ER and have been implicated in facilitating the replication of various positive-strand RNA viruses.[96−98] Furthermore, both RTN1 and RTN3 have been specifically detected in the very low density lipoprotein (VLDL) transport vesicle (VTV);[99] VTV is a key component of the VLDL secretory pathway, which plays an essential role in the production and the release of the infectious HCV particles.[100] Therefore, NS5A interactions with RTN1 and RTN3 suggested novel and potentially crucial roles of the two host proteins in the replication and/or release stages of the HCV lifecycle.

We performed cellular assays to assess the impact of RTN1 and RTN3 siRNA knockdowns on HCV replication and release. Since the HCV-production systems using the HCV JFH1 infectious strain (genotype 2a) isolates alone are capable of both efficient replication and the production of the infectious HCV particles, JFH1 was used to infect the Huh7OK1 cell line 24h after transfection with each siRNA (see Materials and Methods). The infected cells were harvested after 72 h postinfection, and the expression of each host protein was assessed by qRT-PCR (Figure 4A). The viral titer was significantly decreased by individual and double knockdowns of RTN1 and RTN3 (Figure 4B). However, RTN1 and RTN3 knockdowns had no effect on the intracellular viral RNA levels in the HCV infected cells (Figure 4C), suggesting that RTN1 and RTN3 regulate HCV propagation but not HCV replication.

CONCLUSIONS

We describe here our observations of PPIs between HCV NS5A and host proteins. By employing a multifold approach involving an experimental Y2H assay and literature mining, we derived a comprehensive set of experimentally determined binary interactions between NS5A and host proteins. We proceeded to map the combined NS5A−host interactions onto an overall interaction network, which comprised a repertoire of connections, which potentially enable NS5A to link up with and modulate the components of the host cellular networks. We then employed a network-based approach to understand the biological context of these connections in HCV pathogenesis with the help of the TargetMine data warehouse.

A functional analysis of the PPI networks highlighted NS5A interactions with several well connected host factors (hubs) and centrally located "bottlenecks" in the host cellular networks that function in cellular pathways associated with immune system and cell signaling, cellular adhesion and cell transport, cell growth and cell death and ER homeostasis among others. The "bottlenecks" include several proteins that were previously implicated in HCV pathogenesis, thereby suggesting that NS5A interactions with centrally connected host factors may enable the virus to influence strongly the host cellular processes in HCV infection. Notably, many bottlenecks were mapped to pathways associated with the infectious diseases induced by diverse bacterial and viral pathogens of the human host. These observations thus suggest the presence of some common themes underlying the onset of various human diseases associated with pathogenic infection in humans, a better understanding of which may be helpful in optimizing broad spectrum approaches to counteracting a wide range of pathogenic infections.

Cellular assays based on siRNA knockdowns in the HCV infected and replicon cells demonstrated RTN1 and RTN3, ER-localized NS5A interacting proteins, to be novel regulators of HCV propagation, but not replication, and thus promising novel candidates for anti-HCV therapy.

Our analysis therefore provides further insights into the role of NS5A−host interactions in HCV infection, a deeper understanding of which may aid in the identification of new clinically relevant targets for optimizing the therapeutic strategies to manipulate HCV−host interactions and thus more effectively combating HCV infection. Our analysis also emphasizes the importance of elaborate network-based computational approaches that integrate diverse biological data types in investigating host−pathogen interactions.

ASSOCIATED CONTENT

Supporting Information

Supporting methods, figures, and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: kenji@nibio.go.jp (K.M.); lokesh@nibio.go.jp (L.P.T.). Tel: +81-72-641-9890. Fax: +81-72-641-9881.

Author Contributions

¶L. P. Tripathi and H. Kambara contributed equally to this work.

Notes

The authors declare no competing financial interest.

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VIRAL HEPATITIS

Ca^{2+}/S100 proteins regulate HCV virus NS5A–FKBP8/FKBP38 interaction and HCV virus RNA replication

Joji Tani[1,*], Seiko Shimamoto[2,*], Kyoko Mori[3], Nobuyuki Kato[3], Kohji Moriishi[4], Yoshiharu Matsuura[5], Hiroshi Tokumitsu[2], Mitsumasa Tsuchiya[2], Tomohito Fujimoto[2], Kiyohito Kato[1], Hisaaki Miyoshi[1], Tsutomu Masaki[1] and Ryoji Kobayashi[2]

1 Department of Gastroenterology and Neurology, Kagawa University Faculty of Medicine, Kagawa, Japan
2 Department of Signal Transduction Sciences, Kagawa University Faculty of Medicine, Kagawa, Japan
3 Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan
4 Department of Microbiology, Division of Medicine, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Yamanashi, Japan
5 Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

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Correspondence
Ryoji Kobayashi, MD, PhD, Department of Signal Transduction Sciences, Kagawa University Faculty of Medicine, 1750-1, Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan
Tel: +81-87-891-2249
Fax: +81-87-891-2249
e-mail: ryoji@med.kagawa-u.ac.jp

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Abstract

Background & Aim: FKBP8/FKBP38 is a unique FK506-binding protein with a C-terminal membrane anchor and localizes at the outer membranes of mitochondria and the endoplasmic reticulum. Similar to some immunophilins, such as FKBP51, FKBP52 and Cyclophilin 40, FKBP8/FKBP38 contain a putative Calmodulin-binding domain and a tetratricopeptide-repeat (TPR) domain for the binding of Hsp90. Both Hsp90 and the non-structural protein 5A (NS5A) of the hepatitis C virus (HCV) interact specifically with FKBP8/FKBP38 through its TPR domain, and the ternary complex formation plays a critical role in HCV RNA replication. The goal of this study is to evaluate that the host factor inhibits the ternary complex formation and the replication of HCV *in vitro* and *in vivo*. *Methods:* S100 proteins, FKBP38, FKBP8, HCV NS5A, Hsp90, and calmodulin were expressed in E.coli and purified. *In vitro* binding studies were performed by GST pull-down, S-tag pull-down and surface plasmon resonance analyses. The effect of S100 proteins on HCV replication was analysed by Western blotting using an HCV NS3 antibody following transfection of S100 proteins into the HCV replicon harbouring cell line (sO cells). *Results: In vitro* binding studies showed that S100A1, S100A2, S100A6, S100B and S100P directly interacted with FKBP8/FKBP38 in a Ca^{2+}-dependent manner and inhibited the FKBP8/FKBP38–Hsp90 and FKBP8/FKBP38–NS5A interactions. Furthermore, overexpression of S100A1, S100A2 and S100A6 in sO cells resulted in the efficient inhibition of HCV replication. *Conclusion:* The association of the S100 proteins with FKBP8/FKBP38 provides a novel Ca^{2+}-dependent regulatory role in HCV replication through the NS5A–host protein interaction.

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease, which frequently progresses to cirrhosis and hepatocellular carcinoma (1, 2). HCV represents a global public health problem, affecting approximately 170 million people worldwide, which is more than 3% of the world population (3, 4). HCV is a member of the positive strand RNA viruses, belongs to the family Flaviviridae, genus Hepacivirus, and contains a genome of approximately 9.6 kb in length, which encodes a large polyprotein precursor of approximately 3000 amino acids (5, 6). The polyprotein is cleaved by host and viral proteases to release the individual enzymes and proteins that mediate virus replication, assembly and release, producing viral structural proteins (Core, E1 and E2), a putative viropore protein (p7) and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) (5, 6). Importantly, the NS5A of HCV plays a critical role in HCV replication and is an attractive target for antiviral therapy of HCV infection (7). NS5A is a multifunctional 56–58 kDa serine phosphoprotein and interacts with a number of cellular proteins thereby affecting numerous host functions, including the modulation of signal transduction pathways, suppression of apoptosis and modulation of transcription (8–10). Recently, FK506-binding protein 8 (FKBP8)/FK506-binding protein 38 (FKBP38) was shown to interact with NS5A and to regulate HCV replication (11, 12),

*These authors contributed equally to this work.

suggesting that the immunophilins are promising therapies for chronic hepatitis C. FKBP8/FKBP38 binds to both NS5A and heat-shock protein 90 (Hsp90) through the tetratricopeptide-repeat (TPR) domain, and the ternary complex (FKBP8/FKBP38–Hsp90–NS5A) plays a critical role in HCV replication (11, 12). TPRs are loosely conserved 34 amino acid helix–turn–helix sequence motifs that mediate protein–protein interactions (13). This property enables TPR-containing proteins to function as scaffold proteins and allows them to be involved in a variety of cellular functions (14, 15).

The S100 protein family is composed of at least 25 members that share two EF-hand motifs, a 25–65% amino acid sequence homology and a molecular weight of 10–12 kDa (16–18).

Recently, we demonstrated that S100A2 and S100A6 interacted with the TPR domains of Hsp70/Hsp90-organizing protein (Hop), kinesin light chain (KLC) and Tom70 in a Ca^{2+}-dependent manner, leading to the dissociation of the Hsp90–Hop–Hsp70, KLC–JIP1 and Tom70–Hsps interactions *in vitro* and *in vivo* (19). Further studies have revealed an interaction of S100A1 and S100A2 bound to FK506-binding protein 52 (FKBP52) and cyclophilin 40 (Cyp40), which contain a TPR domain; in the presence of Ca^{2+}, this interaction led to the inhibition of the Cyp40–Hsp90 and FKBP52–Hsp90 interactions (20).

Because S100 proteins interact with TPR motifs (19, 20), we explored the potential role for S100 proteins in the regulation of the FKBP8/FKBP38–HSP90 and/or FKBP8/FKBP38–NS5A interactions and thereby the control of HCV replication. In this study, we demonstrate that Ca^{2+}/S100 proteins modulate replication of HCV via two different mechanisms. Firstly, S100A1, S100A2, S100A6, S100B and S100P interact with the TPR domain of FKBP8/FKBP38 and compete with Hsp90 binding to FKBP38. Secondly, these S100 proteins inhibit the interaction between NS5A and FKBP8/FKBP38. Using HCV replicon harbouring cells (sO cell: HCV O strain of genotype 1b) (21, 22), the overexpression of S100A1, S100A2 and S100A6 with A23187 treatment has shown a significant decrease in NS3 expression. These observations indicate that Ca^{2+}/S100 proteins could modulate HCV replication by inhibiting the interaction between FKBP8/FKBP38 and its binding partners.

