Protein Kinase D negatively regulates Hepatitis C virus maturation through phosphorylation of Oxysterol Binding Protein and Ceramide Transfer Protein.

*Yutaka Amako, *Gulam H Syed, and Aleem Siddiqui

Department of Medicine, Division of Infectious Diseases, Moores Cancer Center, University of California, San Diego, La Jolla, CA 92093

Address correspondence to: Aleem Siddiqui, Department of Medicine, Division of Infectious Diseases, Stein 409, University of California, San Diego, 9500 Gilman Dr, 0711, La Jolla, CA 92093. Phone: 858 822 1750, Fax: 858 822 1749. Email: asiddiqui@ucsd.edu.

Hepatitis C virus (HCV) RNA replicates its genome on the specialized ER modified membranes termed membranous web and utilizes lipid droplets for initiating the viral nucleocapsid assembly. Previous studies have shown that HCV maturation and/or egress pathway requires host sphingolipid synthesis. Ceramide transfer protein (CERT) and oxysterol binding protein (OSBP) play crucial role in sphingolipid biosynthesis. Protein kinase D (PKD), a serine/threonine kinase is recruited to the trans-Golgi network (TGN), where it influences vesicular trafficking to plasma membrane by regulation of several important mediators via phosphorylation. PKD attenuates the function of both CERT and OSBP by phosphorylation at their respective Ser132 and Ser240 residues (phosphorylation inhibition).

Here, we investigated the functional role of PKD in HCV secretion. Our studies show that HCV gene expression downregulated PKD activation. PKD depletion by shRNA or inhibition by pharmacological inhibitor Go6976 enhanced HCV secretion. Over-expression of constitutively active form of PKD suppressed HCV secretion. The suppression by PKD was subverted by the ectopic expression of nonphosphorylatable serine mutants S132A CERT or S240A OSBP. These observations imply that PKD negatively regulates HCV secretion/release via attenuating OSBP and CERT functions by phosphorylation inhibition.

This study identifies the key role of the Golgi components in HCV maturation process.
genome replicates within a ribonucleoprotein complex, on the ER-derived modified membranous structures termed as “membranous web” (6-8). The viral replication complex is assembled in close proximity to cytosolic lipid droplets (LDs) and this arrangement promotes subsequent steps of viral assembly/morphogenesis. HCV alters host lipid metabolism and causes the redistribution and accumulation of LDs around the perinuclear region (9,10). The viral core protein closely associates with LDs and recruits NS5A and these interactions are critical for efficient viral assembly process (11). Evidence suggests that HCV secretion is linked to cellular very low-density lipoprotein (VLDL) secretion (12). HCV secretion is inhibited by silencing apolipoprotein B-100 (ApoB), ApoE and ApoC1 as well as inhibition of MTP activity (13-15). These and other data strongly argue for the utilization of the VLDL secretory pathway by HCV for its maturation/secretion (12,16). Although the VLDL secretion pathway is not completely characterized, it is believed to occur through the Golgi network (17,18). The exact pathway that results in the association of HCV nucleocapsids (either enveloped or non-enveloped) with the VLDL particles en route to the Golgi compartment remains to be characterized. Similarly the role of lipid droplets in HCV morphogenesis remains to be clearly understood.

OSBP is a sterol sensor and facilitates trafficking of cholesterol or hydroxycholesterol from ER to Golgi (19,20). OSBP binds to both VAP-A on the ER and phosphatidylinositol 4-phosphate (PI4P) on the Golgi to form “membrane contact site” (MCS) to facilitate lipid transfer between opposing surfaces (21). CERT, which shares functional homology with OSBP, regulates the transport of ceramide from ER to the Golgi, where the ceramide is converted to sphingolipids (22). OSBP modulates CERT activation and translocation to the Golgi, and thereby integrates sterol homeostasis to sphingolipid biosynthesis (21,23). We previously showed that OSBP mediates HCV secretion while binding to NS5A and VAP-A (24). Inhibition of CERT function effectively suppressed HCV release without affecting RNA replication (25). These studies indicate that these lipid transport proteins, CERT and OSBP directly contribute to the HCV morphogenesis/secretion.

PKD is a serine/threonine kinase and exists in three distinct isoforms (PKD1, PKD2, PKD3). PKD regulates multiple cellular processes including cell survival, adhesion, motility and differentiation (26,27,28). In addition, PKD promotes the fission of cargo vesicles from the TGN and thus regulates the secretion of these vesicles from TGN to the plasma membrane (26,28,29). PKD is recruited to the Golgi through the interaction between diacylglycerol (DAG) and its cysteine-rich C1a domain (27-29). The Golgi associated PKD is activated by a novel PKC isoform PKCη by phosphorylation of serine residues in the “activation loop” of PKD (30). At the TGN, PKD activates PI4KIIIβ to generate PI4P, which mediates the Golgi localization of CERT and OSBP proteins via binding to their PH
domains. PKD-mediated phosphorylation of CERT at Ser132 and OSBP at Ser240 impairs their Golgi localization and inhibits their functions in integrating the cholesterol and sphingomyelin (SM) metabolism (31,32). While active PKD is known to promote secretion of small cargo proteins (i.e. VSV-G), little is known about how PKD modulates the transport of large cargos like viral vesicles or encapsidated viral core particles in TGN. In this study, we investigated the functional role of PKD in the HCV maturation and/or secretion process with an emphasis on its substrates, CERT and OSBP.

