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# Alanine scanning mutagenesis of hepatitis C virus E2 cysteine residues: Insights into E2 biogenesis and antigenicity

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ABSTRACT

Envelope glycoprotein 2 (E2) of hepatitis C virus contains 18 conserved cysteine (Cys) residues in its ectodomain. By cysteine-alanine mutagenesis and function analysis, six Cys in H77 E2 (C494, C508, C552, C564, C607 and C644) were found to be indispensable for recognition by conformation-dependent mAb H53. Removal of any of these Cys residues did not affect E2 heterodimerization with E1, but notably reduced E1E2 transmembrane transportation. These Cys together with C429 and C503 were required for conformation-dependent mAb H48 recognition. All of the above Cys except C607 were required for H77 and Con1 E2 binding to CD81. None of individual mutation of above Cys affected the ability of E2 to induce neutralizing antibodies in mice. Mouse antibodies mainly recognize E2 linear epitopes and are unrelated to epitopes recognized by human E2 antibodies. The findings provide new insights for understanding the biogenesis of functional HCV envelope proteins and HCV neutralizing immunity.



## Introduction

Hepatitis C virus (HCV) is an enveloped virus of the *Flaviviridae* family with a single-stand positive RNA genome (Poynard et al., 2003). HCV infection is a major cause of chronic liver disease that frequently leads to cirrhosis and hepatocellular carcinoma (Kanwal et al., 2011). Although the recent clinical application of two HCV NS3 protease inhibitors improved the efficacy of standard treatment with pegylated alpha interferon combining ribavirin (Serfaty et al., 2012; Poordad et al., 2011), drug resistant mutants have been reported (Romano et al., 2012). More importantly, no vaccine is available so far.

HCV envelope proteins, consisting of E1 and E2, form a non-covalent heterodimer and are believed to be the basic functional unit that mediates HCV cell entry (Lavie et al., 2007; Op De Beeck et al., 2004). Increasing data demonstrated that E2 protein plays a key role in mediating HCV interaction with cellular receptors and inducing membrane fusion (Ploss and Evans, 2012; Zeisel et al., 2011). E2 is a type-I transmembrane protein, within its ectodomain contains 18 cysteine (Cys) residues strictly conserved across the seven major genotypes. Krey et al. (2010) specified the nine disulfide bonds formed by 18 cysteine residues within HCV E2 ectodomain and predicted E2 tertiary organization as a class II fusion protein related to flavi- and alphaviruses with three non-overlapping domains. Recently, McCaffrey et al. (2012) reported that some cysteine residues perform indispensable role in HCVpp incorporated E2 reactivity with a conformation dependent monoclonal antibody (mAb) H53, E2 binding to HCV receptor CD81 and E1E2 heterodimerization, and all the cysteine residues in E2 protein of H77 strain of genotype 1a are required for cell entry of HCVpp and HCVcc. Fenouillet et al. (2008) reported that E2 disulfide bonds masked antigenic domains and reducing status contributed E2 to induce neutralizing antibodies.

In McCaffrey's report (2012), the characterization of cysteine mutant E2 proteins was performed in the context of pseudoparticles incorporated E2. Nevertheless, assembly characteristics of HCVpp are basically different from authentic HCV particles. More importantly, in this report, at least six cysteine mutations abolished E2 incorporated into pseudoparticles, in this scenario, the significance of cysteine residues in E2 folding and function could not be comprehensively assessed. In Fenouillet's report (2008), effect of disulfide bonds on E2 antigenicity was investigated using E2 protein treated with reducing agents, so the disulfide bonds

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that masked antigenic domains could not be specified. In this study, we performed a comprehensive alanine-scanning mutagenesis of the 18 cysteine residues to investigate the functional role of each cysteine residue for folding and transmembrane transportation of intracellular *de novo* synthesized E2 and for antigenicity of E2 protein.

## Results

### *Effect of Cys-Ala mutation on E2 reactivity with two conformation-dependent mAbs*

HEK 293 T cells were transfected with wild type or each Cys-Ala mutant E1E2 plasmid and E2 and E1 protein was determined by western blotting and GNA capture ELISA. The detective antibodies used were E2 pAb, E2 mAb C6H and E1 mAb A4 (recognizing a linear epitope in E1 protein). None of the individual Cys-Ala mutations affected E2 and E1 expression (Fig. 1A and B). The cell lysates were then subjected to reactivity assay with two conformation-dependent mAbs H53 and H48. H53 is specific for a conformation epitope in correctly folded E2, no matter alone or noncovalently associated with E1, which is believed to represent native prebudding forms of the HCV envelope (Deleersnyder et al., 1997). This epitope is independent of CD81-binding sites. McCaffrey et al. (2012) recently determined the effect of Cys-Ala mutation on H53 reactivity with HCVpp incorporated E2 protein. This assay could not discriminate the effects caused by the quantity of E2 incorporated into pseudoparticles or H53 reactivity of E2 protein itself. In the present study, C494A, C508A, C552A, C564A, C607A and C644A mutations nearly completely abrogated E2 binding to H53, and C597A showed a significantly reduced reactivity with H53 (Fig. 1C). H48 is a conformation-dependent mAb that disturbs interaction between HCV envelope protein and HCV receptor CD81 (Op De Beeck et al., 2004). C429A, C494A, C503A, C508A, C552A, C564A, C607A and C644A mutations abolished E2 binding to H48, whereas the rest mutants displayed reactivity to H48 comparable with wild type level (Fig. 1C).