Materials and methods

Materials

Phenyl–Sepharose and glutathione–Sepharose were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Nickel–nitrilotriacetic acid agarose was purchased from Qiagen. S-protein agarose was purchased from Merck. Antibodies were obtained as follows: anti-S100A1 (Novus Biologicals, Littleton, Colorado, USA), anti-S100A2 (ANOVA, Santa Clara, CA, USA), anti-S100A6 (Proteintech Group Inc., Chicago, IL, USA), Anti-HCVNS3 (Leica Microsystems, Wetzlar, Germany), anti-β-actin (BioVision, Milpitas, CA, USA) and horseradish peroxidase-conjugated antimouse or antirabbit IgG antibody (GE Healthcare). All other chemicals were obtained from standard commercial sources.

Plasmids

The following plasmids were previously described: pME18S-S100A1, -S100A2, -S100A6, pET-Calmodulin (pET-CaM), pET-CaM-Glutathione-S-transferase (pET-CaM-GST) and pET16b-Hsp90 (19, 20, 23, 24). Human FKBP38 and FKBP8 complementary DNAs (cDNAs) were purchased from Open Biosystems and subcloned into pET30a. For the expression of FKBP38 C-terminal deletion mutants, polymerase chain reaction (PCR) fragments encoding residues 1–166, −297, −315, −325 and −335 were also cloned into pET30a. To create a plasmid of the pET30a-FKBP38 carboxylate clamp mutant (K250E/R254E), the plasmid was generated using inverse PCR using pET30a-FKBP38 (1–335) as a template. pETUbHis-NS5A was kindly provided by Dr Craig E. Cameron (25). The sequence integrity of the all inserts was confirmed through automated sequence analysis (Applied Biosystems, Foster City, CA, USA).

Preparation of recombinant proteins

All recombinant proteins were produced in *Escherichia coli* strain BL21(DE3) or BL21(DE3) CodonPlus-RIL (Novagen, Darmstadt, Germany). S100 proteins (S100A1, S100A2, S100A4, S100A6, S100A10, S100A11, S100A12, S100B and S100P) were expressed and prepared as described previously (26, 27). Calmodulin (CaM) was prepared as described by Hayashi and colleagues (28). C-terminally GST-tagged CaM linked by a Gly_6 spacer (CaM-GST) was prepared as described (23, 24). A C-terminally His-tagged NS5A, lacking the N-terminal 32 amino acid residues of the membrane-anchoring region (NS5A-His), was expressed via the pET-ubiquitin expression system, in which the NS5A-His was fused with ubiquitin at the C-terminus and was cleaved by a ubiquitin-specific protease, Ubp1, in *E. coli* as described by Huang and colleagues (25). The His-tagged Hsp90 and NS5A were purified via the nickel–nitrilotriacetic acid (NTA)-agarose method according to the manufacturer's protocol. Protein expression of N-terminally His_6 and S-tagged FKBP38 (His_6-S-tag -FKBP38) and FKBP8 (His_6-S-tag-FKBP8) was induced with the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM and further incubation at 16°C for 4 h. The His_6-S-tag-FKBP38 mutants (1–166, −297, −315, −325) and the His_6-S-tag-FKBP8 mutants (1–382) were purified using NTA agarose. The His_6-S-tag-FKBP38 (1–335), His_6-S-tag-FKBP8 (1–392) and His_6-S-tag-FKBP38-K250E/R254E mutants were purified with CaM-GST-coupled glutathione–Sepharose

columns as follows. The bacterial pellet from 200 ml of culture was resuspended in 10 ml of Buffer A (20 mM Tris-HCl, 200 mM NaCl and 5 mM dithiothreitol, pH 7.5), lysed using ultrasonic disruption and centrifuged at 35 000 *g* for 30 min at 4°C. The purified CaM-GST (3 mg) and $CaCl_2$ (2 mM final concentration) were mixed with the supernatant. The resulting mixture was applied to a glutathione – Sepharose column (1 ml bed volume; GE Healthcare) and then the column was washed with 5 ml of Buffer A supplemented with 0.2 mM $CaCl_2$. His_6-S-tag-FKBP38 or His_6-S-tag-FKBP8 was eluted using Buffer A supplemented with 0.5 mM ethylene glycol tetraacetic acid (EGTA). The concentration and purity of the isolated proteins were determined with a Bradford assay (Bio-Rad, Hercules, CA, USA) and Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE).

Pull-down assay

To assess the binding of S100 proteins, Hsp90 and NS5A to FKBP8/FKBP38, His_6-S-tag-FKBP8/FKBP38 and either S100 proteins, Hsp90 or NS5A, were mixed with S-protein-agarose beads (20 μl) in Buffer B (20 mM Tris-HCl, 150 mM NaCl and 0.02% Tween 20, pH 7.5) in the presence of 1 mM $CaCl_2$ or EGTA. The reaction mixtures (200 μl) were incubated for 60 min at 25°C. After the resin was washed three times with 1.0 ml of Buffer B, the resin was boiled in SDS-sample buffer (30 μl). Next, the samples were subjected to SDS-PAGE and visualized with Coomassie Blue staining. The details of the experimental conditions are described in the figure legends.

Surface Plasmon Resonance (SPR)

Binding kinetics were analysed using a SPR Biacore 2000 system (Biacore AB, Little Chalfont, Buckinghamshire, UK). CM5 research grade chips, N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Biacore amine coupling kit) were used for the amine coupling of the recombinant N-terminally His_6 and S-tagged FKBP8 (His_6-S-tag-FKBP8) (4540RU) to the dextran surface of the CM5 chip. Recombinant S100A1, S100A2, S100A6, S100B, S100P and CaM (1.25 μM, 625 nM, 313 nM, 156 nM and 78 nM respectively) were injected over the sensor surface at a flow rate of 20 μl/min in HBS-P buffer (10 mM Hepes, 150 mM NaCl and 0.005% Surfactant P20, pH 7.4) containing 1 mM $CaCl_2$. The S100 proteins were allowed to interact with the surface of the sensor chip for 2.5 min, after which HBS-P buffer was injected over the sensor surface to monitor the dissociation of the S100 protein. At intervals based on the sample injection, an FKBP8-coupled sensor chip was regenerated with HBS-P buffer supplemented with 2.5 mM EGTA and 0.75% n-Octyl-ß-D-glucopyranoside. The response curves were prepared for fit using a subtraction of the signal generated simultaneously on the control flow cell. Biacore sensorgram curves were evaluated in BIA-evaluation 4.1 using a 1:2 binding model for the S100 proteins. A 1:1 Langmuir model was used for the CaM binding.

Cell culture, transfection and preparation of cell lysates

HuH-7-derived cells harbouring HCV replicon harbouring cells (sO cells) were maintained in Dulbecco's Modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) containing 0.3 mg/ml G418 (Promega, Fitchburg, MA, USA) supplemented with 10% fetal bovine serum (Invitrogen) in a humidified 5% CO_2 incubator at 37°C as described (21). Transient transfections were performed using Fugene 6 (Roche, Mannheim, Germany) according to the manufacturer's instructions. The sO cells (10 cm dish) were transfected with pME18S-S100A1, pME18S-S100A2 or pME18S-S100A6 (each 7 μg). After 8 h of incubation with the transfection reagents, the medium was changed and cells were grown until 100% confluent. Next, the cells were treated with or without A23187 (5 μM) for 6, 12 and 24 h and were then lysed via the addition of 1 ml of the lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Nonidet-P40, 10% glycerol, pH 7.5), briefly sonicated and centrifuged at 15 000 *g* for 30 min at 4°C. The supernatants were subjected to SDS-PAGE, followed by Western blot analysis.

Results

Interaction between the members of the S100 protein family and FKBP38

Previously, we demonstrated that S100A1, S100A2 and S100A6 interacted with the TPR domains of Hop, Tom70, CyP40, FKBP52 and KLC in a Ca^{2+}-dependent manner and led to the disruption of the TPR protein –client protein interactions *in vitro* and *in vivo* (9, 20). Because S100 proteins interact with TPR motifs, we explored the potential role of S100 proteins in regulating the functions of FKBP8/FKBP38. Firstly, we examined the interaction of the S100 proteins with FKBP8/FKBP38. FKBP8/FKBP38, a TPR-containing non-canonical member of the immunosuppressive drug FK506-binding protein (FKBP) family, consists of four structural and functional domains. The FK506-binding domain is located in the N-terminal half, followed by a TPR domain, a putative CaM-binding site and a transmembrane domain. Human FKBP8 is identical to FKBP38 except for the extra 58 amino acid residues at the N-terminus. The domain structure organization of FKBP8/FKBP38 is shown in Figure 1a. Because our preliminary binding analyses demonstrated that the last 10 residues immediately upstream of the transmembrane domain (^{326}AWSIPWKWLF^{335}) in FKBP38, but not the putative CaM-binding site, compose the actual

