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A Novel Human Radixin Peptide Inhibits Hepatitis C Virus Infection at the Level of Cell Entry

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Abstract

Hepatitis C virus infection of hepatocytes is a multistep process involving the interaction between viral and host cell molecules. Recently, we identified ezrin–moesin–radixin proteins and spleen tyrosine kinase (SYK) as important host therapeutic targets for HCV treatment development. Previously, an ezrin hinge region peptide (Hep1) has been shown to exert anti-HCV properties in vivo, though its mechanism of action remains limited. In search of potential novel inhibitors of HCV infection and their functional mechanism we analyzed the anti-HCV properties of different human derived radixin peptides. Sixteen different radixin peptides were derived, synthesized and tested. Real-time quantitative PCR, cell toxicity assay, immuno-precipitation/western blot analysis and computational resource for drug discovery software were used for experimental analysis. We found that a human radixin hinge region peptide (Peptide1) can specifically block HCV J6/JFH-1 infection of Huh7.5 cells. Peptide 1 had no cell toxicity or intracellular uptake into Huh7.5 cells. Mechanistically, the anti-HCV activity of Peptide 1 extended to disruption of HCV engagement of CD81 thereby blocking downstream SYK activation, which we have recently demonstrated to be important for effective HCV infection of target hepatocytes. Our findings highlight a novel functional class of anti-HCV agents that can inhibit HCV infection, most likely by disrupting vital viral-host signaling interactions at the level of virus entry.

Keywords

Anti-viral peptide; Ezrin; HCV J6/JFH-1 virus; Spleen tyrosine kinase (SYK); Moesin; Radixin

Introduction

Hepatitis C virus infection (HCV) is a major health burden globally with over 170 million people chronically infected (Global Surveillance and Control of Hepatitis C 1999). Without

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treatment, most HCV infections progress to chronic liver disease, liver fibrosis, cirrhosis, hepatocellular carcinoma and ultimately death. Over the past decade significant progress has been made in the development of potent treatments against HCV infection including interferon- α , ribavirin, NS3–NS4 protease inhibitors and HCV neutralizing antibodies (Soriano et al. 2009; Bacon and Khalid 2011; Edwards et al. 2012). Despite these breakthroughs, the emergence of drug resistance to current therapy due to the high mutation rate of the HCV virus (Susser et al. 2009; Shang et al. 2013) means that novel classes of anti-virals are still needed. Targeting essential host molecules has emerged as an attractive strategy to avoid virus resistance as well as the potential of yielding broad spectrum anti-virals against multiple virus families which use similar host proteins for infection.

The HCV virus, a single stranded positive sense RNA virus of the *Flaviviridae* family, primarily infects primate hepatocytes using host cell molecules for entry, some of which include CD81 (Pileri et al. 1998), scavenger receptor b1 (Scarselli et al. 2002), claudin (Evans et al. 2007), occludin (Ploss et al. 2009) and others (Rice 2011). Given the importance of these host molecules in HCV entry to hepatocytes, numerous therapeutic agents are currently being developed to block their function. Small human derived anti-viral peptides are attractive because of their relative low cost, minimal side effects, low likelihood of viral resistance and easy adaptability to combination therapy (Cui et al. 2013; Li et al. 2011; Choocheep et al. 2010). In the context of HCV, one study identified the human ezrin peptide, Hep1, to display strong anti-HCV properties in vivo in HCV-HIV co-infected patients (Salamov et al. 2007) highlighting the potential antiviral properties of other ezrin family derived peptides. Additionally we recently found that human ezrin, moesin and radixin proteins differentially regulate HCV infection and replication (Bukong et al. 2013). Chronic HCV infection significantly decreased moesin and radixin expression in Huh7.5 cells and liver biopsies from HCV infected patients (Bukong et al. 2013). Artificial over expression of moesin or radixin in Huh7.5 cells prior to HCV J6/JFH-1 infection significantly suppressed HCV infection (Bukong et al. 2013). The remarkable observation that ezrin–moesin–radixin (EMR) proteins can modulate HCV infection and the lack of functional studies on how the ezrin hinge region peptides function provide a rational platform for assessing the antiviral mechanism of other hinge region EMR peptides, specifically radixin which is highly expressed in the liver (Kikuchi et al. 2002).