Our studies show that PKD negatively regulates HCV secretion via the attenuation of OSBP and CERT through phosphorylation of their specific serine residues. HCV infection mitigates PKD activation. RNA interference of PKD expression and inhibition of PKD activity led to an increase in HCV secretion. Over-expression of constitutively active form of PKD caused suppression of HCV secretion. This suppression by PKD was subverted by the ectopic expression of CERT S132A mutant or OSBP S240A mutant. These studies identify the key role of the Golgi network in HCV maturation process.

**Experimental Procedures**

**Plasmids** - The plasmids pJC1 and p7-RLuc2A were kind gifts of Drs. F. Chisari (The Scripps Research Institute, CA) and C Rice (Rockefeller University, NY) respectively. p7-RLuc2A is derived from JC1 (33). N-terminally HA-tagged human PKD1 expression vectors, HA.PKD, HA.PKD.K/W, HA.PKD.S738E/S742E and ΔPH were obtained from Dr. Alex Toker through Addgene (Cambridge, MA). pcDNA3-STrEp tagged (Strep)-PKD1 expression vectors were generated by PCR mediated cloning using HA-PKD vector as template. Following pair of oligonucleotides was used for PCR. Fwd; 5’-cggtatcatgctggacctggagccacccgcagttcgagaaagcgcctecgggtctg-3’ and Rwd; 5’-caactttgcactgcaagcc-3’. PCR product was digested with BamHI and HindIII, then inserted into corresponding sites of pcDNA3-HA.PKD vectors. Strep tag affinity procedure was according to manufacturer’s instructions (IBA, Gottingen, Germany). pSUPER-shRNA expression vector for targeting PKD1 expression was developed as previously described (34). Following pairs were inserted between Bgl II and Hind III sites of pSUPER vector. shPKD1-1: 5’-gatcccccatgctgtggggctggtacttcaagagatcaccaccccccacagcattttttgaa-3’ and 5’-agctttttcacaattatgtctggggtcttgactctctttgagagatcagccccacagcatgggaagaaagagagatcaccaccccccacagcattttttgaa-3’. shPKD1-2: 5’-gatcccgctctgcattgtgtggtttctctcctcagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagag
following primers; S240A; 5’-gctctgcagcgttctctcGCtgagctggagtccctgaa-3’ and 5’-ttcagggactccagctcaGCgagagaacgctgcagagc-3’. PCR product was digested with Afe I and Bcl I, and cloned into corresponding sites of the vector.

*Lentiviral Packaging and Transfection-* pCSII-EF1-MCS and a set of packaging plasmid vectors, including pCMV-VSV-G-RSV-Rev and pCAG-HIVgp, were kind gift from Dr. Hiroyuki Miyoshi (Riken BioResource Center, Japan). cDNAs encoding Strep-tagged PKD1, FLAG-OSBP and FLAG-CERT were recloned into multi cloning site of pCSII-EF1-MCS using oligolinkers and conventional molecular cloning techniques. HEK293FT cells were used as packaging host to obtain lentiviral vectors. Packaging transfection was performed using TransIT-LT1 reagent (Mirus Bio, WI), accordingly to manufacturer’s instructions.

*Reagents and Antibodies-* Anti-FLAG monoclonal antibody, clone M2 was purchased from SIGMA. Anti-PKD1 polyclonal antibody was obtained from Cell Signaling. Anti-phospho-PKD1 (PKC sites, rabbit monoclonal, EP1493Y) and anti-phospho-PKD1 (auto-phosphorylation site, rabbit polyclonal) were purchased from Novus Biological and Cell Signaling, respectively. The anti-PKD pMOTIF antibody was a generous gift of Dr. A Toker (Harvard Medical School) (38). Mouse monoclonal anti-NS5A antibody was a kind gift of Dr. C Rice (Rockefeller University, NY). Mouse monoclonal anti-Core antibody was purchased from Affinity Bioreagents. Rabbit polyclonal anti-human albumin and anti-calnexin antibody are from MP Biomedicals and Santa Cruz Biotechnology, respectively. PKC inhibitor Go6983 and PKC/PKD inhibitor Go6976 were purchased from Calbiochem. Inhibitors were dissolved in dimethyl sulfoxide (DMSO) (Hybri-Max, Sigma-Aldrich, St. Louis, MO) and used at a final concentration of DMSO not exceeding 0.1% (v/v).

*Immunoprecipitation-* For immunoprecipitation of FLAG-CERT proteins, transfected cells in 60 mm dish were lysed in 1ml of lysis buffer (0.25% deoxycholic acid, 0.1% Triton X-100, 150 mM NaCl, 100 mM Tris/HCl pH8.0, plus phosphatase inhibitors). After brief sonification and centrifugation, 0.5 ml of cleared lysate was incubated with 2 ug of anti-FLAG monoclonal antibody M2 (Sigma-Aldrich, St. Louis, MO) for 1h at 4C. Immune-complexes were captured with Protein G Sepharose 4FF beads (GE Healthcare) by subsequent incubation at 4C for 1h. Captured immune-complexes were washed for 5 times with 1ml of lysis buffer and 2 times with tris-buffered saline. Western blot analysis was performed as described previously (24).