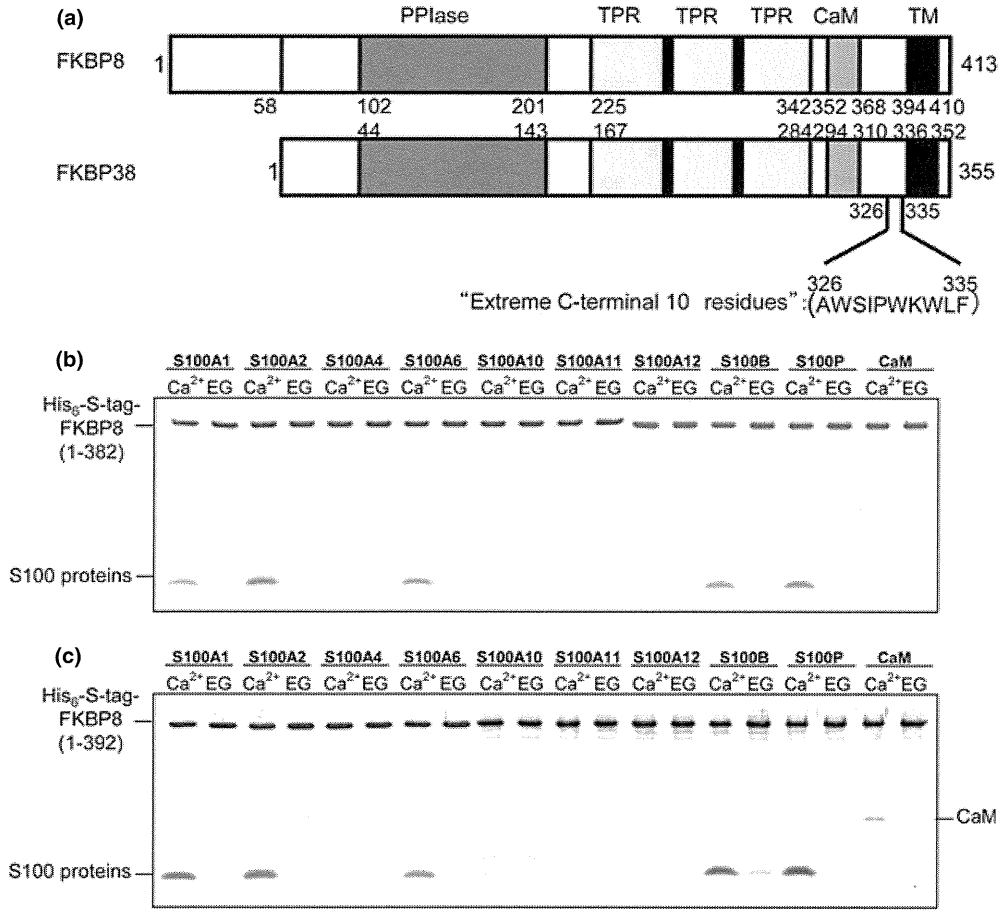
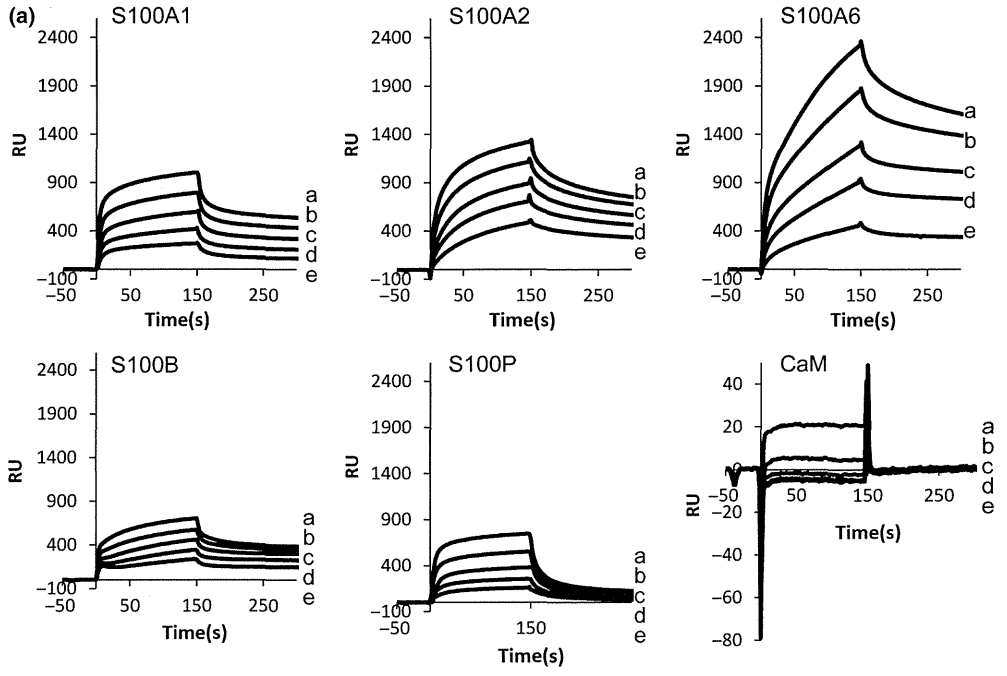


Fig. 1. Interaction of FKBP8 and S100 proteins. (a) Schematic representation of FKBP8 and FKBP38. The peptidyl prolyl *cis/trans* isomerases (PPIase), tetratricopeptide repeat (TPR), calmodulin-binding site (CaM), 'extreme C-terminal 10 residues' and transmembrane domain (TM) are shown. The number refers to the amino acid positions of FKBP8. (b) The S-tag pull-down assay was performed using His_6-S-tag-FKBP8 (1–382) and the S100 proteins. (c) The S-tag pull-down assay was performed using His_6-S-tag-FKBP8 (1–392) and the S100 proteins. The His_6-S-tag-FKBP8 (20 μg) and S100 proteins (20 μg) were incubated with S-protein agarose beads in the presence of either 1 mM $CaCl_2$ or EGTA for 1 h. Beads were washed and samples were separated with 10% Tricine–SDS-PAGE and stained with Coomassie Blue.

Ca^{2+}/CaM-binding site, we prepared a recombinant His_6-S-tag-FKBP8 (1–382) and a His_6-S-tag-FKBP8 (1–392). A precise analysis of the S100 proteins–FKBP38 binding will be described elsewhere (S. Shimamoto, H. Tokumitsu, M. Tsuchiya, Y. Kubota and R. Kobayashi, unpublished data). To test the direct binding of the S100 proteins to FKBP8, we performed a pull-down assay using the S100 proteins with His_6-S-tag-FKBP8 (1–382) or His_6-S-tag-FKBP8 (1–392) in the presence of 1 mM $CaCl_2$ or EGTA. The bound proteins were analysed using Tricine–SDS-PAGE. As shown in Figure 1b, c, S100A1, S100A2, S100A6, S100B and S100P bound strongly to His_6-S-tag-FKBP8 (1–382) or His_6-S-tag-FKBP8 (1–392) in a Ca^{2+}-dependent manner, whereas CaM did not bind to His_6-S-tag-FKBP8 (1–382). In addition, S100A4, S100A10 and S100A11 bound weakly to His_6-S-tag-FKBP8 (1–392).

To examine the real-time binding kinetics of S100A1, S100A2, S100A6, S100B, S100P and CaM to FKBP8, the recombinant His_6-S-tag-FKBP8 (1–392) was immobilized on a sensor chip surface and the protein complex formation was analysed using SPR (Fig. 2a). The binding curves of S100A1, S100A2, S100A6, S100B and S100P were fit to the 1:2 ligand-binding model, whereas the binding curve of CaM was fit to the 1:1 ligand-binding model. The association rate constant (Ka) of S100A1, S100B and S100P to the immobilized FKBP8 (1–392) is indistinguishable and the association of S100A6 and S100A2 occurred more slowly compared with that of other S100 proteins. This suggested an underlying interaction mechanism that was different among the S100 proteins (S100A1, S100B, S100P vs. S100A2, S100A6) (Fig. 2b). The binding concentration of the S100 proteins was measured using a sensorgram. The resonance unit (RU) correlates with the amount of analyte bound (1 RU = 1 pg/mm^2), and the amount of the S100 proteins and CaM binding to FKBP8 (1–392) were significantly different



(b) The binding affinity of FKBP8 and S100 protein as analyzed by the Biacore

Analyte	ka1 (1/Ms)	kd1 (1/s)	ka2 (1/Ms)	kd2 (1/s)	KA1 (1/M)	KA2 (1/M)	KD1(M)	KD2 (M)
S100A1	1.08×10^{5}	0.98×10^{-4}	0.38×10^{5}	0.16×10^{-4}	1.11×10^{8}	0.2×10^{10}	9.03×10^{-9}	4.19×10^{-10}
S100A2	0.90×10^{5}	7.15×10^{-4}	0.61×10^{5}	0.08×10^{-4}	0.13×10^{8}	0.73×10^{10}	0.79×10^{-9}	1.37×10^{-10}
S100A6	0.68×10^{5}	5.31×10^{-4}	0.15×10^{5}	0.12×10^{-4}	1.29×10^{8}	0.13×10^{10}	7.76×10^{-9}	7.78×10^{-10}
S100B	5.47×10^{5}	1.17×10^{-4}	2.60×10^{5}	0.27×10^{-4}	4.68×10^{8}	0.97×10^{10}	2.14×10^{-9}	1.03×10^{-10}
S100P	1.21×10^{5}	1.60×10^{-4}	1.99×10^{5}	0.08×10^{-4}	0.76×10^{8}	2.45×10^{10}	0.13×10^{-9}	0.41×10^{-10}
CaM	0.36×10^{5}	0.10×10^{-4}			3.53×10^{8}		2.83×10^{-9}	

Fig. 2. Analysis of FKBP8 and S100 protein binding by *Surface Plasmon Resonance (SPR)*. (a) Recombinant His_6-S-tag-FKBP8 was immobilized to the dextran surface of the CM5 chip in 20 mM ammonium acetate, pH 4.2, until 4540 response units (0.3 pmol) were bound and a stable base line was obtained. Recombinant S100A1, S100A2, S100A6, S100B, S100P, S100A12 and CaM were injected at various concentrations (a: 1.25 μM, b: 625 nM, c: 313 nM, d: 156 nM and e: 78 nM). The response curves were prepared for fitting by subtraction of the signal generated simultaneously on the control flow cell. (b) Biacore sensorgram curves were evaluated using BIAevaluation 3.0 using a 1:1 Langmuir model (for CaM) or 1:2 binding model (for the S100 proteins). The calculated kinetic parameters of S100 protein binding to FKBP8 were presented.

(S100A6 > S100A2 > S100A1 ≥ S100P > S100B >> CaM) (Fig. 2).

