A Novel Function of CD81 in Controlling Hepatitis C Virus Replication

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Running title:
CD81 level determines HCV replication efficiency

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Abstract

The mechanisms of HCV replication remain poorly understood and cellular factors required for HCV replication are yet to be completely defined. CD81 is known to mediate HCV entry. Our study uncovered an unexpected novel function of CD81 in HCV lifecycle that is important for HCV RNA replication. HCV replication occurred efficiently in infected cells with high-level CD81. In HCV infected or RNA transfected cells with low CD81 expression, initial viral protein synthesis occurred normally but efficient replication failed to proceed. The aborted replication could be restored by transient transfection of CD81 expression plasmid. The CD81-dependent replication was demonstrated in both HCV infectious cell culture and HCV replicon cells of genotype 1b and 2a. We also showed that CD81 expression is positively correlated with the kinetics of HCV RNA synthesis, but inversely related to the kinetics of viral protein production, suggesting that CD81 may control viral replication by directing viral RNA template function to RNA replication. Thus CD81 may be necessary for the efficient replication of HCV genome in addition to its role in viral entry.
Introduction

Hepatitis C virus (HCV) infection affects about 170 million people worldwide. Chronic HCV infection is an important cause of liver diseases, leading to cirrhosis and hepatocellular carcinoma (2, 18). The therapy for chronic HCV infection to date is suboptimal and associated with many side effects (12, 13). The mechanisms of HCV replication and persistent infection remain poorly understood (3, 31).

HCV carries a positive- and single-stranded RNA genome consisting of approximately 9600 nucleotides (36). HCV encodes 10 proteins and exploits cellular factors for replication (24, 32, 35, 41). However, many crucial host factors required for HCV RNA replication remain undefined. HCV RNA genome, like other positive-stranded RNA viruses, serves as templates for both viral proteins translation and RNA replication (4, 15, 28), which are expected to be asynchronous in vivo as the template pool is constantly replenished from ongoing HCV infection and replication (4). However, a coordinated translation/transcription process would be predicted if the use of HCV RNA as template is subjected to cellular factor control that directs HCV RNA for specific template function and synchronizes translation/transcription process. CD81 has diverse functions in various biologic processes (23, 39, 48) and is known to mediate HCV entry (10, 30, 34, 49). CD81 has recently been suggested to play a role in post-entry events (8). In this study we identified CD81 as a key cellular factor required for efficient HCV RNA replication and no efficient RNA replication could occur in HCV infected or RNA transfected cells with low level CD81. Our data also showed that utilization of HCV RNA as templates for viral protein and RNA synthesis is mutually exclusive and
suggested that HCV RNA template function for RNA replication may be subjected to CD81 control.
Materials and Methods

Plasmids. HCV JFH1 full cDNA sequence cloned into the pUC19 plasmid (pJFH-1) and the JFH1 subgenomic replicon plasmid (pSGR-JFH1/Luc) have been described previously (46). pJ6/JFH1-p7RLuc2A plasmid (20) was a gift of Charlie Rice (Rockefeller University, NY). pHCV-FLuc-3’-UTR containing the firefly luciferase gene driven by the HCV IRES was provided by Michael Niepmann (40). Human CD81 gene-containing plasmid (pCDM8) (33) is a gift of Shoshana Levy (Stanford University, CA).

CD81 mutant construct was generated by PCR based mutagenesis that converted methionine coding codon (AGT) to a stop codon (TAG) in the reading frame. The primers were: sense: atc aag tac ctg ctc ttc gtc tag aat ttc gtc ttc tgg ctg; anti-sense: tgt tct tga gca ctg agg tgg tca aag cag. Lack of CD81 expression was verified by CD81 staining after transfection with this construct.

Cell lines and culture. Huh 7.5 cells (7) maintained in our laboratory contain less than 50% CD81 positive population. To obtain cell populations with different percentages of CD81 positive cells, three populations were selected from Huh 7.5 cell by FACS sorting: CD81 high-level population (CD81-H) with 90% CD81-positive cell, CD81 low-level 1 (CD81-L1) with 10% CD81-positive cells and CD81 low-level 2 (CD81-L2) with no detectable CD81 expression. All three populations were subjected to the same culture condition as the Huh7.5 cells. They were expanded, cryopreserved and thawed for subsequent experiments. Continuous passages of these cells were avoided because of concern for altered phenotype during long-term culture. Typically, these cells were not passaged for more than ten times after thawing. CD81 expression profiles of the three populations were monitored by FACS for CD81 expression after re-establishment.
of cryopreserved cells and during experiments. The phenotype of CD81 expression remained stable during this short-term culturing condition. For FACS analysis, cells were trypsinized and then washed with MACS buffer containing PBS, BSA, EDTA and 0.09% azide, pH7.2 (Miltenyi Biotec Inc, Auburn, CA). Approximate 10⁶ cells were suspended in 100 µl MACS buffer and mixed with 10 µl FITC-conjugated anti-human CD81 (JS-81, BD Pharmingen) or 10 µl FITC-conjugated mouse IgG1 as isotype control (BD Pharmingen) and incubated on ice for 20 min and washed twice with MACS buffer and suspended in 400 µl MACS buffer for FACS analysis. FACS for Sr-B1 expression was performed with mouse monoclonal anti-human CLA-1 (BD Transduction laboratories).

