolished with the mutation of D535A whereas only a variable drop in binding was observed for G530A. AP33 does not use G530 and D535 as contact residues for its epitope. The AP33 epitope does however overlap with the first CD81 binding site and so AP33 can exhibit competition with the CD81 molecule directly at this epitope. Any competition between AP33 and the CD81 protein at other CD81 binding sites could be due to steric hindrance. It is therefore interesting that D535A in the third CD81 binding site affected binding of multiple CD81 competing antibodies to their epitopes when G530A or G530A-D535A did not. Taken together, soluble E2-D535A is a promising target for biopanning or as a vaccine immunogen as it retains native form while abolishing many CD81 based epitopes.

4.3. Discussion

Previous studies have shown that HVR1 occludes the first putative CD81 binding site due to its proximity to this region and to an observed improvement in CD81 binding when HVR1 is removed (Bankwitz, Steinmann et al. 2010; Prentoe, Jensen et al. 2011). Roccassecca and McCaffreys papers both demonstrated that in the absence of all three HVR's, soluble E2 showed improved CD81 binding (Roccasecca, Ansuini et al. 2003; McCaffrey, Boo et al. 2007). More recently however, McCaffrey further showed a contradictory loss of CD81 binding with any deletion of HVR's other than HVR1 (McCaffrey, Gouklani et al. 2011). Contrary to previous results, these last data imply that HVR1 has no influence on CD81 binding and that the removal of HVR2 or 3 are predominantly detrimental to this function. An important difference in the latest data from McCaffreys group and the previous literature is the use of E1E2 whereas the previous data was based on E2 alone. This has functional significance as the production of the E1E2 heterodimer has been shown to have interdependence between E1 and E2 in a manner that does not appear to affect production of E2 alone (Choukhi, Wychoski et al. 1998; Bartosch, Dubuisson et al. 2003; Brazzoli, Helenius et al. 2005). So, the different relationships observed by McCaffrey in E2-CD81 binding when E1 is involved, may be showing an involvement of E1 in the final conformation of E2 that could also be impacting on CD81 binding. The full functional relationship between E1 and E2 still has many elements

that are not fully defined. So, to understand the antigenic relationship between E2 and the HVR's, analysing E2 alone may result in some oversight of the full functional importance of parts of E2 with E1, but will allow a clearer interpretation of the effects specifically on E2 if E1 is not present.

The data presented here are in line with Roccasecca and McCaffreys initial finding that improvement in CD81 binding occurs upon deletions of the HVR's (Roccasecca, Ansuini et al. 2003; McCaffrey, Boo et al. 2007). This implies that all hypervariable regions act to shield CD81 binding sites. According to the E2 tertiary structure model proposed by Kreys group (Krey, d'Alayer et al. 2010), this can be envisaged for HVR1 and HVR3/IgVR as they lie close to the first putative CD81 binding site and between the last two CD81 binding sites respectively. HVR2 on the other hand does coincide with the second putative CD81 binding site causing a loss of some of the key residues in this region. This makes it even more interesting that the removal of HVR2 should enhance CD81 binding. What this means is that the removal of the HVR's has caused secondary effects in the E2 protein. If HVR removal can cause improved access to regions that are elsewhere on E2, then there must be some degree of conformational shift to allow the antigenic exposure. Apart from the triple deleted E2 that had a different truncation, both E2 constructs that had HVR2 deleted showed the best improvement in CD81 binding by more than 100%. Therefore, whereas previous groups have indicated that a triple HVR deleted E2 immunogen would provide

optimal epitope exposure, the data here show that HVR2 is the most influential of the three HVR's for advocating exposure of the CD81 binding-sites.

The latest data from McCaffrey and her group also showed the functional importance of the HVR's for HCV infectivity and replication (McCaffrey, Gouklani et al. 2011). Their data showed that without HVR2 or 3, HCV lost the ability to infect by cell to cell transmission and also showed impaired ability to replicate efficiently. Deletion of HVR1 however, hindered replication and entry though adaptive mutations allowed partial restoration of the ability to infect by the pseudo-particle *in vitro* assay. Whilst there are functional differences in the mode of infectivity for cell to cell and cell free infection, consequently affecting the role of E2 in both processes, the possible loss of native structure from the observed loss of function of HCV E2 without HVR's makes a vaccine/immunogen virus model lacking HVR's less favourable. A functional factor that affects HVR2 and 3 but not HVR1 is the presence of glycans; HVR2 containing glycan 5 and HVR3 containing glycan 9, both having a moderate effect on *in vitro* infectivity, release from cells and sensitivity to neutralisation (reviewed in (Helle, Duverlie et al. 2011). Taken together, the loss of the glycans within the HVR's be partly responsible for the effects demonstrated in McCaffreys most recent paper (McCaffrey, Gouklani et al. 2011). However, this would only be applicable to the deletion of HVR2 or 3, highlighting that other factors

are also involved in the detriment of viral function upon removal of the HVR's.

Infectivity assays using pseudo particle virions expressing E1E2 of various point mutations have shown that either G530 or D535 were crucial for entry as less than 5% infection was observed relative to wild type when these particular mutations were present. Likewise, CD81 binding was almost completely abolished when either residue was replaced with an alanine (Owsianka, Timms et al. 2006). So, whilst these two residues are functionally essential for HCV, here we have shown that they are not essential for E2 conformation, which makes E2 with either of these residues mutated acceptable as an immunogen in a vaccine model or as the antigen target in biopanning.

The S2 cell line has previously been used to produce HCV E2 protein for the purpose of modelling its structure (Krey, d'Alayer et al. 2010). Elsewhere and here, S2 expressed E2 has been deemed an acceptable surrogate for the *in vivo* protein. Biopanning allows the isolation of novel antibodies according to the choice of antigen target. Preliminary data has been provided by Prentoes group (Prentoe, Jensen et al. 2011) showing an obstructive role of E2 HVR1 in shielding the conserved and functional CD81 binding site. There has also been evidence from other groups that collectively show the HVR's sterically hide epitopes (Roccasecca, Ansuini et al. 2003; McCaffrey, Boo et al. 2007). So far in this project, we have seen that deletion of the HVR's allows optimal

exposure of the CD81 binding sites. To derive non-CD81 antibodies, the CD81 binding site focus of the E2 protein therefore needs to be counteracted. So the form of E2 to be used as antigen bait in biopanning to identify non-CD81 epitope accessibility should not be the HVR deleted model.

5. Acidic environment specifically modulates E2 epitope exposure

5.1. Preface

As previously mentioned, the specificities of antibodies derived from biopanning can be shaped by the conditions imposed during the selection process (reviewed in (Bradbury, Sidhu et al. 2011)). Blocking of epitopes recognised by existing antibodies can direct the focus of antibody selection away from those antibodies acting at epitopes defined by the competing antibodies. Alternatively, specific mutagenesis of the target protein can eliminate binding of antibodies targeting dominant epitopes. Antibodies that bind to specific conformers of target proteins have been isolated from different methodologies without adopting specific constraints on the selection pool. Thompson and his group isolated their antibody that specifically binds to the fusion-active form of the West Nile virus envelope protein by immunisation of mice with an un-manipulated protein construct (Thompson 2009). Likewise, antibodies identified elsewhere that bind to post attachment forms of viral envelope proteins thus inhibiting fusion, were isolated from hybridomas that had been generated following immunisation with native viral protein (Imai, Sugimoto et al. 1998; Edwards and Dimmock 2001). Taken together, if antibodies directed at fusion active forms of viral envelope proteins can be isolated for related viruses, and if

adapting selection conditions in biopanning can direct the specificity of antibodies to be isolated, it could be possible to derive antibodies against HCV E2 in its fusion active form to potentially act at a post attachment stage to inhibit fusion and thus entry. Indeed, functionally different conformers of caspase-1 protein have been used in phage display to specifically select antibodies targeting the active form of the protein (Gao, Sidhu et al. 2009). Therefore, to identify an antibody by biopanning that targets an entry intermediate of HCV E2, the conditions to be used in the biopanning process need to be defined such that the E2 target molecule can best represent the *in vivo* fusion active form.

Current literature has shown pH to be a pivotal factor in HCV fusion (Lavillette, Bartosch et al. 2006; Tscherne, Jones et al. 2006; Haid, Pietschmann et al. 2009). There are also indications that E2 exhibits conformational changes (Keck, Op De Beeck et al. 2004; Op De Beeck, Voisset et al. 2004) during the entry process as occurs for other viruses (Maeda, Kawasaki et al. 1981; Sattentau and Moore 1991; Despres, Frenkiel et al. 1993; Lescar, Roussel et al. 2001). Antibody binding has been demonstrated to result in a subsequent change to the antigenic profile of E2 thereby providing indirect evidence of conformational changes in the E2 protein (Keck, Op De Beeck et al. 2004). Similarly, E2 epitope exposure was also affected by redox status as the E2 immunogenicity was distinctly improved upon mild reduction of the soluble protein (Fenouillet, Lavillette et al. 2008). The related Pestivirus

Bovine Viral Diarrhoea Virus (BVDV) was shown to be unaffected by exposure to reducing agents in a neutral pH environment and yet in an acidic environment, the reducing agents inactivated the virus (Krey, Thiel et al. 2005). It is therefore important to understand the complex interplay between various factors to induce functional changes in envelope proteins so that we can fully appreciate the role of conformation of envelope proteins during the entry process.

Taken together, it is likely that E2 conformation and thus epitope exposure is affected by environmental conditions that facilitate entry/ fusion *in vivo*. Therefore, given that acidic pH is a prerequisite for completion of fusion, and conformational changes in E2 are also likely to be involved, it is likely that changes in the E2 protein conformation during the entry process enable exposure of conserved sites as occurs for other acid-dependent conformation-responsive viruses (Sattentau and Moore 1991; Smit, Moesker et al. 2011). Therefore, the antigenic state of variably acidified E2 was investigated to decipher conditions for optimal mimicking of the fusion active state of E2 to be used as a biopanning target.

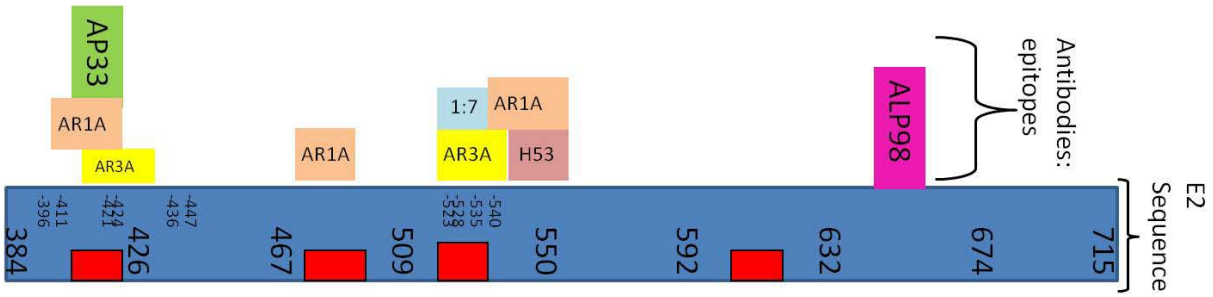

Figure 5.1 Schematic representation of the E2 epitopes analysed for variable exposure in acidic conditions.
Also marked in red boxes are the highly conserved CD81 binding sites for which AP33, 1:7, AR1A and AR3A epitopes overlap with.

5.2. Results

5.2.1. E2 remains stable under acidic incubation

To assess the conformational effect of soluble E2 acidification, it was first necessary to verify that the protein remained stable under acidic conditions. An obstacle to this was the possibility that pH could affect the coating/binding of the E2 protein to the microtiter plate, over the course of the assay, thus affecting the absolute amount of E2. So, the first assay was to look at the relative amount of E2 that remained present in the plate following incubations of various pH levels (see Figure 5.2).

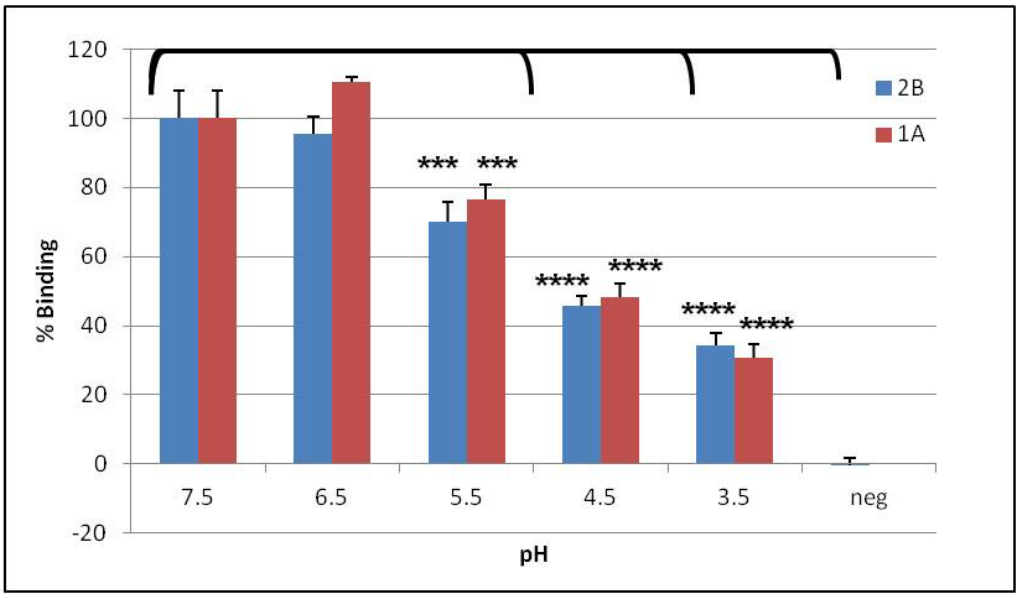

Figure 5.2 The effect on protein coating was looked at using two different strains of E2 from different genotypic groups (2B and 1A). Soluble E2 was coated in an ELISA with incubations of various pH levels probed with an antibody specific for the C-terminal strep tag. The negative control (neg) had no E2 coated. One way ANOVA with Bonferroni post test relative to neutral pH 7.5 statistical analysis (***= $p<0.001$, ****=$p<0.0001$).

Using an antibody specific to the tag associated with the soluble E2 protein, a pH dependant loss of binding was observed. This data would indicate that the acidic pH proportionally affects the E2 protein and/or the associated tag where the epitope lies. Equally this data did not distinguish whether the apparent loss of binding was due to an effect on the protein affecting binding, an effect on the antibody affecting binding to the epitope or if it indicated a loss of absolute E2 present in the assay. Therefore, the same assay was repeated with an antibody that recognises a linear epitope at the C terminus of the E2 protein shortly before the junction to the strep-tag (see Figure 5.3).