S100 protein binding to FKBP38

Previous co-immunoprecipitation studies indicated that both NS5A and Hsp90 bound to the TPR domain of FKBP8 and that the interaction between NS5A and FKBP8 did not affect complex formation with Hsp90 (11, 12). Hsp90 has been reported to bind to the two-carboxylate clamp positions within the TPR domain of FKBP8/FKBP38 through its EEVD motif (11, 29), whereas NS5A binds to the FKBP8-TPR through its Val/Ile^{121} residue (12). To define the binding domain of the S100 proteins in FKBP38, the various length FKBP38 and point mutants fused with S-tagged FKBP38 (1–166), FKBP38 (1–297), FKBP38 (1–315), FKBP38 (1–325), FKBP38 (1–335) and KR (where the

250 Lys, 254 Arg in the TPR domain were replaced with Ala) were constructed (Fig. 3a). Each mutant protein was incubated with S100A1, S100A2, S100A6, S100B, S100P, CaM, Hsp90 and NS5A. NS5A and all the S100 proteins bound to all the FKBP 38 truncation mutants and the KR mutant, but did not bind to FKBP38 (1–166) (Fig. 3b). These data indicate that the S100 proteins interact with the C-terminal portion [including TPR and putative CaM-binding domain) of FKBP38 and do not interact with the FK506-binding domain (i.e. FKBP38 (1–166)] (Fig. 3b). In contrast, the last 10 C-terminus residues of FKBP38 are required for the binding of CaM, and the two-carboxylate clamp is essential for the binding of Hsp90. Taken together, these observations prove the direct interaction of NS5A, Hsp90 and the S100 proteins with FKBP38 and imply the existence of similar, but not identical, binding sites of the S100 proteins, NS5A and Hsp90 within FKBP38.

S100 proteins interfere with the interactions of TPR-mediated FKBP8/FKBP38–HSP90 and FKBP8/FKBP38–NS5A

To study whether S100 proteins interfere with the binding of Hsp90 and NS5A to FKBP8/FKBP38, we performed competitive pull-down assays. Fixed amounts of His_6-S-tag-FKBP8/FKBP38 (25 μg) and Hsp90 (25 μg) were mixed with an increasing amount of the S100 proteins (0–50 μg), and a S-tag pull-down was performed (Fig. 4). SDS-PAGE gels demonstrate the displacement effect of the S100 proteins on the FKBP8–Hsp90 (Fig. 4a) and FKBP38–Hsp90 interactions (Fig. 4b). Furthermore, increasing the amount of the S100 proteins (S100A1, S100A2, S100A6, S100B and S100P) strongly inhibited the binding of His_6-S-tag-FKBP8/FKBP38 with Hsp90, whereas a weaker inhibition was detected with CaM. A significant inhibition was not observed with S100A12 as a negative control. To prove

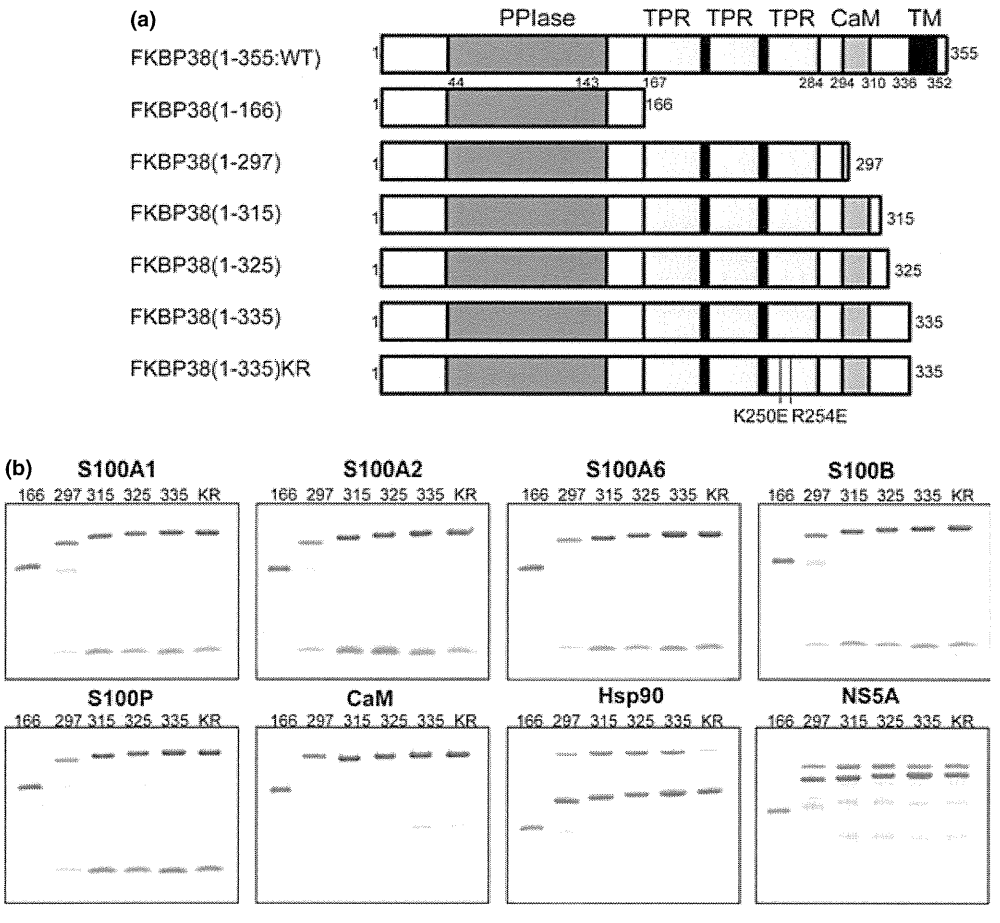


Fig. 3. Interaction of the FKBP8/FKBP38 mutants with the S100 proteins, Hsp90 and NS5A. (a) Schematic diagrams of the FKBP38 mutants. The number refers to the amino acid positions of FKBP38. The peptidyl prolyl *cis/trans* isomerases (PPIase), tetratricopeptide repeat (TPR), calmodulin-binding site (CaM) and transmembrane domain (TM) are shown. (b) The S-tag pull-down assay was performed using His_6-S-tag-FKBP38-WT and it's mutants. The His_6-S-tag-FKBP38 proteins (20 μg) and S100A1, S100A2, S100A6, S100B, S100P, CaM, Hsp90 or NS5A (25 μg each) were incubated with the S-protein agarose beads in the presence of 1 mM $CaCl_2$. Beads were washed and the eluted samples were analysed via a 10% Tricine–SDS-PAGE, followed by Coomassie Blue staining.

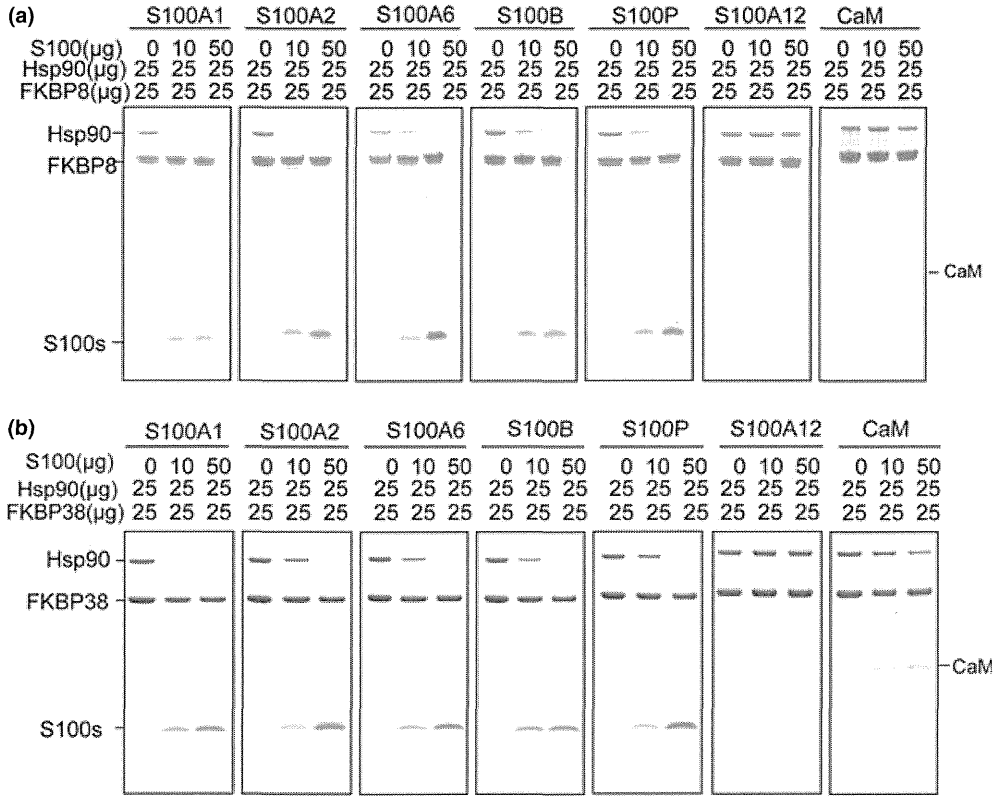


Fig. 4. S100 proteins competitively inhibit Hsp90 binding to FKBP8/FKBP38 *in vitro*. His_6-S-tag-FKBP8 (a, 25 μg) or His_6-S-tag-FKBP38 (b, 25 μg), Hsp90 (25 μg) and increasing amounts of the S100 proteins or CaM (0, 10 and 50 μg) were incubated with S-protein agarose beads in the presence of 1 mM $CaCl_2$ for 1 h. The S-tag pull-down assay was performed as described. Beads were washed and the eluted samples were analysed using a 10% Tricine–SDS-PAGE, followed by Coomassie Blue staining.

this further, a competitive binding assay was also performed with NS5A and His_6-S-tag-FKBP8/FKBP38 (Fig. 5). The addition of purified S100A1, S100A2, S100A6, S100B, S100P and CaM to the binding reactions substantially reduced the amount of NS5A retained on the immobilized His_6-S-tag-FKBP8 (Fig. 5a) and His_6-S-tag-FKBP38 (Fig. 5b). A significant competition was not observed with S100A12 (Figs 4 and 5).