In the present study, we investigated the anti-viral potential and mechanism of action of a human-derived radixin hinge region peptide (Peptide 1). We found that Peptide 1 could modestly inhibit HCV infection by disrupting host-viral signaling events at the level of virus entry. Therefore, EMR hinge region peptides such as the molecule compound Peptide 1 represent a novel functional class of anti-HCV agents.

Materials and Methods

Cell Lines and HCV J6/JFH-1 Virus

The RIG-I deficient human hepatoma Huh7.5 cell line and Huh7.5 cell line harboring Con1 HCV full length replicon (Genotype 1b), a gift from Dr. Charles Rice, were cultured as previously described (Blight et al. 2002). Infectious HCV J6/JFH-1 virions were generated as previously described using the pFL-J6/JFH-1 plasmid (Lindenbach et al. 2005) kindly

provided by Dr. Charles Rice (Rockefeller University, New York, NY, USA) and Dr. Takaji Wakita (National Institute of Infectious Diseases, Tokyo, Japan). Virus quantification for multiplicity of infection (MOI) determination in culture supernatants was determined as previously described (Bukong et al. 2012).

Synchronized HCV J6/JFH-1 Virion-Based Fusion and Infection Assay

Synchronized fusion binding and virus infection assay was carried out as recently described (Sourisseau et al. 2013) with some modifications. Briefly, Huh7.5 cells were incubated for 3 h at 4 °C with HCV J6/JFH-1 viral inputs (MOI of 10) in 1 mL culture medium with and without peptide pretreatments as indicated. Cells were then extensively washed with cold complete medium to remove unbound virions and incubated with or without further treatment as indicated at 37 °C for 24 h. The high virus titre for infection was used so as to obtain detectable amounts of specific phosphorylated proteins which are not attainable with a low MOI. The level of HCV J6/JFH-1 infectivity was analyzed 24 h after infection by western blot analysis for HCV NS3 protein.

Quantitative Real-Time Polymerase Chain Reaction Analysis

Real-time quantitative polymerase chain reactions (RT-qPCR) were performed using the CFX96 Real-Time System (Bio-Rad Laboratories, Inc, Hercules, CA, USA) and iTaq SYBR Green Supermix with ROX (Bio-Rad, cat # 172-5851) using 18S RNA as a housekeeping gene. Relative HCV RNA expression was determined using the comparative delta-Ct method. The following primers were used for HCV real-time quantitative PCR:

HCV-Forward Primer: 5'-TCTGCGGAACCGGTGAGTAC-3' (Bukong et al. 2012)

HCV-Reverse primer: 5'-TCAGGCAGTACCACAAG GCC-3' (Bukong et al. 2012)

Antibodies and Reagents

The following antibodies and reagents were used: anti-HCV core antibody (Abcam Cat # ab2740), anti-NS3 antibody (Abcam cat # ab13830); anti-SYK phospho (pY323) (Epitomics cat # 2173-1); anti-beta Actin antibody [AC-15] (Abcam, Cat # ab6276); goat anti-mouse IgG-HRP (Santa Cruz Cat. # sc-2005); and goat anti-rabbit IgG-HRP (Santa Cruz cat # sc-2004). Anti HCV radixin consensus peptide were designed from the hinge region of radixin modeled after the anti-HCV Ezrin Hep1 peptide, as shown with the EMR hinge region sequence alignment (Fig. 1a). The sequences used were human ezrin (GenBank accession number NP_003370), human moesin (GenBank accession number NP_002435), and human radixin (Gen-Bank accession number NP_001247422). Main-anti HCV peptide sequences used included: Hep 1: TEK KRRETVEREKE; Peptide 1: NEKKKREIAEKEKE and negative control Peptide 16: RIEREKEELMERLK. Peptides were synthesized by GenScript with a purity of >90 %. All peptides were initially dissolved in dimethyl-sulfoxide (DMSO) (Sigma-Aldrich cat. # 472301) at a concentration of 1 mg/mL for use as stocks and diluted further to indicated concentration with DMEM complete medium as indicated for experimental treatments. Peptide properties were assessed using the GenScript peptide calculator and computational resource for drug discovery (<http://crdd.osdd.net>).

