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SHORT COMMUNICATION

Functional analysis of hepatitis C virus E2 glycoproteins and virus-like particles reveals structural dissimilarities between different forms of E2

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Structure–function analysis of the hepatitis C virus (HCV) envelope glycoproteins, E1 and E2, has been difficult due to the unavailability of HCV virions. Truncated soluble forms of E2 have been used as models to study virus interaction with the putative HCV receptor CD81, but they may not fully mimic E2 structures on the virion. Here, we compared the CD81-binding characteristics of truncated E2 ($E2_{660}$) and full-length (FL) E1E2 complex expressed in mammalian cells, and of HCV virus-like particles (VLPs) generated in insect cells. All three glycoprotein forms interacted with human CD81 in an *in vitro* binding assay, allowing us to test a panel of well-characterized anti-E2 monoclonal antibodies (MAbs) for their ability to inhibit the glycoprotein–CD81 interaction. MAbs specific for E2 amino acid (aa) regions 396–407, 412–423 and 528–535 blocked binding to CD81 of all antigens tested. However, MAbs specific for regions 432–443, 436–443 and 436–447 inhibited the interaction of VLPs, but not of $E2_{660}$ or the FL E1E2 complex with CD81, indicating the existence of structural differences amongst the E2 forms. These findings underscore the need to carefully select an appropriate ligand for structure–function analysis.

Hepatitis C virus (HCV), a member of the *Flaviviridae* family, has a positive-strand RNA genome which encodes a single polyprotein of approximately 3010 amino acids (aa) that is processed into functional proteins by host and viral proteases (Rice, 1996; Ryan *et al*., 1998). The three structural proteins, namely core and the envelope glycoproteins E1 and E2, are located within the N terminus of the polyprotein, whilst the non-structural proteins reside within the C-terminal part (Clarke, 1997). By analogy with other flaviviruses, the core is presumed to be enveloped in a lipid bilayer containing the viral glycoproteins. The glycoproteins E1 and E2 are believed to be type I integral transmembrane proteins, with C-terminal hydrophobic anchor domains. *In vitro* expression studies have shown that these glycoprotein species interact with each other and accumulate within the endoplasmic reticulum (ER), the proposed site for HCV assembly and budding (Choukhi *et al*., 1998; Cocquerel *et al*., 1998; Deleersnyder *et al*., 1997; Dubuisson, 2000; Dubuisson *et al*., 1994; Ralston *et al*., 1993). ER localization signals have been mapped to the C-terminal transmembrane domains of the two glycoproteins, removal of which results in their secretion from expressing cells (Cocquerel *et al*., 1998, 1999; Duvet *et al*., 1998; Flint & McKeating, 1999; Michalak *et al*., 1997). Previous reports have shown that expression of the structural proteins of HCV genotype 1b in insect cells leads to assembly of virus-like particles (VLPs) (Baumert *et al*., 1998, 1999). Interestingly, however, such VLP formation has not been observed in mammalian cells expressing the same viral structural proteins.

The mechanism(s) of HCV entry into its target cell is currently unknown, although the E2 glycoprotein is thought to play a major role in virus–cell attachment (Rosa *et al*., 1996). The E2 glycoprotein extends from aa 384 to 746 of the polyprotein and it carries regions of extreme variability (Kato *et al*., 1992; Mizushima *et al*., 1994; Ogata *et al*., 1991; Weiner *et al*., 1991). The most variable region (HVR-1) is located within the N-terminal 27 residues (aa 384–411) of E2, and a second region, HVR-2, resides in the 476–482 segment. Antibodies specific for epitopes within HVR-1 have been reported to inhibit the binding of E2 glycoprotein to cells and to block HCV infectivity *in vitro* and *in vivo* (Farci *et al*., 1996; Habersetzer *et al*., 1998; Rosa *et al*., 1996; Shimizu *et al*., 1996; Zibert *et al*., 1995). Because of the lack of a suitable cell culture system for *in vitro* propagation of HCV and the