S100 proteins have an inhibitory role in the replication of HCV RNA

The *in vitro* data described above suggest that Ca^{2+}/S100 proteins bind to the TPR domain of FKBP8/FKBP38, and lead to the inhibition of interactions of the FKBP8/FKBP38–Hsp90 and FKBP8/FKBP38–NS5A. Next, to examine whether these interactions are involved in HCV replication, the effect of the overexpression of the S100 protein in combination with ionophore treatment was studied in the HCV replicon harbouring cell line (sO cells) by measuring the amount of the NS3 protein (Fig. 6). We transfected the sO cells with S100A1 (Fig. 6a), S100A2 (Fig. 6b) and S100A6 (Fig. 6c). Following the transfection, the cells were treated with or without A23187 (5 μM) for 6, 12 and 24 h, and the cellular level of NS3 was examined via Western blot analysis using an anti-NS3 antibody. In the control replicon cells, the amount of NS3 did not change in the presence or absence of A23187 treatment. When the S100 proteins were overexpressed, the amount of NS3 was significantly decreased over the incubation period compared with the levels observed in the control cells (*lane 4* vs. *lane 2*). This suggested that increasing the concentration of intracellular Ca^{2+} stimulated the S100 proteins–FKBP8/FKBP38 complex formation, which inhibited the NS5A–FKBP8/FKBP38 and Hsp90–FKBP8/FKBP38 interactions and led to suppress the rate of NS3 production. Collectively, these results demonstrate that the S100 proteins may function as negative regulators of HCV replication in a Ca^{2+}-dependent manner.

Discussion

Hepatitis C virus replication occurs in the cytoplasm and is mediated by a membrane-associated replicase complex consisting of the NS3/NS4A, NS4B, NS5A and NS5B proteins and cellular proteins (host factors) (30).

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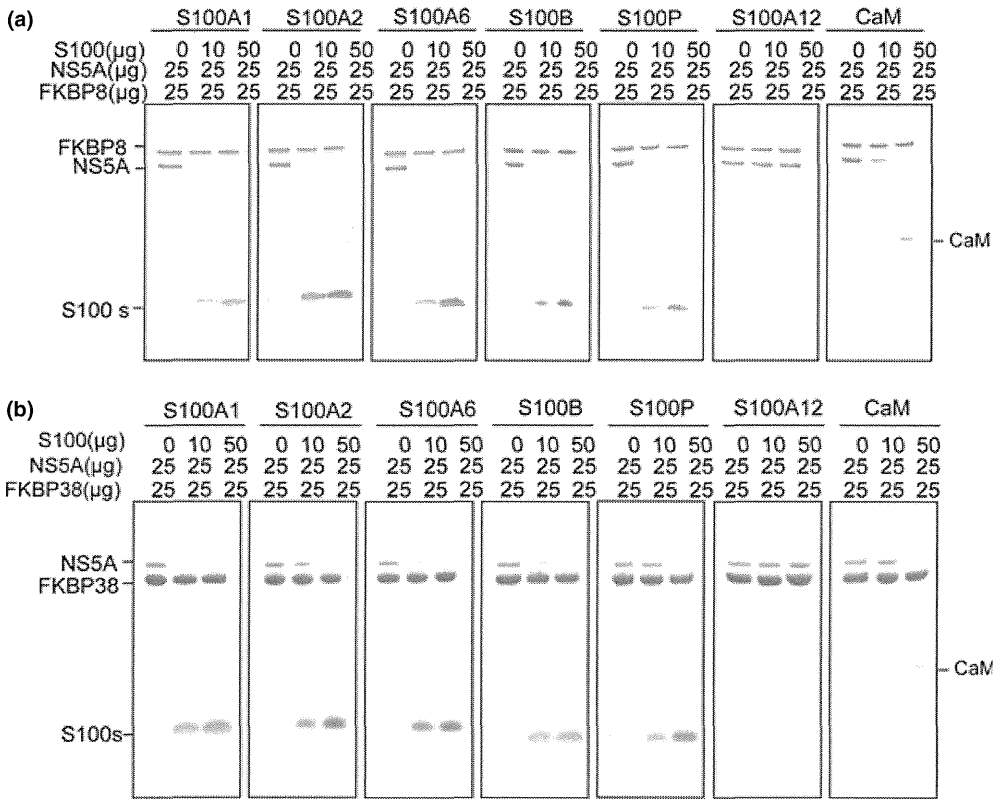

Fig. 5. S100 proteins competitively inhibit NS5A binding to FKBP8/FKBP38 *in vitro*. His_6-S-tag-FKBP8 (a, 25 μg) or His_6-S-tag-FKBP38 (b, 25 μg), NS5A (25 μg) and increasing amounts of the S100 proteins or CaM (0, 10 and 50 μg) were incubated with S-protein agarose beads in the presence of 1 mM $CaCl_2$. The S-tag pull-down assay was performed as described. Beads were washed and the eluted samples were analysed using a 10% Tricine–SDS-PAGE, followed by Coomassie Blue staining.

Importantly, the NS5A of HCV plays a critical role in HCV replication and is an attractive target for antiviral therapy of HCV infection (7).

NS5A is a multifunctional 56–58 kDa serine phosphoprotein and interacts with a number of cellular proteins thereby affecting numerous host functions, including the modulation of signal transduction pathways, suppression of apoptosis and modulation of transcription (8–10). Several cellular proteins, such as hVAP-A (31), hVAP-B (32), FKBP8 (11, 12), Hsp90 (11, 12), Hsp70 (33) and cyclophilin A, B (34, 35) are involved in the replication process of HCV. A recent report indicated that NS5A specifically interacts with FKBP8 via its TPR domain and recruits Hsp90 to the replicase complex; this complex formation is critical for the replication of HCV and thus geldanamycin was able to inhibit the RNA replication in a dose-dependent manner (11). *In vitro* pull-down assays revealed that geldanamycin inhibited the binding of FKBP8 to Hsp90 and/or NS5A domain I. In addition, the interaction between NS5A and FKBP38 disrupts FKBP38-mediated mTOR regulation (10). Moreover, NS5A inhibits apoptosis, potentially via the stabilization of the FKBP38–Hsp90 interactions (36).

TPR proteins are involved in many protein–protein interactions (14, 15); in particular, several cochaperones, including Hip, Hop and the cyclophilins, interact with Hsp70 or Hsp90 through TPR domains (37–40). The intracellular $Ca2^+$ signalling cascade is composed of many molecular components including a large family of EF-hand $Ca2^+$-binding proteins such as calmodulin (CaM), neuronal calcium sensor proteins and S100 proteins (41).

The S100 protein family is composed of at least 25 members that share two EF-hand motifs, a 25–65% amino acid sequence homology, and a molecular weight of 10–12 kDa (16–18).

S100 proteins are proposed to have intracellular and extracellular roles in the regulation of many cellular processes such as cell motility, cell-cycle progression, transcription, protein phosphorylation and tumour progression or suppression (16–18). Recently, we demonstrated that S100A2 and S100A6 interacted with the TPR domains of Hop, KLC and Tom70 in a Ca^{2+}-dependent manner, leading to the dissociation of the Hsp90-Hop-Hsp70, KLC-c-Jun N-terminal kinase-interacting protein-1 (JIP-1) and Tom70–Hsps interactions (19). Further studies revealed that the interaction

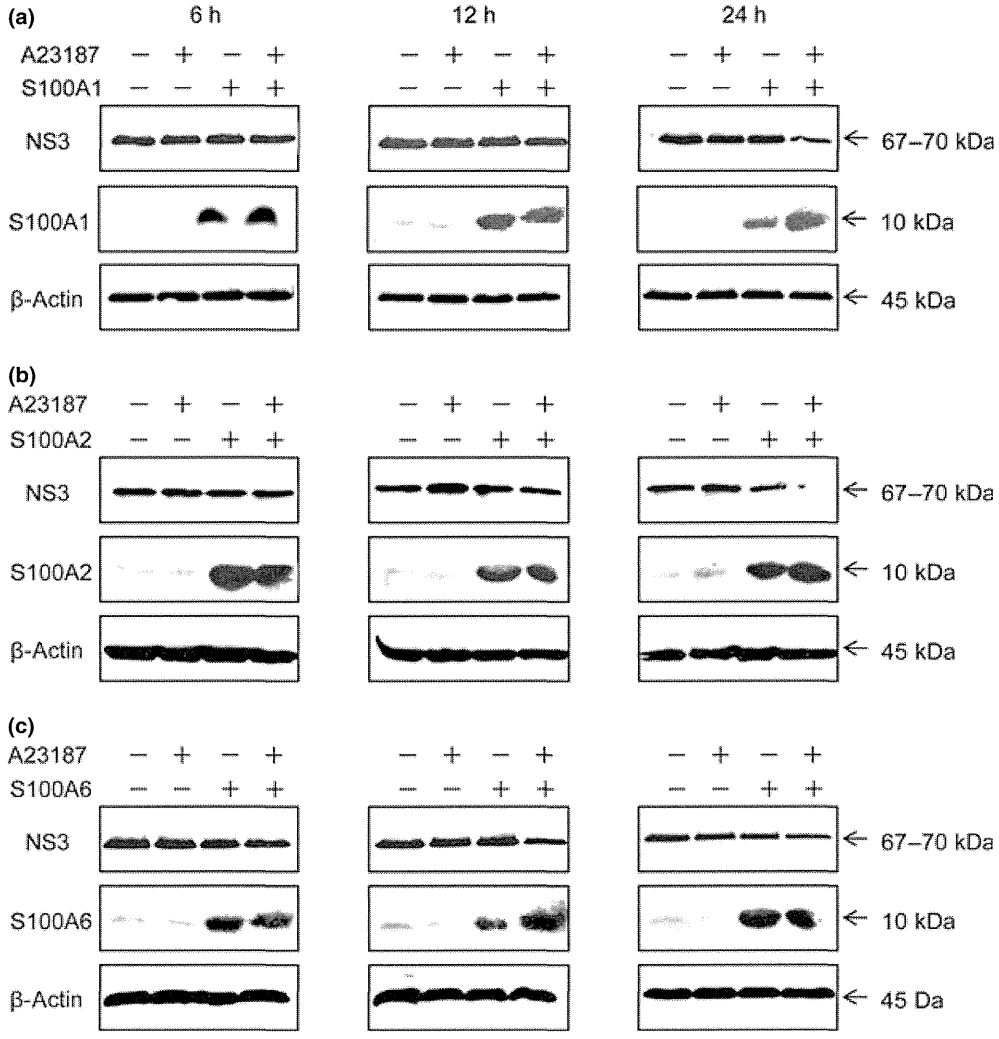

Fig. 6. S100 proteins suppress the cellular level of NS3 in HCV replicon harbouring cells (sO cells). HCV replicon harbouring cells (sO cells) were transiently transfected with (+) or without (–) S100A1 (a), S100A2 (b) and S100A6 (c) as indicated. After A23187 (5 μM) treatment, cell lysates were prepared at different time points (6, 12 and 24 h). The amount of NS3 and S100 proteins was analysed via Western blot analysis with the indicated antibodies. Equal amounts of lysates (20 μg of protein) were loaded in each lane for each time point. β-Actin was used as a control for equal loading.

of S100A1 and S100A2 with FKBP52 and Cyp40, which contain TPR domains, in the presence of Ca^{2+} led to the inhibition of the interactions of Cyp40–Hsp90 and FKBP52–Hsp90 (20).