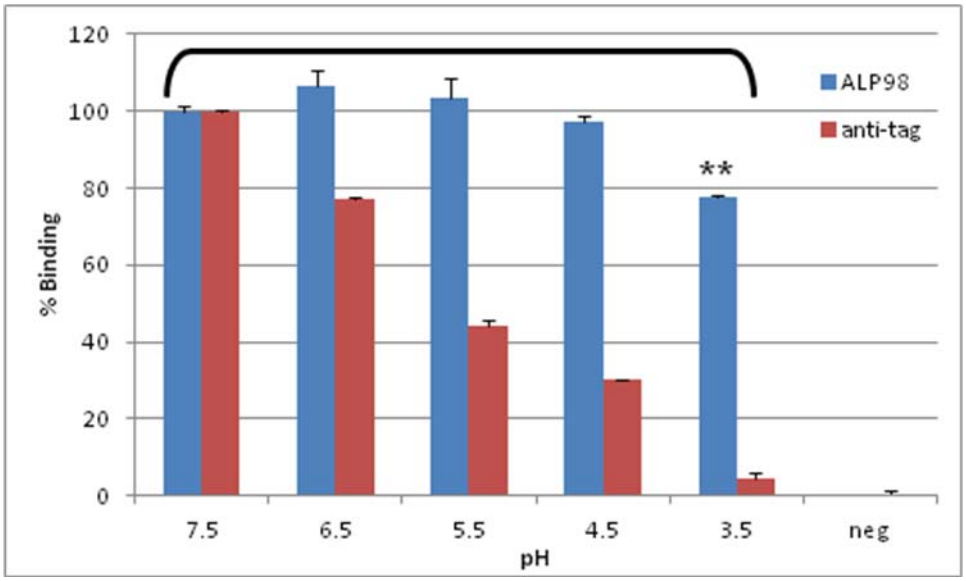

Figure 5.3 Strep-tag dependant and independent detection of soluble E2 over a range of pH levels.
Acidic incubations were performed in ELISA format to assess the effect of pH on uncoating of an ELISA plate when coated with soluble E2. ALP98 recognises a linear epitope at residues 644-651. One way ANOVA with Bonferroni post test relative to neutral pH was performed for ALP98 data (**=p<0.01)

ALP98 is a murine antibody that potently binds a linear epitope at the C terminus of the E2 protein (Owsianka, Clayton et al. 2001). However, this antibody does not recognise genotype 2 and so was only compared

to the anti-strep tag binding for genotype 1 soluble E2. Figure 5.3 shows that the pH-dependant loss of binding by the anti-strep antibody is restricted to that antibody and not a universal effect. The signal derived by ALP98 remains centred around 100% up to pH 4.5, after which there is a 20% loss of signal at pH 3.5. Binding effects on the ALP98 antibody due to pH are therefore less prominent than for the anti-tag antibody, or possibly compensated by additional effects that improve binding kinetics. The loss of signal at pH 3.5 is possibly due to some un-coating of E2 from the plate or an effect on E2 itself. So, if ALP98 binding activity is un-/minimally affected by pH, from pH 7.5 to 4.5, the soluble E2 remains coated to the ELISA plate.

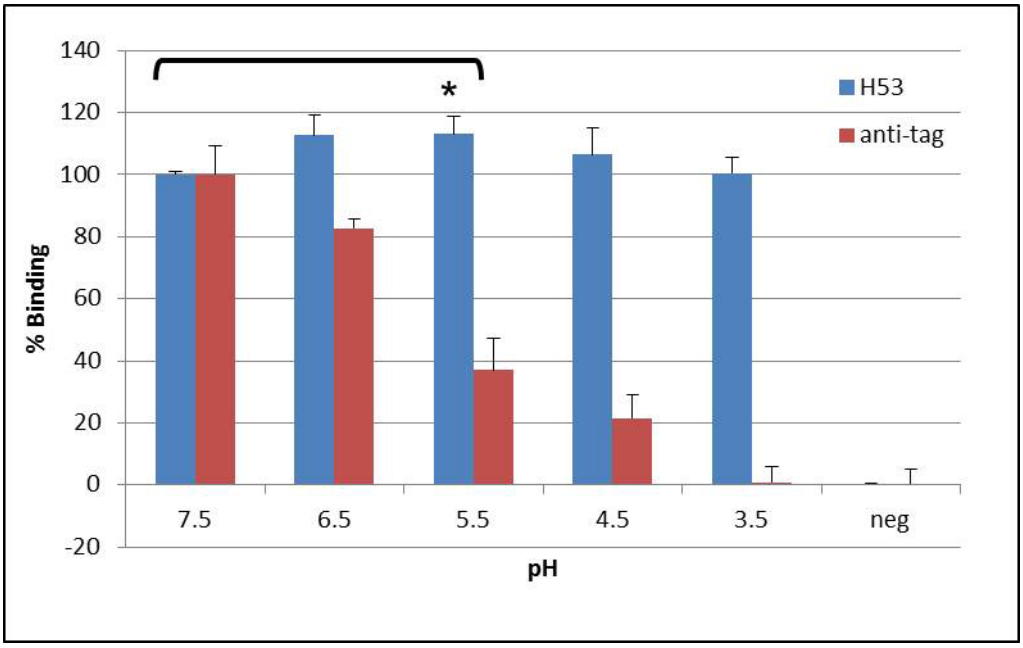

Figure 5.4 Strep-tag dependant and independent detection of soluble E2 1A20.8 over a range of pH levels.

Acidic incubations were performed in ELISA format to assess the effect of pH on E2 conformation. H53 recognises a CD81 binding site independent and conformation-dependant epitope with contact residues 540 and 550 in domain I. One way ANOVA was performed relative to neutral pH with Bonferroni post test alpha as 0.05 (*=p<0.05).

Having shown that acidification of E2 does not appear to cause significant loss of protein from the assay, the relative stability of the E2 protein in the acidic environment was tested with the conformation sensitive murine antibody H53 (see Figure 5.4). Indeed, there was no loss of binding by the antibody H53 over the various acidifications. The loss of binding shown by the anti-tag antibody indicates that there was indeed a lowering of pH and so taken together, if the antibodies used here are not subject to changes in binding activity due to acidification, no loss of conformation in E2 has been observed. Such pH related effects on antibody binding would need to be determined before a final conclusion. However, as the pH was reduced, an improvement of binding by antibody H53 occurred peaking at around pH 5.5, indicating a possible effect in binding kinetics. Unlike ALP98, H53 did not show any loss of binding at pH 3.5. So, either the implied loss of E2 by ALP98 at pH 3.5 does not affect H53 or the favourable binding kinetics demonstrated with H53 compensates for loss of E2 present at the lower pH.

5.2.2. Acid exposure induces permanent effects

To assess the conformational effects on E2 due to acid exposure, any trends observed need to be a true reflection of E2 associated changes and not an indication of factors such as effects on the probing antibody. In order to clarify this, the effect on binding kinetics was compared when E2 was acidified then neutralised prior to coating onto an ELISA plate versus acidification during the incubation with primary (probing)

antibody (see Figure 5.5). Equal amounts of protein were coated onto the plate from each set-up in which acidification was at pH 5.5 known to be crucial for HCV entry and thus most likely to induce any functionally important conformational shifts in the E2 protein.

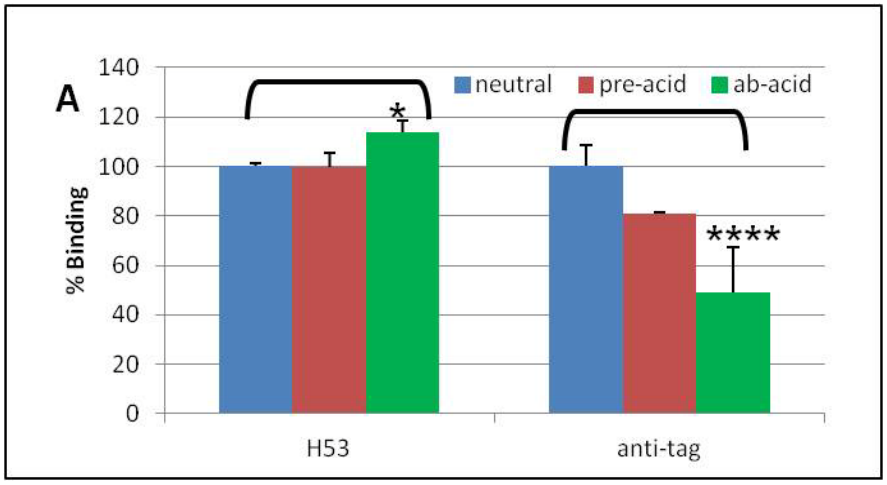

Figure 5.5 Binding of soluble E2 1A20.8 in different ELISA set-ups. E2 either remained at neutral pH throughout the assay (neutral; blue bars), acidified at pH 5.5 then neutralised prior to coating (pre-acid; red bars) or acidified to pH 5.5 during the antibody incubation (ab-acid; green bars) and detected with either the conformation sensitive antibody H53 or an antibody targeting the attached tag. Statistical significance was tested with a one way ANOVA with Bonferroni post test analysis (*, $p<0.05$, ****, $p<0.0001$).

In Figure 5.2, Figure 5.3 and Figure 5.4 there was an evident loss of binding with anti-tag specific antibody following incubation at pH 5.5 compared to no acidification/neutral conditions (pH 7.5). Figure 5.5 shows that whilst up to 50% loss of binding can be observed by the anti-tag antibody in the presence of acidic pH 5.5, only ~20% of this effect is in a permanent antibody-independent manner. The E2 itself however, may not necessarily exhibit the same effect in the two acidification set ups as the attached tag. Therefore, the conformation dependant antibody H53 was used to see if the observed effect on binding is retained when E2 is acidified prior to coating, and thus a permanent

effect on E2. Figure 5.4 shows that there was an increase in binding signal of around 15% for H53 when the antibody incubation occurred at pH 5.5. This increased binding however was not reflected with acidification prior to coating as seen in Figure 5.5, therefore indicating that change in binding of up to 15% is either due to a transient pH effect on E2 and thus the epitope, or effects on the H53 antibody and/or binding kinetics. Literature looking at the stability and biological activity of murine (Matikainen 1984; Jiskoot, Beuvery et al. 1990; Vemeer and Norde 2000) and human antibodies (Castle, Karp et al. 2002; Ejima, Tsumoto et al. 2007) has consistently shown a lack of conformational or binding effects above pH 4, requiring lower pH and long incubations of at least 16 hours or temperatures above 50^{o}C to see significant changes. Therefore, in the pH range 7.5-4.5, effects observed in the assays performed in this project can confidently be attributed to be a product of changes in E2 before the antibody (though the antibodies used will still be susceptible to effects due to the pH to an undetermined and individually varying degree). The data in Figure 5.5 indicates that a significant portion of the acid induced effects on E2 are transient as they were not fully retained following acidification prior to E2 being coated on to the ELISA plate. Therefore to test conformation effects on E2 due to acidic pH, the effect on antibody binding needs to be tested in the presence of acidic pH (when any such conformation effects are most likely to be present).Lastly, the acidification of E2 with the incubation of the probing antibody was logistically much less difficult to automate over a range of pH levels and so taken together,

acidification with antibody incubation was used as the test assay format over acidification prior to coating.

5.2.3. Antigenicity of E2 is altered by pH-induced effects in a strain specific manner

Conformational changes in HCV E2 have so far been assumed to occur during the entry process. In the absence of being able to crystallise the E2 protein, there is a lack of structural appreciation of conformational differences between its pre and post-fusion form. To try and clarify what conformational changes may occur, the antigenic profile of soluble E2 was assessed with four antibodies recognizing epitopes of a conformational and linear nature. The first two antibodies AP33 and 1:7, target epitopes focused on the first and third CD81 binding sites respectively. The second pair of antibodies, AR1A and AR3A, are broad acting and target discontinuous epitopes that overlap with two or more of the highly conserved CD81 binding sites; the former being non neutralising and the latter potently neutralising. The binding signal achieved with each antibody was plotted relative to incubation at neutral pH (Figure 5.6 & Figure 5.7).

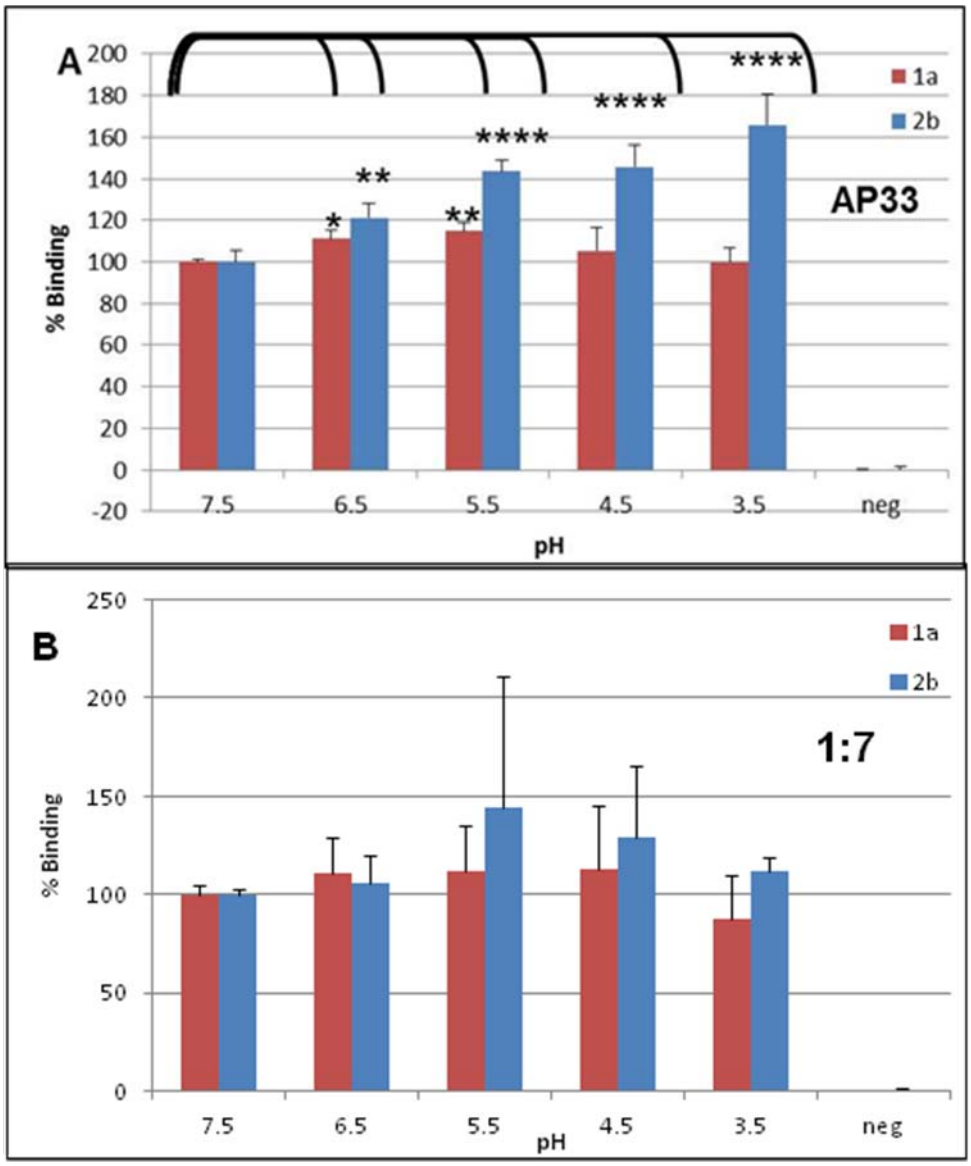

Figure 5.6 Antibody detection of E2 of two strains (1A20.8 in red bars and 2B2.8 in blue bars) over a range of pH level incubations.

A) AP33 is a neutralising antibody that has an epitope focused on the first CD81 binding site. B) 1:7 is a neutralising antibody with an epitope focused on the third CD81 binding site. Statistical testing is by one way ANOVA all relative to neutral pH 7.5 (*= p<0.05, ** P<0.01, ***= p<0.001, ****= p<0.0001).