*Cell culture-* Huh7.5.1 cells are a kind gift of Dr. Frank Chisari (The Scripps Research Institute, CA). Huh7 and Huh7.5.1 cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine sera, 100 U/ml penicillin, 100 g/ml streptomycin and 1mM MEM non-essential amino acids (Invitrogen, Carlsbad, CA).

*In vitro RNA transcription and RNA transfection-* Viral genomic RNAs were synthesized *in vitro* by
**RESULTS**

**HCV infection impairs PKD1 protein activity.**

PKD regulates the trafficking of secretory vesicle by promoting the fission of these vesicles from trans-Golgi network (28). In this study, we investigated the functional role of PKD in the transport of HCV particles. First, we analyzed the expression levels and kinase activity of PKD1 in HCV infected cells. PKC\(\eta\) is a novel PKC isoform known to activate PKD1 at the TGN by phosphorylating Ser\(^{738}\) and Ser\(^{742}\) residues in the PKD1 activation loop (30,41,43). The activated PKD1, then auto-phosphorylates the Ser\(^{910}\) residue in its C-terminus, which correlates with the kinase active form of PKD1 (42,43). We infected Huh7.5.1 cells with culture derived HCV virus particles at multiplicity of infection (MOI) of 0.01 and 0.1. Six days postinfection, the whole cell
lysates were analyzed by immunoblotting using phospho-serine antibodies that specifically recognize the phosphorylated Ser\textsuperscript{738} and Ser\textsuperscript{742} (PKC site) and Ser\textsuperscript{910} (PKD autophosphorylation site). The results show that the PKD1 kinase activation is impaired in HCV infected cells, although total PKD1 expression levels in uninfected and HCV infected cells are the same (Fig. 1A). Consistent with the above data, the reduction of PKD1 phosphorylation is more pronounced in cells infected with the high titer of HCV virus relative to those infected with a lower titer (Fig. 1A, MOI 0.1 vs MOI 0.01). It has been shown that inhibition of PKD1 activity is associated with the decline in the general secretion capacity of cell (28). In agreement with the above results we observed a decline in secretion of secretory horseradish peroxidase (ssHRP-Flag) from HCV infected Huh7.5.1 cells compared to the uninfected cells (Fig. S1). This also holds true for infected and uninfected cells expressing PKD and its mutants. Together, these results suggest that HCV infection leads to impairment of PKD1 activity and in subsequent reduction in the secretory capacity of the host cell.

**PKD1 inhibition enhances HCV virion release.**

Next, we evaluated the functional significance of PKD1 in HCV infection. We used two experimental approaches to analyze the effect of PKD inhibition or suppression of HCV replication and/or virion secretion. The first approach consisted of using pharmacological inhibitor of PKD. Go6976 is an ATP competitive PKC inhibitor which suppresses both PKC\textgreek{a} (IC\textsubscript{50}=2.3 nM) and PKC\textgreek{b}I (IC\textsubscript{50}=6.2 nM) and also strongly inhibits PKD activity (IC\textsubscript{50}=20 nM) (15). Whereas PKC inhibitor Go6983 inhibits PKC\textgreek{a} (IC\textsubscript{50}=7 nM), PKC\textgreek{b} (IC\textsubscript{50}=7 nM), PKC\textgreek{y} (IC\textsubscript{50}=6 nM), PKC\textgreek{g} (IC\textsubscript{50}=10 nM) and PKC\textgreek{z} (IC\textsubscript{50}=60 nM), but not PKD (IC\textsubscript{50}=20 M) (44). Thus, we used Go6983 and Go6976 to selectively elucidate the specific effect of PKD inhibition as opposed to the effect observed as consequence of inhibition of other PKC isoforms. Huh7.5.1 cells were transfected with *in vitro* transcribed RNA of HCV chimera (p7-Rluc2A) containing renilla luciferase (Rluc) reporter gene inserted between p7 and NS2 of the monocistronic JC1 RNA genome (33). The transfected cells were cultured in the presence of physiologically effective concentrations of these inhibitors to elucidate their effect on HCV life cycle. Quantification of luciferase reporter activity in the lysates of the transfected cells was used to assess HCV RNA replication. Whereas the secretion of infectious HCV viral particle was assessed by infecting naïve Huh 7.5.1 cells with culture supernatants collected at 72h posttransfection and measured reporter activity in the infected cells 48h postinfection. Interestingly, the PKC/PKD inhibitor Go6976 enhanced HCV secretion in a dose dependent manner, whereas the PKC inhibitor Go6983 had no notable effect on HCV secretion (Fig. 2B). The ΔE1-E2 mutant, which contains a large in-frame deletion within the coding sequence of E1 and E2 envelope proteins in the p7-Rluc2A genome and therefore incapable of producing infectious virus particles, was used as a negative control for the HCV secretion assay.
In addition, we observed a gradual dose-dependent decline in replication of both p7-Rluc2A and its ΔE1E2 counterpart upon treatment with Go6976 (Fig. 2C and D). However, with the broad-spectrum PKC inhibitor Go6983, the results on replication of p7-Rluc2A and the ΔE1E2 deletion mutant were rather inconsistent. The intracellular infectivity of Go6976 treated cells was also less than that of untreated control and Go6983 treated cells (Fig. S2A), whereas the extracellular infectivity increased upon Go6976 treatment (Fig. S2B). Together, these data suggest that the accelerated secretion of HCV observed upon inhibition of PKD is not a consequence of enhanced rate of virus maturation/assembly. This implies that under normal physiological conditions, PKD negatively regulates HCV secretion.