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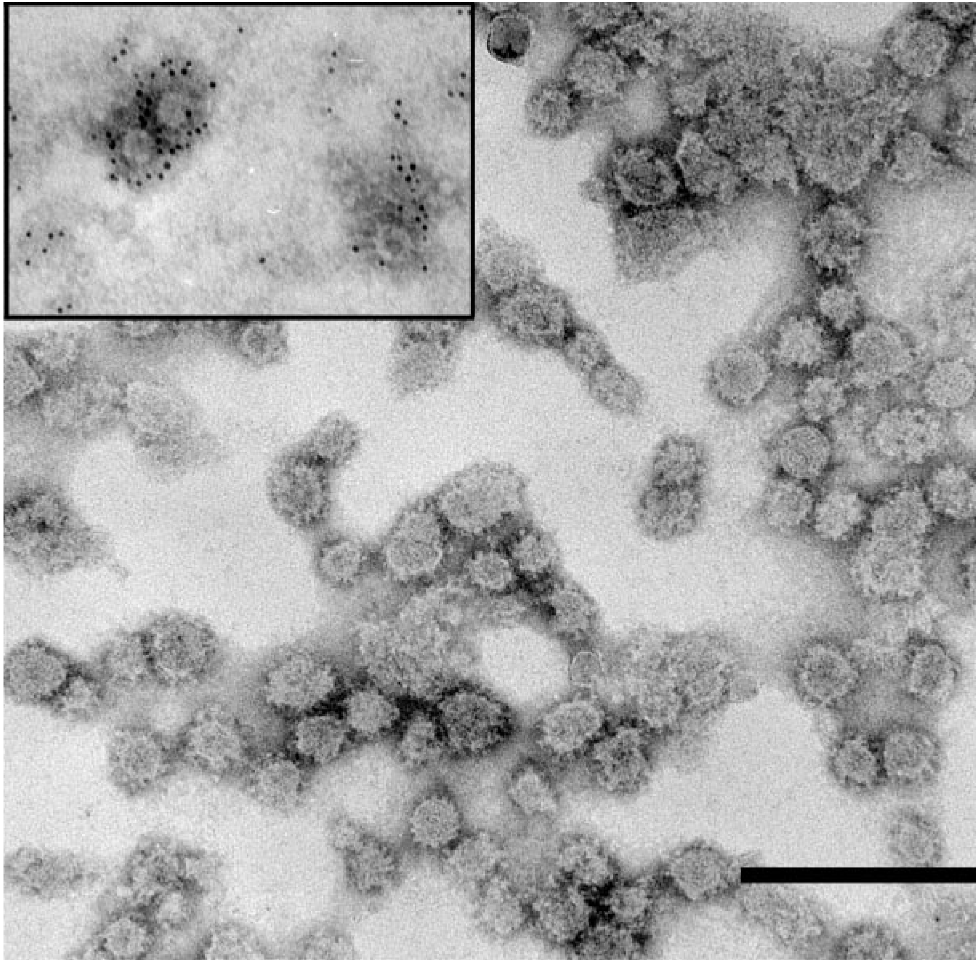