In this study, we demonstrated that specific members of the S100 proteins, such as S100A1, S100A2, S100A6, S100B and S100P, specifically bind to the TPR domain of FKBP8/FKBP38 in a Ca^{2+}-dependent manner and lead to the inhibition of the Hsp90–FKBP8/FKBP38 interaction. The charged residues in the FKBP8/FKBP38-TPR domains are predicted to form the so-called two-carboxylate clamp form salt bridges with the EEVD of HSP90, and point mutations in the carboxylate clamp diminished the binding (11). Because S100 proteins interfered with the binding of Hsp90 to FKBP8/FKBP38, we anticipated that they bound to the amino acid residues composing the carboxylate clamp. However, S100 proteins bound to the alanine mutants of the carboxylate clamp, although Hsp90 binding was clearly inhibited (Fig. 3b). The results suggested that the mode of interaction of the S100 proteins with FKBP8/FKBP38 is different from the FKBP8/FKBP38–Hsp90 electrostatic interaction.

To explore further the interaction between FKBP8/FKBP38 and the S100 proteins, we investigated whether the S100 proteins inhibited the NS5A–FKBP8/FKBP38 interactions. Surprisingly, these S100 proteins effectively disrupted the NS5A–FKBP8/FKBP38 interactions. The binding site of S100 proteins may be close enough to physically interfere with the Hsp90 and NS5A binding to FKBP8/FKBP38. We have previously shown that the S100 proteins bind to TPR domains of Hop, KLC,

Tom70, Cyp40 and FKBP52, and result in the inhibition of the ligand–TPR protein interactions (19, 20). Notably, there is a preference in S100 protein binding among the TPR proteins. For example, S100A2 bound to KLC, FKBP52 and PP5 more tightly, whereas S100A6 preferably bound to Hop, Cyp40 and Tom70. The reason for the selectivity of S100 protein binding to TPR proteins is not fully understood. Currently, it is not possible to predict the residues that are important for the S100–TPR protein interactions.

Because the ternary complex formation of Hsp90, NS5A and FKBP8/FKBP38 is essential for the replication of HCV, it is important to determine whether the S100 proteins function as 'HCV-replication regulator' in intact cells. Using the HCV replicon harbouring cells (sO cells), the overexpression of S100A1, S100A2 and S100A6 in combination with ionomycin treatments showed a significant decrease in the protein level of NS3. The result suggests that the increase in intracellular Ca^{2+} by the treatment of ionomycin stimulated the binding of the S100 proteins and FKBP8/FKBP38, and inhibited the replication of HCV *in vivo*.

In conclusion, our results are the first to demonstrate that the association of the S100 proteins with FKBP8/FKBP38 provides a Ca^{2+}-dependent regulatory mechanism for the replication of HCV through the regulation of the formation of the NS5A–FKBP8/FKBP8–Hsp90 complex. These findings provide a new intracellular Ca^{2+}-signalling pathway via the interactions of the S100 protein–TPR motif.

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Disclosures

The authors disclose no competing interests

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Article

Antimicrobial Peptide LL-37 Produced by HSV-2-Infected Keratinocytes Enhances HIV Infection of Langerhans Cells

Youichi Ogawa,[1] Tatsuyoshi Kawamura,[1,*] Takamitsu Matsuzawa,[1] Rui Aoki,[1] Peter Gee,[4] Atsuya Yamashita,[2] Kohji Moriishi,[2] Kenshi Yamasaki,[3] Yoshio Koyanagi,[4] Andrew Blauvelt,[5] and Shinji Shimada[1]
[1]Department of Dermatology
[2]Department of Microbiology
Faculty of Medicine, University of Yamanashi, Yamanashi 409-3898, Japan
[3]Department of Dermatology, Tohoku University Graduate School of Medicine, Sendai 980-8575, Japan
[4]Laboratory of Viral Pathogenesis, Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan
[5]Oregon Medical Research Center, Portland, OR 97223, USA
*Correspondence: tkawa@yamanashi.ac.jp
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SUMMARY

Herpes simplex virus (HSV)-2 shedding is associated with increased risk for sexually acquiring HIV. Because Langerhans cells (LCs), the mucosal epithelium resident dendritic cells, are suspected to be one of the initial target cell types infected by HIV following sexual exposure, we examined whether and how HSV-2 affects HIV infection of LCs. Although relatively few HSV-2/HIV-coinfected LCs were detected, HSV-2 dramatically enhanced the HIV susceptibility of LCs within skin explants. HSV-2 stimulated epithelial cell production of antimicrobial peptides (AMPs), including human β defensins and LL-37. LL-37 strongly upregulated the expression of HIV receptors in monocyte-derived LCs (mLCs), thereby enhancing their HIV susceptibility. Culture supernatants of epithelial cells infected with HSV-2 enhanced HIV susceptibility in mLCs, and this effect was abrogated by blocking LL-37 production. These data suggest that HSV-2 enhances sexual transmission of HIV by increasing HIV susceptibility of LCs via epithelial cell production of LL-37.

INTRODUCTION

Epidemiologic studies have indicated a strong association between the acquisition of HIV and other sexually transmitted diseases (STDs) (Galvin and Cohen, 2004). This link is especially evident in cases of genital ulcer diseases (GUDs), with a 2- to 11-fold increase in the rate of HIV acquisition in the presence of GUD (Cameron et al., 1989; Fleming and Wasserheit, 1999). It is widely recognized that herpes simplex virus type 2 (HSV-2) is a major cause of GUDs, and more than 50 epidemiologic studies have now indicated that HSV-2 shedding is associated with increased risk for acquiring HIV (Wald and Link, 2002). The risk ratio of HIV acquisition for a person with genital herpes is enhanced from 2 to 4 when compared with a person without genital herpes, and potentially 50% of new HIV infections are considered to be attributable or worsened by HSV-2 infection (Wald and Link, 2002).

During sexual transmission of HIV, virus crosses mucosal epithelium and is eventually transmitted to regional lymph nodes, where it establishes permanent infection. Many studies have shown that Langerhans cells (LCs) are one of the important initial cellular targets for HIV, and that this particular type of dendritic cell (DC) plays a crucial role in disseminating HIV (de Witte et al., 2007; Kawamura et al., 2005; Lederman et al., 2006; Shattock and Moore, 2003). LCs are present within genital skin (e.g., outer foreskin) and mucosal epithelium and, after contact with pathogens, readily emigrate from tissue to draining lymph nodes. Immature resident LCs express surface CD4 and CCR5, but not surface CXCR4 (Zaitseva et al., 1997). These LCs are readily infected ex vivo with R5 HIV, but not with X4 HIV (Kawamura et al., 2000, 2008; Reece et al., 1998; Zaitseva et al., 1997). These findings are consistent with previous epidemiologic observations, which have found that the majority of HIV strains isolated from newly infected patients are R5 HIV strains (Zhu et al., 1993). It has been reported that persons with *CCR5* homozygous defects are largely protected from sexually acquiring HIV (Liu et al., 1996).

Clinical trials performed over the last several years have shown that circumcision greatly reduces the probability of penile HIV transmission, suggesting that the foreskin is an important portal of HIV entry (Auvert et al., 2005; Bailey et al., 2007; Gray et al., 2007). Although the mechanism leading to protection remains undefined, several ex vivo experiments with foreskin explants have indicated that CD4 T lymphocytes and LCs within foreskin epidermis are initial target cells for HIV (Fahrbach et al., 2010; Ganor et al., 2010; Grivel et al., 2011; Zhou et al., 2011).

