Raf-1 kinase associates with Hepatitis C virus NS5A and regulates viral replication

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Abstract **Hepatitis C virus (HCV) is a positive-strand RNA virus that frequently causes persistent infection associated with severe liver disease. HCV nonstructural protein 5A (NS5A) is essential for viral replication. Here, the kinase Raf-1 was identified as a novel cellular binding partner of NS5A, binding to the C-terminal domain of NS5A. Raf-1 colocalizes with NS5A in the HCV replication complex. The interaction of NS5A with Raf-1 results in increased Raf-1 phosphorylation at serine 338. Integrity of Raf-1 is crucial for HCV replication: inhibition of Raf-1 by the small-molecule inhibitor BAY43-9006 or downregulation of Raf-1 by siRNA attenuates viral replication.**
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Keywords: HCV; NS5A; Raf-1; Replication

1. Introduction

Hepatitis C virus (HCV) is a positive-strand RNA virus of approximately 9600 bp. Acute infection is often mild or inapparent. However, the virus persists in the majority of the cases. Persistent infection is associated with a high risk of liver cirrhosis and/or hepatocellular carcinoma [1].

The viral RNA codes for one large open reading frame (ORF) of approximately 3000 amino acids that is posttranslationally processed by cellular and viral proteases. The N-terminus encodes the structural proteins core, E1 and E2 and the C-terminus encodes the nonstructural proteins p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B [2].

The study of the life cycle of HCV has been hampered by the lack of an efficient cell culture system. This limitation was at least partially overcome by the introduction of selectable replicons that allow the analysis of HCV replication in cell culture [3]. Although cells harboring the full-length replicon RNA do not produce any infectious HCV particles, the replication complex is readily formed at the endoplasmatic reticulum [4]. NS5A is an integral part of the replication complex. Mutations

in NS5A affect the rate of HCV replication [5,6]. This indicates that NS5A is not only physically present in the replication complex but adopts a central role during HCV replication.

NS5A is capable of interacting with a number of host cell proteins [7]. Some of these interaction partners seem to be relevant in terms of triggering a deregulation of host cell signal transduction, such as Grb2 [8], phosphatidylinositol 3-kinase [9] or p53 [10]. Among the multitude of cellular proteins that interact with NS5A, only the human vesicle-associated protein hVAP-A is known to be necessary for viral replication [11].

The identification of cellular interaction partners of NS5A that are functionally relevant for replication is of great interest, both with regard to understanding HCV biology and with regard to the development of novel antiviral strategies.

2. Materials and methods

2.1. Materials

The NS5A gene was kindly provided by Dr. Kunitada Shimotohno (Kyoto University, Japan) and was derived from a genotype Ib isolate (Gene Bank Accession Number D16435). *Strep*Tactin sepharose was obtained from IBA (Goettingen, Germany). HCV replicon cell lines HuH-7 I_{377}/NS3-3′/wt/9-13 [3] and HuH-7 I_{389}/NS3-3′/LucUbiNeo-ET [12] were generously donated by Dr. Ralf Bartenschlager (University of Heidelberg, Germany). Antibodies were obtained from the following sources: HA (Santa Cruz), V5 (Invitrogen), Raf-1 (BD Transduction Laboratories and Santa Cruz), phospho-Raf (Ser 338) (Upstate), phospho-MEK (Cell Signaling Technology), active-MAPK (Promega), ERK2 (Santa Cruz). The rabbit-derived NS5A-specific antiserum was generated in our laboratory. BAY43-9006 and U0126 were purchased from Calbiochem. The recombinant baculovirus coding for GST-Raf-1 was provided by Dr. Walter Kolch (Beatson Institute for Cancer Research, Glasgow, UK).

2.2. Affinity chromatography

A fusion protein of NS5A with an N-terminal Strep tag and a C-terminal V5-epitope was isolated from a bacterial expression system and was immobilized on a *Strep*Tactin column (IBA, Goettingen) equilibrated with buffer W (100 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA). The column was incubated with hepatoma cell lysate (HuH-7) to allow potential interaction partners to bind to the immobilized NS5A. After a washing step, Strep–NS5A–V5 was eluted by buffer E (buffer W supplemented with 5 mM desthiobiotin). As controls, immobilized Hepatitis-B virus core protein (HBc) (Strep–HBc) and an unloaded *Strep*Tactin column were used.