Huh7-25 clone, and subgenomic HCV replicon cell lines Huh7/Rep-Feo (genotype 1b) and pSGR-JFH1-C4/1 (genotype 2a) have been described (1, 25, 43). Another subgenomic HCV JFH1 replicon cell line (SGR-JFH1-FLuc/Neo expressing FLuc-neo as the reporter was generated similarly. All replicon cells were maintained in the presence of 500 µg/mL of G418 (Invitrogen).

**siRNA and transfection.** CD81 siRNA pool (siGenome SMARTpool duxel ID: D-017257-01, 03, 04 and 05) and non-target control 2 siRNA (ID: D-001810-02-05) were purchased from Dharmacon Inc (CO). siRNA transfection was carried out in 12-well plates by Oligofectamine reagents (Invitrogen) and the final siRNA concentration was 50 nM.

**Production of HCV pseudovirus and HCV JFH1 virus and viral inoculation.** HCV pseudovirus (H77 strain) was a gift of Rice CM (Rockefeller University, New York). HCVpp infection and measurement of infectivity have been described previously (14). For transfection of JFH1 HCV RNA, cells were plated onto a 6-well plate at
1.5x10^5 cells per well for overnight. Cells were washed twice with PBS after the medium
was removed and 1ml Opti-MEM (Invitrogen) was added. Then the transfection mix
containing 1000 µl Opti-MEM, 12 µl DMRIE-C (Invitrogen) and 10 µg in vitro
transcribed JFH1 RNA was added to cells and incubated for 4 hrs. Then Opti-MEM was
replaced with the regular DMEM medium. The medium containing high titers of HCV
(from day 3 to day 7) were collected as an initial inoculum. For HCV infection, Huh7.5
cells were plated onto 6-wells plates at 1.5x10^5 cells per well for overnight. The cells
were inoculated with cell culture derived HCV (HCVcc) as described above in HCV
RNA transfection section. The inoculum was removed at day 1 post inoculation (pi) and
the infected cells were transferred to 10 cm dish at day 3 pi. The medium was collected
and pooled together from day 4 to 7. The medium containing high titer HCV was
aliquoted and stored at -80°C as inoculum. The inoculum was used an MOI of 200:1
(based on HCV RNA genome copies per cell). In our experience, one infectious unit is
equivalent to about 200-500 HCVcc RNA genome copies.

**HCV RNA and core antigen assays.** Trypsinized cells were washed with PBS
once and RNA was extracted by using Trizol (Invitrogen). RNA was resuspended in 50
µL DEPC-H2O and the final concentration was adjusted at 0.1 µg RNA /µL. 3 µL RNA
was used for quantitative-RT-PCR and HCV RNA copies were expressed as per µg RNA.

For extraction of RNA in the medium, 250 µL medium was mixed with 750 µl Trizol LS
(Invitrogen) as described by the manufacture. RNA was re-suspended in 50 µl DEPC-
H2O. 3 µL RNA was used for q-RT-PCR and HCV RNA copies were normalized as per
mL medium. HCV RNA q-RT-PCR was described previously (42). Primers and Taqman
probe for CD81 RNA quantification were: sense primer: agg gct gca cca agt gc; anti-
sense primer: tgt ctc cca gct cca gat a; Taqman probe sequence: 5’ 6-FAM caa gta cct gct 1
cct cgt ctt caa 3’TAMRA. For minus-strand RNA detection we applied the method of
Lanford et al (27). A sense primer (gcg tta gta tga g7c tac agc at nt position 87-110 of
5’ UTR of JFH1 genome) was used to synthesize cDNA from the minus-strand RNA
templates at 70°C by rTth DNA polymerase (Applied Biosystems). The reverse
transcription activity of rTth was blocked by adding the buffer chelating MnCl₂ and the
DNA polymerase activity was facilitated by including MgCl₂ containing buffer after RT
reaction. The Q-PCR reaction was performed as the detection of total HCV RNA.

For intracellular core determination, approximately 300,000 cells were suspended
with 100 µL lysis buffer, pH 7.5 (20 mM Tris, 1% NP40, 1% Na Deoxycholate, 0.1%
SDS and 1x protease inhibitor cocktail) and incubated on ice for 20 min. The supernatant
was transferred to a new tube after a brief spin to remove cell debris. 10 µL supernatant
was diluted 10-fold for core protein ELISA (Ortho). The total intracellular core protein
was expressed as atto mole per well. A similar amount of uninfected cells at each time
point were harvested and prepared at the same way as the infected cells for negative
control in ELISA tests. For extracellular core determination, 5 µL of culture supernatant
was diluted in 20-fold and used for core ELISA. The core protein in the medium was
expressed as atto mole/mL.