We also evaluated the effect of these inhibitors on VLDL secretion by Western analysis of ApoB and ApoE proteins in the culture supernatants. Our observations reveal that treatment with PKD inhibitor Go6976 modestly decreased ApoB and ApoE secretion when compared to secretion by untreated control cells. VLDL secretion occurs via the Golgi (17,18). Inhibition of PKD1 activity is associated with a decline in the general secretion capacity of cell occurring through the Golgi compartment (Fig. S1B) (28). Similarly, VLDL secretion was also inhibited by PKD inhibitor Go6976 (Fig. S4).

In the second approach, we employed shRNA-mediated downregulation of PKD1 using DNA vector-based shRNA expression system to analyze its effect on HCV life cycle. Using two different PKD-specific shRNAs, we could achieve a modest (~50%) reduction of PKD1 protein expression (Fig. 3A and B). The PKD1-shRNA vector transfected Huh7.5.1 cells were electroporated with in vitro transcribed HCV-JCI genome and HCV virus particle secretion in culture supernatants assessed by performing the foci forming unit assay (14,24). Downregulation of PKD expression was associated with concomitant increase in secretion of infectious HCV virus particles (Fig. 3C), although the levels of HCV replication determined by quantitative-(q)RT-PCR analysis of intracellular RNA remained unchanged (Fig 3D). Overall this data, in concert with the data presented in Fig.2, supports the notion that PKD inhibition or downregulation has positive effect and leads to an increase in the secretion of infectious HCV virus particles.

**Overexpression of dominant-positive PKD1 suppresses HCV secretion.** We, next, analyzed the effect of various functional domain mutants of PKD1 on HCV replication and secretion. We used the lentiviral expression system to overexpress various PKD1 mutants. Transduction of HCV infected Huh7.5.1 cells with the dominant positive S738/742E or pleckstrin domain (PH) lacking ΔPH PKD mutants hindered HCV secretion (Fig. 4A), although PKD wild type or the S738E and ΔPH PKD mutants modestly increased the HCV replication (Fig 4B). Both S738/742E and ΔPH PKD mutants are kinase-active forms of PKD (28). In contrast, the kinase-inactive/dead (K612W) PKD1 mutant did not enhance HCV
secretion (Fig. 4A). The earlier results (Fig. 2B and Fig. 3C) suggested that inhibition or downregulation of PKD1 is associated with enhanced rates of virus secretion suggesting that PKD kinase activity exerts a negative effect on HCV secretion. The dominant positive and ΔPH mutants of PKD1 are constitutively active kinases and hence their overexpression negatively impacted HCV secretion whereas the overexpression of kinase-inactive PKD mutant had no obvious affect on the virus secretion.

**PKD1 negatively impacts HCV secretion through CERT and OSBP phosphorylation.** Among the multiple substrates of PKD1, OSBP and CERT are key players in the Golgi lipid trafficking and biogenesis (21,32). As we have discussed above both CERT and OSBP positively contribute to HCV maturation/secretion. Recent reports show that PKD phosphorylates OSBP at Ser\(^{240}\) and CERT at Ser\(^{132}\) and inhibits their activity (phosphorylation inhibition) (31,32). We speculated that PKD1 negatively impacts HCV secretion through phosphorylation inactivation of CERT and OSBP. To evaluate this point, we have analyzed the status of PKD specific OSBP phosphorylation at ser\(^{240}\) residue by using PKD pMOTIF antibody. Huh 7.5.1 cells transduced with lentivirus encoding FLAG-tagged OSBP were infected with JCI virus at an MOI of 0.1. Four days post-infection, cellular lysates were immunoprecipitated with anti-Flag antibody and immunoblotted using pMOTIF antibody. The results show that PKD mediated phosphorylation of OSBP in HCV infected cells was significantly less than of uninfected control cells (Fig. 4F), thus confirming that HCV infection impairs PKD1 activity. Hence, we assume that the serine mutants of CERT and OSBP that do not serve as PKD substrate would therefore alleviate or subvert the negative regulation of PKD1 on HCV virus particle secretion. To test this hypothesis, we analyzed the effect of coexpressing PKD1 with the respective S240A and S132A serine mutants of OSBP and CERT on HCV secretion. Huh7.5.1 cells were infected with lentivirus encoding wild-type PKD1 and followed by superinfection with lentivirus encoding the respective serine mutants of OSBP or CERT. The transduced cells were then subjected to electroporation with *in vitro* transcribed RNA of p7-Rluc2A at 24h, and infectivity of culture supernatants collected at 72 h post-electroporation was evaluated. Coexpression of the CERT or OSBP serine mutants respectively with PKD1 led to a two-fold increase in the infectious HCV virus particles released in the culture supernatants compared to that of PKD1 expression alone (Fig. 4C), in contrast to the HCV replication levels that remained fairly constant in all cases (Fig. 4D). This modest 2-fold increase is significant considering the presence of endogenous wild type forms of OSBP and CERT. In addition, we observed that the expression of wild type CERT and OSBP also partially increased HCV secretion, although not as significantly as the expression of mutant forms (Fig. 4C). Similar results were obtained when wild type or S132A CERT mutant was expressed in the absence of ectopic PKD expression (Fig.
Together, these data support our hypothesis that PKD1 negatively influences HCV secretion through the phosphorylation inhibition of OSBP and CERT proteins that otherwise serve as positive regulators of HCV virus secretion.