2.3. Immunoprecipitation, immunocomplex assay and immunofluorescence microscopy

Immunoprecipitation and immunofluorescence microscopy were performed as described [4,13]. Immunofluorescence staining was analyzed by confocal laser scanning microscopy.

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The immunocomplex assay was performed as previously described [14].

2.4. Quantitative immunoblotting

Naïve HuH-7 cells or the HCV-replicon cell lines HuH-7 I$_{377}$/NS3-3′/wt/9-13 and HuH-7 I$_{389}$/NS3-3′/LucUbiNeo-ET were lysed in lysis buffer (100 mM Tris pH 7.5, 137 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM PMSF, 2 μg/ml leupeptin, 5 μg/ml aprotinin). Lysates were cleared by centrifugation and subjected to SDS–PAGE and subsequent Western blotting using rat-derived anti-phospho-Raf-1 (S338) (Upstate) and mouse-derived anti-Raf-1 (Transduction Labs). Phospho-Raf-1 and Raf-1 were visualized using IRDye800-conjugated anti-rat IgG (Rockland) and Alexa-Fluor 680-conjugated anti-mouse IgG (Molecular Probes). Immunostaining was quantified using the Odyssey system (Li-Cor).

2.5. HCV replication assays

To monitor HCV replication, bicistronic subgenomic reporter replicon cell lines HuH-7 I$_{389}$/NS3-3′/LucUbiNeo-ET were used as described before [12].

2.6. Silencing of Raf-1 by RNAi

To silence Raf-1 by RNA interference, commercially available siR-NAs (Ambion) were used. HCV replicon cells were transfected with 200 nM of siRNA using siPORT Lipid (Ambion) according to the manufacturer's instructions. Cells were harvested 48 h post transfection.

3. Results and discussion

3.1. Raf-1 binds to NS5A

To identify novel binding partners of NS5A, purified NS5A was immobilized on an affinity chromatography column. The column was loaded with HuH-7-derived cell lysate to allow potential interaction partners to bind to immobilized NS5A. Specifically binding proteins were analyzed by Western blotting. Since NS5A has been shown to interfere with the MAP kinase cascade and the NF-κB pathway [8,15], we focused on proteins involved in these signaling cascades. Based on this approach, Raf-1 was found to specifically interact with NS5A (Fig. 1A).

This was confirmed by coimmunoprecipitation experiments using cellular lysates derived from Sf9 cells that had been infected with recombinant baculoviruses. The coimmunoprecipitation experiments show that Raf-1 is coprecipitated with NS5A and vice versa (Fig. 1B and C).

The next set of experiments was performed to analyze whether the interaction of Raf-1 with NS5A is reflected by a colocalization in HCV replicon-containing cells. Although these cells do not produce any infectious particles, HCV replication complexes are formed at the endoplasmatic reticulum [4]. In these cells, NS5A displays a punctuate staining (Fig. 2A) that arises from the clustering of the nonstructural proteins in the replication complexes. The confocal laser scanning microscopy and the intensity profile show that Raf-1 colocalizes with NS5A in the replication complexes (Fig. 2A).