**Single cell-based q-RT-PCR assay.** The assay has been described previously for
single cell HBVcccDNA quantification (50, 51). Briefly, trypsinized cells were
suspended in the DMEM medium and counted. The initial cell concentration in the
suspension was approximately 10⁵/ml. The cell suspension was then subjected to two
steps and 100 fold dilutions with buffer containing 150 mM Tris-HCL pH 8.0, 1mM

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EDTA and 10 mM NaCl. Then the cell suspension concentration was further adjusted to
100 cells per ml. 10 µl cell suspension containing approximately a single cell was
manually distributed to each of the 96 wells in the plate. 10 µl proteinase K solution was
added to each well (final concentration: 2 mg/mL) and incubated at 50 C for 60 min and
the proteinase K was inactivated at 75 C for 15 min. 10 µL solution were transferred from
each well to a new plate. One plate was used for HCV RNA, and the other was for CD81
RNA q-RT-PCR.
Results

Divergent levels of HCV RNA in various CD81-expressing cells after HCV infection. To investigate HCV infection and replication efficiency in cells with different CD81 expression, two cell populations, CD81-high (CD81-H) and CD81-low 1 (CD81-L1) containing 90% and 10% CD81 positive cells respectively, were first isolated from Huh 7.5 cell through cell sorting. A third cell population containing barely detectable CD81 expression was isolated from CD81-L1 cells and designated as CD81-L2. The difference in CD81 expression level detected by FACS was also confirmed by CD81 RNA quantification among three cells (Fig.1A and 1B). CD81-H and CD81-L1 cells not only differ by the percentage of CD81 positive cells, but also differ in CD81 expression intensity by relative mean fluorescence intensity (up to 4-fold)(Fig. 2B). The CD81 expression profiles of the various cell lines by FACS were similar to a previous paper, in which Huh7-derived clones with variable CD81 expression levels were selected (1). CD81 expression did not increase appreciably after permeabilization of cells before staining, indicating that most of the expression was on the cell surface (Fig. S1). No major difference in SR-BI expression level was detected among the parental Huh 7.5 cell and its derived cell lines (Fig. S2). Each cell population was inoculated with the same size inoculum (MOI of 200:1 based on HCV genome copies per cell). The intracellular HCV RNA was determined at day 6 post-inoculation (pi) by quantitative RT-PCR (q-RT-PCR). As shown in Fig. 1C, a >200-fold difference in HCV RNA level was found between CD81-H and CD81-L1 cells, whereas no HCV RNA was detectable in CD81-L2 cells (detection limit of $10^3$ copies/mL). The difference was initially thought to result from the difference in percentage of CD81 positive cells, assuming that only cells with
detectable cell surface CD81 expression could be infected by HCV (1). It would take multi-rounds of infections to have all 90% of CD81 positive cells infected in CD81-H cells. We reasoned that a 9-fold difference in CD81-positive cells might not explain the >200 fold difference in HCV RNA levels between CD81-H and CD81-L1 cells and an even bigger difference between CD81-H and CD81-L2 cells, suggesting that the infected CD81-H cells might support higher HCV replication in addition to the difference in the number of infected cells.

Additional role of CD81 in HCV RNA replication other than viral entry.

There are two possible explanations (or a combination of both) for the higher HCV RNA level in infected CD81-H cells. CD81-H cells support more efficient HCV entry, or CD81-H cells support more efficient HCV RNA replication. To investigate these two possibilities, CD81-H, CD81-L1 and CD81-L2 cells were tested for HCV entry efficiency by the HCVpp assay (6, 14). Three cell lines did not show any significant differences in HCVpp infectivity (Fig. 2A), despite a 4-fold difference in CD81 relative mean fluorescence intensity (rMFI)(Fig. 2B) among the populations tested. It is noteworthy that CD81 expression level in CD81 positive cells fluctuates periodically. CD81 expression level showed a two-fold reduction in rMFI (Fig. 2B) in retesting of CD81-H cells with HCVpp infection. No corresponding reduction of HCVpp entry efficiency was observed. HCVpp infectivity was significantly suppressed by anti-CD81 antibodies in all cells tested, indicating that CD81 is indeed important for viral entry (Fig. 2A). Although the three cell lines did not show major differences in HCVpp infectivity, the CD81-L2 cells did show somewhat lower luciferase activities in the HCVpp assay than the CD81-H cells, suggesting that the very low level of CD81 may be affecting viral entry.
To further investigate the viral entry efficiency, each cell population was inoculated with HCVcc. HCV infected cells were harvested by trypsin treatment and extensive washing to eliminate surface-bound virus (8) at 4 h pi and intracellular HCV RNA was determined. There was no difference in HCV RNA level among the three populations (Fig. 2C).

The HCVcc entry data was confirmed by infection with HCVcc containing the luciferase reporter gene (20). The luciferase activity was measured at 24 h pi and no difference was detected among the three cell populations tested (Fig. 2D). Both HCVpp and HCVcc data suggest that cells with high CD81 levels do not facilitate more HCV entry. This finding prompted us to consider that high level CD81 might not be sufficient to increase viral entry efficiency if other cellular factors (37) involved in HCV entry, like SR-BI (5), were limiting (Fig. S2). It has been shown that HCVcc or HCVpp can enter into cells with low level CD81 (1, 26, 52) and cells with different CD81 levels have no noticeable differences in initial HCV infection (26). It is conceivable that a threshold of CD81 expression is necessary for viral entry and beyond this threshold, more efficient viral entry does not occur. This observation of a CD81 threshold level has already been suggested previously (26). Thus, our data suggests that the high-level of HCV RNA in CD81-H cells may result from a more efficient HCV RNA replication.