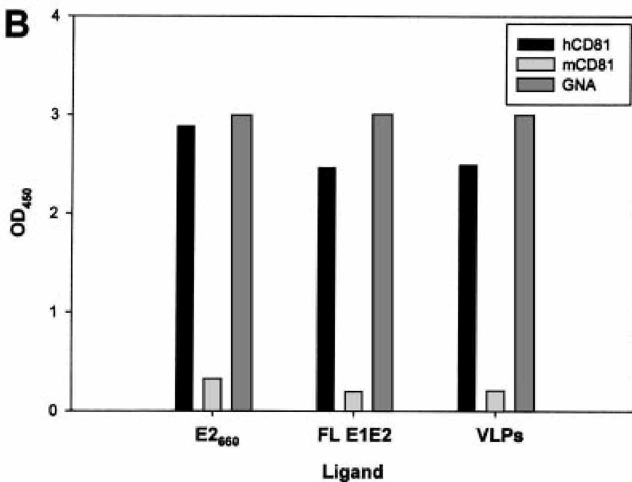

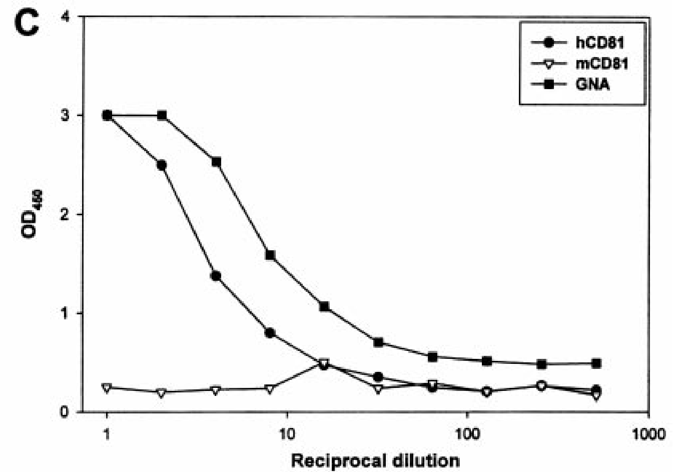

Fig. 1. (A) Electron micrograph of negative-stained HCV VLPs isolated from Sf cells infected with rbac-B45. Inset: VLPs placed on the EM grid were incubated first with anti-E2 MAb AP33 for 4 h at room temperature, washed with PBS, and then incubated with anti-mouse IgG conjugated with 5 nm gold particles (Nanoprobe, UK). The grid was washed, stained with Nanovan (Nanoprobe) for 2 min, drained and examined in the EM. Bar, 200 nm. (B) Interaction of various HCV glycoprotein forms with CD81. As described in the text, saturating amounts of $E2_{660}$, FL E1E2 or VLPs were captured in ELISA dishes coated with GST–hCD81, GST–mCD81 or GNA lectin. Bound antigens were visualized with an anti-E2 antiserum (R646), anti-rabbit IgG–HRP conjugate and TMB (Sigma) substrate. Absorbance values were determined at 450 nm (Dynatech). (C) Dose-dependent binding of HCV VLPs to CD81. Decreasing concentrations of VLPs were captured with GST–hCD81, GST–mCD81 or GNA lectin and the bound antigen was detected as described in (B) above.

Table 1. MAb-mediated inhibition of HCV E2–CD81 interaction

MAbs 7/59, 6/82a, 6/16, 9/86a, 9/27, 3/11, 2/69a, 1/39, 11/20c, 7/16b, 6/41a, 2/64a, 9/75 and 6/53 were developed in rat (Flint *et al*., 1999; C. Maidens and others, unpublished). The conformation-sensitive mouse MAbs H53 (Cocquerel *et al*., 1998) and H60 were kind gifts from Jean Dubuisson. The remaining MAbs were generated in mice immunized with a mammalian cell-expressed HCV strain Gla E1E2 (Patel *et al*., 1999, 2000), a detailed characterization of which will be described elsewhere (A. H. Patel). Values in the shaded area of the Table are for six inhibitory MAbs recognizing epitopes within the E2 aa region 412–447.

MAb	Epitope	Inhibition of CD81 binding*		
		$E2_{660}$	FL E1E2	VLP
7/59	HVR1 (384–395)	−	−	−
6/82a	HVR1 (384–395)	−	−	−
6/16	HVR1 (384–395)	−	−	−
9/86a	HVR1 (Conf.†)	−	−	−
9/27	HVR1 (396–407)	−	+	+
3/11	412–423	+	+	+
AP33	412–423	+	+	+
2/69a	432–443	−	−	+
1/39	436–443	−	−	+
11/20c	436–447	−	−	+
7/16b	436–447	−	−	+
AP320	464–471	−	−	−
AP436	464–475	−	−	−
6/41a	480–493	−	−	−
2/64a	524–531	−	−	−
9/75	528–535	−	+	+
6/53	544–551	−	−	−
AP266	644–651	−	−	−
ALP98	644–651	−	−	−
ALP11	644–651	−	−	−
ALP1	648–659	−	−	−
H53	Conf.	−	−	−
H60	Conf.	−	−	−

* See Fig. 2 legend for assay conditions. Only MAbs with 80% or higher blocking activity relative to the respective no MAb control were scored as +.
† Conf., conformation-sensitive epitopes.

unavailability of virions in sufficient quantities, it has not yet been possible to assess the precise role of the glycoproteins in cell attachment and entry. Instead truncated, secreted versions of E2 have been used as soluble mimics of the native forms present on virus particles. Pileri *et al*. (1998) reported that both a truncated soluble form of E2, and HCV virions bound to the large extracellular loop (LEL) of the cell surface protein CD81, suggesting a potential receptor candidate for HCV. The interaction between CD81 and truncated forms of E2 or the E1E2 heterodimer was confirmed and further analysed by several groups (Chan-Fook *et al*., 2000; Flint *et al*., 1999, 2000; Hadlock *et al*., 2000; Meola *et al*., 2000; Patel *et al*., 2000; Petracca *et al*., 2000; Wunschmann *et al*., 2000). However, a comparative antigenic characterization of the various forms of HCV glycoproteins (including VLPs) binding to CD81 has not been reported. From the point of view of structure–function analysis, it is important to consider whether antigenic and/or structural differences exist between soluble and native forms of glycoproteins and to ensure that appropriate ligands are selected carefully to mimic accurately the native viral forms. In contrast to the soluble or non-particulate forms of HCV structural proteins, those present in VLPs may be more likely to represent the native, virion-like conformation(s).