**HCV infection induces morphological changes of TGN.** Previous studies showed the functional reliance of HCV on TGN localizing PI4P-binding proteins such as CERT, OSBP, and PI4KIIIβ (24,25). These proteins serve as PKD substrates and contribute to the integrity and functionality of TGN in sorting and shipping vesicular cargo to their intended destination. Little is known about the functional role of TGN in the HCV morphogenesis. In attempts to gain insight into this aspect, we investigated the morphological changes of TGN in HCV infected cells and those overexpressing the various mutants of PKD1. The HCV infected Huh7 cells were transduced with lentiviruses encoding wild type, kinase-inactive (KD), or dominant active (CA) forms of PKD1. At 48h post-transduction the cells were subsequently processed for immunofluorescence staining of TGN using anti-TGN46 antibody, and counterstained with anti-Strep tag (PKD1) and anti-HCV E2 antibody. We observed that in HCV infected Huh7 cells the TGN46 staining pattern was dispersed and displayed a distorted/fragmented pattern of Golgi staining in contrast to the characteristic perinuclear staining pattern observed in uninfected Huh7 cells (Fig. 5, top row, cells are labeled with yellow vs red asterisk). Quantitative analysis revealed that nearly 90% HCV infected cells displayed a dispersed or fragmented pattern of the Golgi in contrast to 10% of uninfected cells (Fig. S3). As reported previously (28), the ectopically expressed wild type (WT) or dominant active (CA) PKD showed partial localization at the TGN in uninfected Huh7 cells, whereas the kinase-dead/inactive PKD mutant strongly localized to the TGN which displayed a condensed staining pattern because of block in vesicle fission (Fig 5, 1st column). The ectopic expression of wild type (WT) or dominant active (CA) PKD in HCV infected Huh7 cells resulted in partial restoration of the dispersed TGN staining pattern typically observed in HCV infected cells (Fig. 5, 2nd and 4th row, see cells marked with white asterisks). In contrast, the expression of the kinase-inactive PKD1 mutant (KD) in HCV infected cells did not lead to any restoration of TGN organization (Fig. 5, 3rd row, see cells marked with white asterisks). These observations highlight the potential of HCV gene expression in causing the Golgi fragmentation to positively promote the viral particle morphogenesis_secretion reminiscent of the Golgi fragmentation observed during Herpes virus or Chlamydia infections (45-47). As described above, the overexpression of dominant active PKD hindered HCV virus secretion and in agreement with this result, we observed a partial restoration of the Golgi fragmentation in HCV infected cells expressing dominant active or wild type PKD. Together, these observations suggest that Golgi distortion/fragmentation may aid HCV maturation_secretion.
DISCUSSION

The establishment of productive HCV infection culture system has enabled investigators to pursue inquiries relating to the HCV entry, replication, maturation and secretion processes of viral life cycle (40,48,49). Several studies attempted to delineate cellular pathways leading to release of viral particles. These studies correlated viral export with lipoprotein trafficking and revealed the reliance of HCV viral particle trafficking on VLDL secretory pathway (13-15,16,50). It was shown that silencing ApoB and ApoE or inhibiting MTP activity abrogated HCV secretion. In earlier studies ApoE and ApoB were found on the surface of infectious viral particles, suggesting that lipoproteins associate with viral particles to produce lipoviroprotein particles (51-54). It is not known exactly the stage at which HCV viral particles associate with lipoproteins. These findings raise the possibilities of viral entry via LDL or SR-B1 receptors and indeed there have been studies, which support this view (51,52,55,56). These studies further link the functional significance of viral maturation/secretion pathway in contributing to subsequent binding/entry events of viral life cycle (55,57).