It was worth noticing that mutation of C552, C494, C508 or C564 abolished H53 reactivity, but mutation of their corresponding partner C429, C486, C503 or C569 did not affect H53 recognition at all. Similarly, mutation of C494 or C564 abrogated H48 recognition, but neither mutation of their partner cysteine residues (C486 or C569) affected H48 reactivity. To investigate whether that is caused by aberrant match of the unpaired cysteine or alteration of local conformation, E2 proteins containing simultaneous Ala replacement of two cysteine residues within one disulfide bond were prepared and their reactivity with two mAbs was assayed. Results showed that disulfide bonds 1, 3, 4, and 5 were strictly indispensable for both H48 and H53 recognition. Deletion of disulfide bond 7 did not affect H48 reactivity, but moderately reduced H53 reactivity. Deletion of disulfide bond 8 reduced both H48 and H53 reactivity. Compared with individual cysteine residues mutation in disulfide bond 8, pairwise mutation of C607 and C644 partially recovered H53 and H48 recognition. Disulfide bonds 2, 6 and 9 were dispensable for H48 and H53 reactivity (Fig. 1D).

### *Effect of individual Cys-Ala mutation on E2 binding to CD81*

Interaction between HCV E2 and cellular CD81 is a pivotal step in the complicated process of HCV entry. CD81 binding of Cys-Ala mutant E2 in the context of full-length envelope protein was detected using CD81 LEL capture ELISA. Similar with H53 reactivity assay of HCVpp-incorporated E2 performed by McCaffrey et al. (2012), the CD81 binding assay of HCVpp-incorporated E2 in that report could not distinguish the effect between the quantity of pseudoparticles incorporated E2 and CD81 binding activity of E2 itself. Based on CD81 LEL binding ELISA, C429A, C494A, C503A, C508A, C552A, C564A, and C644A mutant of intracellular H77 E2

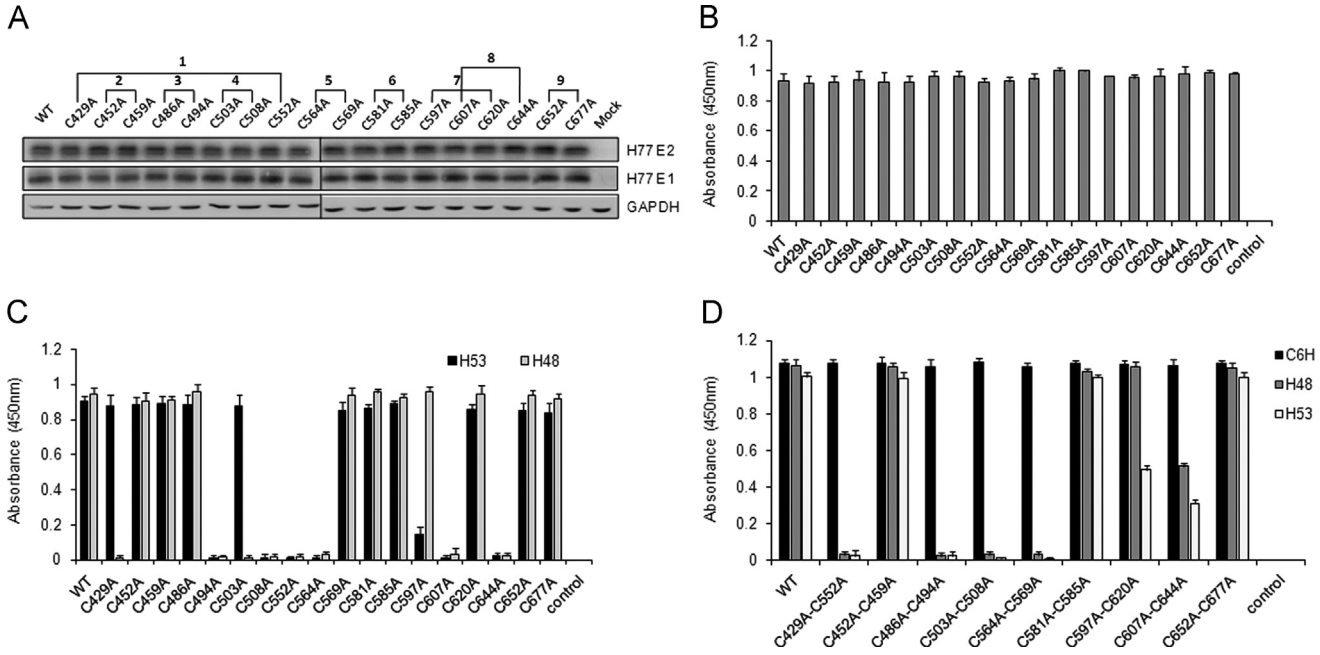


**Fig. 1.** Effect of Cys-Ala mutations on reactivity of H77 E2 protein with conformation-dependent mAbs H48 and H53. (A) HEK 293T cells were transfected with plasmids containing wild type or individual Cys-Ala mutant H77 E1E2 expression plasmid and control mock plasmid, respectively. Forty-eight hr later, the cells were harvested, and intracellular E1 and E2 were determined by western blotting. The detective antibodies were E2 pAb and E1 mAb A4, respectively, and GAPDH was assayed as an internal standard. (B) Reactivity of E2 protein in 293T cell lysates with a linear epitope mAb C6H was detected via GNA capture ELISA. (C) Reactivity of E2 protein in 293T cell lysates with mAbs H48 and H53 was detected via GNA capture ELISA. (D) HEK 293T cells were transfected with wild type or pairwise cysteine mutant H77 E1E2 plasmids. Reactivity of E2 protein in 293 T cell lysates with mAbs C6H, H48 and H53 was analyzed via GNA capture ELISA. The ELISA data are the means ± standard deviation of three independent experiments.