Western Blot Analysis

For protein western blot analysis, treated cells as described were washed twice in ice cold phosphate buffer saline (PBS) then lysed in RIPA buffer (Boston Bio-products cat # BP-115) supplemented with protease inhibitor cocktail (Roche Cat. # 11836153001). Protein samples for western blot analysis were mixed with Laemmli's buffer (Boston Bioproducts Cat. # BP-110R) and boiled for 5 min then subjected to 10 % SDS-PAGE gel electrophoresis under reducing conditions. Resolved proteins were transferred onto a nitrocellulose membrane then probed with the indicated primary antibodies followed by an appropriate HRP-conjugated secondary IgG antibody as previously described (Bukong et al. 2012). Protein bands were analyzed using the Fujifilm LAS-4000 luminescent image analyzer (GE Healthcare Biosciences, Pittsburgh, PA, USA). Quantification of western blot band density normalized to the actin band density was done using the NIH ImageJ software (Schneider et al. 2012).

Immunofluorescence Microscopy

Huh7.5 cells were cultured on glass cover slips. After peptide treatment as indicated cells were fixed for 10 min with 2 % paraformaldehyde in PBS. Cover slips were washed three times with PBS then mounted on slides using mounting medium with Dapi (Invitrogen cat. # P36935). Images were then acquired using an Olympus BX51 fluorescence microscope and the Nikon NIS-Element BR3.10 software (Olympus, Pennsylvania, USA).

Statistical Analysis

Data are presented as mean + standard error of the mean (SEM). Results presented are representative of at least three independently repeated experiments and microscopic observations of at least 10 fields per independent experimental slide sequentially analyzed to minimize spectral bleed through artifacts.

Statistical analysis was done using the two-tailed student *t* test or the Mann–Whitney test for at least 3 independently repeat experiments. *p*-values less than 0.05 were considered statistically significant.

Results

Rationale and Design of Human Radixin Hinge-Region Peptides as Potential Anti-HCV Inhibitors

Previous studies including ours have revealed the important role of EMR proteins in regulating RNA virus infection at the cell entry level (Naghavi et al. 2007; Haedicke et al. 2008; Bukong et al. 2013). Recently, a human derived ezrin hinge region peptide (Hep1) has been shown to possess anti-HCV properties in HCV-HIV co-infected patients in vivo (Salamov et al. 2007). Based on these observations we surmised that other peptides from the hinge region of other EMR proteins, specifically radixin, might possess potent anti-viral properties. Further, radixin is highly expressed in the liver (Kikuchi et al. 2002) and significantly decreases in hepatocytes during chronic HCV infection of hepatocytes (Bukong et al. 2013). Sequence alignment of EMR hinge region peptide to match an anti-HCV ezrin Hep1 peptide (Salamov et al. 2007) served as the basis for the design of potential anti-HCV

radixin peptides (Fig. 1a). Sixteen peptides including Hep1 were initially screened for potential anti-HCV activity (Fig. 1b).

Radixin Hinge Region Peptide (Peptide 1) Blocks HCV J6/JFH-1 Infection in Huh7.5 Cells at the Level of Cell Entry

Radixin hinge region peptides identified by EMR hinge region sequence alignments were screened for potential anti-HCV properties. Using similar peptide concentrations (1 µg/mL) as previously described (Salamov et al. 2007), we found that radixin hinge region both radixin peptides, Peptide 1 and Peptide 6 pre-treatment of Huh7.5 cells prior to HCV J6/JFH-1 infection (MOI of 1) could significantly suppress infection of Huh7.5 cells as demonstrated by decreased HCV RNA expression (Fig. 2). Peptide 1 was more effective than Peptide 6, and the previously reported Hep1 peptide (Gepon) and IL-28, an anti-viral interferon, also inhibited HCV replication. Despite the potent anti-viral property of Peptide 6 we focused on Peptide 1 which similar to Hep1 did not show high peptide hydrophobicity like Peptide 6 (Supplementary Fig. 1). Peptide 1 did not show any significant toxicity to cells (Supplementary Fig. 2). Using synchronized HCV J6/JFH-1 infection assay, the capacity of Peptide 1 to either block HCV virus entry or replication in Huh7.5 cells was directly investigated (Fig. 3a). Huh7.5 cells were treated with or not with Peptide 1 for 1 h prior to HCV J6/JFH-1 exposure for 3 h at 4 °C. Western blot analysis of HCV NS3 proteins in Huh7.5 cells from these experiments clearly demonstrated that peptide 1 most likely functions at the level of HCV entry (Fig. 3a), as Peptide 1 treatment of Huh7.5 cell after virus entry did not show reduced HCV NS3 protein expression (Fig. 3a). This conclusion was further strengthened by the observation that Peptide 1 treatment of Con1 full length replicon cells for up to 72 h had no effect on HCV RNA replication (Fig. 3b). Con1 full length replicons support HCV replication without production of infectious viral particles, thus viral entry is not involved in this in vitro HCV model. Additionally, carboxyfluorescein (FAM) conjugated Peptide 1 did not show any intracellular uptake into Huh7.5 cells compared to the cell permeable anti-cancer peptide Buforin IIb (Lee et al. 2008) (Supplementary Fig. 3).