To analyze the interaction of Raf-1 and NS5A in more detail, deletion mutants of Raf-1 and NS5A were generated. After transient transfection of HuH-7 cells with these mutants, their subcellular localization was determined by confocal laser scanning microscopy. Raf-1 which lacks the catalytical domain (Raf C4) [16] is exclusively found in the nucleus. Expression of this mutant does not affect the extranuclear localization of NS5A (Fig. 2B) suggesting that this mutant does not bind to NS5A.
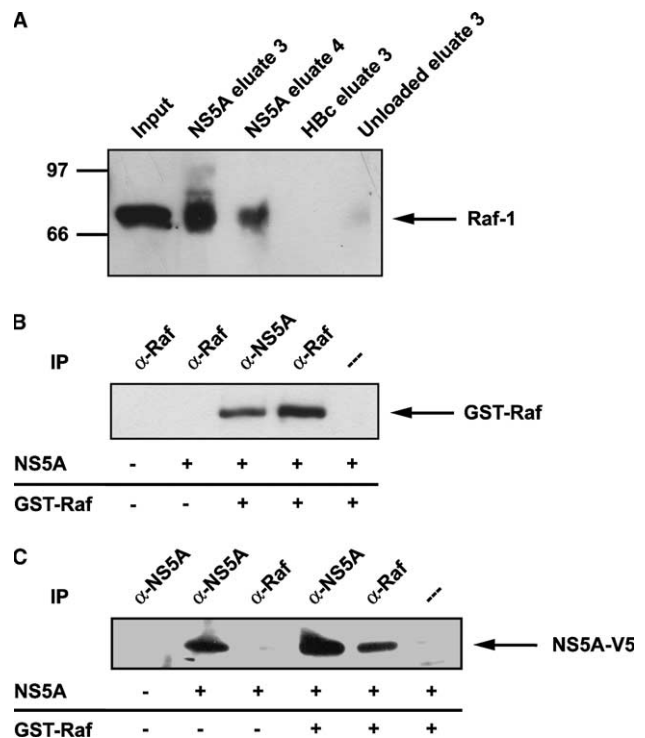

Fig. 1. NS5A interacts with Raf-1. (A) Strep–NS5A–V5 was immobilized on a StrepTactin sepharose column. Immobilized NS5A was incubated with HuH-7 cell lysate. NS5A was eluted together with potential binding partners. As controls, a column loaded with Hepatitis B Virus Core (HBc) and an unloaded column were included. Eluates were analyzed for coelution of Raf-1 by Western blotting using a Raf-1-specific antiserum (Santa Cruz). (B, C) Strep–NS5A–V5 and GST-Raf-1 were expressed in Sf9 cells. Sf9 cells were lysed and subjected to immunoprecipitation as indicated. Immunoprecipitates were analyzed by Western blotting using either a Raf-1-specific antiserum (Santa Cruz) (B) or a V5-specific antiserum (Invitrogen) (C).

On the other hand, deletion of the N-terminal domain of NS5A that mediates attachment to the endoplasmatic reticulum was performed (NS5A 211-449). N-terminal deletion mutants of NS5A are found in the nucleus [17]. Nuclear localization of this NS5A deletion mutant resulted in a translocation of Raf-1 from the cytosol to the nucleus (Fig. 2C). This indicates that the domain between amino acids 211 and 449 of NS5A is sufficient to mediate interaction with Raf-1. Furthermore, this experiment shows that the interaction between Raf-1 and NS5A is strong enough to relocalize Raf-1 to a subcellular compartment that is usually devoid of Raf-1 (nucleus) [18].