Figure 5.6 show the epitope exposure over a pH scale from 7.5 to 3.5 for soluble E2 of genotypic strain 1A20.8 and 2B2.8 with antibodies AP33 and 1:7. What is immediately evident for AP33 is a difference in pattern between the two strains as the pH is decreased. Epitope exposure for AP33 showed a different profile for the two strains of E2: E2 of strain 1a

showed a peak epitope exposure at pH 5.5 then returning close to 100% at the lowest pH, whilst strain 2b showed an improvement in epitope exposure proportional to the increase in acidity. This has two consequences. Firstly, the antigenic state of E2 is pH-affected and secondly such trends are also strain specific. However, the epitope for 1:7 however showed no significant changes in epitope exposure over the pH range tested here.

The second pair of epitopes examined were discontinuous epitopes overlapping with multiple CD81 binding sites, but with only one of them being neutralizing; neutralising antibody AR3A and AR1A as non-neutralizing (see Figure 5.7). Given that parts of these epitopes overlap, there is some degree of competition between these two antibodies (Law, Maruyama et al. 2008), however the differences between these epitopes denote that the antibodies have differing phenotypes. The antigenic profile of E2 using these antibodies, like 1:7, did not show a significant pattern over the pH range, or a distinctly different profile from the two strains of E2 tested. However, AR1A did show a significant drop in epitope exposure at the lowest pH tested for both E2 strains.

Taken together, the data here support that acidic pH induces changes in the antigenic profile in a strain and epitope-specific manner. Given that pH 5.5 has been shown to be the optimal pH for fusion to occur in the endosome, a pH of 5.5 was taken forward as a promotive factor to

induce a fusion active state in the soluble E2 for biopanning to select fusion related antibodies.

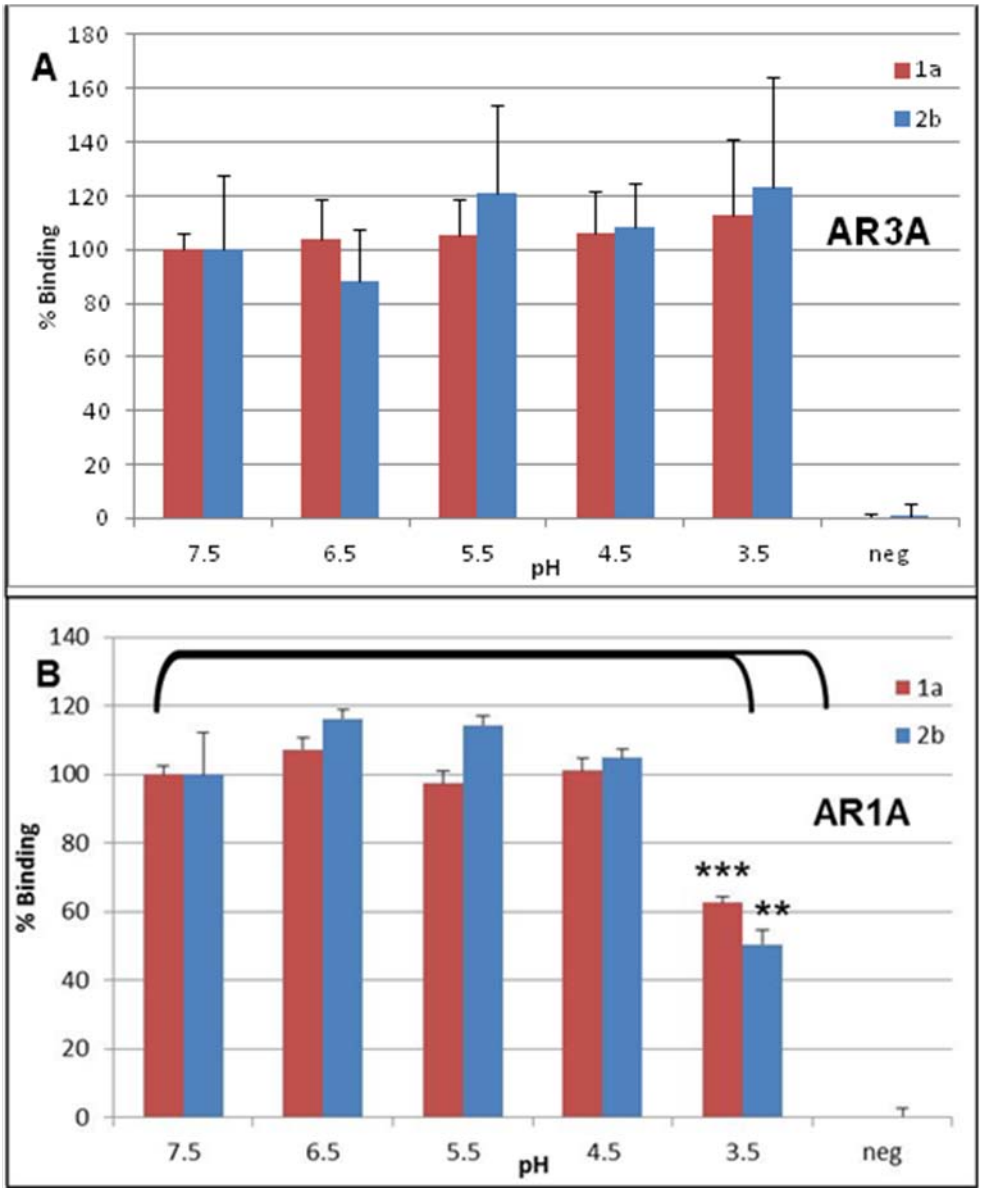

Figure 5.7 Antigenic testing over a pH range of 7.5-3.5 with antibodies recognising discontinuous epitopes.
A) Conformation-sensitive neutralising antibody AR3A, which has a discontinuous epitope spanning the central domain I and residues near the first and third CD81 binding sites. B) Non-neutralising conformation sensitive antibody AR1A has a discontinuous epitope that coincides with the first and second CD81 binding site with other contact residues close to the third. Statistical significance is from a one way ANOVA relative to neutral pH 7.5 for each strain (**= $p<0.01$, ***$p<0.001$).

5.3. Discussion

The strep tag attached to the E2 molecule consisted of 8 amino acids, one of which is a histidine. The consequence of this amino acid is that as the pH level lowered, there was protonation of the histidine which could sterically affect the ability of the anti-tag antibody to recognise its epitope. Therefore, the pH-dependant loss of binding by the anti-tag antibody over a pH range did not truly reflect a loss of absolute strep-tag associated E2 in the assay but instead indicated the degree of acidification as the more acidic the environment, the greater the proportion of histidine residues in the pool of E2-tag molecules present became protonated. Figure 5.5 shows that effects of protonation of the E2 and associated tag were only partly retained following acidification prior to coating compared to acidification in the presence of the anti-tag antibody, and thus the acid related effects observed are either partly reversible or partly a reflection of effects on the antibody itself, if not both. For the E2 conformation sensitive antibody H53, there was no apparent difference in binding between the neutral E2 control and the E2 acidified prior to coating E2. This indicates that either any E2 effects due to acidification were reversible, or any changes did not affect H53 binding its epitope. When acidification occurred in the presence of the antibody, however, a 13% difference was observed therefore indicating that up to 13% of binding is affected by transient changes in epitope or that an acidic environment affects binding kinetics by up to 13%. Figure 5.5 shows that for the anti-tag antibody, a minimum of 12% difference

in binding could be attributed to permanent changes in its epitope on E2. However, given the differences in individual antibody-epitope binding in response to pH effects, a universal standard is not appropriate for the panel of antibodies.

The question of monoclonal antibody stability in pH varying environments has been addressed previously with a consistent outcome. Preliminary studies by Matikainens group showed that in ELISA format, murine antibodies retained significant binding even after incubation at pH 4-9 for over 16 hours (Matikainen 1984). Further to this a multitude of assays were used to assess antibody activity and stability following 32 days incubation at pH3-10 followed by neutralisation (Jiskoot, Beuvery et al. 1990). Collectively, ELISA, flow cytometry, gel permeation chromatography, fluorescence polarisation and quenching, IEF and western blot analysis showed only loss of antibody activity from incubation at the extreme pHs. Fluorescence spectroscopy, ultraviolet CD, birefringence, and calorimetric methods showed significant changes in conformation of murine monoclonal antibodies only at pH below 3.5 with temperatures above 50^{o}C (Jiskoot, Beuvery et al. 1990; Vemeer and Norde 2000). This data from murine antibody analysis suggests that pH effects observed in this project are likely due to E2 epitope related changes, however differences between individual antibodies and species differences cannot be ignored. Indeed, one analysis of human monoclonal antibodies showed no change in stability or binding following incubations at pH 4 or above (Castle, Karp et al.

2002). Only after 24 hours of incubation at pH 3.5 was blocking activity affected or antigen binding seen to be reduced by more than 40%. Circular dichroism (CD), differential scanning calorimetry (DSC), and sedimentation velocity have also been used to assess stability and conformation of human monoclonal antibodies exposed to pH 2.7-3.9 as can occur during purification process (Ejima, Tsumoto et al. 2007). Overall no gross conformation changes were observed, and following 24 hours of incubation at acidic pH in 4 ^{o}C near UV CD-spectra showed the acid conformation was stable. Taken together, the literature is consistent with the view that antibodies undergo limited conformational change and so support pH effects observed here to be attributable to changes in the E2 epitopes.

The entry process of flaviviruses such as dengue virus and West Nile virus are known to include conformational changes in the envelope proteins that facilitate fusion. The data presented here show that E2 firstly remains stable and secondly exhibits changes in epitope exposure in a locally restricted manner as an effect of exposure to an acidic environment. Therefore, as related virus envelope proteins expose conserved sites specifically during the entry-fusion process, HCV E2 is also likely to do so in response to a lowering of pH in the endosome or linked with other factors/aspects of the entry. The proposed fusion peptide of HCV E2 is located in domain II close to the second CD81 binding site (Krey, d'Alayer et al. 2010). The only antibody tested here with an epitope involving residues in this region was AR1A which only

showed an effect at the lowest pH where upon residues other than histidine can become protonated. Continuing this analysis with antibodies that target epitopes in the vicinity of the fusion loop would give a better indication of how the locality of the fusion loop may change its exposure during the entry process.

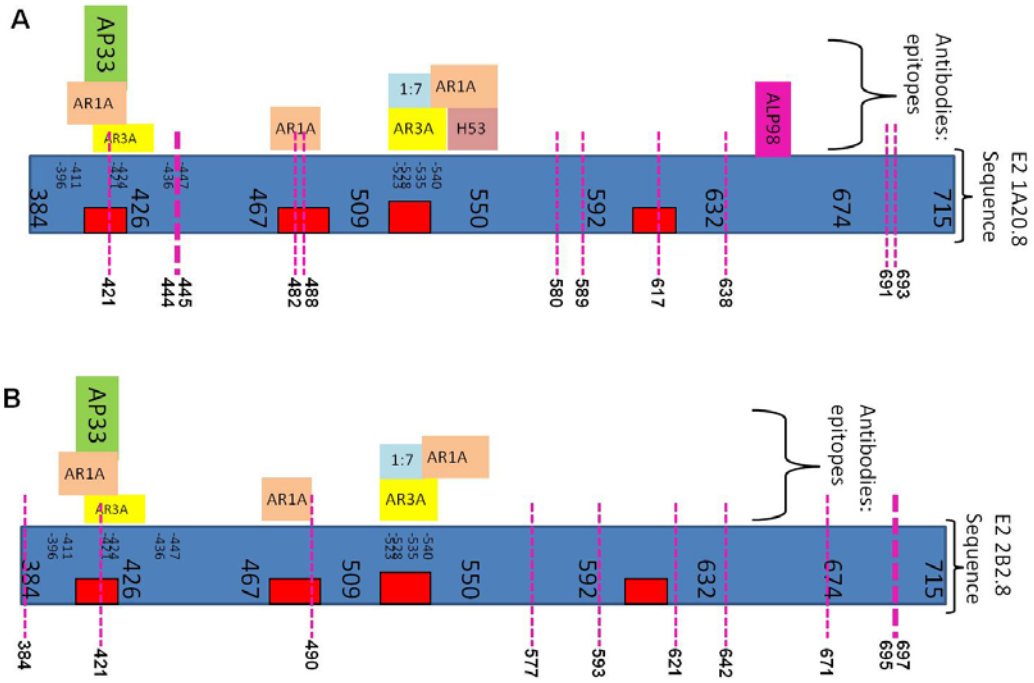

Figure 5.8 Schematic representation of the location of histidine residues in the linear E2 form for E2 strains 1A 20.8 (A) and 2B2.8 (B).

Red boxes mark the location of the four putative CD81 binding sites, purple dashed lines the histidine residues and the coloured boxes the active epitopes for each strain.

Figure 5.8 shows the relative positioning of all histidine residues, CD81 binding sites and epitopes examined in this study. Between pH 7.5 and 4.5, the only amino acid that would become protonated in this range is histidine and only the AP33 epitope for strain 2B2.8 was significantly acid affected in this pH range. There are multiple examples of histidine protonation causing low pH associated structural changes (Lazar,

Marshall et al. 2003; Nordlund 2003), even within the endosome and so this event is thought to play a critical role for viral fusion (Chen, Lee et al. 1998; Bressanali, Stiasny et al. 2004; Da Poian, Carneiro et al. 2005; Roussel, Lescar et al. 2006). A difference in the location of the histidine residues between the two strains of E2 is that a histidine at residue 384 in strain 2B2.8 is not present in strain 1A20.8. According to the proposed tertiary structure of E2 (Krey, d'Alayer et al. 2010), residue 384 is in closer proximity to the AP33 epitope than the two histidines of strain 1A20.8 at residues 444 and 445, which are buried deep in the centre of domain I. Taken together, it is possible that the acid related effect of the AP33 epitope exposure in strain 2B2.8 is a secondary consequence of the protonation of the histidine at residue 384. To further investigate this, specific mutagenesis of this residue to another polar amino acid such as glutamic acid or aspartic acid and also to a non-polar amino acid such as glycine to repeat the antigenic testing would elucidate how protonation of specific residues affects exposure of certain epitopes.

Genotypic differences in HCV E2 function have been observed elsewhere (Prentoe, Jensen et al. 2011; Tarr, Urbanowicz et al. 2011). Likewise, as an assessment of function, binding to CD81 protein also varied over the various genotypic strains looked at (Albecka, Montserret et al. 2011). Taken together, the inter-strain difference in binding profile observed here is thus likely to be a real effect and not solely an artefact of the assay.

6. A phage display selection method using a novel target

6.1. Preface

Biopanning is an *in vitro* display technology for isolating novel ligands for a given peptide. Such a ligand may be in the form of an antibody or conversely in the form of an epitope that a given antibody recognizes. Phage display is the most popular biopanning format and has indeed been used to isolate the main broad acting neutralizing antibodies targeting HCV E2 known to date. Various vectors exist to create libraries for use in biopanning; though all the E2-antibodies derived from phage display mentioned here came from libraries of the same vector (Johansson, Voisset et al. 2007; Law, Maruyama et al. 2008; Perotti, Mancini et al. 2008; Mancini, Diotti et al. 2009; Giang, Dorner et al. 2012). In 1991 a new phagemid system was published that allowed the creation of combinatorial Fab libraries on the surface of the M13 phage particle (Barbas, Kang et al. 1991). Monovalent display of the Fab molecules fused to pIII capsid protein along with the use of VCSM13 helper phage is the basis of the type 3+3 library formats that have generated anti-E2 antibodies. In this vector, the light chain and heavy chain are produced separately and come together associated to the protein product of gene III to which the heavy chain is fused (see Figure 6.1 and Figure 6.2)

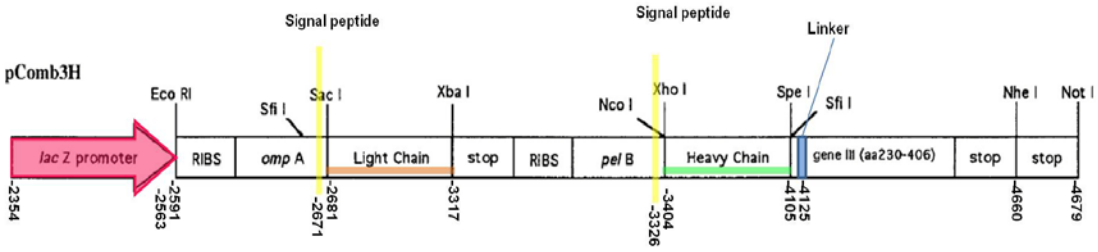

Figure 6.1 Linear sequence format of the pComb 3H vector with gene inserts for Fab combinatorial library of 3+3 format as used here and elsewhere (Barbas and Wagner 1995).