To study whether cells with higher CD81 support more efficient RNA replication, we investigated whether HCV RNA replication efficiency is correlated with CD81 level at the single-cell level. Both HCV RNA and CD81 RNA levels were determined in the same cells harvested on days 3 and 6 pi by a single cell-based RNA quantification technique (50, 51). Fig. 3A showed distribution patterns of CD81 RNA and HCV RNA...
copies per cell at day 6 pi. The highest level of CD81 RNA per cell was 3600 copies while the lowest was under 100 copies. HCV replication efficiency differed significantly among individual cells. The highest level of HCV RNA per cell was 89,600 copies. Average HCV RNA copies per cell increased by 13-fold from 320 to 4300 copies in cells with an average of 400 CD81 RNA copies between day 3 and 6 pi (Fig. 3B). In cells with an average of 1800 CD81 RNA copies, the average HCV RNA copies increased by more than 200-fold from 130 to 35,000 copies over the same time period (Fig. 3C). The relationship between CD81 RNA and HCV RNA levels in the same individual cells were further analyzed. As shown in Fig. 3C, there was a positive correlation between CD81 RNA and HCV RNA level in the same cells on day 6 pi (p<0.0001), suggesting that HCV RNA replication efficiency is dependent on CD81 level in individual cells. In contrast, the percentage of HCV infected cells, as demonstrated by single-cell assay, did not show a corresponding difference between high and low CD81-expressing cell populations (Fig 3D). In fact, a somewhat higher percentage of lower CD81-expressing cells were positive for HCV RNA.

**CD81-L cells do not support efficient viral replication after viral protein synthesis.** To determine whether CD81-L cells support efficient HCV RNA replication, CD81-H, CD81-L1 and CD81-L2 cells were transfected with JFH1 subgenomic replicon RNA containing the luciferase gene. The cells were harvested for determination of luciferase activities and HCV RNA levels at various time points. As shown in Fig. 4A (left panel), there were no differences in luciferase activities among all cells tested up to day 2 post-transfection. However, efficient HCV RNA replication was only detected in CD81-H but not in CD81-L cells as reflected by the decreasing HCV RNA levels in
CD81-L and Huh7-25 cells (Fig. 4A, right panel). To exclude the possibility that the requirement of high-level CD81 for HCV RNA replication is a unique feature of the Huh 7.5 cell lines we selected, a different Huh7 cell clone (Huh7-25) with little CD81 expression (1)(Fig. S4A) was tested and similar result was obtained as the CD81-L2 cells. Equally efficient protein translation in CD81-L1 and CD81-L2 cells was further demonstrated by transfection of a construct containing luciferase reporter gene under the control of the HCV IRES (40)(Fig.4B).

We investigated the difference in RNA replication efficiency during the first 24–h period after cells were transfected with JFH1 subgenomic RNA or JFH1 genomic RNA. As shown in Fig. 4C and 4D, a 10-fold or more difference in intracellular or extracellular HCV RNA level was detected between transfected CD81-H and CD81-L2 cells. In addition, HCV minus-strand RNA was detected only in the CD81-H cells. These data indicate that these cells support protein synthesis equally after RNA transfection, but cells with low level CD81 do not support efficient HCV RNA replication. It also raises the interesting question that HCV protein reporter constructs, like the luciferase, may not necessarily reflect the HCV RNA replication status in certain situation.

Viral protein synthesis and RNA replication were also tested in infected cells. Intracellular core protein and HCV RNA levels were analyzed at different time points after infection of CD81-H, CD81-L1 and CD81-L2 cells. Consistence with Fig.4A, the core protein levels were similar among infected CD81-H, CD81-L1 and CD81-L2 cells on days 2 and 3 (Fig.5A), suggesting that CD81-L cells are capable of supporting viral protein synthesis after viral entry. The newly synthesized core protein detected by ELISA was further verified by core staining-positive cells in these cells. As many as 27% of
CD81-L2 cells became core-positive on day 3 (Fig. S3), but not on day 1 (data not shown), suggesting that the detected core protein was newly synthesized in infected CD81-L2 cell. However, no HCV RNA replication occurred in infected CD81-L2 cells while only a modest HCV RNA replication was detected in CD81-L1 (Fig. 5B). These data suggest that efficient HCV RNA replication did not occur in CD81-L cells despite an active viral protein synthesis. In contrast, efficient HCV RNA replication occurred in CD81-H cells with HCV RNA level 10-fold and 1000-fold greater than that of CD81-L1 cells at 24 h and on day 6 pi, respectively (Fig. 5B), suggesting that viral replication proceeds efficiently in CD81-H cells.

The difference in HCV RNA replication levels between CD81-L1 and CD81-H was further demonstrated by analyzing the distribution of newly synthesized core protein. A higher amount of core protein was detected intracellularly in CD81-L1 cells from day 1 to 3 pi while more core protein was detected extracellularly in CD81-H cells during the same time period (Fig. 5C), suggesting that CD81-L1 cells were infected by HCV and supported viral protein synthesis. However, the synthesized core protein was mainly intracellular and accumulated to a higher level in CD81-L1 cells. In contrast, the synthesized core protein in CD81-H cells was efficiently exported to the medium, probably in the form of virions as a result of active HCV RNA replication. This observation was further supported by the kinetics of intracellular and extracellular HCV RNA levels after infection. Both intracellular and extracellular HCV RNA levels in CD81-H cells were 100-fold higher than that of CD81-L1 cells (Fig. 5D).