An enveloped viral particle is topologically identical to a cargo vesicle, while they are en route to cell surface for egress. Large cargos, such as chylomicrons, some viruses and procollagen, reach TGN by atypical route. The egress pathway of these cargos from TGN to cell surface has not been characterized. PKD isoforms differentially regulate the basolateral transport of proteins from the TGN towards the plasma membrane and also modulate the transport of cargo in polarized cells (26,47,58). PKD regulates Golgi lipid homeostasis through the phosphorylation of PI4KIIIβ, CERT and OSBP substrates. PKD1 phosphorylates PI4KIIIβ to stimulate lipid kinase activity, leading to the production of PI4P at the Golgi. PI4P in turn contributes to the formation of PI4P-rich vesicles to carry cargo proteins. Thus, PI4KIIIβ, which is localized in the Golgi, regulates anterograde transport of cargo proteins destined for the plasma membrane (15,36). Recent work shows that PKD- mediated phosphorylation of CERT attenuates its function in SM synthesis and phosphorylation of OSBP causes its dislocalization from the Golgi (32). These activities of PKD impinge a regulatory role in the maintenance of Golgi homeostasis. Both CERT and OSBP play crucial roles in HCV secretion at different levels. A functional role of SM synthesis and Golgi-localized OSBP in HCV secretion has been previously reported (24,25). Mutations in OSBP PH domain including its deletion abrogated HCV secretion (24). PKD phosphorylation of CERT and OSBP at respective serine residues collectively cripples their functions. Our studies show decreased HCV secretion upon ectopic overexpression of dominant active PKD mutants (CA and ΔPH) are consistent with the role of PKD in negatively regulating OSBP and CERT functions. Hence, mutating the serine residues of CERT and OSBP that serve as PKD substrates should counter this negative influence of PKD. We
observed that over-expression of the serine mutants of CERT (S132A) and OSBP (S240A) subverted the negative effect of PKD1 on HCV secretion (Fig. 4C). We further observed that overexpression of both wild type CERT and OSBP also marginally increased HCV secretion (Fig. 4C). Since CERT and OSBP both serve as PKD substrates, transient overexpression of these proteins may overwhelm the basal capacity of PKD-mediated phosphorylation resulting in the presence of some unphosphorylated active CERT and OSBP proteins that can eventually enhance HCV secretion. In addition, inhibition of PKD activity by PKD-specific inhibitor Go6976 enhanced the rate of viral release without any effect on intracellular RNA replication, thus indicating that this inhibition is exerted at post-translation/replication steps of viral life cycle (Fig. 2B and C). Based on a body of literature on PKD, OSBP and CERT in Golgi trafficking, a recent model was proposed by Toker and colleagues, which integrates the functions of these key players in Golgi trafficking (32). We extrapolate these observations in the context of HCV infection and accordingly propose the following model (Fig. 6). In this model, HCV gene expression causes downregulation of PKD activation (autophosphorylation). Mechanisms of this downregulation are not known. PKD phosphorylation of OSBP disables its association with TGN. OSBP phosphorylation may lead to inactive state in which the interaction between its PH domain and PI4P is inhibited which will abrogate further functions including its interactions with CERT. Similarly, PKD phosphorylation of CERT decreases its PH domain’s interaction with PI4P, which will lead to decreased transport of ceramide and hence attenuation of SM synthesis. In either case, the phosphorylation of OSBP and CERT leads to inactivation of these proteins leading to abrogation of their principal functions that ultimately manifest in the overall inhibition of HCV secretion process in the TGN. Recent report demonstrates that HCV particles are relatively enriched in SM, cholesterol and cholesterol ester (59).

Inhibition of PKD has a negative effect on general secretion of protein occurring through the Golgi compartment (Fig. S1). PKD inhibition led to a modest decrease in the VLDL secretion (Fig S4). This observation suggests that PKD affects VLDL secretion like other secretory proteins trafficking through Golgi network. This study, however, revealed that PKD inhibition has a positive effect on HCV secretion via CERT and OSBP phosphorylation. Several studies suggest that HCV secretion parallels VLDL secretion (13-16,50). At this point, it is not known what fraction of VLDL is recruited for HCV secretion and at what stage of its transport that HCV piggybacks, if it piggybacks, or associates with lipoprotein particles.

HCV gene expression appears to cause a general distortion of Golgi compartment (Fig. 5, row 1, compare cells with yellow vs red asterisks). Based on this observation, we conclude that this altered TGN pattern imparts a positive influence on HCV maturation process reminiscent of Golgi
fragmentation aiding Herpes virus morphogenesis or Chlamydia infection (45-47). These changes modestly affected general secretion of proteins in HCV infected cells (Fig. S1).

Several recent reports have highlighted the negative regulation of PKD in cell migration and motility. PKD is considered a central regulator of cofilin signaling network via direct phosphorylation of slingshot I like (SSHIL) protein (60). PKD also phosphorylates cortactin at Ser298, which leads to its reduced F-actin binding (61). So active PKD inhibits the functions of SSHIL and cortactin and kinase-inactive forms strongly enhance motility and invasiveness mediated by these proteins. The unrecognized function of PKD as a regulator of polarized cell motility and F-actin polymerization reinforces its mode of action via phosphorylation inhibition of these functions. Therefore, keeping in with this mode of action, PKD negatively regulates HCV secretion and release. Understanding the various steps of HCV morphogenesis will open newer avenues for the design of antiviral strategies.