In primate models of simian immunodeficiency virus (SIV) infection, there is controversy regarding which cells in the genital mucosa are initially infected by SIV. Studies have demonstrated that the primary infected cells present in the lamina propria of the cervicovaginal mucosa 48–72 hr after intravaginal exposure to SIV are T cells or submucosal DCs, but not epithelial LCs (Spira et al., 1996; Zhang et al., 1999). When vaginal tissue was examined within 1 hr following vaginal inoculation, however, up to

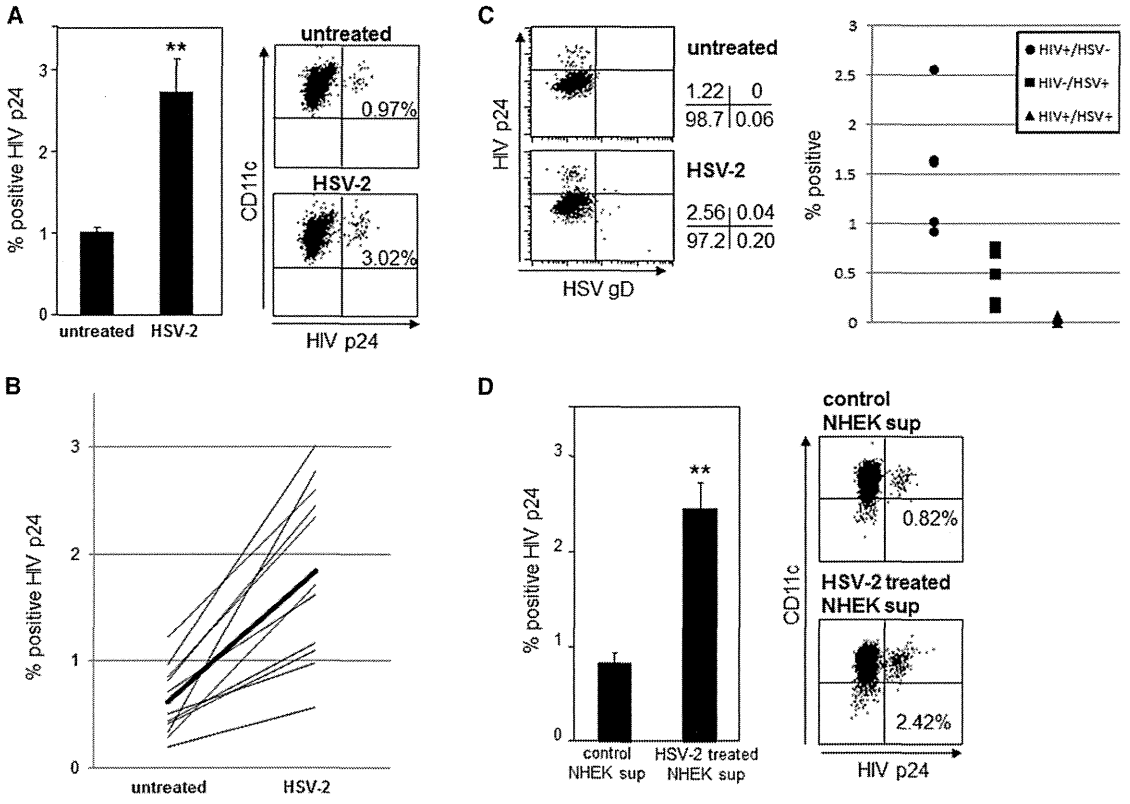

Figure 1. HSV-2-Infected Epithelial Cells Augment HIV Infection in LCs

(A–C) Epithelial sheets were preincubated with HSV-2 and then exposed to R5 HIV. Epithelial sheets were floated on culture medium to allow migration of LCs from the explants. Emigrating cells from the epidermal sheets were collected 3 days following HIV exposure. HIV-infected LCs were assessed by HIV p24 intracellular staining in langerin$^+$ CD11c$^+$ LCs (A) and the results of 11 separate experiments with different donors are summarized (B). HSV– and/or HIV-infected LCs were assessed by HIV p24 and HSV gD intracellular staining in CD11c$^+$ LCs, and the results from five different donors are summarized (C).

(D) mLCs were preincubated with the supernatants from NHEKs treated with or without HSV-2, and then exposed to R5 HIV. HIV p24$^+$ cells were assessed in langerin$^+$ CD11c$^+$ mLCs at day 7.

(A, C, and D) Representative flow cytometric analyses are shown. (A and D) Results are shown as means ± SD (n = 3) (**$p < 0.01$). See also Figure S1.

90% of SIV-infected cells were found to be LCs (Hu et al., 2000). Because only a single layer of columnar epithelium guards the endocervix and the transformation zone, the mucosal barrier can be easily breached by mechanisms such as the microtrauma associated with sexual intercourse, which provides immediate access to target cells, especially CD4$^+$ T cells, in the submucosa (Haase, 2010). Indeed, it has been shown that following mucosal exposure to high doses of SIV, virus can gain access through breaks in the mucosal epithelial barrier and infect resting CD4$^+$ T cells in the submucosa (Haase, 2010). Since molecules targeting CCR5 completely protected against mucosal transmission of SHIV (Lederman et al., 2004), CD4/CCR5-mediated de novo infection of LCs and/or CD4$^+$ T cells is considered to be a major pathway involved in sexual transmission of HIV.

Several mechanisms have been proposed to explain enhanced sexual transmission of HIV during active STD infection, including breakdown of epithelial barriers (i.e., ulceration) with direct inoculation of HIV into the blood (Cunningham et al., 1985), presence of inflammatory leukocytes that act as targets (Zhu et al., 2009), and coinfection of cells by HIV and STD pathogens. Biological mechanisms responsible for greater HIV transmission rates in the presence of genital herpes infections, however, are as of yet unknown. Recently, we and others have suggested that HIV susceptibility of LCs could be directly enhanced by pathogens and indirectly enhanced by inflammatory factors during STD, thereby leading to more likely sexual transmission of HIV (de Jong et al., 2008; Ogawa et al., 2009). In this report, we found that HSV-2 primarily infected epithelial cells and then markedly enhanced HIV infection in adjacent LCs. Interestingly, mechanistic studies revealed that LL-37 produced by HSV-2-infected epithelial cells upregulated CD4 and CCR5 on the surface of bystander LCs, thereby enhancing HIV infection in these cells. These findings may lead to new strategies designed to block sexual transmission of HIV.

RESULTS

HSV-2 Indirectly Enhances HIV Susceptibility in LCs via Interaction with Epithelial Cells

We first examined whether HSV-2 modulates HIV susceptibility of LCs by using an ex vivo skin explant model, whereby resident LCs within epithelial tissue are exposed to HIV and then allowed to emigrate from tissue, thus mimicking conditions that occur following mucosal exposure to HIV (Kawamura et al., 2000).

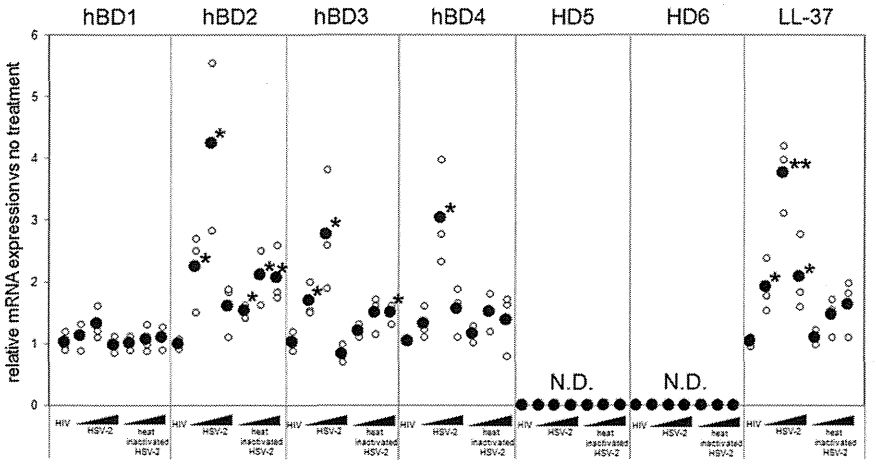

Figure 2. HSV-2 Increases Expression of Human β Defensin-2, Defensin-3, Defensin-4, and LL-37 in Normal Human Epithelial Cells

HIV-1 (10^5 $TCID_{50}$), HSV-2 ($1 \times 10^4 - 1 \times 10^6$ PFU), and heat-inactivated HSV-2 ($1 \times 10^4 - 1 \times 10^6$ PFU) were exposed to NHEKs for 1 hr and then washed twice. Cells were incubated in culture medium for 12 hr, and the mRNA expression of indicated AMPs was determined by qPCR. Results are shown as mean relative mRNA expressions (○) from three different experiments and mean the average (●) of those (*$p < 0.05$; **$p < 0.01$). See also Figure S2.

Epidermal sheets obtained from suction blister roofs were exposed to HSV-2 strain G at 1×10^6 PFU/tissue for 1 hr, and then exposed to R5-tropic HIV-1_{BaL} for 2 hr. Three days later, to quantify numbers of HIV-infected LCs at the single-cell level, cells emigrating from the explants were stained with anti-CD11c, anti-langerin, and anti-HIV p24 mAbs. Intracellular staining for HIV p24 represents productive HIV replication within LCs, since expression can be completely blocked by AZT (Kawamura et al., 2003). The numbers of LCs emigrating from individual explants were determined, and the mean yield ± SD was HSV−/HIV−; $1.04 \pm 0.25 \times 10^4$, HSV−/HIV+; $1.13 \pm 0.31 \times 10^4$, HSV+/HIV−; $1.25 \pm 0.27 \times 10^4$; and HSV+/HIV+; $1.21 \pm 0.25 \times 10^4$ (n = 3). Thus, the number of LCs recovered from the skin explants was not significantly affected by HSV-2 or HIV exposure. However, preincubation of epithelial sheets with HSV-2 significantly increased the percentage of HIV $p24^+$ cells within $langerin^+$ $CD11c^+$ LCs approximately 3-fold as compared to LCs emigrating from nonexposed epithelial sheets (Figure 1A). The results of 11 separate experiments with different skin donors are summarized in Figure 1 B (mean percentage HIV $p24^+$ LCs ± SD = 0.61 ± 0.31 with no HSV-2; 1.85 ± 0.79 with HSV-2 preincubation, p = 0.0002, n = 11). To assess the ratio of individual HSV-2- and/or HIV-infected LCs emigrating from explants, cells were collected from cultures and stained with anti-CD11c, anti-HSV gD, and anti-HIV p24 mAbs. A recent study in mice showed that HSV impeded emigration of infected LCs by inducing apoptosis and by blocking E-cadherin downregulation (Miller et al., 2011b). Consistent with this finding, the percentage of HSV-2-infected emigrating LCs was much less when compared with LCs that remained within explants at day 3 (Figure 1C and see Figure S1 online). More importantly, we found that HSV-2/HIV-coinfected emigrating LCs were rarely detected (mean percentage of HIV-1 $p24^+$/HSV-2 gD^+ LCs ± SD = 0.04 ± 0.02, n = 5, Figure 1C), suggesting that HSV may not directly modulate HIV susceptibility in LCs. Indeed, supernatants from epithelial cell cultures of normal human epidermal keratinocytes (NHEKs) treated with HSV-2 also increased the percentage of HIV p24+ monocyte-derived LCs (mLCs), even though mLCs were not exposed to HSV-2 (Figure 1D). Of note, the magnitude of HIV susceptibility in mLCs enhanced by HSV-treated NHEK supernatants was comparable to that in emigrating LCs in the epithelial explant experiments. Enhancement of HIV infection by supernatants from HSV-2-treated NHEKs was dependent on the dose of HSV-2, and supernatants from heat-inactivated HSV-2-treated NHEKs did not affect HIV susceptibility in mLCs (Figure S2). HIV susceptibility in mLCs was also enhanced when we infected NHEKs with a second HSV-2 strain, 186 (data not shown). Taken together, these results suggest that HSV-2 indirectly mediates HIV infection of epidermal LCs by a soluble factor or factors released by HSV-2-infected epithelial cells.

