Tight Junction Proteins Claudin-1 and Occludin Control Hepatitis C Virus Entry and Are Downregulated during Infection To Prevent Superinfection∗§

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A tight junction (TJ) protein, claudin-1 (CLDN1), was identified recently as a key factor for hepatitis C virus (HCV) entry. Here, we also show that another TJ protein, occludin, is also required for HCV entry. Mutational study of CLDN1 revealed that its tight junctional distribution plays an important role in mediating viral entry. Together, these data support the model in which HCV enters liver cells from the TJ. Interestingly, HCV infection of Huh-7 hepatoma cells downregulated the expression of CLDN1 and occludin, preventing superinfection. The altered TJ protein expression may contribute to the morphological and functional changes observed in HCV-infected hepatocytes.

Recently, considerable progress has been made in elucidating the molecular mechanisms by which hepatitis C virus (HCV) infects human liver cells. The current accepted model of HCV infection is that virus particles associated with lipoproteins, found circulating in the bloodstream, use glycosaminoglycans and/or the LDL receptor on host cells as initial attachment factors. After binding, the HCV particle interacts with SR-BI and CD81 and is subsequently relocalized to the tight junction (TJ) protein claudin-1 (CLDN1) (6). Next, the HCV particle becomes internalized via clathrin-mediated endocytosis, followed by viral fusion, which likely occurs in early endosomes. Some critical information, however, is missing in such a model with regard to the role of CLDN1. (i) The interaction between CLDN1 and incoming HCV virions has yet to be verified experimentally; (ii) the precise site of viral entry needs to be determined given that CLDN1 predominantly localizes to TJs in polarized cells; and (iii) the potential involvement of other TJ proteins in HCV entry remains untested. We have shown previously that the TJ-like CLDN1 distribution correlates with cellular permissiveness to HCV infection (19). In the current study, we intend to define the importance of junctional CLDN1 and other TJ proteins in HCV entry.

TJ protein OCLN is required for HCV entry. As hepatocytes are highly polarized in vivo, we first sought to investigate whether HCV entry mimics the major group B coxsackievirus (CVB) entry, in which CVB enters polarized epithelial cells through TJs by a complex mechanism requiring attachment to occludin (OCLN) and the induction of caveolar endocytosis (3). To this end, we utilized synthetic interference RNA (siRNA) or packaged retroviruses to deliver short-hairpin-based RNA (shRNA) to knock down the expression of TJ proteins CLDN1, OCLN, ZO-1, JAM-1, and CAR (CVB receptor) to examine the roles of each of the proteins during HCV infection. Targeted sequences of the siRNAs and shRNAs are presented in the supplemental material. As shown in Fig. 1A and B, depletion of OCLN affected neither the expression level nor the localization of CLDN1; however, depletion of ZO-1 by siRNA modestly reduced the CLDN1 level (Fig. 1A). We then performed the infection assay according to a previously established procedure (19). Reduction of CLDN1, OCLN, and ZO-1 expression inhibited entry of human immunodeficiency virus (HIV)-HCV pseudotypes (HCVpp), but not vesicular stomatitis virus G pseudotypes (VSVpp), into Huh7.5.1 (Fig. 1C; see Fig. S1 in the supplemental material). Similar results were observed using cell culture-grown HCV (HCVcc) encoding firefly luciferase (Fig. 1C). Notably, depletion of ZO-1 by shRNA targeting a different region of ZO-1 had minimal effect on HCVpp entry (see Fig. S1 in the supplemental material), suggesting that ZO-1 is unlikely to be directly involved in HCV entry. The observed effect of ZO-1 knockdown on viral entry in Fig. 1C could be due to the modest reduction in CLDN1 level, as ZO-1 is known to determine where claudins are polymerized in TJ strand formation (18).

Next, we examined whether any portion of OCLN, a four-transmembrane protein with a relatively long C-terminal tail, may replace CLDN1 in mediating HCV entry. Specifically, we generated chimeric proteins containing the two extracellular loops from one of these proteins and the C-terminal domain from the other. Chimeric proteins containing the two CLDN1 extracellular loops and the OCLN C-terminal domain were still able to render 293T cells susceptible to HCVpp, but this was not the case for the alternative chimeric protein (see Fig. S2 in the supplemental material). Together, these results imply that CLDN1 and OCLN function distinctly in mediating HCV entry.