REFERENCE


FOOTNOTES

We are grateful to Dr. T. Wakita (National Institute of Infectious Diseases, Tokyo, Japan) for kindly providing the infectious JDH1 molecular clone, Charles Rice (Rockefeller University, New York) for p7-Rluc2A and monoclonal anti NS5a antibody, Frank Chisari (TSRI, La Jolla, CA) for Huh7.5.1 cells and JC1, Dennis Burton (TSRI) for recombinant human monoclonal anti-E2 antibody, Vivek Malhotra (CGR, Barcelona, Spain), Alex Toker (Harvard Medical School) for pMOTIF antibody. This study was supported by NIH grants DK077704 and AI085087 (A.S.).

*These authors contributed equally. +Present address: Institute of Molecular and Cellular Biology, University of Leeds, Leeds, UK.

Abbreviations used are: HCV, hepatitis C virus; PKD, protein kinase D; CERT, ceramide transfer protein; OSBP, oxysterol binding protein; TGN, trans-Golgi network; VLDL, very low-density lipoprotein.

FIGURE LEGENDS

Fig. 1. HCV infection down-regulates PKD1 activation in Huh7.5.1 cells (A) Analysis of PKD expression in HCV infected cells. Huh7 and Huh7.5.1 cells were infected with culture derived HCV particles by the indicated multiplicity of infection (M.O.I). Six days after infection, whole cell lysates were analyzed by Western blot assay. Phosphorylated serine residues at 738 and 742 (PKC sites) or 910 (auto-
phosphorylation site) were probed using phospho-serine substrates-specific antibody. Both phosphorylations represent enzymatically active form of PKD1. (B) Quantitation of PKD1 expression and activation. Detected protein band intensities (shown in panel A) were calculated by 1D Image Analysis Software (Kodak). PKD protein band intensity was normalized to calnexin. The values were represented by percentage relative to uninfected control.

Fig. 2. PKD inhibitor Go6976 promotes HCV secretion in a dose-dependent manner. (A) Effect of PKD/PKC (Go6976) and PKC (Go6983) inhibitors on protein expression by Western blot assays. Huh7.5.1 cells were mock transfected or transfected with in vitro transcribed p7-Rluc2A RNA, as indicated. Transfected cells were treated with or without 1 µM of Go6976 or Go6983 2 days after transfection and incubated for three additional days. Electroblotted membranes were probed with the following antibodies; anti-PKD, anti-phospho-PKD1 (PKC sites), anti-NS5A and anti-Calnexin. (B) Effect of PKC (Go6983) and PKC/PKD (Go6976) inhibitors on HCV virion release. Cultured supernatants were used to infect naïve Huh7.5.1 cells and cellular lysates were evaluated for renilla luciferase activity. This protocol detects the HCV virion release. The E1-E2 mutant (ΔE), which contains an in-frame deletion within the E1 and E2 coding sequence and hence incapable of producing infectious virus particles, was used as a negative control in the HCV virion release assay. (C) Effect of PKC (Go6983) and PKC/PKD (Go6976) inhibitors on HCV replication. Intracellular luciferase activities were measured for samples described in B. This analysis represents intracellular viral RNA replication. (D) Effect of PKC (Go6983) and PKC/PKD (Go6976) inhibitors on HCV replication. p7-Rluc2A containing E1E2 deletion (ΔE1E2) was employed to monitor RNA replication in the presence of inhibitors. This mutant is defective in HCV particles formation.

Fig. 3. PKD1 depletion promotes HCV secretion. Huh7.5.1 cells were transfected with plasmid vectors encoding shRNA as indicated. Four days after transfection, cells were electroporated with in vitro transcribed HCV JC1 RNA and incubated for three days. Whole cell lysates were subjected to Western blot assays (A). Panels represent expression of total PKD1, albumin, HCV NS5A, respectively. (B) The level of PKD1 depletion was quantified by 1D Image Analysis Software (Kodak). (C) HCV secretion/release was measured by foci-forming unit assay as described in Materials and Methods. (D) HCV replication determined by qRT-PCR analysis of HCV genome equivalents (GE) per µg total intracellular RNA.

Fig. 4. PKD regulates HCV secretion via phosphorylation of CERT and OSBP
(A) Effect of PKD overexpression on HCV secretion. Huh7.5.1 cells were infected with lentiviral vectors
encoding PKD1 (WT), dominant active (S738/742E), PH domain deletion (ΔPH) mutant, and kinase-inactive mutant (K612W), as indicated. At 24h post-infection cells were electroporated with in vitro transcribed p7-Rluc2a RNA. At 72h post-electroporation the culture media used for HCV secretion assay. Graph bars represent rates of HCV secretion in a relative manner. (B) Effect of PKD overexpression on HCV replication. Cellular lysates used in ‘A’ were used to determine replication by renilla luciferase activity. (C) Effect of overexpression of CERT and OSBP wild type and mutants on HCV secretion. Huh7.5.1 cells were co-infected with a lentiviral vector encoding PKD1 wild type as well as those encoding WT or S132A CERT or S240A OSBP respectively. At 24h post infection cells were electroporated with p7-Rluc2A RNA and HCV secretion assay performed as described above. (D) Effect of overexpression of CERT and OSBP wild type and mutants on HCV replication. Samples used in ‘C’ were used to determine replication by renilla luciferase activity. (E) Western blotting of cell lysates from samples in panel ‘C’. The Western analysis shows the expression analysis of PKD1, OSBP, CERT, NS5A and calnexin in the lysates. (F) Detection of PKD-specific phosphorylation of OSBP at ser240. Huh7.5.1 cells were transduced with lentivirus encoding Flag-tagged OSBP. At 24h post transduction the cells were infected with HCV JCI virus at a MOI of 0.1, 4 days later the cell lysates were immunopercipitated with anti-FLAG antibody and immunoblotted with PKD pMOTIF antibody. The cell lysates were probed for the expression of FLAG-OSBP and HCV NS5A. -actin serves as protein loading control.