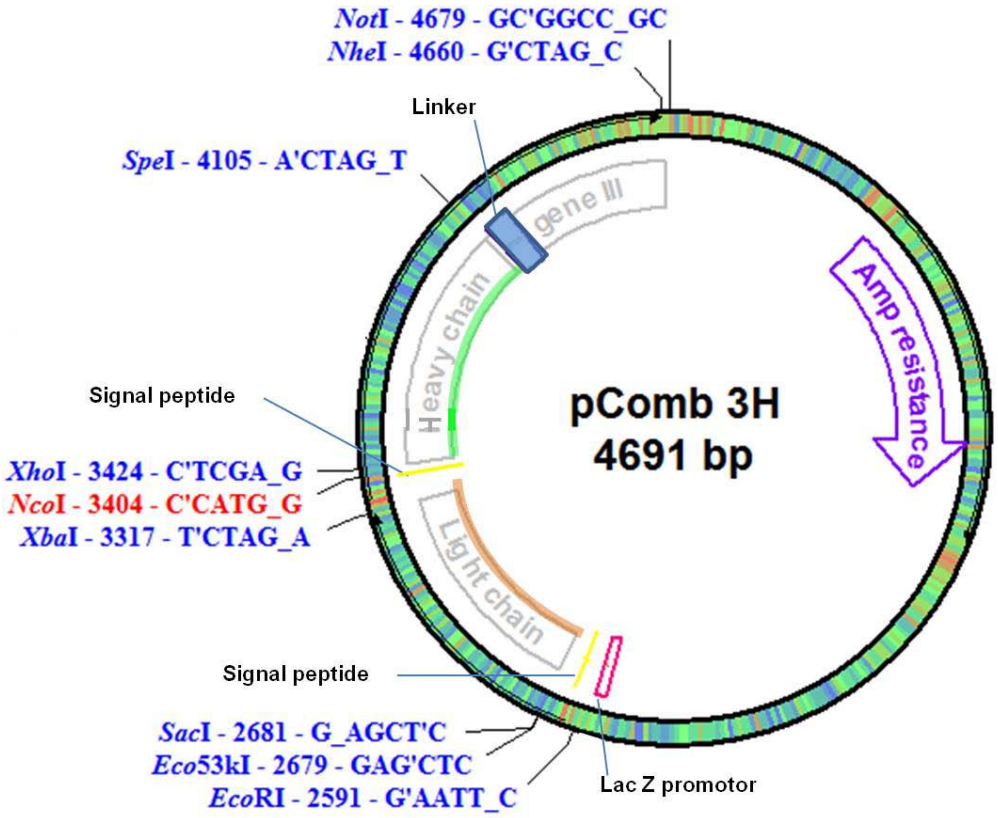

Figure 6.2 pComb 3H phagemid vector layout.
Depicted are gene inserts, restriction sites, leader sequences, promoter and linker (fusing the heavy chain to the gene III protein product) as in Figure 6.1.

In recent years novel antibodies have been identified targeting envelope proteins of related and similar viruses to HCV. West Nile virus is related to HCV and has envelope proteins that undergo conformational changes during the entry process exposing key epitopes that can be targeted to block fusion and completion of the entry process (Thompson 2009). Similarly, antibodies that target cryptic epitopes found on envelope proteins only exposed after the initiation of the entry process have been isolated for HIV (Zwick, Labrijn et al. 2001). As discussed earlier, should HCV E2 also undergo conformational changes during entry, there is the potential to uncover novel epitopes that can be targeted as an antiviral strategy. Earlier discussion has elaborated the current data on E2 and the evidence for conformational responses to factors such as pH. In section 5 we saw that the exposure of conserved epitopes that overlap with CD81 binding sites was positively affected by an acidic environment. Indeed the CD81 binding sites are highly conserved and most importantly, the first and third site looked at most closely are centrally located in the E2 protein according to the current model (Krey, d'Alayer et al. 2010). Therefore, the evidence so far suggests that in a fashion similar to viral breathing described elsewhere (Dowd 2009), HCV E2 also shows temporally improved exposure of central and conserved regions, however, in specific response to prolonged pH exposure. Therefore, novel epitopes might also be exposed with this antigenic effect. To increase the chances of targeting a novel epitope in the biopanning format, conditions to induce the exposure of such epitopes need to be defined. Given the data, here and elsewhere,

acidification of E2 was taken as a prerequisite condition of the antigen target for the biopanning process. Also, to improve the likelihood of identifying a novel epitope that is not dependent on the CD81 binding sites seen so far to dominate the E2 broadly neutralising epitopes, the D535A E2 was chosen to be the antigen target.

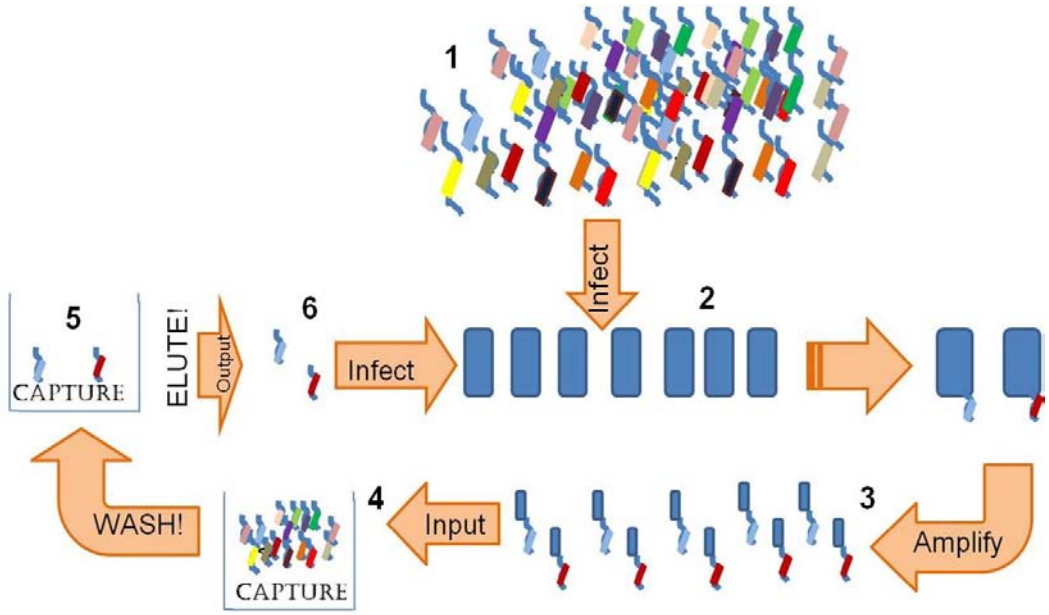

Figure 6.3 Illustration of the selection process as a cycle of steps. The starting point 1 pre-made library of phage particles each expressing a different Fab clone associated with the gene III product. The selection cycle begins with infection of a bacterial culture such as XL1-blue by the bacteriophage and amplification by growth of the phage producing-bacteria depicted as stage 3. Phage particles are isolated from the bacterial culture to apply to the panning process in step 4-6. This may be in the form of antigen-coated ELISA wells or as magnetic beads. After incubation to allow adhesion between the antigen capture and the phage particle, wells are washed to remove the unbound phage leaving only specifically bound phage particles in step 5. Finally, the pool of specific phage is eluted in step 6 and is ready to directly infect a new bacterial culture to initiate a new round of panning.

The phage display protocol in this project included a two-hour incubation of the antigen target and the Fab- expressing phage particles. The binding event between the phage-displayed Fab molecule and the E2 target was in the acidic environment of pH 5.5. To counteract any possible secondary effects on the phage and Fab due to the acid

environment, a second group in which the E2 target was acidified prior to mixing with phage-Fab was panned at neutral pH.

6.2. Results

6.2.1. Phage infectivity is not affected by acidity

The phage display process designed in this project involved a prolonged incubation of phage in an acidic environment. To verify that this incubation would not impair function of the phage particle, infectivity of the phage with or without incubation at pH 5.5 (as used in the panning process) was compared with all other parameters matched between the two test groups for both libraries panned.

Table 6.1 Comparison of phage infectivity following test incubations. Library phage were incubated for 2-3 hours at pH 5.5 or pH 7.5 (non-treated) before infecting XL1-Blue bacteria at MOI >30 to plate on 1.5% agar plates supplemented with 50-100 µg/ml of selection antibiotic ampicillin. Titers were derived in duplicate and averages recorded. Unpaired t-test comparison of means produced non-significant p value of 0.3099 for the lambda library and 0.688 for the kappa library.

	Phage titer (cfu/ml)			
	Lambda		Kappa	
	Acidified	neutral	Acidified	Neutral
Average	2.098×10^{10}	7.635×10^{10}	2.1×10^{9}	1.8×10^{9}
P value	0.3099		0.688	

The recovered titer of bacteria infected with equimolar aliquots of phage, were found to be in the same log range for both libraries showing no significant difference according to the unpaired t-test comparison of means. The phage were thus concluded not to be functionally impaired by the acidic incubation. Any further difference to be observed between

the two test groups in the biopanning process could then be attributed to effects on the expressed Fab particles themselves.

6.2.2. Titers show restriction and enrichment

Four to five rounds of biopanning were performed in which the two test groups showed a restriction in titers followed by a further expansion, thus indicating enrichment (see Figure 6.4). Though this was much less pronounced for the kappa library panning, the recovery of titer in the final panning within the acid group still occurred. However, the neutral test group of the kappa library panning had a titer reduced to zero at pan 3 and thus a fourth panning did not occur.

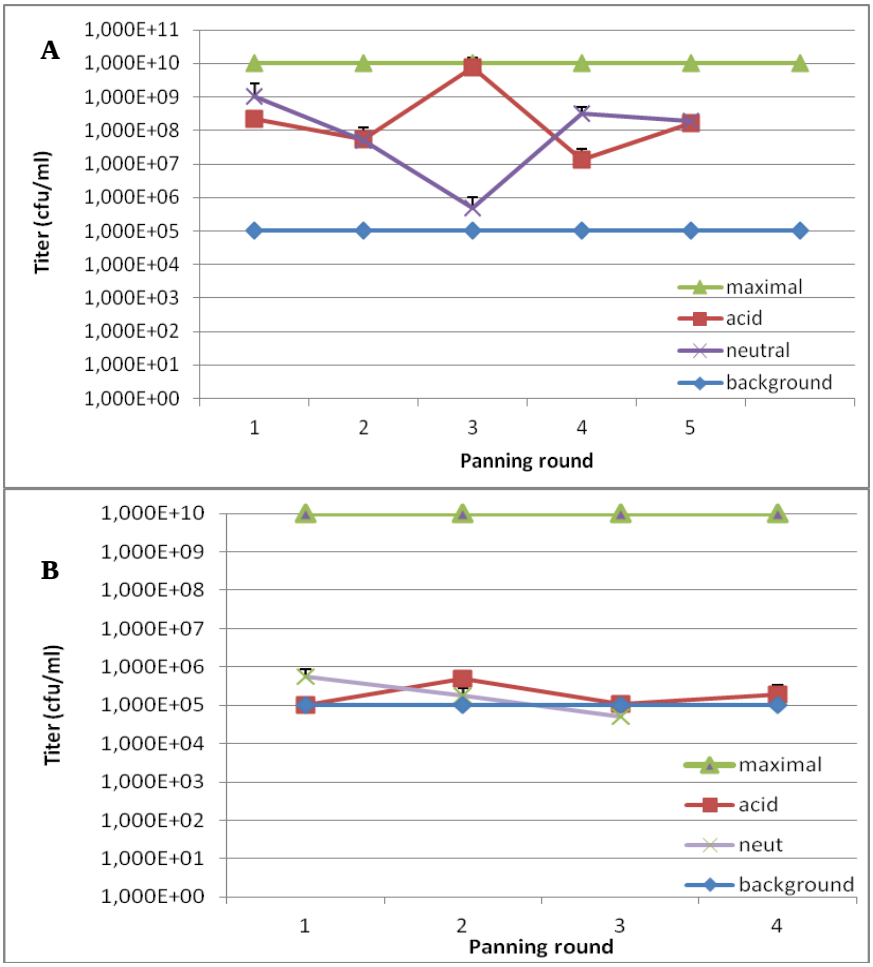

Figure 6.4 Biopanning eluate phage titers after each round of selection recorded in triplicate shown here as averages.

Five rounds of panning were performed against the lambda library (A) and four with the kappa (B), with the selection event occurring in two formats: either in the presence of pH 5.5 (acid; red line), or at neutral pH (7.5; purple bar) where the E2 target protein had been exposed to pH 5.5 for 20 minutes then neutralisation before application to the panning to distinguish permanent over temporal acid induced E2 effects.

6.2.3. Screening by cell lysate ELISA does not distinguish erroneous clones

Screening of phage eluates following the biopanning can be performed with multiple assay formats. A popular method is to use the bacterial cell lysates following overnight culture growth of the phage-producing bacteria as the source of expressed Fab in an ELISA. In this format the capture can be the same antigen target used in the panning or a molecule specific for the phage particle or Fab, such as anti-pIII or anti–fd antibody respectively. Using the antigen target as a capture allows verification of the specificity of the selected Fab, and the anti-fd antibody as a capture allows an assessment of the relative amount of Fab/phage molecules present (given that the cell lysate is a crude sample and so has a variable amount of Fab/phage molecules). With these data, the ratio between the signals derived from the different captures can be calculated to assess the relative affinities between different Fabs.

Table 6.2 Calculation of relative affinity of Fab molecules from a selection of test bacterial cell lysates.

Clone	D535A antigen (ag)		Anti fd capture (cap)		Ratio (ag/cap)
	Average (OD 620 nm)	St. Dev.	Average (OD 620 nm)	St. Dev.	
N4	0.4175	0.0276	0.4075	0.0389	1.0245
N10	0.4000	0.1414	0.4875	0.0148	0.8205
A1	0.2735	0.0134	0.2765	0.0063	0.989
A6	0.8375	0.1732	0.7450	0.0693	1.124
1:7	3.9380	0	4.0655	0.0686	0.968

A ratio greater than 1 was taken as an acceptable affinity of the Fab molecule for the antigen as the relative specific binding would be greater than the binding signal reflecting amount of phage present.