3.2. Raf-1 activity is increased in HCV replicon-containing cells

The data described above show that Raf-1 interacts with NS5A. This prompted us to analyze whether NS5A affects Raf-1 activity. Raf-1 activity is highly correlated with its phosphorylation status: phosphorylation at serine 338 is a critical step in the activation of Raf-1 [19]. Therefore, we investigated whether Raf-1 phosphorylation at serine 338 is altered in NS5A-producing replicon cell lines (HuH-7 I$_{389}$/NS3-3′/LucUbiNeo-ET and HuH-7 I$_{377}$/NS3-3′/wt/9-13). Fig. 3A shows that Raf-1 phosphorylation at serine 338 is significantly increased in both HCV-replicon cell lines in comparison to naïve HuH-7 cells. To rule out that the increase in Raf-1 phosphor-
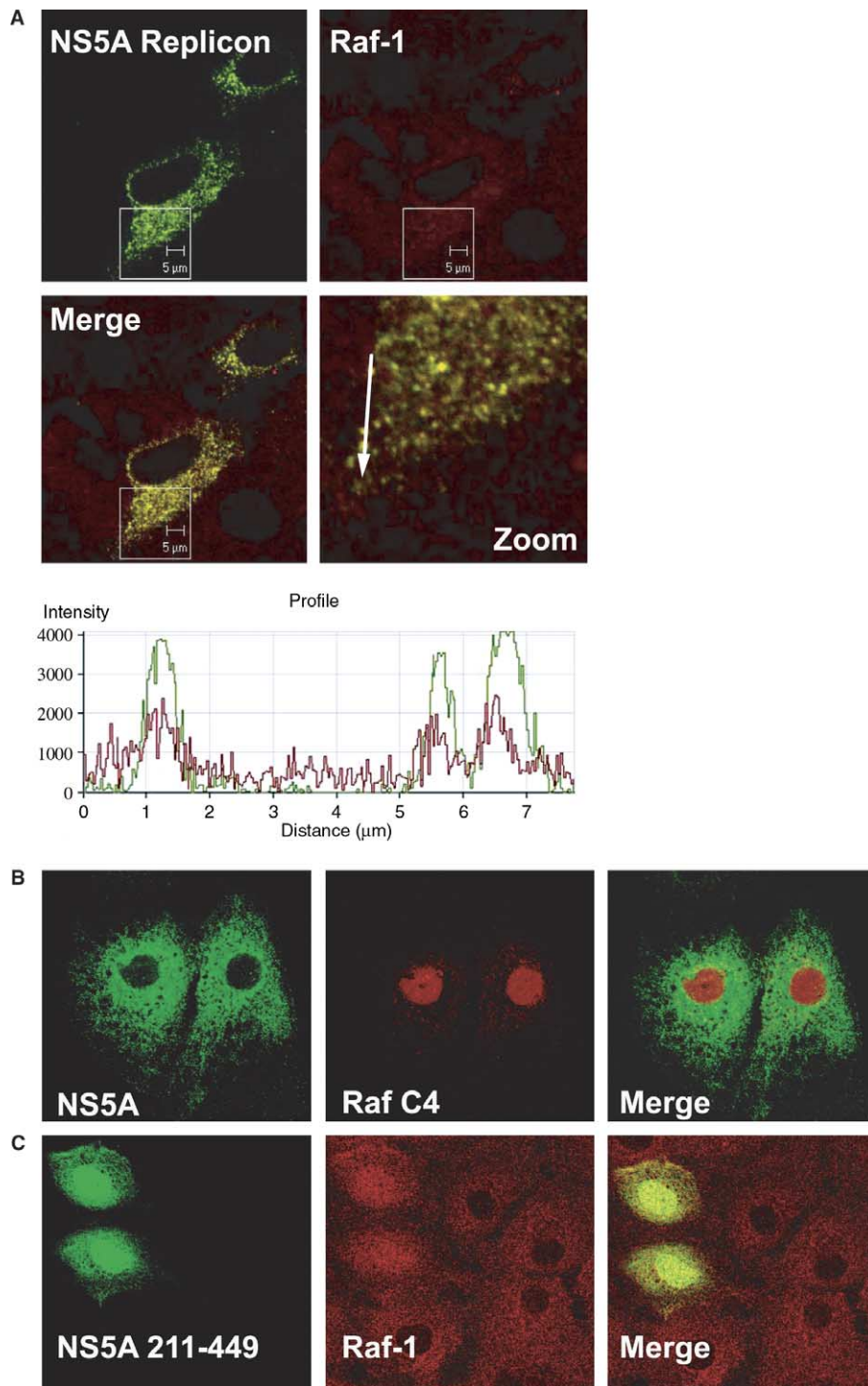
Fig. 2. Raf-1 is colocalized with NS5A in the replication complex. (A) HCV replicon cells (HuH-7 I$_{377}$/NS3-3′/wt/9-13) were stained for NS5A using an NS5A-specific antiserum and Raf-1 using anti-Raf-1 (BD Transduction Labs). Samples were analyzed by confocal laser scanning microscopy. The lower panel shows the intensity profile corresponding to the region spanned by the white arrow in the upper panel (NS5A in green and Raf-1 in red). (B,C) HuH-7 cells were transfected with pcDNA–HA–NS5A–V5 and pRK5-Raf C4 (B) or pcDNA–Strep–NS5A(211–449)–V5 (C). NS5A was detected using an NS5A-specific antiserum. Endogenous Raf-1 and heterologously expressed Raf C4 were detected using anti-Raf-1 (BD Transduction Labs).