Ectopic expression of CD81 leads to efficient HCV RNA replication in CD81-L cells. To test directly whether high-level CD81 is required for efficient HCV RNA
replication, CD81-L2 cell was first transfected with JFH1 subgenomic replicon or JFH1 genomic RNA, and then transfected with CD81 expression plasmid or vector 1 day later. As shown in Fig. 4D and 6A, CD81-L2 cells showed decreasing HCV RNA level after HCV RNA transfection, suggesting a lack of HCV RNA replication. In contrast, following CD81 DNA transfection, CD81-L2 cells showed significantly increasing HCV RNA levels, similar to CD81-H cells, suggesting that HCV RNA replication was restored in CD81-L2 cells after CD81 transfection. To further explore the role of CD81 in viral replication, CD81-L2 cells were first infected with HCV, and after infection cells were washed extensively and replated onto 6-well plates on day 1 pi. The replated cells were then transfected with CD81 expression plasmid on day 2 pi. Cells were harvested at various time points to determine HCV RNA levels. As shown in Fig. 6B, infected CD81-L2 cells did not support HCV RNA replication at all. In contrast, CD81-L2 cells transfected with CD81 after inoculation demonstrated a rapid increase in HCV RNA level. The kinetics of the increase suggests this early increase likely occurs at the replication level. It is unlikely that this rapid increase is the effect of CD81 transfection on HCV entry because CD81-mediated HCV entry should occur within one hour of infection (11, 17). However the later increase probably represents enhanced CD81-dependent viral spread. These data provide direct evidence that a high level of CD81 is required for efficient HCV RNA replication, and the inability of HCV to replicate in CD81-L2 is a result of low-level CD81. This experiment also supports the above finding that HCV can enter into CD81-low cells but fails to initiate replication efficiently. Huh7-25 cells were also tested in this replicon transfection experiment. As shown in Fig. 6C, JFH1 subgenomic replicon RNA only replicated in Huh7-25 cells transfected
with CD81 DNA, but not in cells with vector DNA or no DNA transfection, confirming that the Huh7-25 clone (similar to the CD81-L2 cells) does not support efficient HCV RNA replication. Like CD81-L2 cells, Huh7-25 cells showed little virus production after infection with JFH-1 HCVcc, However, a robust viral production was detected in the infected Huh7-25 cells that were later transfected with CD81 expression plasmid (Fig. 6D). To determine that CD81-mediated entry did not account for this increase, CD81 blocking antibody was added to the medium after transfection. CD81-H cells were used as a control and showed that addition of CD81 antibodies after infection substantially inhibited viral spread (Fig. 6D, top panel), suggesting that the recently reported CD-81 independent cell-to-cell transmission (45, 47) does not play a major role here. In contrast, Huh7.25 cells transfected with CD81 showed a substantial increase (2 logs) in HCV RNA levels despite the addition of CD81 antibodies on the day of CD81 transfection (Fig. 6D, bottom panel). A further increase (1 log) was noted without the addition of CD81 antibodies after CD81 transfection, probably due to the effect on viral spread.

Modulation of HCV replication by CD81 in HCV replicon cells. To further study the effect of CD81 expression on HCV replication, several subgenomic replicon cells (25, 43) were studied. Since subgenomic replicon cells harbor HCV RNA sequences covering only the NS3-NS5 region, infectious virus is not produced and viral spread does not occur. HCV RNA level in a JFH1 subgenomic replicon cell line with low CD81 level (26)(Fig. S4B) was studied by transiently transfecting CD81 expression plasmid. After CD81 transfection, a 3-6 fold increase of HCV RNA levels was observed in this cell line (Fig. 7A). Control transfection with empty vector or CD81 mutant had no effect on HCV replication.
Two other subgenomic replicon cell lines Huh7/Rep-Feo (1b) (43) and Huh7/pJFH-Feo (2a) (25) had high-level CD81 (Fig. S4C and D) and were treated with CD81 siRNA (Dharmacon) to reduce CD81 expression by 80% or more. HCV RNA level was reduced by 4-6-fold in CD81 siRNA-transfected cells, whereas control siRNA transfection had no effect (Fig. 7B and C). The data showed that replication of both JFH1 (genotype 2a) and 1b RNA is CD81-dependent. Lowering of HCV RNA level as a result of the specific reduction of CD81 by siRNA confirmed the causative relationship between CD81 level and HCV replication efficiency.

The CD81-dependent HCV RNA replication was further illustrated by the kinetics of HCV RNA and CD81 RNA levels in the 1b and 2a subgenomic replicon cells with high CD81 expression. CD81 levels in those cells fluctuated and so did the HCV RNA levels. A positive correlation between the two kinetics was observed (Fig. 7D), suggesting the importance of high-level CD81 in efficient HCV replication.

