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Significance of palmitoylation of CD81 on its association with tetraspanin-enriched microdomains and mediating hepatitis C virus cell entry

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ABSTRACT

CD81, a co-receptor for hepatitis C virus (HCV), is a member of the tetraspanin superfamily and is heavily palmitoylated in the juxtamembrane cysteine residues. Palmitoylation plays an important role in protein–protein interactions and association with cholesterol-rich domains of membranes. In this study, Huh7 cells expressing wild-type or palmitoylation-defective CD81 were generated to analyze whether palmitoylation of CD81 is involved in HCV cell entry. Our data showed that de-palmitoylation of CD81 dramatically reduced its association with tetraspanin CD151, but did not influence CD81 partition in detergent-resistant membranes. Moreover, de-palmitoylated CD81 decreased the host cell susceptibility to HCV. Notably, CD151-specific antibodies and siRNA inhibited HCV cell entry, and detachment of CD81 with CD151 decreased the lateral movement of virus particle/CD81 complex to areas of cell–cell contact. These results suggest that palmitoylation of CD81 should facilitate HCV entry, at least in part, by regulating the association of CD81 with tetraspanin-enriched microdomains.

Introduction

Approximately 180 million people are infected worldwide by hepatitis C virus (HCV). A majority of those people are at risk for the development of chronic infection, which induces end-stage liver disease such as liver cirrhosis and hepatocellular carcinoma (Lauer and Walker, 2001). However, treatment options for chronic hepatitis C are limited and a vaccine is not available. Due to the serious consequences of its infection in humans, much effort has being made to understand the basic mechanisms of HCV cell entry and infectivity.

HCV cell entry is a multi-step process mediated by several receptors, involving scavenger receptor class B type I (SR-BI), tetraspanin CD81, and tight junction proteins claudin-1 (CLDN1) and occludin (OCLN) (Stamataki et al., 2008). CD81 is a member of the tetraspanin superfamily defined by four transmembrane domains, a conserved CCG motif, and four cysteine residues that form critical disulfide bonds in the large extracellular loop (LEL). CD81 interacts with HCV envelope (E2) glycoprotein via a series of discontinuous amino acid residues in LEL (Flint et al., 2006). Antibodies directed against CD81 as well as a soluble form of the CD81 LEL, are able to inhibit HCV entry into hepatocytes both *in vitro* (Bartosch et al., 2003; Molina et al., 2008) and *in vivo* (Meuleman et al., 2008). In conjunction with viral entry, a role for CD81 in post-binding steps of HCV infection has been demonstrated. Following virus binding, CD81-mediated signals allow the lateral movement of virus particle/CD81 complex and its delivery to areas of cell–cell contact (Brazzoli et al., 2008), which may initiate viral internalization process (Harris et al., 2010).

Tetraspanins associate with tetraspanin and non-tetraspanin proteins to many biological functions, including cell–cell adhesion, cell migration, signaling and proliferation (Hemler, 2008). These associations are thought to occur mainly in membrane compartments such as lipid rafts and constitute tetraspanin-enriched microdomains (TEMs). In most cell lines, CD81 is associated in a high stoichiometry with its partner proteins EWI-F or EWI-2 (Charrin et al., 2003). Both are members of the EWI family, a small Ig-domain family whose members have a single transmembrane domain and several extracellular Ig-domains, as well as a very short cytosolic tail (Stipp et al., 2001). Recently, it was shown that a truncated form of its partner EWI-2 (EWI-2wint) blocked HCV infection (Rocha-Perugini et al., 2008). This inhibitory effect depends on the interaction of EWI-2/EWI-2wint with CD81 (Montpellier et al., 2011). In addition to their direct and robust interaction with partner proteins, tetraspanins also associate with each other dynamically and less

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stoichiometrically. In human hepatocytes, CD81 is associated with CD151 and some other tetraspanins (Rocha-Perugini et al., 2009). However, it is not known whether these interactions play a role in HCV infection.

Palmitoylation occurs at the eight juxtamembrane cysteine residues of CD81 (Delandre et al., 2009). Covalent attachment of palmitate is a post-translational modification that influences protein–protein interactions and association with cholesterol-rich domains of membranes (Linder and Deschenes, 2003). The palmitoylation of the juxtamembrane cysteine residues is present in many tetraspanins, including CD9, CD63, CD81 and CD151 (Charrin et al., 2002). Mutagenesis of these cysteines, for example, abolished the palmitoylation of CD9 (Kovalenko et al., 2004) and of CD151 (Berditchevski et al., 2002), thus reducing their association with other tetraspanins.

Tetraspanins are mainly present in part in a detergent-resistant membrane (DRM) domain. These DRM are reported to be highly ordered cholesterol-enriched microdomains, also termed lipid rafts. These ordered membrane microdomains play an important role in the early steps of virus infection by providing a convenient platform for virus–receptor interactions (Lu et al., 2008; Carter et al., 2009; Medigeshi et al., 2008). Targeting of proteins such as src kinases (Dunphy and Linder, 1998), influenza hemagglutinin (Scheiffele et al., 1997) and caveolin (Uittenbogaard and Smart, 2000) to lipid rafts is dependent on palmitoylation. However, whether palmitoylation alters the association of CD81 with TEMs, and location of CD81 in lipid rafts, and whether these interactions play a role in CD81 mediating HCV cell entry remains to be addressed.