ylation was due to any other component of the HCV replication complex, HuH-7 were transiently transfected with an NS5A expression plasmid and analyzed accordingly. Fig. 3B

shows that Raf-1 phosphorylation is increased in cells producing NS5A alone as well. To corroborate this finding, Raf-1 kinase activity was analyzed in immunoprecipitates isolated
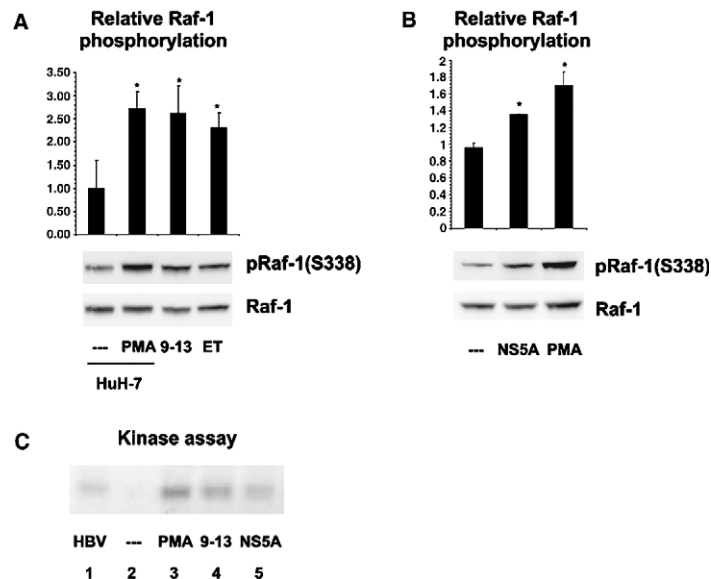
Fig. 3. Raf-1 phosphorylation is increased in HCV replicon-containing cells. (A) HCV replicon cells HuH-7 I$_{389}$/NS3-3′/LucUbiNeo-ET or HuH-7 I$_{377}$/NS3-3′/wt/9-13 and naïve HuH-7 cells were analyzed by Western blotting using anti-phospho-Raf-1 (S338) (Upstate) and anti-Raf-1 (BD Transduction Labs) in parallel. Immunostaining (anti-phospho-Raf-1 (S338)/anti-Raf-1) was quantified using the Odyssey system (Li-Cor). The bar graph shows the summary of three independent experiments. (∗) indicates a significant increase with regard to naïve HuH-7 cells ($p < 0.05$). (B) HuH-7 cells were transfected with NS5A or with a control plasmid (—) and stimulated with PMA (100 ng/ml) as indicated. Immunostaining (anti-phospho-Raf-1 (S338)/anti-Raf-1) was analyzed and quantified as described in (A). (C) Cell lysates were prepared from HuH-7 I$_{377}$/NS3-3′/wt/9-13 (lane 4) and HuH-7 cells that were transfected with a full-length HBV genome (lane 1), with a control plasmid (lanes 2 and 3) or with NS5A (lane 5) and stimulated with 100 ng/ml PMA (lane 3). Raf-1 was precipitated from these lysates and Raf-1 activity was determined by immunocomplex assay (using [γ-^{32}P]ATP and recombinant MEK as a substrate). Phosphorylated MEK was visualized by SDS–PAGE and autoradiography.

from transiently transfected HuH-7 cells or from HCV-replicon cells. Fig. 3C shows that Raf-1 kinase activity is significantly increased in NS5A-producing cells. This indicates that binding of NS5A to Raf-1 triggers Raf-1 activation.

In contrast to this, analysis of MEK phosphorylation in HCV replicon cell lines revealed no difference between HCV replicon cells and naïve HuH-7 cells (data not shown). This suggests that although Raf-1 is activated in HCV replicon cells, the MAP kinase cascade downstream of Raf-1 is unaffected.