In this study, we investigated the CD81-binding characteristics of various forms of HCV glycoproteins. Recombinant vaccinia virus (v1-836) or baculovirus (rbac-B45) expressing aa 1–836, representing the entire structural region (core, E1 and E2) plus p7 and the N-terminal 27 aa of NS2 of the infectious clone of HCV genotype 1a strain H77c (Yanagi *et al*., 1997) were generated. Full-length (FL) E1E2 complex was produced by infecting COS-7 cells with v1-836 for 24 h; the cells were then washed in PBS, lysed in lysis buffer (20 mM Tris–HCl, pH 7·4; 150 mM NaCl; 1 mM EDTA; 0·5% Triton X-100), and the lysate used as a source of FL E1E2 for assays described in this study. The soluble form of E2, $E2_{660}$, was produced in COS-7 cells using a recombinant vaccinia virus ($vE2_{660}$) described previously (Patel *et al*., 2000). The medium of COS-7 cells infected with $vE2_{660}$ was used as a source of $E2_{660}$. Expression of HCV structural proteins in mammalian or insect cells infected with the appropriate recombinant virus was confirmed by immunofluorescence, Western blot and immunoprecipitation assays (data not shown).

Insect cells [*Spodoptera frugiperda* (Sf)] were infected with rbac-B45 to produce HCV VLPs, which were partially purified by sucrose gradient centrifugation essentially as described by Baumert *et al*. (1998). Electron microscopic (EM) analysis of VLPs revealed a population of particles with radii ranging from 20 to 44 nm, the morphology of which was consistent with that predicted by analogy to related pesti- and flaviviruses. Particles were at least partially enveloped with spikes protruding from the surface (Fig. 1 A). The presence of HCV E2 glycoprotein on the surface of VLPs was confirmed by immunogold electron microscopy (Fig. 1 A, inset). Similarly, the E1 glycoprotein was also shown to be present on the surface of VLPs (data not shown). A detailed structural and antigenic characterization of the VLPs will be described elsewhere.

To evaluate interactions of HCV glycoproteins used in this study with CD81, we used an antigen capture ELISA described previously (Flint *et al*., 1999; Patel *et al*., 2000). Briefly, GNA (*Galanthus nivalis*) lectin (Sigma), which has been shown to bind HCV glycoproteins to high specificity (Cardoso *et al*., 1998; Ralston *et al*., 1993), or the GST fusion protein containing the LEL of human or mouse CD81 (GST–hCD81 or