Fig. 5. HCV infection induces morphological changes of TGN. Diffused pattern of TGN (green) is seen associated with HCV infection. HCV infected and uninfected Huh7 cells were transduced with respective lentiviral vectors encoding empty ORF (no), PKD wild type (WT), PKD K612W (KD) or PKD S738/742E (CA) as indicated on the left. Left column; Merged images of TGN (green) and strep-tagged PKD (Red) in uninfected Huh7 cells. 4X4 panels on right show images of TGN46 (Green), Strep-tagged PKD (red) and HCV envelope (E2) (white) staining as indicated in HCV infected Huh7 cells. The extreme right column shows merged view of TGN and PKD. All panels were counterstained for nuclei with DAPI (blue). In the upper row, cells highlighted with yellow and red asterisk marks represent HCV infected and uninfected cells respectively.

Fig. 6. Model depicting the role of Protein Kinase D in HCV secretion. PKD1 regulates HCV secretion by regulating enzymatic activities of CERT and OSBP thorough phosphorylation inhibition. OSBP and CERT form “membrane contact site” between ER and the TGN by binding to both VAP-A and PI4P. CERT protein transports ceramide from ER to TGN, where transported ceramides are converted to sphingolipids. OSBP transports cholesterols and oxysterols to TGN. These transported lipids contribute to
the formation of microdomain enriched with cholesterols and sphingolipids. HCV particles have been shown to be sensitive to sphingomyelinase and methyl-b-cyclodextrin treatments (cholesterol depletion) (25). CERT inhibitor (HPA-12) attenuates HCV secretion. OSBP facilitates HCV secretion (24). PKD1 phosphorylates OSBP to sequester it from TGN. PKD1 phosphorylates CERT to inactivate its enzyme activity. In this study, PKD1-specific siRNA enhanced HCV secretion. PKD1 inhibition by PKD-specific inhibitor Go6976 also promoted HCV secretion. Over-expression of dominant active form of PKD1 suppressed HCV secretion. Co-expression of OSBP S240A or CERT S132A mutants subverted this suppression. PKD1 negatively regulates HCV maturation/secretion pathway through the phosphorylation of CERT and OSBP. To circumvent these effects, HCV down-regulates PKD activation (Figure 1).
Amako et al., Fig. 1

A

<table>
<thead>
<tr>
<th></th>
<th>Huh7.5.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>0.01</td>
</tr>
</tbody>
</table>

NS5A

PKD1 (Total)

Calnexin

p-PKD1 (PKC sites)

p-PKD1 (auto)

Calnexin

B

Huh7.5.1

<table>
<thead>
<tr>
<th></th>
<th>Total PKD1</th>
<th>p-PKD (PKC)</th>
<th>p-PKD (auto)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Infection control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M.O.I = 0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M.O.I = 0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Amako et al., Fig. 3
Amako et al., Fig 4

- **A**
  - Bar graph showing RLU values for ΔE1E2 and p7-Rluc2A with mutations Empty, WT, S738/742E ΔPH, and K612W.

- **B**
  - Bar graph showing RLU values for ΔE1E2 and p7-Rluc2A with mutations Empty, WT, S738/742E ΔPH, and K612W.

- **C**
  - Bar graph showing RLU values for CERT WT, CERT S132A, OSBP WT, and OSBP S240A.

- **D**
  - Bar graph showing RLU values for CERT WT, CERT S132A, OSBP WT, and OSBP S240A.

- **E**
  - Table showing expression levels for st-PKD, FLAG-CERT, and FLAG-OSBP.

- **F**
  - Western blot images showing proteins PDK, OSBP, CERT, NS5A, and Calnexin with HCV treated with - and + conditions and FLAG-OSBP treated with - and + conditions.
Amako et al., Fig. 5

No HCV infection  HCV Infection

No  

WT  

KD  

CA  

Merged  

TGN46  PKD  HCV E2  Merged
Cholesterol/Sphingolipid-dependent virion maturation

PKC

Gö6976

Gö6983

PKD1

CERT

OSBP

PtdIns(4)P

HCV

MCS

ER

TGN

Cytosol

Ceramide

Sphingolipid

Cholesterol

Phosphatidylinositol 4-phosphate

Amako et al., Fig. 6

PH domain

FFAT

Lipid-binding domain

Lipid transporter protein
Protein kinase D negatively regulates hepatitis C virus maturation through phosphorylation of oxysterol binding protein and ceramide transfer protein
Yutaka Amako, Gulam H. Syed and Aleem Siddiqui

J. Biol. Chem. published online February 1, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M110.182097

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2011/02/01/M110.182097.DC1