**CD81 in HCV RNA template function.** Like other positive-strand RNA viruses, the use of the same viral RNA as templates by viral protein translation and RNA transcription in HCV life cycle should be mutually exclusive. The experimental data supporting this notion in positive-strand RNA viruses were generated from in vitro cell-free system (4, 15). The template pool is constantly replenished as in vivo infection proceeds and the two processes are expected to be asynchronous. However, a clear pattern for a single dominant template function at a time would also be predicted if the template function is subjected to control by cellular factor(s) allowing the coordinated protein translation and RNA transcription. In deed, we observed a pattern for mutually exclusive use of HCV RNA as templates for viral protein translation versus RNA
replication in infected cell culture when the kinetics of HCV RNA and core protein level
were analyzed. As shown in Fig. 8, the kinetics of the intracellular HCV RNA and the
core protein level were opposite at each time point: an increasing intracellular HCV RNA
level was accompanied by a decreasing core protein level and vice versa. Our data not
only supports the mutually exclusive use of HCV RNA for each template function in
vivo, but also suggests that cellular factors may direct HCV RNA to one template
function at a time. When the kinetics of the intracellular HCV RNA, core protein, and
CD81 levels were analyzed, changing CD81 RNA level was positively correlated with
the changing HCV RNA level, but was inversely related to the core protein level (Fig. 8).
The positive and inverse correlations among CD81, HCV RNA and viral protein kinetics
suggest that CD81 may be one of the cellular factors directing HCV RNA to the
replication process. The viral protein level appears normal while HCV RNA level is
decreasing when CD81 level is low, suggesting that templates can still be used for viral
protein translation, but not for RNA synthesis. This is probably why no difference was
detected in the luciferase activities or core protein levels in CD81-L cells from those of
CD81-H at the early phase of transfection or infection. Thus, CD81 may control HCV
RNA replication, possibly through directing HCV RNA templates function to RNA
replication.
Discussion

CD81 is known to mediate viral entry in HCV infection (10, 30, 34, 49) and also implicated in cell-to-cell transmission of HCV infection (45, 47). Our study showed that significant differences existed in HCV RNA levels after HCV infection among CD81-H, CD81-L1 and CD81-L2 populations and could not be simply explained by the CD81 entry function. This observation prompted us to investigate whether CD81 is required for additional steps in HCV lifecycle, such as RNA replication. Using a variety of techniques and cell lines, we uncovered a novel function of CD81 in HCV lifecycle that is indispensable for HCV RNA replication. CD81 is a tetraspanin family member and is enriched in the lipid rafts of membranous compartments of the cells, where HCV RNA replication is believed to take place (9, 16). The requirement for CD81 participation by HCV replication process can be facilitated by the physical proximity of CD81 to HCV replicating site.

To explain our data and the proposed dual functions of CD81 in the context of HCV infection and replication, we reason that a low threshold amount of CD81 is required for the HCV entry function but a much higher level of CD81 is necessary for efficient HCV replication subsequent to viral entry. Koutsoudakis et al (26) previously reported that about 70,000 CD81 molecules in a cell appear to be the threshold for viral entry. Our data suggest that the three cell lines with very different levels of CD81 allow similar viral entry but appear to support divergent efficiencies of HCV replication that correlate well with the CD81 levels. It is interesting to note that CD81-L2 cells, despite having very low level of CD81, can still support viral entry, although at a somewhat
lower level than the higher CD81 expressing cells. The CD81 expression level of the L2
cells is probably just around the “threshold level” for viral entry.

Our data provides evidence for the mutually exclusive use of HCV RNA as
templates for either RNA replication or protein synthesis in infected cell culture. Two
lines of evidence support that the use of HCV RNA for RNA replication is subjected to
cellular factor control, such as CD81. One is the absence of efficient RNA replication
after viral protein translation in HCV-infected and RNA transfected CD81-L1 and L2
cells, suggesting that RNA replication could not spontaneously occur when the CD81
level was low. The other evidence is that a clear pattern for one dominant template
function at a time was shown in infected CD81-H cells, suggesting that there is a
coordinated process that directs HCV RNA molecules to RNA replication function. It is
likely that cellular factors are involved in directing viral RNA molecules to two distinct
template functions. On the other hand, viral protein synthesis is negatively correlated to
CD81 and HCV RNA levels. These data suggest that the template function of HCV RNA
is controlled by cellular factors like CD81, which directs HCV RNA to its replication
function instead of protein translation. However it is not clear how CD81 exerts this
function.

CD81 may directly assist in the assembly of HCV replicase complex including
NS5B, contributing to viral RNA replication. Alternatively, CD81 may be indirectly
linked to a cellular pathway that is critical for efficient viral replication, Brazzoli et al, in
a recent study, showed that the engagement of CD81 during HCV infection activates the
Raf/MEK/ERK signaling cascade and that this pathway affects post-entry events of the
HCV lifecycle, presumable at the replication step (8). Further experiments are needed to elucidate the molecular mechanism of this novel CD81 function.