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OCLN coprecipitates with HCV E2 in HCVcc-infected hepatoma cells. HCV entry is dependent upon clathrin-mediated endocytosis (1). By screening a pool of pharmacological inhibitors, we found that dynasore, an inhibitor of dynamin, which interacts with OCLN-based cellular structure (10), nearly abolished HCVpp entry (see Table S1 in the supplemental material). Dynamin plays an essential role in receptor-mediated endocytosis via clathrin-coated pits and caveolae (4). Since Huh7 and its derivatives are known to be naturally deficient in dynamin (made by Dharmacon, Ambion, and IDTDNA) at the final concentration of 40 nM by the RNAiMax reagent (Invitrogen) for 24 h. Cells were incubated for an additional 24 h, and the specific knockdown of each protein was verified by immunoblotting (A) and confocal microscopy (B)

FIG. 1. Depletion of OCLN blocks HCV entry. Huh7.5.1 cells (2 × 10⁵) were transfected with the indicated siRNA oligonucleotides (made by Dharmacon, Ambion, and IDTDNA) at the final concentration of 40 nM by the RNAiMax reagent (Invitrogen) for 24 h. Cells were incubated for an additional 24 h, and the specific knockdown of each protein was verified by immunoblotting (A) and confocal microscopy (B). (C) Cells derived from the steps in panel A were infected with the indicated viruses for 2 h and further incubated for 24 to 48 h prior to the luciferase assay. Data plotted are the means ± standard deviations of the results. Bald virus was packaged using the same HIV core construct without the Env-encoding gene, and HCVcc-Luc represents an HCVcc virus with a luciferase gene inserted into its genome. The results shown are representative of three independent experiments.
cell surface but not be concentrated at cell junctions (extra-junction type), were much less efficient in rendering 293T cells susceptible to HCVpp infection than was the wild-type protein (Fig. 3A to C). Furthermore, when confluent 293T cells transfected with wild-type CLDN1 were split and reseeded to avoid the formation of cell-cell contact, they became far less permissive to HCVpp infection despite cell surface expression of CLDN1 (unpublished results). Together, these data suggest that the tight-junctional localization of CLDN1 is critical for viral entry and that HCV entry may require a delicate molecular architecture of multiple proteins, occurring only at TJs.

HCV infection downregulates CLDN1 and OCLN expressions, preventing superinfection. Next, we examined the expression levels of CLDN1 and OCLN during HCV infection and found that they were downregulated following infection (Fig. 4A). Flow cytometric analysis confirmed the downregulation of cell surface CLDN1, but not CD81, following infection (Fig. 4B and data not shown). Consequently, HCVcc-infected cells became refractory to HCVpp infection (Fig. 4C). In order to elucidate which viral protein may be causing this downregulation, we coexpressed individual HCV proteins with CLDN1 in 293T cells. Here, expression of HCV structural proteins, Core and E1E2, significantly inhibited the expression of CLDN1 from a cotransfected plasmid. Deletion of the first 50 amino acids of the Core protein nullified this inhibition (Fig. 4D).

The downregulation of CLDN1/OCLN following HCV infection provides an appealing explanation for the exclusion of HCV superinfection, a state in which infected cells become resistant to future infection, allowing the host cells to contain the infection. It has been documented that HCV infection does result in the exclusion of superinfection, and this was demonstrated to be not due to reduction in CD81 or SR-BI level on cell surface but, rather, mediated primarily by interference at the level of HCV RNA translation and subsequent viral replication (16). While our findings certainly add more pieces to the understanding of the mechanisms by which exclusion of superinfection occurs, they are directly conflicting with a recent report in which Reynolds et al. reported that CLDN1 expression was slightly upregulated in HCV JFH1-infected Huh7.5 cells using confocal microscopy (14). We are unable to explain the discrepancy except that different detection methods, and more importantly, different incubation periods, were noted between the two studies. In fact, it appears that HCV infection may lead to a rather global reduction in TJ proteins, as even the CAR protein level decreased in HCVcc-infected...
orders (17). Indeed, the lack of CLDN1 has been linked to neonatal sclerosing cholangitis syndrome (7). Future work should evaluate hepatocellular CLDN1/OCLN expression levels in well-controlled groups during HCV disease progression.

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