The control clone was the Fab molecule of 1:7 shown in section 4.2.3 to have minimal affinity for E2 D535A used here as the antigen target. Therefore, given the low expected affinity of 1:7 to D535A, the fact that 1:7 showed a ratio of less than 1 supported the cut-off level for the ratio of binding to distinguish non/low-binders being not too low. Four plasmid DNA test clones were picked out using this analysis: 2 from each test group (acidic and neutral incubation). One from each group was considered a possible specific binder according to the ratios calculated (see Table 6.2). To subsequently assess this method of screening, these four clones were separated on an agarose gel following digestion with Sac I to verify that they were the correct size. According to the vector sequence illustrated in Figure 6.2, there should be a single Sac I site that should produce a size of ~4.7 kb. Two of the clones, A6 and N10, showed a size difference between the digested and undigested groups indicating presence of the Sac I site. N4, however, did not appear to be digested by Sac I indicating that the phagemid is erroneous though the size could be correct at around 5 kb. However, if the phagemid was not digested, the inferred size seen in Figure 6.5 cannot be taken as true. On the other hand, clones A6 and N10 that did appear to be digested by Sac I showed a resultant size of around 3.5 kb and 4 kb respectively, both of which were far short of the expected size.

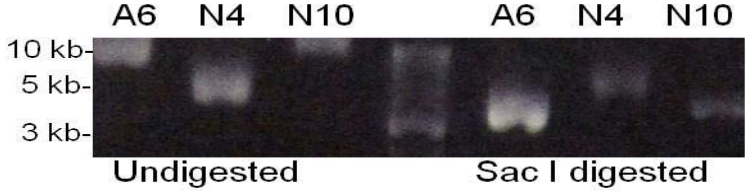

Figure 6.5 Sac I restriction digest of three phagemid clones that were selected by cell lysate ELISA binding ratio calculation.

According to Table 6.2, clones A6 and N4 were possible specific binders and yet the restriction digest showed grave errors in these phagemids. The test sample in the ELISA was the cell lysate from the bacteria in which these phagemids were expressed and therefore also contained many other proteins, malformed and otherwise. Therefore, the crudeness of the sample could affect the clarity of the assay.

A PCR-based screen was designed to test for the presence of intact phagemid clones using two sets of primers that could identify the presence of the light chain, heavy chain and the full segment of light chain and heavy chain together (see Table 2.5 and Figure 6.1). This would produce bands of around 700 base pairs for the individual chains and a band around of 1,200 base pairs for the full segment containing both chains.

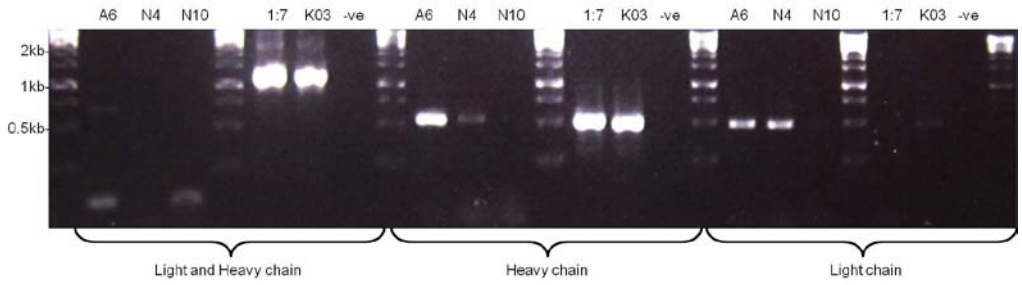

Figure 6.6 PCR screening of three phagemid clones to verify presence and correct size of gene inserts.
Heavy and light chain gene inserts were amplified for test clones were A6, N4 and N10, from the lambda library panning with positive size controls 1:7 and K03 from the kappa library.

Two control clones were used: 1:7 and a non-E2 binding clone also of the same vector named K03. The PCR screen showed that all three clones tested proved erroneous whereby they either lacked a gene insert

or showed an incorrect size for both genes together. The control clones were of kappa lineage and so for the light chain showed no amplification due to the use of a lambda-specific primer. This indicates an absence of cross-contamination that could produce false positives. Taken together, the PCR screen can discriminate erroneous clones that are not complete or deletion containing, which would make a beneficial means of initial screening before more defined analysis of specific Fab reactivity.

6.2.4. PCR screening of panning eluates

Bacterial cultures of the panning eluates, which represented a mixed population of phagemid, were applied to the PCR screen devised (see Figure 6.6). The final three pans from the lambda library panning were tested; acid group pan 3, 4 and 5 (A3, A4, and A5 respectively), along with neutral group pan 3, 4 and 5 (N3, N4, and N5 respectively). Primers specific to either the kappa or lambda light chains were used for screening this distinguishing between the two. The control clones 1:7 and K03 however, contained kappa light chains and so the PCR screen using lambda light chain primers for the test clones did not specifically amplify their light chains though these clones had been tested elsewhere with the correct primer to know that the correct pair did work and that the control clones were correct. Use of the lambda light chain primer with the kappa controls provided a control for contamination of samples thus indicating their purity. PCR screening of panning eluates from the kappa library panning was also performed using all eluates available.

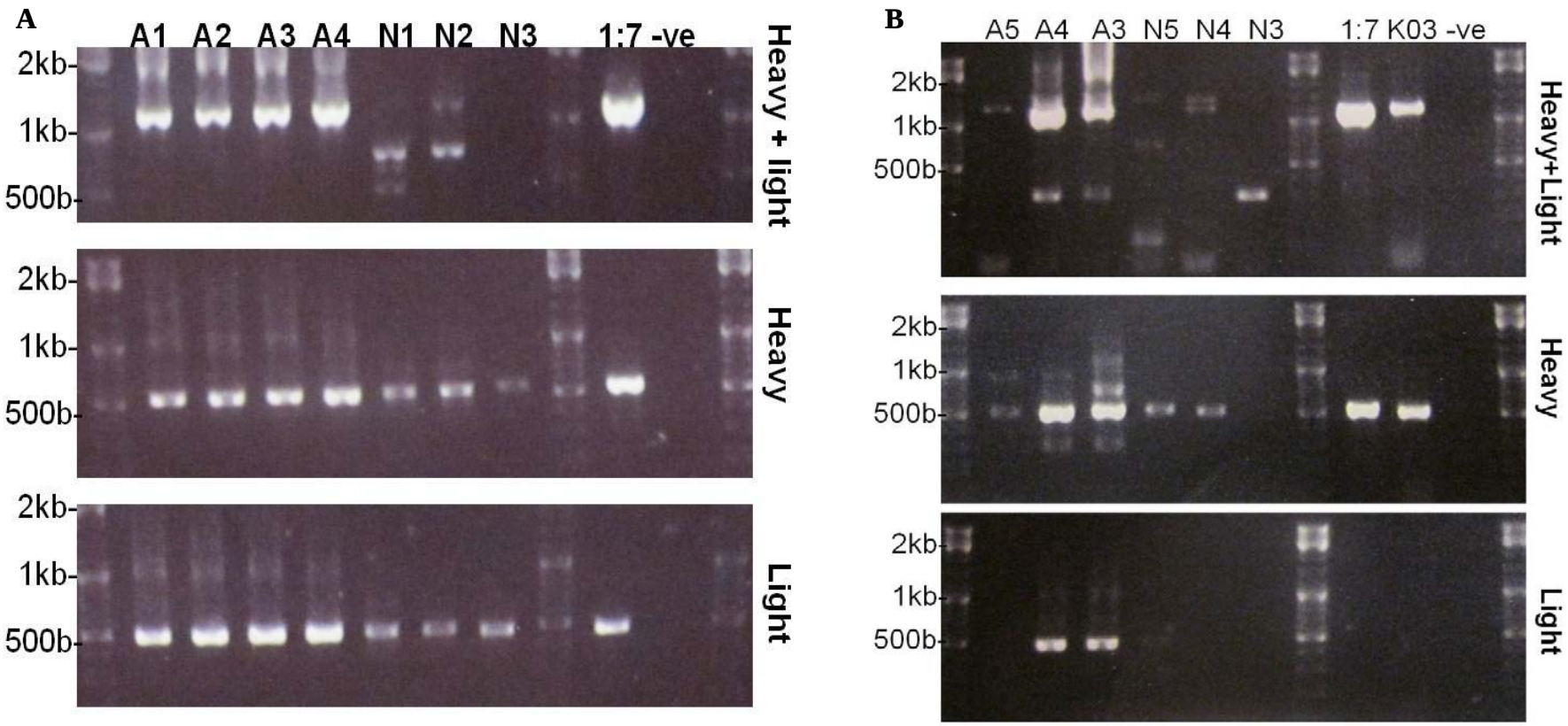

Figure 6.7 PCR screen of panning round eluates (1-5) from the Kappa (A) and Lambda (B) library, acid and neutral panning condition groups (A / N).

Amplifications of the heavy and light chain gene inserts were performed with the 1:7 (kappa) clone as a positive control for insert size.

Figure 6.7 A and B shows that the control clones 1:7 and K03 were of expected sizes for the heavy and light with heavy chain segment amplification (~600 base pairs and ~1,200 base pairs respectively), along with no contamination for the lambda light chain amplification. The eluates of the neutral pH biopanning group showed a lack of intact clones across the 3 pans analysed from both libraries as no light chains were amplified from the lambda library, along with a lack of correct heavy with light chain in both libraries. For this reason, the neutral pH panning groups were discarded from further screening. From the acid group pans of the lambda library, pan 5 lacked light chains and the band for the amplification of light with heavy chain being very faint implied a very low number of phagemid vectors containing the full correct insert. Pan 3 and 4, however, distinctly showed the correct size bands for all 3 PCR amplifications. Only the amplification of both the light and heavy chain showed surplus bands matching the size of a single chain, possibly a product of non-specific binding as it was fainter, or equally indicating the presence of a smaller proportion of vectors containing a deletion in the insert. The panning eluates A4 and A3 of the lambda panning acid group were thus taken forward as candidate samples for further screening. Presence of correct clones throughout the screen of the kappa library acid panning eluates supported the final pan (A4) to also be taken forward for further screening. Taken together, the PCR screen provides a satisfactory method of vetting a mixed population of phagemid clones for intact and correctly sized inserts of both Fab genes.

6.2.5. Evidence of enrichment of specific clones

Given the presence of incorrect clones being found after panning, enrichment of correct clones was then tested to complement the titers recorded. Figure 6.8 shows the PCR screen applied in section 6.2.3 to the lambda and kappa phage library pre-panning (pan 0) as an indication of the frequency of intact clones in the starting libraries. Thirty individual clones from each library were initially tested using the PCR screen to verify the presence of chain segments of the correct size in the phagemid vectors. From the Lambda library, only 2 of the 30 clones showed correct segments for the heavy, light and both chains together. This gave a surprisingly low rate of ~7 % intact clones in the starting library. The Kappa library showed a higher quality with 30 % intact clones for heavy, light and both genes together. To know that the biopanning resulted in enrichment of intact clones, a higher percentage of intact clones would be need to be present after panning.

Individual clones from the panning eluates of the Lambda acid group pan A3 and A4, along with the Kappa acid group A4 were also tested with the PCR screen of the heavy and light chain together (see Figure 6.9 and Figure 6.10). Lambda Pan 3 showed 70 % intact segments with varying strength of the visible band and some lanes showing surplus bands of smaller sizes. Looking only at the lanes that contained a single band of the correct size, the proportion is lowered to 25 %. However, this is still over three times that of the initial proportion found in the un-panned library shown in Figure 6.8. At pan four, however, there are

no visible bands of the correct size. Given the presence of the correct-sized band when looking at the pan eluate as a whole in Figure 6.7, the result is thus more likely to be showing that the proportion of intact clones in A4 was reduced to less than 1 in 20/ 5% and so would not be showing any enrichment of intact clones. Taken together, the acid group pan A3 showed an enrichment of intact clones and so was taken forward as the eluate to be screened for specific reactivity against the antigen target.

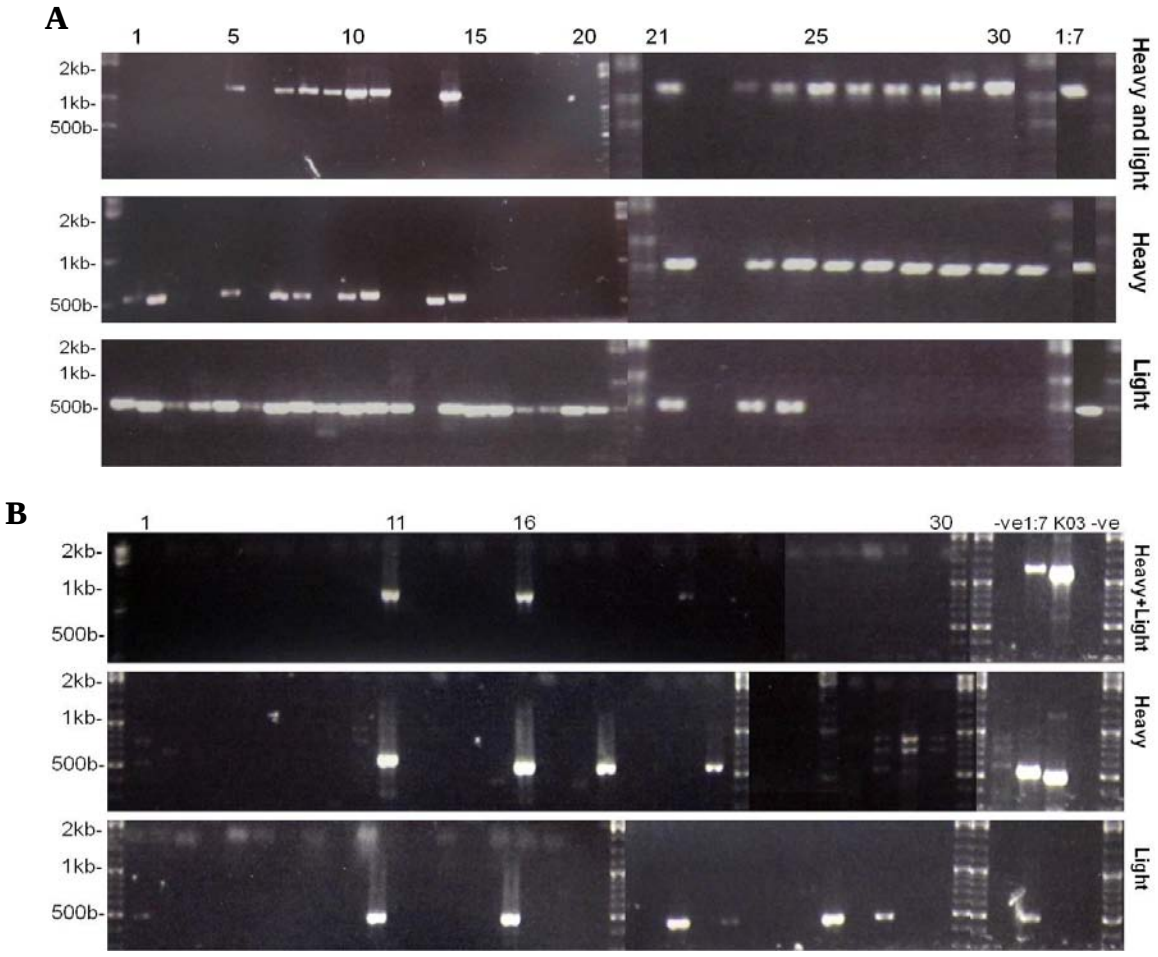

Figure 6.8 PCR screen of 30 individual phagemid clones from the Kappa (A) and Lambda (B) library phagemid pool pre-panning.
Clones 1:7 and K03 act as positive controls for insert size.