CD81 is reported to have diverse functions in biologic process. For instance, CD81 is implicated in metastasis of cancer cells (21). CD81 can influence adhesion, morphology, activation, proliferation, and differentiation of B, T, and other cells (22). In parasite infection, hepatocyte CD81 is required for plasmodium falciparum and plasmodium yoelii sporozoite infectivity (39). CD81 is also implicated in modulation of infectivity, enhancement of viral gene expression and promotion of virus assembly, budding and cell-to-cell spread in HIV lifecycle (19, 38, 44). Identification of this novel CD81 function in HCV replication indicates that CD81 plays a more pleiotropic role in HCV lifecycle besides its well-defined role in viral entry. Our data suggest that CD81 has duel function in HCV infection: a low threshold level of CD81 required for viral entry and a higher level of CD81 necessary for efficient HCV RNA replication. The dependence of HCV replication on CD81 creates an inherent vulnerability for HCV replication. Thus CD81 functions could be explored for potential therapeutic development because of the multiple roles of CD81 in HCV infection, as had been explored in a recent study in the Alb-uPA/SCID mouse model engrafted with human hepatocytes (29).
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Demonstration of in vitro infection of chimpanzee hepatocytes with hepatitis C virus


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**Figure Legends**

FIG. 1. CD81 expression and HCV RNA levels among the three Huh7.5 cell populations. CD81 cell surface expression and CD81 RNA level of the three cell lines were monitored regularly and the representative profiles are shown. (A) Percentages of CD81 positive cells detected by FACS (blue: CD81; red: isotype IgG1). The relative mean fluorescence intensity (rMFI: ratio of CD81 MFI over IgG1 control MFI in the same cell population) of each cell population is shown. (B) CD81 RNA expression level determined by q-RT-PCR. (C) Intracellular HCV RNA level in CD81-H, CD81-L1 and CD81-L2 cells, detected by q-RT-PCR at day 6 post-infection (pi) with JFH1 HCVcc. nd: not detectable. Mean values (triplicates) ± standard deviations (s.d.). Data from one of three independent experiments are shown.

FIG. 2. Similar efficiency of viral entry among the three Huh7.5 cell populations. (A) Luciferase activities measured at 48 h after HCVpp inoculation. Mean values (triplicates) ± s.d. (B) The CD81 relative mean fluorescence intensity among the three cell populations were monitored in this experiment. Significant differences in RMFI among the cell populations exist: p=0.0084 CD81-H (test 1) vs (test 2); p= 0.0026 and p=0.0056 CD81-H (test 1) vs CD81-L1 and vs CD81-L2, respectively. (C) Intracellular HCV RNA level determined at 4 h pi by q-RT-PCR. Cells were harvested by 8 min trypsin treatment that removes almost all bound viruses, but not internalized viruses (data not shown).
FIG. 3. Single-cell correlation of HCV RNA and CD81 levels. Huh7.5 cells were infected with HCVcc and subjected to single-cell-based qRT-PCR assay. (A) The distribution of CD81 and HCV RNA copies/cell measured at day 6 pi in individual cells. (B) HCV and CD81 RNA levels were determined on day 3 and 6. Cells were divided into those with 100-1000 (top panel) and those with 1000-10000 (bottom panel) CD81 copies/cell, and plotted for their corresponding HCV RNA copies/cell. (C) Correlation of HCV RNA with CD81 RNA copy numbers in individual cells on day 6 pi was shown (r=0.58133, p<0.0001, by Microcal Software Inc. MA). (D) Percent of HCV infection in cells with different CD81 levels. Infected cells were harvested at day 3 pi and HCV RNA and CD81 RNA copies in the same cells were determined. The percentage of infection in each group was calculated by dividing the number of cells with detectable HCV RNA by the number of the total cells in each of three populations that contained <100, 100<1000, and >1000 copies of CD81 RNA per cell.

FIG. 4. Protein synthesis and RNA replication among cell populations with different CD81 level. (A) Luciferase activities (left panel) and HCV RNA levels (right panel) measured at different time points after JFH1 subgenomic replicon RNA (pSGR-JFH1/Luc) transfection of Huh7.5 cells. Mean values (triplicates) ± S.D. (B) Luciferase
activities of Huh7.5 cells after transfection with a construct containing luciferase gene

Intracellular total and minus strand RNA level at 24 h after pSGR-JFH1/Luc RNA transfection. Cells were transfected and harvested at 4 and 24 h. The total and minus HCV RNA was determined by Q-RT-PCR. The minus-strand HCV RNA assay has a detection limit of about 500-1000 copies/µg of total RNA. ND: not detected. (D)

Intracellular and extracellular RNA levels at 24 h after transfection with JFH1 genomic RNA. CD81-H and CD81-L2 cells were first transfected with JFH1 RNA, and cells and medium were harvested at 4 and 24 h later for determination of HCV RNA levels.

Another set of CD81-L2 cells were transfected with CD81 expression plasmid at 24 h after JFH1 RNA transfection and harvested 24 h later (48 h after JFH1 RNA transfection) for HCV RNA determination as above. Mean values ± S.D. One of two independent experiments is shown.

FIG. 5. HCV core protein and HCV RNA levels in the three CD81 cell populations. (A) Intracellular core protein levels of infected CD81-H, CD81-L1 and CD81-L2 cells were determined by ELISA at day 2 and 3 pi. (B) Intracellular HCV RNA levels of the three cell lines were measured by q-RT-PCR. Intracellular (top) and extracellular (bottom) core (C) and HCV RNA (D) levels at various time points pi were measured. Mean values ± S.D. One of three independent experiments is shown.