3.3. Inhibition of Raf-1 modulates HCV replication

Since Raf-1 phosphorylation was increased in the presence of NS5A, we analyzed whether the inhibition of Raf-1 affects HCV replication in the replicon cell line HuH/LucUbiNeo-ET [12]. To that end, Raf-1 activity was modulated by the means of a small-molecule inhibitor (BAY43-9006) [20] or by siRNA. Inhibition of Raf-1 by 10 µM BAY43-9006 or by siRNA resulted in a significantly decreased HCV replication (Fig. 4A–D).

The next set of experiments was performed to control whether Raf-1 exerts its effect on HCV replication via the MAP kinase signaling cascade. If this was the case, inhibition of MEK (which acts immediately downstream of Raf-1) should result in decreased HCV replication as well. In accordance with the observation that NS5A does not trigger activation of MEK, replication was largely unaffected by the presence of a small-molecule inhibitor of MEK (U0126) (Fig. 4B). The inhibitory effect of U0126 and BAY43-9006

on the MAP kinase signaling cascade was confirmed by Western blotting using anti-phospho-MEK and anti-phospho-ERK (Fig. 4A).

The effect of Raf-1 inhibition on viral replication could be explained by at least two models: (i) Raf-1 might modify some component of the replication complex by phosphorylation. This could either be a cellular component or a viral protein. (ii) Alternatively, the Raf-1-mediated effect on replication could be explained as a consequence of Raf-1 signaling towards downstream targets other than the MEK/ERK cascade. Although alternative signaling pathways initiated by Raf-1 are still poorly understood, it is emerging that Raf-1 exerts its anti-apoptotic effect by a distinct signaling pathway that culminates in the displacement of BAD from Bcl-2 at the outer mitochondrial membrane [21]. Interestingly, PAK1-dependent phosphorylation of Raf-1 at serine 338 constitutes the major stimulus for the mitochondrial signaling pathway of Raf-1 [22]. Therefore, NS5A might induce a conformational change in Raf-1 that leads to the PAK1-dependent phosphorylation of serine 338, thereby triggering a cell survival signal that is required to sustain viral replication. In contrast, inhibition of Raf-1 may compromise its anti-apoptotic action and thereby affect HCV replication.