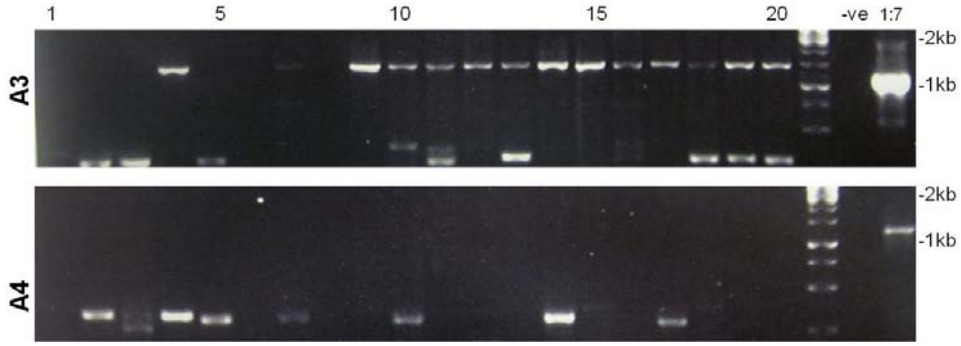

Figure 6.9 PCR screening of phagemid clones from the final two panning rounds of the lambda library.
Heavy with light chain gene inserts for 20 individual phagemid clones from pan group A3 and A4 of the Lambda library panning were compared to the positive control 1:7.

Analysis of the final panning output from the kappa library panning is shown in Figure 6.10. Out of twenty clones, 50% were shown to have intact light chain, heavy chain and both genes together of a correct size. This is a 20% improvement, which is much less than seen for the lambda library even though the initial kappa library contained a higher proportion of correct clones. Overall, however, enrichment of correct clones was evident for the acid test panning groups from both libraries.

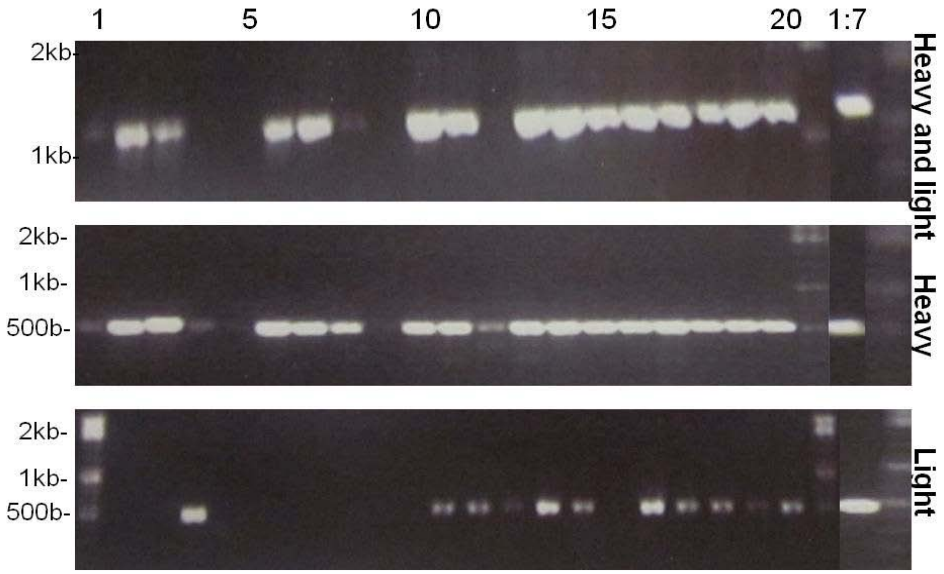

Figure 6.10 PCR screening of kappa library phagemid clones.
Amplifying the heavy, light and heavy with light chain gene segments of selected clones from panning group Acid-4, with 1:7 as positive control clone.

6.2.6. Reactivity of panning eluates by ELISA

Panning eluate purified phage were tested in ELISA to verify specific reactivity against E2. One eluate from the lambda neutral panning group was included for comparison and the Fab clone 1:7 acted as the positive control. Figure 6.11A shows that of the lambda panning, neutral panning group 5 (N5) and acid panning group 4 (A4) had significantly less specific reactivity than 1:7 clone, unlike acid panning group 3 according to a one way ANOVA. This data thus supports the decision to screen the eluate from panning 3 of the lambda acid panning group for E2-specific clones. The final panning from the Kappa library showed improved reactivity to E2 over the previous panning compared to background binding and was therefore also screened further (see Figure 6.11B).

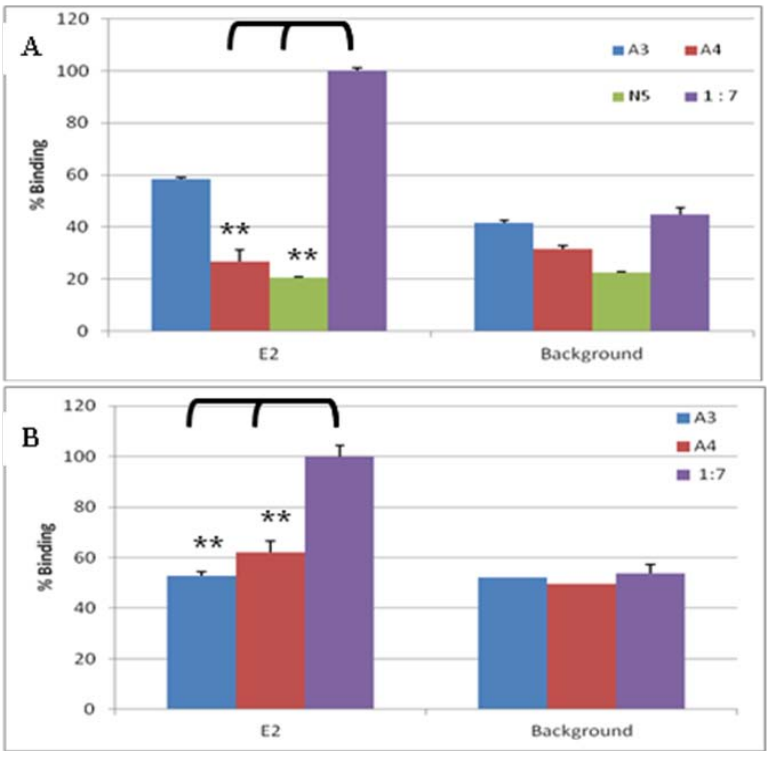

Figure 6.11 ELISA testing the reactivity of the Lambda (A) and Kappa (B) library panning eluates against E2 1A20.8.
Statistical significance was tested with a one way ANOVA with Bonferroni post test analysis (** = $p<0.01$).

6.2.7. Individual selected clones show positive reactivity against E2 in ELISA

PCR analysis of phage clones showed 7 phage clones from the lambda library panning correctly containing intact heavy and light chain gene inserts (see Figure 6.9). Reactivity was tested in ELISA format against wild-type E2 of genotype 1a against which the positive control 1:7 shows optimal binding, against D535A E2 as used in the panning process, and also against blocked wells to indicate non-specific binding. The positive control, known to lose binding ability where residue D535 is mutated, showed an expected loss of binding against D535A that matched the non-specific binding to the blocked well (See Figure 6.12). The clone K03 is a non-E2 binding clone and so indicates non-specific binding. Test clones 9, 12, 14 and 15 showed significant differences binding to wild-type E2 compared to the non-specific binding to the blocked wells and D535A E2. Along with the crudeness for the assay possibly masking the full extent of such relationships, these data were taken as indicative of potential for greater reactivity in the form of IgG and therefore, it was concluded that all clones analysed here were possible candidates for subcloning into a mammalian expression system to be produced as IgG for further reactivity and specificity testing.

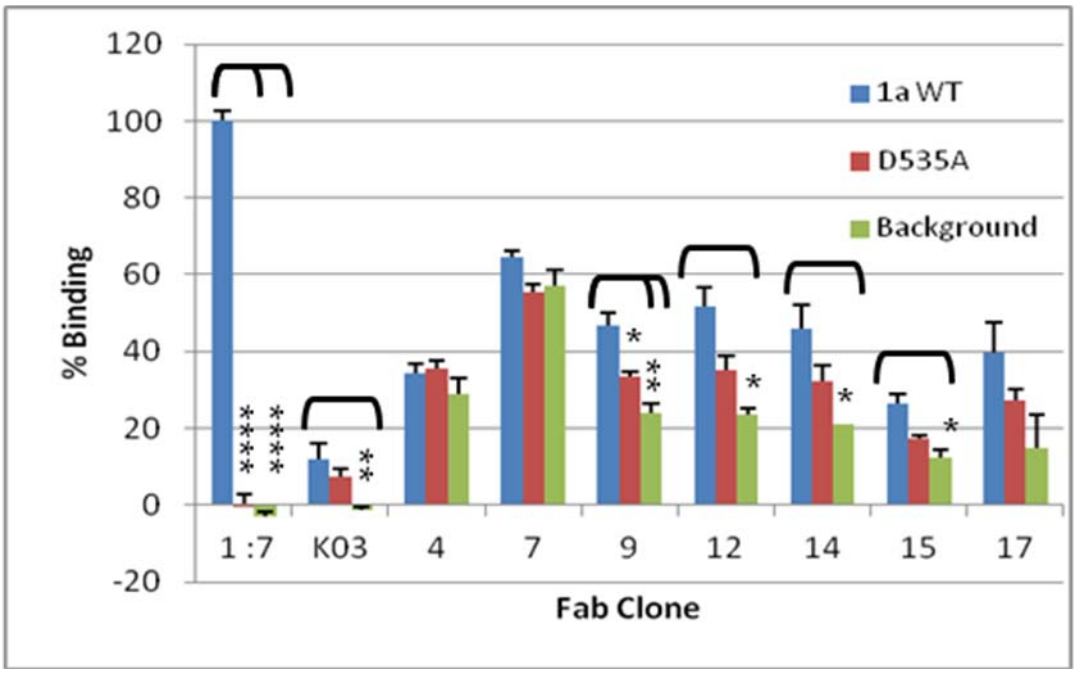

Figure 6.12 Reactivity of selected phage Lambda clones to E2 wild-type protein or D535A target protein in ELISA.
1:7 binding to wild-type E2 provided the positive control set at 100% with blocked wells indicating non-specific binding marking the baseline 0%. All other data are plotted relative to 1:7. Statistical significance was tested with a one way ANOVA with Bonferroni post test analysis (*,=p<0.05, **=p<0.01, ***=p<0.0001, ****= p<0.0001)

Figure 6.13 shows the ELISA testing of the kappa library panning clones. The positive control 1:7 in this assay showed a significantly higher binding to the wild type E2 than to the panning target D535A, though binding to D535A was still much higher than the non-specific binding observed by the negative wells. Inter-assay variability was thus taken to be a large factor in the difference in binding pattern observed by the 1:7 control clone in Figure 6.13 and Figure 6.12. However, looking at the test clones from the kappa group, an increased level of binding to the target E2 protein can be seen over the wild-type E2 protein, indicating preferential binding for a majority of the clones tested. A high degree of variability can be seen in these data by the large error bars

although binding to both E2 proteins was consistently greater than to the negative wells with some tests reaching statistical significance. Taken together, no kappa clones analysed were discounted from being possible candidates for specific reactivity in the IgG format.

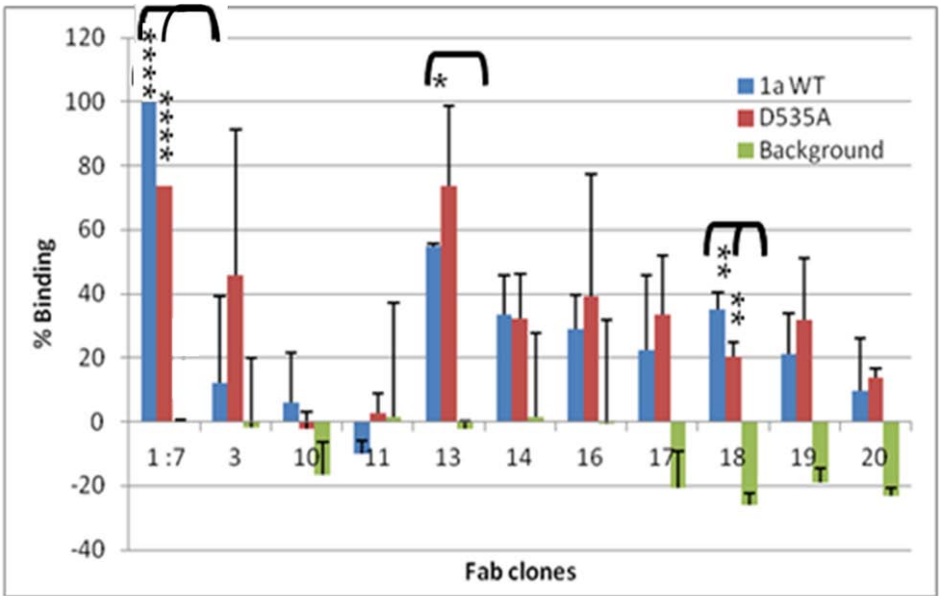

Figure 6.13 Reactivity of selected phage kappa clones to E2 wild-type protein or D535A target protein in ELISA.
Data calculated and plotted relative 1:7 as in Figure 6.12.

6.2.8. Selected clones show preferential binding at acidic pH in ELISA

Section 6.2.7 showed that the selected phage from both library pannings contained significant reactivity to the E2 molecule; wild type for 4 of the lambda clones and/or the D535A target used in the biopanning process for kappa 2 of the clones. Binding in acid or neutral pH was compared in order to clarify how much of this mixed reactivity was dependent on the acidic environment. Firstly, the binding of the mixed population phage panning eluates were tested. Figure 6.14 shows the lambda

library eluates with a preferential binding to the target E2 D535A in neutral pH over acidic with significance for pan group A4, whereas the panning eluates from the kappa library panning showed a preferential binding to the target protein in acidic pH for both eluates tested, also significantly for the second pan group tested (see Figure 6.14). However, a mixed population could mask the binding activity of the minority of preferred clones and so the final clones from each library panning experiment were also tested for acid-dependent binding.