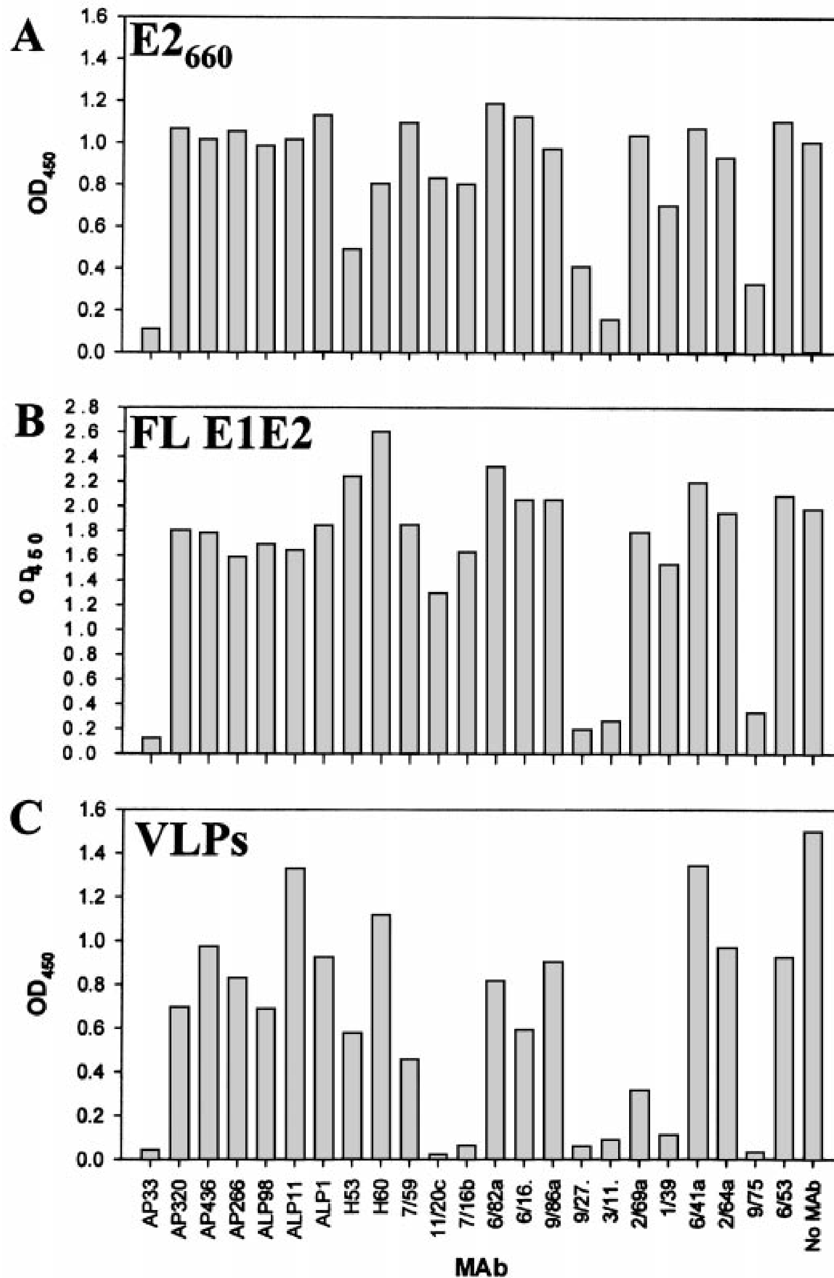

Fig. 2. MAb inhibition of binding of various HCV glycoprotein forms to CD81. $E2_{660}$ (A), FL E1E2 (B) or VLPs (C) were pre-incubated with saturating concentrations of various MAbs or PBS (No MAb control) for 1 h at room temperature, and the antigen–MAb complex captured with GST–hCD81 coated on ELISA dish. The bound complex was detected using anti-E2 antiserum R646 as described in the legend to Fig. 1 (B).

GST–mCD81, respectively) were used to coat Immulon II ELISA plates (Dynal). After blocking with 4% milk powder, saturating amounts of $E2_{660}$, FL E1E2 complex or VLPs were allowed to bind to GNA or recombinant CD81 and the bound antigen was detected using a rabbit polyclonal anti-E2 antiserum R646. As shown in Fig. 1 (B), all three ligands bound to hCD81 and GNA. Furthermore, binding of VLPs to both hCD81 and GNA was dose-dependent (Fig. 1 C). Similar dose-response curves were determined for $E2_{660}$ and FL E1E2 (data not shown).

We next evaluated a panel of well-characterized mouse and rat monoclonal antibodies (MAbs) specific for epitopes in E2 [Table 1; Flint *et al*. (1999); A. H. Patel and others, unpublished] for their ability to inhibit the interaction with human CD81 of the various HCV glycoprotein ligands. $E2_{660}$, FL E1E2 complex or VLPs were pre-incubated with various MAbs and ligand–MAb complex formation was demonstrated in a GNA lectin-capture assay followed by detection with an anti-species IgG–horseradish peroxidase (HRP) conjugate (data not shown). The ligand–MAb complex was allowed to bind GST–hCD81 in the ELISA capture assay described above and the bound proteins were detected using the anti-E2 polyclonal serum R646 followed by anti-rabbit IgG–HRP conjugate. Several MAbs were able to block binding of the glycoproteins