FIG. 6. Transient expression of CD81 restores HCV RNA replication in CD81-L2 and 7-25 cells. (A) JFH1 subgenomic HCV RNA (pSGR-JFH1/Luc) was transfected into
CD81-H or L2 cells in triplicate followed by transfection of CD81 expression plasmid (CD81-L2+CD81 tf) or a control plasmid (CD81-L2+Vector tf) 24 h later. HCV RNA was harvested and determined immediately and 2 day after CD81 transfection. (B) CD81-L2 cells were infected with HCVcc and 24 h later transfected with CD81 expression plasmid. No tf: no transfection; Control tf: transfection with a control plasmid. Intracellular HCV RNA was then quantified at various time points. (C) JFH1 subgenomic HCV RNA was transfected into Huh7-25 cells in triplicate followed by transfection of CD81 expression plasmid (7-25+CD81 tf) or a control plasmid (7-25+Vector tf) 24 h later. HCV RNA was harvested and determined immediately and 2 day after CD81 transfection. (D) CD81-H (top) and Huh 7-25 cells (bottom) were infected with HCVcc and Huh7-25 cells were transfected with CD81 expression plasmid 24 h later. Both cells were treated with anti-CD81 antibody at various time points (anti-CD81 day 0: anti-CD81 antibody was added together with HCVcc; anti-CD81 day 1: CD81 antibody was added on day 1 pi; CD81 tf: HCVcc infection followed by transfection with CD81 1 day later; CD81 tf+anti-CD81: HCVcc infection followed by transfection of CD81 1 day later with the addition of anti-CD81). HCV RNA levels in the culture supernatant were determined at various time points. One of three independent experiments is shown.

FIG. 7. Modulation of CD81 level changes HCV RNA replication efficiency. (A) JFH1 subgenomic replicon cells with low CD81 level (JFH1 replicon #1) were transfected with CD81 expression plasmid and HCV RNA levels were determined on days 3 and 4 after transfection. Fold increase in HCV RNA level after transfection is shown. CD81 KO: CD81 plasmid with mutated start codon. (B and C) Two HCV subgenomic replicon cell
lines 1b (B) and JFH1 #2 (C), both of which have high CD81 expression, were treated with CD81 siRNA to silence CD81 expression. CD81 RNA (top) HCV RNA (bottom) levels were determined on days 2 and 3 later. NC: negative control (non targeted # 2) siRNA. (D) CD81 and HCV RNA levels were determined in the 1b (top) and JFH #2 (bottom) subgenomic replicon cells at various time points after plating. Mean values ± s.d. One of three independent experiments is shown.

FIG. 8. Relationship of the intracellular HCV RNA and core protein level to CD81 levels during viral replication. CD81-L1 cells were infected with HCV and harvested at various time points. Intracellular HCV RNA, CD81 RNA and core protein levels were determined. Amole/well: attomole/well. Mean values ± s.d. One of two independent experiments is shown.
Figure 1

A

Huh7.5  CD81-H  CD81-L1  CD81-L2

rMFI: 2.1  rMFI: 4.5  rMFI: 1.3  rMFI: 1.1

CD81 Staining Intensity

% Cells

0 20 40 60 80 100

B

CD81 RNA

copies/µg RNA

CD81-H  CD81-L1  CD81-L2

1.0E+03  1.0E+04  1.0E+05  1.0E+06  1.0E+07

C

HCV RNA

copies/µg RNA

CD81-H  CD81-L1  CD81-L2

ND

1.0E+04  1.0E+05  1.0E+06  1.0E+07  1.0E+08  1.0E+09
Figure 2

C
Intracellular HCV RNA (copies/µg RNA)

D
Luciferase activity of HCVpp

Luciferase activity of HCVcc-Rluc infection

Relative CD81 MFI ratio
Figure 3

A

Percent of cells

CD81 RNA copies per cell

HCV RNA copies per cell

CD81

HCV

RNA copies per cell

B

Average HCV and CD81 RNA (copies per cell)

CD81 (100-1000)

CD81 (1000-10000)

Day 3 Day 6

C

Percent of HCV infected cells

HCV/RNA copies per cell (log)

CD81 RNA copies per cell

D

Percent of HCV infected cells

CD81 RNA copies per cell

r=0.58133
p<0.0001

<100 100-1000 >1000

<100 100-1000 >1000

<100 100-1000 >1000
Figure 4

A. Luciferase activity per well
B. HCV RNA (copies per µg RNA)

C. HCV RNA (copies per µg RNA)

D. HCV RNA (copies/mL)
Figure 5

A. Intracellular core protein (amole/well) and HCV RNA (copies/µg RNA) on Days post-infection.

B. Intracellular HCV RNA (copies/µg RNA) and HCV core protein (amole/well) at 4h, 24h, Day 2, and Day 6.

C. Intracellular and Extracellular HCV core protein (amole/well or mL) over Days post-infection.

D. Intracellular and Extracellular HCV RNA (copies/µg RNA or mL) over Days post-infection.
Figure 7

A. JFH1 replicon #1 (low CD81)

B. JFH1 replicon #2 (high CD81)

C. HCV 1b replicon (high CD81)

D. HCV 1b replicon (low CD81)
Days post-infection
intracellular HCV RNA, CD81
RNA (copies/µg RNA), or
HCV core protein (amole/well)