Further experiments using Peptide 1 revealed a dose-dependent effect (Fig. 3c) indicating the anti-HCV potency of this peptide. Additionally Peptide 1 demonstrated similar anti-HCV properties compared to Hep1 and a very low dose of interferon α (Fig. 3D).

Radixin Hinge Region Peptide Blocks Viral Entry by Blocking HCV Engagement of CD81

Recent reports including ours have demonstrated that engagement of CD81, a key host receptor for HCV, leads to ezrin and radixin phosphorylation via spleen tyrosine kinase (SYK) activation (Bukong et al. 2013; Coffey et al. 2009). Additionally, we recently showed that disruption of downstream signaling events after CD81 engagement leading to SYK activation blocks HCV J6/JFH-1 infection of Huh7.5 cells (Bukong et al. 2013). The observation that Peptide 1 inhibited HCV infection led us to speculate that this peptide might be disrupting signaling events necessary for effective HCV entry into a target cell. In support of this hypothesis, pre-treatment of Huh7.5 cells with Peptide 1 but not the control Peptide 16 followed by HCV J6/JFH1 exposure blocked effective HCV engagement of CD81 leading to SYK activation in Huh7.5 cells (Fig. 4). These experiments indicated a

mechanistic role for the novel radix in hinge region Peptide 1 in reducing HCV J6/JFH-1 infectivity at the level of HCV engagement of CD81 entry thereby disrupting SYK phosphorylation which is an important downstream modulator for effective infection (Bukong et al. 2013).

Discussion

The limited efficacy of current treatments against HCV coupled with the alarming disease prevalence has sparked interest in the development of more potent anti-HCV drugs. Currently, most approved clinical therapies target viral HCV components (Ploss and Dubuisson 2012) and by their very nature have higher chances of the virus developing treatment resistance. To overcome this limitation, treatment strategies are now being formulated to target host cellular factors needed by the virus for infection and replication. This approach is extremely attractive because treatment resistance cannot be easily developed and multiple viruses which use similar host molecules can be targeted with a single anti-viral agent.

HCV infection of a target cell is a multistep process involving a number of host cell molecules. Studies have identified host molecules like CD81 (Pileri et al. 1998; Meuleman et al. 2008), claudin 1 (Evans et al. 2007), LDLR (Molina et al. 2007), SR-BI (Scarselli et al. 2002), occludin (Ploss et al. 2009) and others (Rice 2011), all of which are located at the plasma membrane, to be important for HCV infection of permissive cells. Additionally, we have recently identified important therapeutic host molecules and signaling targets downstream of CD81 which can be exploited for HCV treatment (Bukong et al. 2013).

In this report, we demonstrate that a radixin hinge region peptide (Peptide 1) modestly blocks the entry of HCV J6/JFH-1 virus into Huh7.5 cells suggesting a role for this peptide at the very early stage of HCV infection. All the anti HCV peptides which showed anti-viral potential have greater than 75 % hydrophilicity and a net basic charge of 1. Peptide 1 showed a higher anti-viral capacity than Peptide 6 despite similar hydrophilicity and charge possibly due to the higher and dual peptide hydrophobicity of Peptide 6. Because the included sequence of Peptide 1 has a greater than 60 % sequence homology to the anti HCV peptide, Hep1, we cannot exclude similar additional antiviral properties of Peptide 1 in vivo similar to Hep1 (Salamov et al. 2007) which were not explored in this study.