In this study, wild-type, partially or completely palmitoylation-defective CD81 were expressed in CD81-deficient Huh7 hepatoma cells. These cells permitted an examination of the effect of palmitoylation on the interaction of CD81 with TEMs and their role in mediating HCV entry. The data suggest that palmitoylation of CD81 strongly promotes HCV entry, at least in part by regulating the association of CD81 with tetraspanin-enriched microdomains.

Results

Association of CD81 with CD151 is dependent on palmitoylation

To examine the role of palmitoylation in the intermolecular association involving CD81, Huh7 cells were incubated with 2-bromopalmitate (2-BP), which is a potent inhibitor of palmitoylation. As shown in Fig. 1A, 2-BP treatment completely eliminated the incorporation of [^{3}H] palmitate into CD81. When total CD81 content was assayed, a faster migrating band was observed on polyacrylamide gels, which was consistent with the loss of palmitoyl moieties (Fig. 1A). Additional co-immunoprecipitations showed that the levels of CD81-associated CD151 and EWI-2 decreased by almost 95% and 10%, respectively, in 2-BP treated compared to untreated cells (Fig. 1A).

To further demonstrate the role of CD81 palmitoylation in protein–protein interactions, experiments were designed to examine the interactions of palmitoylation-deficient CD81 with CD151 and EWI-2. For transmembrane proteins, palmitoylation typically occurs on intracellular cysteine residues and proximal to transmembrane domains (Resh, 1999). CD81 contains eight juxtamembrane cysteines at position 6, 9, 80, 89, 97, 104, 227 and 228 (Fig. 1B). To evaluate the contribution of palmitoylation to HCV infectivity, CD81-deficient Huh7 cells were stably transfected with wild-type CD81 (wtCD81) or each of three cysteine mutants of CD81. Preliminary characterization of these proteins showed comparable levels of expression by FACS analysis (Fig. 1C). Importantly, CD81m5 incorporated only about 30% of [^{3}H] palmitate compared to wild type CD81, while CD81m8 incorporated only about 10% of [^{3}H] palmitate (Fig. 1C). These observations confirm that intracellular juxtamembrane cysteine residues, proximal to all four TM domains, are palmitoylated in CD81.

The effect of cysteine mutagenesis on associations of CD81 with other tetraspanin web members was analyzed by immunoprecipitation under experimental conditions that maintain tetraspanin–tetraspanin interactions (*e.g.* in 1% Brij97). As shown in Fig. 1D, the amount of CD81 co-immunoprecipitated with endogenous CD151 depended on the number of cysteines in the intracellular and transmembrane domains. Elimination of palmitoylation (CD81m8) almost completely abolished the interaction of CD81 with tetraspanin CD151 (Fig. 1D, upper panel), and slightly reduced the association with its partner, EWI-2 (Fig. 1D, lower panel). Hence, these data suggest that the mutation of juxtamembrane cysteine residues affects the association of CD81 with tetraspanin CD151.

Palmitoylation does not contribute to raft localization of CD81

Since palmitoylation promotes the association of most transmembrane proteins with lipid rafts (Dunphy and Linder, 1998; Scheiffele et al., 1997; Uittenbogaard and Smart, 2000), experiments were designed to investigate whether palmitoylation of CD81 played a role in its partition to DRM. Brij97 lysates of Huh7 cells expressing wild type or mutant CD81 were subjected to density equilibrium centrifugation in sucrose gradients, and the fractions then analyzed by western blotting. As shown in Fig. 2A, wtCD81 appeared in the low density fractions of a sucrose gradient. There was no difference in the distribution of wtCD81 and palmitoylation-deficient CD81, indicating that mutation of juxtamembrane cysteines did not change the flotation properties of CD81 (Fig. 2A). In control experiments, abundant endogenous caveolin-2 was in the low density fractions (Fig. 2A, Fractions 3–5) and CD71 marked the dense fractions (Fig. 2A, Fractions 9–12).

To further dissect the contribution of palmitoylation to CD81 raft localization, lysates from 2-BP-treated and untreated Huh7 cells were subjected to sucrose density gradient centrifugation. After pharmacological block of palmitoylation, wild-type CD81 exhibited similar distribution with sucrose gradients to their palmitoylation-deficient counterparts (Fig. 2B, upper panel). In contrast, 2-BP treatment shifted the distribution of caveolin-2 into the heavier fractions of the sucrose gradient (Fig. 2B, bottom panel).

To verify that the western blotting data reflected plasma membrane localization of CD81 mutants, confocal laser scanning microscopy was performed using antibodies against CD81 and caveolin-2. As shown in Fig. 2C, wtCD81 and CD81 mutants co-localized with caveolin-2. Taken together, these results show that blocking of palmitoylation does not have a significant influence on the raft localization of CD81.

Mutation of juxtamembrane cysteines in CD81 impairs HCV entry

To determine whether palmitoylation of CD81 alters its function as a HCV receptor, each CD81 mutant was analyzed for its capacity to mediate HCV-E2 binding, HCVpp entry and HCVcc infection. To test the capacity of these CD81 variants to interact with HCV glycoproteins, we used a soluble recombinant form of HCV E2 glycoprotein (sE2). As shown in Fig. 3A, sE2 bound to wtCD81 and to all CD81 mutants to similar extents, suggesting that removal of the palmitoylation does not affect binding of CD81 to the HCV envelope.

To examine the effect of CD81 palmitoylation on viral entry, Huh7 cells expressing wild type or mutant CD81 were infected with pseudotyped particles bearing HCV envelope proteins of different genotypes or VSV-G envelopes as control. Compared to