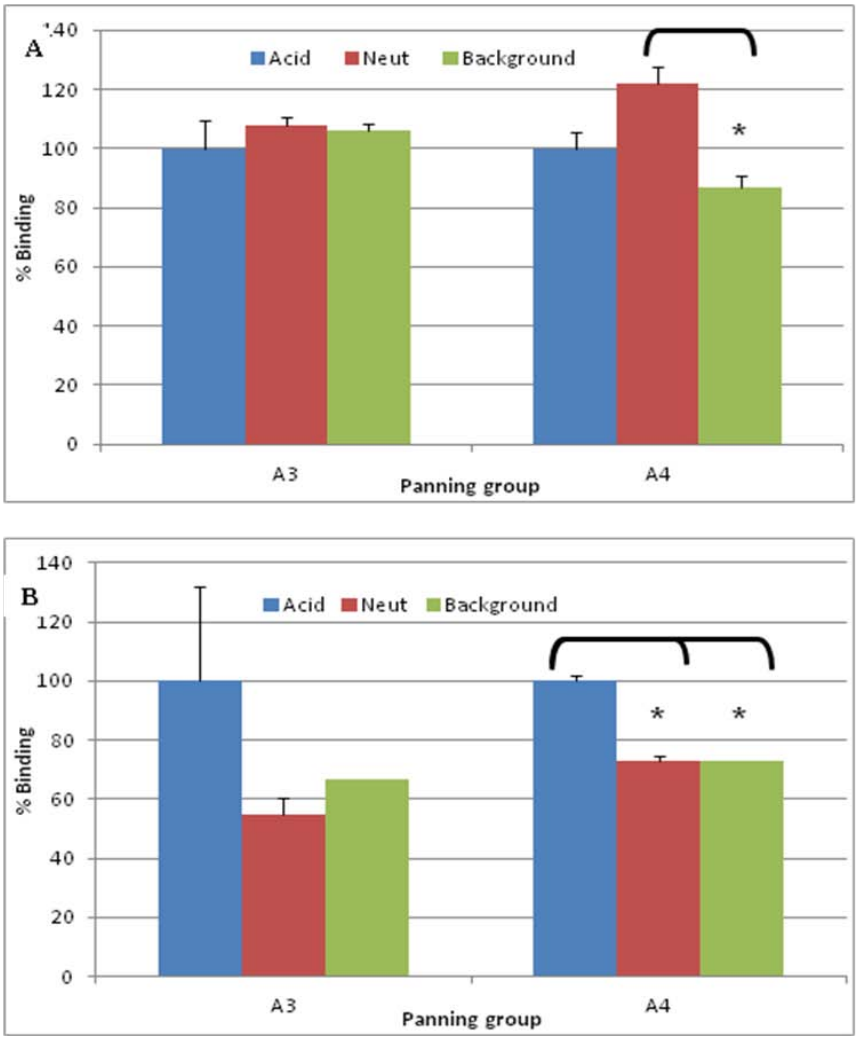

Figure 6.14 Reactivity of the final two panning eluates to the target protein D535A E2 in acidic and neutral binding environment for the lambda (A) and kappa (B) library.
For each panning eluate, binding in acidic conditions as per the panning process was set at 100%. Statistical testing with one way ANOVA (*=p<0.05)

Figure 6.15 shows the relative binding of the final clones from each library panning to the target D535A E2 protein in acidic pH 5.5 as used in the biopanning set up (set as 100% for each clone), compared to neutral pH 7.5 or non-specific binding to blocked ELISA wells. The lambda clones showed consistently preferred binding to E2 in an acidic environment of pH 5.5 although the high variability of the data limits the scope of this effect. The kappa library clones also showed variability in binding though a preferential binding to acidic E2 could be determined for a a majority of the panel. Taken together, though low reactivity and high variability clouded a pattern in binding of the phage-expressed Fab clones to D535A E2 in the different pH environments, there was still evidence of preferential binding at acidic pH. This therefore indicated that the biopanning set up adopted in this project enabled enrichment of Fab clones with improved binding at acidic pH. Time permitting, subcloning of these clones into expression vectors to be produced in an IgG format for further testing would be a great addition to the validation of this project.

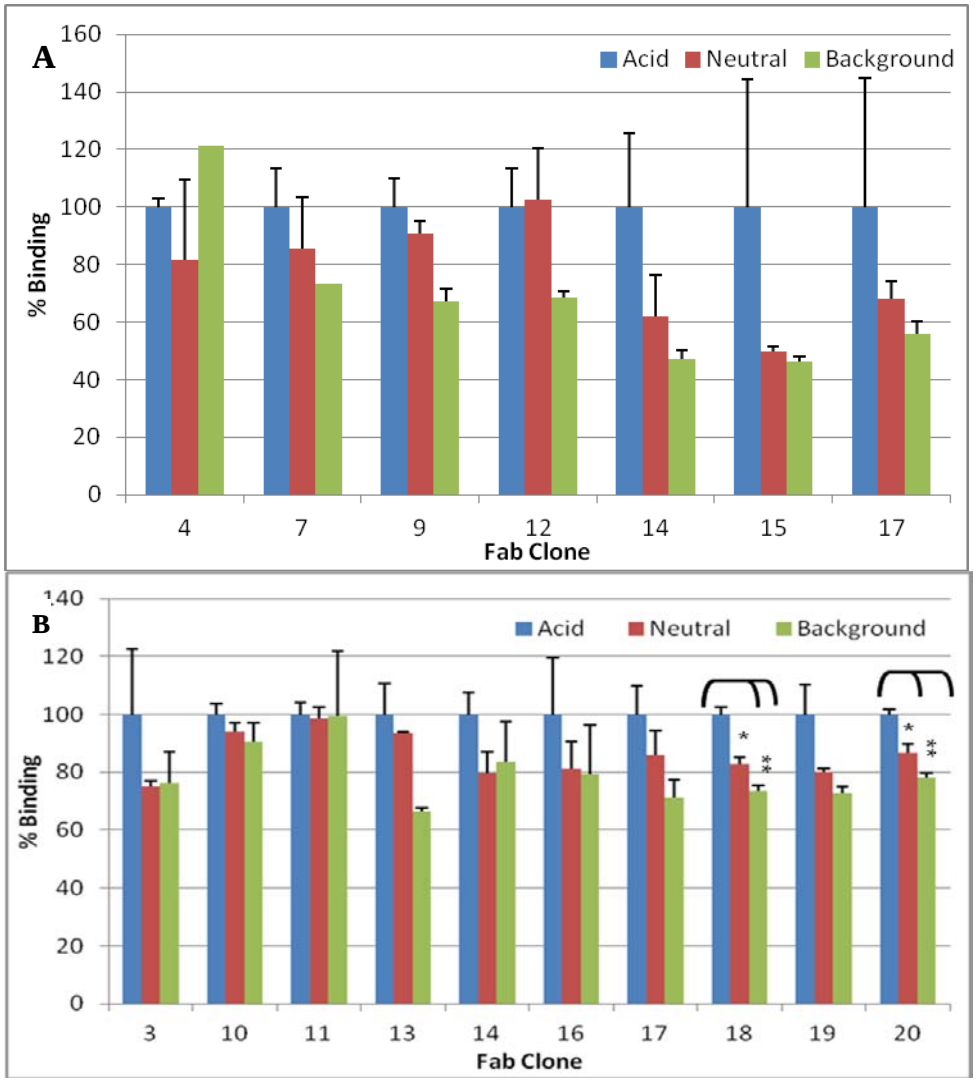

Figure 6.15 Reactivity of selected phage clones to the target E2 protein D535A in acidic condition or neutral pH:
A-lambda library clones and B-kappa library clones, where acidic binding represents 100% for each clone. Statistical testing was performed with one way ANOVA (*=p<0.05, **=p<0.01).

6.2.9. Sequence analysis of the selected paratopes

Sequence analysis can be used to verify the diversity of a given phage display antibody pool or as a means of selection (Spear, Breakfield et al. 2001; Wang, Zhang et al. 2004; Ballard, Holm et al. 2006; Cheng, Zhou et al. 2009). As the libraries had previously been panned using another form of the E2 target protein (Allander 2000), the sequences derived here and previously were compared in Table 6.4. Likewise, given that

the aim of the protocol used in this project was to isolate Fab clones with preferential binding in the acidic environment, a greater degree of hydrophobicity in the paratope could be expected and parameters of hydrophobicity were therefore described (see Table 6.3). Unlike the germline encoded and cross reactive 1st and 2nd complementarity region (CDR), CDR3 is considered to be the key determinent of specificity in the B cell receptor and therefore antibody derived antigen recognition. In fact the CDR3 of the variable heavy chain is sufficient to allow otherwise identical IgM molecules to identify one antigen from another (Xu and Davis 2000). Analysis of the sequences of the CDR3 of a sample of the selected clones and those previously identified (Allander 2000) showed no immediate difference in isoelectric points or net charge at pH 7 was observed as the ranges of the parameters overlapped for all clones.

Table 6.3 Hydrophobicity parameters for a selection of phage clones. Clones Kappa 11-18, & 3, and Lambda 4, 12 were isolated from the current project whereas L1, 1:7 and A8 are clones previously isolated and described by (Allander 2000).

Fab heavy CDR3	Isoelectric point (pH)	Net charge at pH 7
Kappa 11-18	3.1	-1
Kappa 3	9.43	1
Lambda 4, 12	7.65	0.1
L1	4.26	-1
1:7	7.65	0.1
A8	3.88	-1

Table 6.4 Sequence complementarity of clones derived from phage display panning of lambda and kappa libraries.

Fab clone			CDR3 sequence		Proportion hydrophobic residues
			Light chain	Heavy chain	
Lambda	Incorrect clones	P	QSFDSNNLV	-	
		Q	NSRDTSGNNNYV	DGPEENMLIPAAIRYYFDS	
		W	QSYDISLSGNV	DGPEENMLIPAAIRYYFDS	
	New panning	4	QSYDRNFGGWV	VVIPNAIRHTMGYYFDY	0.53
		7	QSYDRNFGGWV	-	
		12	QSYDRNFGGWV	VVIPNAIRHTMGYYFDY	
		14	QSYDRNFGGWV	-	
		15	-	DASMWFAP	
Kappa	Allander 2000	L1	QQYGSPPYT	EVLFGSIKGRYYLEN	0.38
		A8	QQSYTTPRT	SPIKMVQGMMLDAFDI	
		1:7	QQRSDWVT	VVIPNAIRHTMGYYFDY	
		1:11	QQYGSSPRT	KDPPRFCSGGNCYPGFFQQ	
		L3	QHYSTWPLT	TEGSPFGSIKGRYLEN	
		A12	QQYGTPRT	MPYPKHCSRGSCWGWFDP	
		1:5	QLYGNSRWT	RDPPRYCSAGRCYPGF	
	New panning	3	HHYVSSSRT	VRVGRLLVRGYYDYFMDV	0.47
		11	QQYFASPRT	ATGWMLDS	
		13	QQYFASPRT	ATGWMLDS	
		18	QQYFASPRT	ATGWMLDS	

Sequencing of the phagemid clones showed a low level of diversity (see Table 6.4). Dominant clones were seen among the kappa and lambda sequences, alongside some clones for which full sequences proved difficult to attain. This may be due to the quality of the samples, loss of primer binding sites or sub-optimal amplification parameters for the type of sample used (e.g. bacterial culture or colony, or purified DNA). Table 6.4 shows the proportion of hydrophobic residues present in the heavy chain CDR3 sequence among the different sequences presented. For the newly panned groups this variable is close to or above 0.5 meaning there is an average of 50% hydrophobic residues in these sequences. Among the heavy chain CDR3 sequences from the previously panned group, this rate drops to 0.38 which indicates that less than 40% of these sequences are hydrophobic residues. It is possible that whilst the hydrophobicity of the selected CDR3 regions may not differ from the sequences derived without using acidic conditions, the propensity to have hydrophobic residues in the CDR3 regions in the newly selected clones may be an effect of panning in acidic conditions. However, hydrophobic residues have been shown to be pivotal for the binding affinity and structural integrity of antibody paratopes where tyrosine plays a unique role in antigen recognition (Fellouse 2006).

6.3. Discussion

One of the conditions used in the biopanning protocol adopted in this project involved a prolonged incubation of Fab-expressing phage and E2 protein in an acidic pH. In section 5.2.1, it was shown that the HCV-fusion pH of 5.5 did not destabilise the E2 protein. However, the phage particle and the expressed Fab were also exposed to acidity and so equally at risk of acid-induced effects. Analysis of a group of structural environmental bacteriophage (actinophage) showed that in acidic habitats, a minimum of phage could be isolated from conditions below pH 6, whereas the same host species and phage from neutral pH environments contained high levels of natural phage (Sykes and Williams 1981). A more detailed analysis, however, showed that infectivity and adsorption were not hindered at acidic pH but that it was replication that was greatly reduced. Whilst there is a modest amount of literature detailing the activity and stability of phage proteins/enzymes at various pH levels, there is a limited emphasis on the stability/life cycle of the phage particle. The exposure of the phage particle to acidic pH in this project thus relies on the ability of the phage to remain infective following acidic incubation. Table 6.1 shows no difference in phage titer derived from infecting bacteria following incubation at acidic or neutral pH, and thus the phage in this project were not deemed functionally compromised by the conditions used.

Another possible consequence of this biopanning set up was acid-induced effects on the surface-expressed Fab molecule. Section 5 showed acid-related effects in antibody-antigen binding and so from this data, such effects could equally be present in the panning process. Indeed, full human monoclonal antibodies have previously shown improved antigen binding ability at acidic pH, sometimes despite there being almost no binding activity observed at neutral pH (Kumar and Saxena 1992; Raghavan, Bonagura et al. 1995; Lopez, Trevani et al. 1999). Analysing the stability and function of human IgG showed that an exposure to pH 4 or lower for more than 24 hours was necessary for any effects to occur (Castle, Karp et al. 2002). The Fab molecule, however, is likely to be less stable than a full IgG molecule. The fusion-active Fab identified to neutralise West Nile Virus (Thompson 2009) was shown to be functionally indifferent to the pH it was exposed to, thus supporting the notion that the acidic conditions imposed in the biopanning procedure in this project would not hinder the ability of the phage-expressed Fab to bind to the antigen.

Initial screening of the phage clones was performed using bacterial cell lysates as described elsewhere (Barbas and Wagner 1995). This approach, however, did not distinguish clones that contained deletions as illustrated in Table 6.2/Figure 6.5. A PCR-based screening of the library and/or the clones derived has been used elsewhere either to verify the quality of the enrichment after the biopanning process or to screen clones based on gene size before testing for specificity (Spear,

Breakfield et al. 2001; Gnanasekar, Rao et al. 2004; Wang, Zhang et al. 2004; Ballard, Holm et al. 2006; Cheng, Zhou et al. 2009). Therefore, the same approach was adopted here to assess phage clones containing the correct sized genes before testing reactivity. Figure 6.8, Figure 6.9 and Figure 6.10 together show that enrichment of clones containing the correct sized inserts occurred between the initial libraries and the final panning groups analysed. The proportion of correct clones found in both libraries was low, however enrichment was observed for both panning experiments. The maximum proportion of correct clones after panning was estimated to be up to 70%. Ideally, the final proportion would be above 90% and so the panning experiments performed could be deemed suboptimal. Factors affecting this low rate could include the low proportion in the original library and low stringency during the panning process. The proportion of correct clones in the starting library is a product of the efficiency of the sub-cloning performed in the creation of the library and so can be highly variable. Likewise, the stringency adopted during the panning process is a product of human input and cannot easily be assessed in a quantifiable manner, especially as we were applying a novel panning protocol (acidic condition). Taken together, the biopanning experiments analysed here showed enrichment and selection of specific clones, though in future, the biopanning protocol used in this project could benefit from optimisation.

Sequence analysis of the final clones showed repeated CDR3 sequences indicating related clones originating from the same B cell. Differences in

the binding behaviour could therefore be attributed to elements outside of the region analysed such as the framework sequences. These differences, such as in the form of mutations, may exert changes in binding behaviour of the Fab molecule due to differences in the global structure of the molecule. However, true differences in the binding pattern of the clones analysed in this project could be better determined in the format of IgG molecules and testing *in vitro* binding or neutralisation in cell culture. A vector for the expression of human IgG in mammalian cells that could be used is the pDCOrig huIgI1 (Scancell Ltd, Nottingham, UK). For this, subcloning of the individual heavy and light chains currently in the pCOMB 3H vector into pDCOrig vector is followed by transfection of a mammalian cell line whereupon transient expression of the whole IgG molecule can occur. Such data would be able to provide validation of the method used in this study and indeed further work is aimed at achieving this.

The final clones analysed from both libraries were panned in an acidic environment. A total of 9 clones out of 17 analysed showed specific reactivity to E2, 1 of which also showed preferential binding at pH 5.5 (see Figure 6.12, Figure 6.13 & Figure 6.15). Comparison of binding to the E2 target protein D535A and the wild type E2 protein revealed that of the 9 clones that showed specific reactivity to E2, 8 had preferential binding to the wild type E2 rather than the D535A E2 which was used as the target molecule during selection. This data was not the expected outcome; however the data do not discount the preferential binding to

either E2 protein by the Fab clones over background non-specific binding. The single clone that did show specific reactivity to E2 with higher binding to D535A and in acidic conditions was however a novel sequence and so, the method used in this project successfully allowed isolation of a novel fab clone with desired characteristics. Only further experimentation in the IgG format will reveal if these qualities are upheld in the full antibody form.