HSV-2 Augments the Production of Antimicrobial Peptides from Keratinocytes, and LL-37 Enhances HIV Infection in LCs

In STDs, antimicrobial peptides (AMPs), including defensins and cathelicidin, are the key effector molecules of mucosal innate and adaptive immunity. Human vaginal epithelial cells and epidermal keratinocytes can produce human α defensin-5 (HD5), HD6 and human β defensin-1 (hBD1), hBD2, hBD3, hBD4, and the sole cathelicidin in humans, LL-37. Certain defensins (e.g., hBD1) are expressed constitutively, and others (e.g., hBD2 and hBD3) show increased expression in response to inflammation or infection (Klotman and Chang, 2006). Several reports have indicated that several of these AMPs modulate HIV infectivity in peripheral blood mononuclear cells (PBMC) or in $CD4^+$ T cells (Bergman et al., 2007; Klotman et al., 2008; Quiñones-Mateu et al., 2003; Sun et al., 2005). Thus, we investigated whether HSV-2 induced AMPs production in keratinocytes. NHEKs were incubated with HSV-2 ($1 \times 10^4 - 1 \times 10^6$ PFU) or HIV-1 (1×10^5 $TCID_{50}$), and relative mRNA expression levels of AMPs were determined by quantitative RT-PCR. Interestingly, HSV-2 significantly increased the expression of hBD2, hBD3, hBD4, and LL-37, whereas neither HIV nor heat-inactivated HSV-2 affected expression of hBD3, hBD4, and LL-37 (Figure 2).

To determine whether keratinocyte-derived AMPs affect HIV susceptibility of LCs, mLCs were stimulated with AMPs or TNF-α, as a positive control, for 24 hr before exposure to HIV-1. Strikingly, only LL-37 significantly increased the percentage of HIV $p24^+$ mLCs (Figure 3A). This infection-enhancing effect of LL-37 was observed in a dose-dependent manner, utilizing concentrations of LL-37 observed in physiologic conditions (Ong et al., 2002; Yamasaki et al., 2007) (Figure 3B). Interestingly, LL-37 also significantly upregulated surface expression

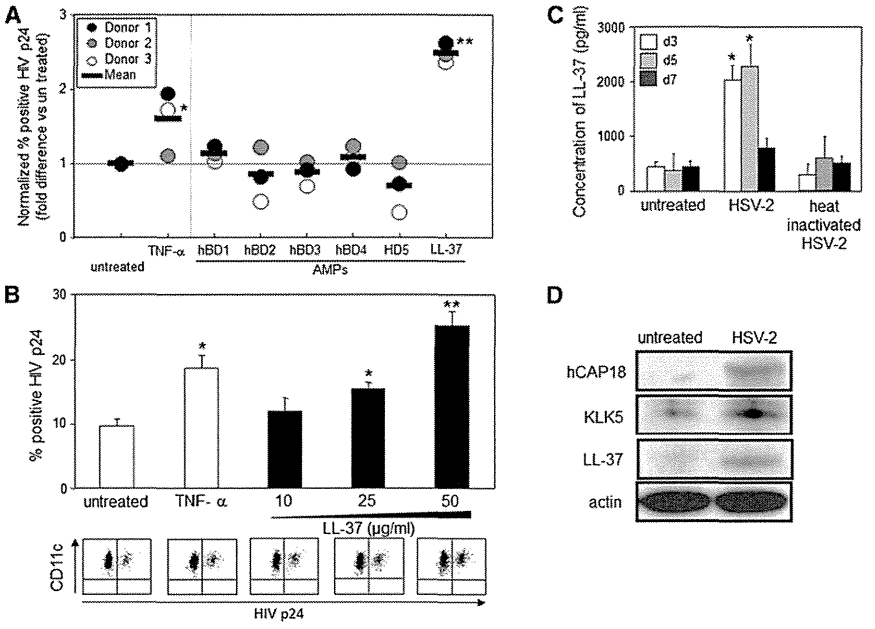

Figure 3. LL-37 Enhances HIV Susceptibility in mLCs

mLCs were stimulated with the indicated AMPs or rhTNF-α 24 hr prior to HIV exposure. To assess HIV infection levels, mLCs were collected 7 days after HIV exposure, and HIV p24$^+$ cells were quantified in langerin$^+$ CD11c$^+$ mLCs.

(A) Each circle indicates the normalized percentage of positive cells for HIV p24; mean values obtained from different donors are shown as horizontal marks.

(B) The percentage of positive cells for HIV p24 in langerin$^+$ CD11c$^+$ mLCs and representative flow cytometric analyses following LL-37 stimulation.

(C) NHEKs were exposed to HSV-2 or heat-inactivated HSV-2. Following culture for the indicated number of days, LL-37 levels were measured in supernatants by ELISA. Results are shown as means ± SD (n = 3) (*$p < 0.05$).

(D) NHEKs were treated with HSV-2 for 3 days and then lysed. The expression of hCAP18, KLK5, and LL-37 was determined by western blot analysis. All data shown represent at least two separate experiments. See also Figure S3.

of CD86, CD83, and CCR7 on mLCs (Figure S3), indicating that LL-37 induces LC maturation.

To confirm whether HSV-2-treated NHEKs could produce LL-37 protein, we measured LL-37 protein levels in culture supernatants from NHEKs treated with medium alone, HSV-2, or heat-inactivated HSV-2 by ELISA. HSV-2 significantly induced production of LL-37 in NHEKs, which peaked at day 5 (Figure 3C). Expression levels of kallikrein 5 (KLK5) have been shown to parallel induction of LL-37 in KCs, since the activity of cathelicidin is controlled by enzymatic processing of the proform hCAP18 to a mature peptide LL-37 by KLK5, a serine protease (Morizane et al., 2010; Yamasaki et al., 2006). Therefore, we measured protein levels of LL-37, hCAP18, and KLK5 in NHEKs treated with medium alone or HSV-2. As shown in Figure 3D, HSV-2 induced production of LL-37 in NHEKs. Similarly, HSV-2 increased expression of hCAP18 as well as KLK5, and these protein levels coincided with the induction of LL-37 in NHEKs (Figure 3D).

To further confirm the participation of LL-37 in this enhancement, we used RNA interference (siRNA) to block LL-37 production. Protein levels were quantified by western blotting followed by densitometry analysis. Transfection of siRNA targeting LL-37 induced an efficient knockdown in NHEKs (55% downregulation; Figure 4A). In line with the results of western blot analyses, siRNA-mediated interference of LL-37 in NHEKs significantly reduced enhancement of HIV infection in mLCs by supernatants from HSV-2-treated NHEKs, in comparison with control siRNA targeting an irrelevant sequence (Figure 4B). Based on these results, we conclude that enhanced HIV infection in mLCs by supernatants from HSV-2-treated NHEKs is, at least in part, mediated by LL-37.

Recently, TNF-α derived from KCs has also been shown to enhance HIV susceptibility of LCs (de Jong et al., 2008; Ogawa et al., 2009). In our experiments, however, TNF-α was not detected in culture supernatants from NHEKs treated by HSV-2 (data not shown), consistent with a recent report (de Jong et al., 2010). In addition, preincubation of supernatants from HSV-2-treated NHEKs with an anti-TNF-α neutralizing mAb, prior to exposing mLCs, did not affect HIV susceptibility in mLCs (Figure S4).

LL-37 Enhances Surface Expression of CD4 and CCR5 on mLCs

Previous studies have revealed that langerin expressed on LCs is a natural barrier to HIV infection because HIV virions captured by langerin are internalized into LC Birbeck granules and degraded (de Witte et al., 2007). In addition, APOBEC3G (A3G) and SAM domain and HD domain 1 (SAMHD1) has been recently shown to function as a potent postentry cellular restriction factor for HIV in DCs or LCs (Hrecka et al., 2011; Laguette et al., 2011; Ogawa et al., 2009; Pion et al., 2006). Therefore, we next examined whether LL-37 affects the expression levels of these molecules in mLCs. LL-37 stimulation did not affect the expression of langerin, A3G, or SAMHD1 (Figures 4C and 4D and Figure S5). In contrast, LL-37 significantly increased surface expression of CD4 and CCR5 on mLCs (Figure 4C). In addition, siRNA-mediated interference of LL-37 in NHEKs significantly reduced the enhancement of surface expression of CD4 and CCR5 in mLCs by supernatants from HSV-2-treated NHEKs, in comparison with control siRNA targeting an irrelevant sequence (Figure 4C). Thus, our results suggest that LL-37 enhances HIV infection in LCs by increasing surface expression of HIV receptors, rather than by modulating restriction factors such as langerin, A3G, or SAMHD1.

Since LL-37 upregulated surface expression of CD4 and CCR5 in LCs, we next examined whether LL-37 specifically enhanced R5-tropic HIV entry into LCs by using single-round infection assays with pseudotyped viruses containing a luciferase reporter and different envelope proteins (Env): Env from either R5 HIV-1 (JR-FL; R5), X4 HIV-1 (IIIB; X4), or vesicular stomatitis virus (VSV-G). As expected, we found that LL-37 pretreatment enhanced the infectivity of mLCs to R5-VSV in