The data described here suggest that the inhibition of cellular signaling might be an effective antiviral strategy. This is an exciting prospect given the fact that an effective cure of HCV is still unavailable. The Raf-1 inhibitor BAY43-9006 is currently being evaluated in clinical trials as an anti-cancer drug [23], suggesting that it might be tolerated well enough to be applicable for antiviral therapy.
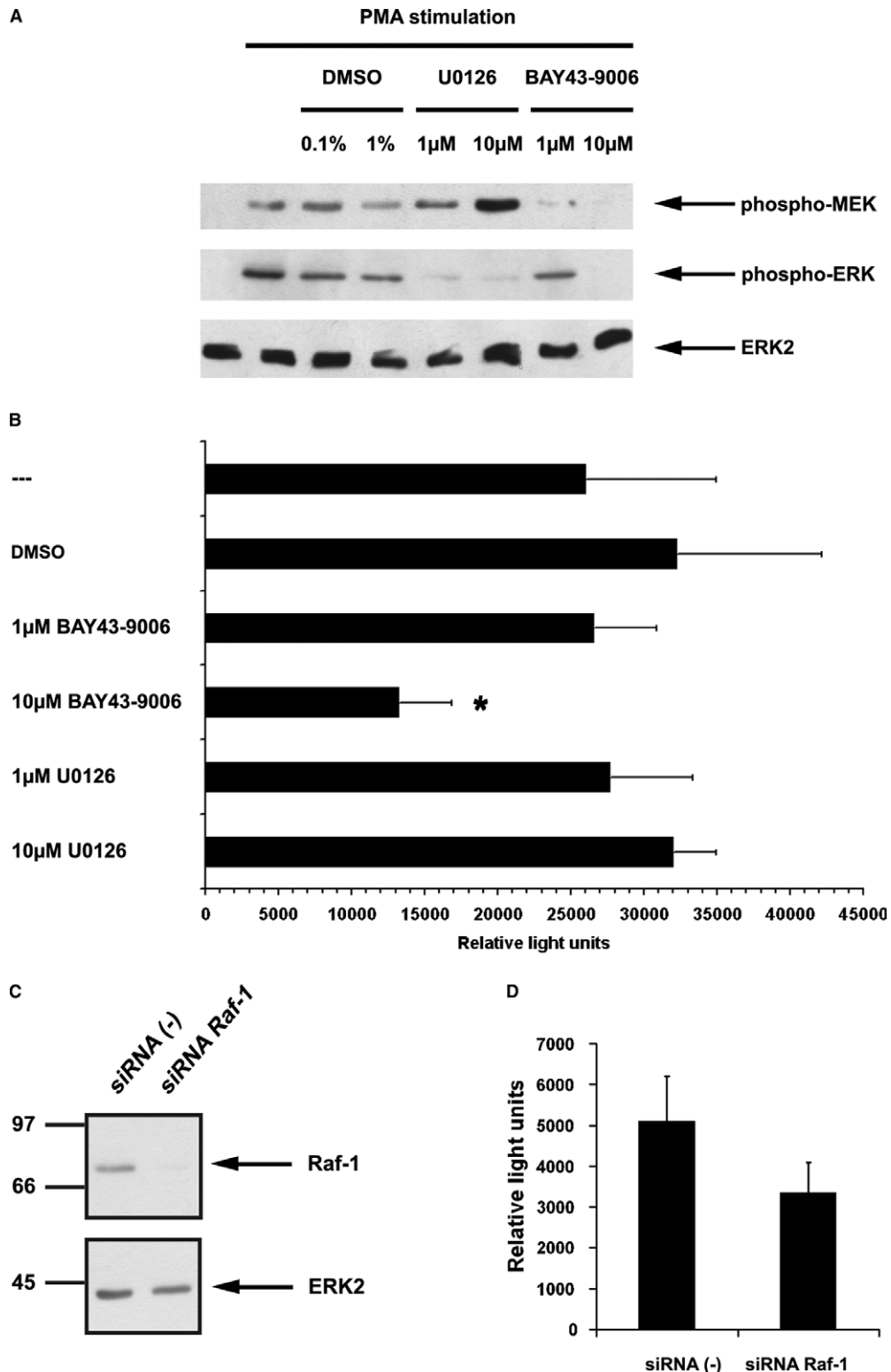
Fig. 4. Inhibition of Raf-1 modulates HCV replication. (A) HCV replicon cells HuH-7 I_{389}/NS3-3'/LucUbiNeo-ET were preincubated with DMSO (0.1%, 1%), U0126 (1, 10 μM) or BAY43-9006 (1, 10 μM) for 30 min and subsequently stimulated with PMA (100 ng/ml) for 20 min as indicated. Phosphorylation of MEK and ERK was analyzed by Western blotting using anti-phospho MEK (Cell Signaling) or anti-active MAPK (Promega). ERK2 levels were monitored using an ERK2-specific antiserum (Santa Cruz). (B) HCV replicon cells HuH-7 I_{389}/NS3-3'/LucUbiNeo-ET were incubated with DMSO (1%), U0126 (1, 10 μM) or BAY43-9006 (1, 10 μM) for 6 h. Cells were harvested immediately thereafter and replication was analyzed by measuring luciferase levels. (*) indicates a significant reduction with regard to untreated cells ($p < 0.05$). (C, D) HCV replicon cells HuH-7 I_{389}/NS3-3'/LucUbiNeo-ET were transfected with a Raf-1-specific siRNA or a negative control siRNA (Ambion). Cells were harvested 48 h post transfection. HCV replication was analyzed by measuring luciferase levels (D). Raf-1 expression was monitored by Western blotting using a Raf-1-specific antiserum (Santa Cruz) (C). As a control, expression of ERK-2 was monitored at the same time (C).

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