to CD81, albeit with varying efficiencies (Fig. 2). MAbs which showed 80% or greater inhibition of binding are used here to highlight the differences in CD81 binding characteristics of various ligands. MAbs AP33 and 3/11 (specific for aa 412–423, Fig. 2; Table 1) inhibited the interaction of all three ligands with CD81. MAbs 9/27 (HVR1, aa 396–407) and 9/75 (aa 528–535) inhibited the interaction of FL E1E2 or VLP with CD81, and had modest affects on the $E2_{660}$–CD81 interaction (Fig. 2A–C, Table1). In addition, MAbs 11/20c, 7/16b (both recognizing aa 436–447 of E2), 2/69a (aa 432–443), and 1/39 (aa 436–443) all efficiently blocked the interaction of VLPs (Fig. 2C), but not $E2_{660}$ or FL E1E2 complex (Fig. 2A, B), with CD81 (Fig. 2A–C). The differential inhibition of CD81 binding is not due to differences in MAb reactivity with the various antigens, since all of the above MAbs recognized the E2 ligands comparably (data not shown). Taken together, these results indicate structural differences between different E2 glycoprotein forms.

The E2 aa regions involved in direct contact with CD81 have not yet been identified. Previous studies have shown that the CD81 binding site within the soluble form of E2 is conformational in nature (Flint *et al*., 1999). Hadlock *et al*. (2000) recently described inhibition of binding of HCV virions or E2 glycoprotein to CD81 by human MAbs specific for a number of conserved conformational epitopes in E2. However, because of the conformational nature of the relevant epitopes it was not possible in that study to ascertain the E2 region(s) involved in CD81 binding.

The inhibitory activity of MAbs could be due to occupancy of epitopes involved in direct contact with CD81 or may simply be a consequence of occlusion by steric hindrance. In addition to highlighting the presence of structural differences between different forms of viral glycoproteins, our results have broadly identified three regions (namely, HVR-1, aa 412–447 and 528–535) within E2 that may modulate E2–CD81 interaction. Of the five anti-HVR1 MAbs, only MAb 9/27 blocked the interaction of FL E1E2 and VLPs (and to a lesser extent $E2_{660}$) with CD81, indicating that HVR1 is probably not involved in direct contact with CD81. This is consistent with our recent data which showed that chimeric E2 glycoproteins, and those lacking HVR1, bind CD81 with an affinity comparable to the parental glycoprotein (Patel *et al*., 2000; Forns *et al*., 2000). Several reports have implicated HVR-1 as having a role in eliciting neutralizing antibodies and as a region responsible for interacting with the cell surface (Farci *et al*., 1996; Rosa *et al*., 1996; Shimizu *et al*., 1996; Zibert *et al*., 1995). Given that CD81 is expressed in a wide range of cell types (Levy *et al*., 1998), it is unlikely to be a determinant of hepatotropism for HCV. Our unpublished results, along with results from others (Agnello *et al*., 1999; Petracca *et al*., 2000), suggest that other candidate receptor(s) for HCV exist, and that involvement of HVR-1 in E2 binding the cell surface via such factors cannot be ruled out.

The epitopes recognized by six of the eight inhibitory MAbs are located within the E2 region 412–447 (shaded area, Table 1), indicating that residues in this 35 aa region may play a crucial role in interaction with CD81. Two of the MAbs, AP33 and 3/11, inhibited the interaction of all three ligands with CD81, indicating that the aa residues 412–423 may directly contact CD81. The remaining four MAbs (recognizing epitopes located between E2 aa 432–447, Table 1) only block the VLP–CD81 interaction (but not $E2_{660}$ or FL E1E2), indicating that the inhibition may be due to steric hindrance. These data are in keeping with our recent studies showing that the aa residues in E2 region 407–524 are important for modulating CD81 interaction (Patel *et al*., 2000). In addition to the 35 aa E2 region described above, MAb 9/75 (recognizing aa 528–535) also blocked the interaction of all three ligands with CD81, implicating this region as a possible second binding site. Interestingly, using fold recognition methods Yagnik *et al*. (2000) recently produced a model of HCV E2 glycoprotein in which they identified aa 474–494 and 522–551 as CD81-binding regions. Our experimental observations do not entirely support this interpretation in that they implicate the aa region upstream of the first CD81-binding domain 1 depicted in the model. However, the inhibitory activity of MAb 9/75 is consistent with the involvement of the second CD81-binding region proposed by Yagnik *et al*. (2000).

In conclusion, the ligand-dependent differential MAb inhibition of E2–CD81 interaction strongly points to differences in the E2 tertiary structure between $E2_{660}$, FL E1E2 and VLPs. It is important to take this into consideration when a particular viral ligand is selected for structure–function analysis.

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