Analysis of the paratope sequence (see Table 6.4) and hydrophobicity (Table 6.3) did not show any particular differences between the selected clones and the previously isolated antibodies from the same kappa library panned (Allander 2000). The likely effect of the panning protocol adopted here would be to select for paratopes with an increased proportion of hydrophobic residues to interact with the cryptic epitopes on E2 likely to become unshielded upon acidification. Indeed, cryptic epitopes as seen in HIV and related viruses are often hydrophobic in nature (Oss 1995) or have a greater tendency to contain hydrophobic residues (David, Borza et al. 2001). Whilst a hydrophobic epitope will bind more strongly to a hydrophobic paratope, an interaction with a non-hydrophobic paratope would be 2-3 times weaker, which is still a functionally important interaction (Oss 1995). In the absence of a hydrophobic paratope being able to interact with its epitope, it is conceivable that the paratope would be likely to form a weak non-specific interaction to evade the aqueous hydrophilic environment. This model could partly explain the relatively low specific

binding by the phage clones as a product of higher non-specific binding seen in Figure 6.11-Figure 6.14. As this project is the first phage display set up to pan specifically in an acidic environment for the selection of acid-dependent Fab clones, it is therefore not possible to easily gauge the realistic impact of the specific and non-specific binding affinity of selected clones as affected by the pH environment.

7. Final discussion

In just over two decades HCV has been isolated, sequenced, cultured and investigated in a multitude of ways. Despite these efforts however, an effective vaccine still evades us and the virus continues to triumph over the natural immune response. Many antibodies that target HCV E2 have been isolated over the last decade, almost all of which target epitopes overlapping with CD81 binding sites. In the early part of this project a panel of soluble E2 constructs were produced in S2 cells. Characterisation of these proteins revealed expected sizes for this cell line although glycosylation was variable and not complete compared to E2 expressed elsewhere in mammalian cells (Dubuisson, Hsu et al. 1994; Fournillier, Wychowski et al. 2001; Op de Beeck, Cocquerel et al. 2001; Slater-Handshy, Droll et al. 2004; Liu, Chen et al. 2007; McCaffrey, Boo et al. 2007). High five and sf9 are two insect cell lines that have been used extensively for the production of several eukaryotic proteins successfully and so could be used for the expression of HCV E2 protein (Li, Brown et al. 2001; Morias and Costa 2003; Andronopoulou, Labropoulou et al. 2006; Douris, Swevers et al. 2006; Munster, Ziegelmuller et al. 2006). Like S2 cells, culturing of this cell line is significantly less time consuming, labour expensive, allows greater yields of highly pure protein and uses cheaper reagents than mammalian cell culture for protein production. As insect derived cell lines, the same issues of glycosylation observed in this project with S2

cells will likely also apply to sf9 and high five cell lines. Therefore, testing the expressed E2 protein from these cell lines would allow evaluation of whether these cell lines are appropriate to use for the production of functionally competent or structurally intact, soluble, secreted HCV E2 protein as a replacement or improvement to S2 cells. Previously, production of HCV E2 in insect cells relied on the baculovirus system which was not able to overcome the need to lyse cells to extract the proteins (Lanford, Notvall et al. 1993; Zhao, Liao et al. 2003). Using this system however it was observed that there could be variation in the glycosylation status of the envelope proteins thus producing variable molecular weight proteins as seen in this project (Lanford, Notvall et al. 1993). Otherwise Zhaos group was able to produce the mammalian standard sized 66 kDa soluble E2 protein in insect cells (Zhao, Liao et al. 2003) thus confirming that it is possible to produce HCV E2 as seen in mammalian cells. Taken together, advancements in expression vectors and cell culture techniques might eventually allow for stable, inducible expression of HCV E2 in insect cells conferring the practical advantages seen so far, matched to the protein characteristics observed when expressed in mammalian cells and ultimately *in vivo*. For now however, the S2 cell line allows production of soluble HCV E2 protein of an acceptable standard for basic experimentation and interpretation.

S2 expressed soluble HCV E2 protein was purified and characterised in section 3 and 4. Antibodies targeting epitopes with conformational

determinants were used in ELISA and Western blot assays to assess the structural integrity of the purified E2 protein. SDS-PAGE analysis was also used to verify size and stability. The glycosylation status of the E2 protein was seen to vary though the overall size of all E2 constructs produced was deemed satisfactory (section 3.2.5). Assessment of the conformational state of the HVR deleted E2 constructs was confirmed with CD81 binding as CD81 has a conformational binding domain on E2 spanning 4 discrete regions (Owsianka, Timms et al. 2006; Rothwangl, Manicassamy et al. 2008). Due to the nature of D535A and G530A being key CD81 binding determinants (Owsianka, Timms et al. 2006), binding to the conformational antibody H53, which cannot recognise E2 of genotype 2 (in which the HVR deletion panel was created) but can recognise genotype 1 (Cocquerel, Meunier et al. 1998), was used instead. Only the double mutant G530A-D535A lost conformation (see Figure 4.7) thus excluding it as a candidate target protein for biopanning. Ideally, more extensive characterisation would be performed in this analysis, such as a greater array of assays or a wider choice of antibodies to further support the conclusion that these E2 constructs retained conformational structure. The literature has shown that both HVR deleted E2 along with G530A and D535A E2 does not retain consistent binding and entry capabilities in cell culture or pseudo particle assays (Owsianka, Timms et al. 2006; McCaffrey, Gouklani et al. 2011) thus hampering the choice of assays applicable. Two such assays that would allow a more accurate interpretation however would be the use of Isothermal Titration Calorimetry (ITC) (O'Brien, Ladbury et al. 2000),

and Surface Plasmon Resonance Immunoassay (SPRI) (Liedberg, Nylander et al. 1983; Rich and Myszka 2007). Using these assays, the binding parameters between E2 and a test antibody can be accurately analysed. Thus comparison between binding efficiency of each E2-antibody pair can be made. The choice of conformational antibodies for these analyses could also be greater, such as the use of the inclusion of the CBH antibodies: some of which are CD81 independent (Hadlock, Lanford et al. 2000). Likewise, the epitope analysis performed in section 4.2.2 and 4.2.3 could be extended by comparing the same deletions/mutations in multiple genotypes. Indeed, it has been shown certain characteristics of HCV differ among the various genotypes (Lavillette, Tarr et al. 2005) and so exploring these differences further to better understand the structural consequences in such a genetically diverse virus would still be valuable in determining the universal characteristics of HCV.

Section 5 looked at the effects of pH environment on HCV E2-antibody binding. The E2 protein tested was seen to remain stable in the acidic conditions known to occur during the entry process (see section 5.2.1). Looking more closely at the transiency of these epitope effects in E2, it was hard to form a definitive interpretation. A non-significant portion of acid-induced binding effects appeared to be retained after neutralisation of E2 when looking at the antibody targeting the strep-tag, whereas a significant effect in antibody binding was observed in the presence of pH 5.5 for this antibody and H53 (section 5.2.2). Inability to

differentiate how much of the acid-induced conformational effect on E2 is permanent or transient led to the choice to analyse epitope binding in the presence of acidic pH along with the design of a panning group in section 6 where the interaction between E2 and Fab expressing phage was in the presence of acid pH 5.5. Given the limited data available on the extent of pH induced conformational effects on HCV glycoproteins and their duration, a valuable preliminary investigation was initiated here that with further work could go towards defining in further detail the events of entry and fusion for E2 from a structural biology perspective. The data gathered in section 5.2.2 would have greatly benefited from expansion to include the full pH and antibody range available when looking at the transiency of conformational E2 effects. Also, more antibodies available targeting epitopes of a conformational nature, neutralising preferably though not necessarily CD81 binding site located, would have improved the possible interpretation. As previously mentioned, the panel of CBH antibodies from the Foung lab would have been applicable (Hadlock, Lanford et al. 2000). The use of ITC or SPRI could also be applied to the analysis of pH induced conformational effects on E2. Using pH as a constant or variable in each E2-antibody pair, it would be possible to more accurately analyse the effect of pre-acidified then neutralised E2 (thus permanent effects only), as well as binding in the presence of various acidic pH levels. Another valuable extension of this work would be to compare these data across genotypic groups. It has already been shown that there are subtle differences in pH dependency during entry across different genotypic groups

(Lavillette, Tarr et al. 2005), and so there is potential for more pronounced differences in the binding behaviour of E2 across the pH range. Such differences may be variances in the threshold of sensitivity to pH or the extent of the binding kinetic effects observed.

Biopanning of a combinatorial phage display library in the pComb3H vector was described in section 6. During this process, difficulties in attaining high titers, efficient screening of the selected clones, as well as testing of these clones was seen. Assessment of the library pre-panning showed a low proportion of intact phagemid clones though this did not prevent earlier use of the library to successfully isolate novel neutralising antibodies (Allander 2000; Johansson, Voisset et al. 2007) (section 6.2.4). Screening methodologies were developed leading to a final pool of potential clones (see section 6.2.3 to 6.2.5). Higher titres of purified phage would have allowed more flexibility in the analysis of the test clones derived such as titration assays for binding affinities. Issues also arose in the sequencing of these clones limiting the interpretation of the diversity of the final panel analysed. Unlike the previous sections, in which affinity was the characteristic that would have benefitted from more accurate experimental assessment, in this final section, determining the specificity of each fab clone to the target E2 protein was equally hard to clearly define. Relatively small differences were observed in binding of the fab clones to the D535A E2 construct used as the target in biopanning compared to the wild type protein and non-specific binding. Comparison of the acidic environment as in the

panning for fab-E2 binding also did not show great differences to binding in neutral pH with only 2 out of 17 clones showing significant preferential binding in acidic conditions. The biopanning method employed in this project would have benefitted from more panning groups to better compare the selection of clones derived from various conditions. The advantage to that would be the ability to discriminate fab clones that can be isolated from binding in acidic and neutral binding conditions or clones that are only isolated in acidic conditions for example. Subtractive selection like this has been used elsewhere to define clones according to sequence or selective characteristics (Gnanasekar, Rao et al. 2004; Hof, Cheung et al. 2006). A subtractive selection panning group for this biopanning project could adopt an exhaustive set up as effectively demonstrated by Giang and his group. This could be panning enrichment/selection with an acidic pH 5.5, followed by selection at neutral pH in which the unbound phage is retained and reused for a further selection at acidic pH. The reversal of pH conditions, could also be performed i.e., selection at neutral pH followed by selection at pH 5.5 where unbound phage is retained to use in one more selection event once more at neutral pH. The first group containing fab clones that can only bind E2 in a pH 5.5 environment, and the second group containing fab clones that can only bind E2 in neutral pH conditions would then theoretically contain two distinct pools of fab clones that could be verified by sequence analysis. Alternatively, fab clones that are able to bind their target at neutral and

acidic pH may be the desired product and thus alternate selection conditions could be used in the same panning group.

Research aimed at finding novel, broadly neutralising antibodies to HIV, influenza virus, flaviviruses, and other viruses has not only produced useful antibodies but also made novel methodologies available for producing novel HCV antibodies. The project described in this dissertation and other projects that produced the main anti-HCV broadly neutralising antibodies used phage display to isolate the desired antibodies. Recent research on antibodies targeting influenza virus used phage display followed by cell-based screening to find the C05 antibody, which has an epitope on the stem of the crucial viral Haemaglutinin envelope protein. The C05 antibody neutralises several virus groups, protecting mice from viral challenge (Ekiert, Kashyap et al. 2012).

RT-PCR using single-plasma-cell extracts and plasma-cell monoculture methods were recently used to isolate novel influenza-neutralising antibodies (Corti, Voss et al. 2011; Whittle, Zhang et al. 2011). Advances in phage-display technologies in the 1990s led to an explosion of research aimed at identifying novel antibodies. Recent growth in techniques based on flow cytometry and cell sorting allowed antibody-producing cells to be systematically isolated. Corti (2011) used cell sorting to isolate more than 10^5 plasma cells in single-cell cultures, which they subsequently screened for desired antibodies (Corti, Voss et al. 2011), identifying the F16 antibody several times in the same donor.

The F16 antibody recognises a conserved epitope and neutralises multiple virus groups by blocking the conformational changes necessary for viral envelope fusion with the cellular membrane.

HIV research has also benefited from recent methodological advances. Walker (2009) screened 1800 donors using a novel, single-B-cell microneutralisation method and isolated two novel antibodies with quaternery structure-dependant conformational epitopes. The neutralising antibodies were about an order of magnitude more potent than other known HIV antibodies and had the same or greater target breadth. Wu (2010) used cell sorting and RT-PCR, along with cell staining and cell-based, single-B-cell capture-screen isolation of antibody-producing cells, to identify novel, broadly neutralising HIV antibodies. They used a resurfaced viral glycoprotein (gp120) to isolate memory B cells specific for the host CD4-binding site. They then used the isolated B cells to identify the VRC01 antibody, a highly potent antibody that neutralised more than 90% of the HIV isolates tested. Another novel approach to HIV research using an alternative sorting strategy based on capturing single B cells that recognise the viral Env protein expressed on the surface of transfected cells (Klein, Gaebler et al. 2012) identified a novel epitope that is not displayed on the soluble form of the Env protein.

The continuing methodological advances for isolating and screening antibody-producing cells have proven fruitful for HIV and influenza

research. The same advances can be used to expand the available pool of broadly neutralising anti-HCV antibodies. For now, phage display is the most productive method used in HCV research and will likely continue to be productive as our understanding of the virus grows. The choice of methodology will always depend on the properties of the virus that we wish to manipulate, however; so the broader research on HCV host-cell entry and infectivity, which is currently far behind the research on HIV and influenza host-cell entry and infectivity, remains crucial to our efforts to identify novel, broadly neutralising anti-HCV antibodies.

8. Final conclusions

This project aimed to isolate novel antibodies targeting HCV E2 non-CD81 epitopes that would retain binding ability during entry and fusion. Characterisation of various E2 constructs revealed novel aspects of E2 epitope exposure and binding following targeted deletion/mutation such that HVR2 is the most influential HVR in improving exposure of the CD81 binding domain. Also HCV E2 is able to maintain stable conformation with G530 or D535 mutated but not both together. A brief analysis of epitope exposure in varying pH environments also revealed potential for genotype and epitope specific conformational changes in response to acidic environment. Fusion binding antibodies have been isolated for viruses other than HCV as discussed earlier providing proof of principle that such a model of an antibody for HCV can be achieved. Equally, antibodies to non-CD81 epitopes have recently been isolated by multiple groups also using tailored phage display methodologies. The same target E2 construct D535A as used here successfully generated non CD81 binding epitopes thereby supporting the notion of such antibodies to be found (Keck, Xia et al. 2012). Also recently, the use of competitive inhibition of the target molecule (E1E2) to occlude sites of known epitopes has been used to isolate novel antibodies binding to "new" areas of the envelope protein (Giang, Dorner et al. 2012). Like these recent studies, this project has used a phage display approach, with the same phagemid vector, using a defined set of conditions to

select for specific qualities of the Fab clone isolated to encourage functional ability in the full antibody format for effectivity during the entry process of HCV, Fab clones isolated in this project showed initial promise for preferential binding to the D535A E2 construct, along with clones showing enhanced binding in acidic conditions. Only one of the isolated Fab clones however significantly showed both characteristics. Taken together, the biopanning setup used here, can potentially allow isolation of antibodies targeting epitopes on HCV E2, optimally exposed during the entry process.

9. References

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