In a recent report we demonstrated the important role of EMR proteins in HCV infection at the level of HCV entry. We found that HCV E2 protein engagement of CD81 led to ezrin phosphorylation via SYK activation. SYK activation of ezrin led to ezrin relocalization with F-actin which we identified as important events necessary for HCV entry and infection of a target cell. Given that Peptide 1 blocked effective engagement of HCV with CD81 leading to downstream inhibition of SYK activation, our novel finding supports a model where EMR hinge region peptides block HCV viral entry and infection. Our novel data supports a mechanism where Peptide 1 can block SYK activation by upstream disruption of HCV interaction with C81, which is a crucial step for effective HCV entry and infection of a susceptible cell (Bukong et al. 2013).

In conclusion, the identification of the radixin hinge region peptide and one of its functional mechanisms now adds a novel anti-viral drug that targets HCV entry. Given the importance of EMR proteins in modulating other viral infections like HIV (Haedicke et al. 2008; Naghavi et al. 2007), this report will also aid in dissecting the anti-viral potential of other EMR peptides against other viral infections. Given that most anti-viral peptides in clinical use target viral factors, the observation that Peptide 1 targets a host molecule and hence reduces the likelihood of developing resistance offers potential clinical advantage of this peptide. Additionally, by virtue of its distinct mechanism of HCV inhibition, Peptide 1 and other such peptides may be used in combination with other anti-HCV drugs for potential synergistic anti-viral effects. Given that we find just a modest reduction in HCV infection with the EMR hinge region peptides assessed we suggest that such peptides should not serve as first line therapy against HCV infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Rational design of potential anti-HCV radixin peptides. **a** Schematic illustration of sequence alignment for EMR hinge region providing a basis for the design of radixin peptides similar to the Hep 1 anti-HCV ezrin peptide. **b** List of radixin hinge region peptides initially screened for potential anti-HCV properties. All peptides were synthesized by GenScript with >90 % purity

Fig. 2.

A human derived radixin hinge region peptide (Peptide 1) suppresses HCV infection.

Huh7.5 cells were pre-treated with the indicated peptide (1 $\mu\text{g/mL}$) for 1 h followed by co-culture with HCV J6/JFH1 virus for 3 h at 4 °C. After 4 h virus and peptides were washed off from cells, and incubated for a further 24 h prior to real time qPCR analysis of HCV RNA. Data are presented as fold inhibition relative to control infections in which cells were treated with dimethyl sulfoxide (DMSO 0.01 %). Results are expressed as mean + standard error of the mean (SEM) and $p < 0.05$ was considered statistically significant by the Mann–Whitney test for four independent repeat experiments

Fig. 3.

Anti-HCV Peptide 1 blocks entry of HCV J6/JFH-1 in Huh7.5 cells. **a, c, d** Synchronization method for HCV infection utilizing a modified infection protocol where virus supernatants are incubated with Huh7.5 cells with or without treatments as indicated for 3 h at 4 °C. The indicated 3 h incubation at 4 °C allows synchronization of HCV J6/JFH-1 attachment to target cells, but not virus entry. Cells were then washed 4 times with cold PBS to remove unbound viruses and incubated for a further 24 h in fresh medium with additional treatment or not as indicated. **a** Western blot analysis of HCV NS3 protein 24 h after synchronized HCV J6/JFH-1 infection with or without peptide or specific treatment as indicated. **b** Treatment of FL replicon cells with anti-HCV peptide 1 and HCV RNA analysis 72 h after peptide treatment. **c** Peptide 1 pre-treatment followed by HCV synchronized infection and western Blot analysis of HCV core protein to determine the dose dependent effect of a consensus moesin–radixin peptide (Peptide 1) 24 h after HCV infection. **d** Western blot analysis of HCV NS3 protein in Huh7.5 cells 24 h after synchronized HCV J6/JFH-1 infection for Peptide 1, Hep1 and negative control Peptide 16. Data is representative of 4 independent experiments expressed as mean + SEM, $p < 0.05$ were considered statistically significant by Mann–Whitney test

Fig. 4. Anti-HCV Peptide 1 blocks HCV infection by disrupting HCV engagement of CD81 thereby inhibiting downstream SYK activation. Huh7.5 cells were pre-treated with the indicated peptide or not as indicated followed HCV J6/JFH-1 (MOI 10) co-culture or not as indicated for 90 min. Cells were then extensively washed five times with cold PBS followed by total cell protein extraction. Extracted proteins were subjected to immunoprecipitation and western blot analysis for phospho-SYK. Data is representative of 4 independent experiments expressed as mean + SEM, and $p < 0.05$ was considered statistically significant by the Mann–Whitney test