厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業）

分担研究報告書

GFP 挿入ウイルスを用いた HCV 感染細胞検出系の確立

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研究要旨　C型肝炎ウイルス（HCV）の培養細胞への感染を簡便に検できる系を構築するため、GFP 遺伝子を HCV のゲノムに挿入し、細胞感染後に GFP を発現するレポーターウイルスシステムの構築を試みた。このシステムは、HCV の感染増殖が可能な細胞を生きたまま分離できることから HCV の感染増殖に関与する宿主側の因子の同定も可能であると考える。

A. 研究目的

C型肝炎ウイルス（HCV）は世界中に多くの感染者が存在し、慢性肝炎から肝細胞癌を発症する重大な感染症の一つである。しかし、その治療は依然インターフェロン（IFN）に頼っているのが現状であり、ウイルス排除率は十分とは言えず副作用や耐性ウイルス出現の問題もある。さらに、高価な薬剤を長期にわたって使用するため、患者負担が大きく医療経済も圧迫している。HCVに対する抗ウイルス剤の探索が進まない大きな原因は、このウイルスの培養が不可能であり、抗ウイルス剤の効果が簡単に評価できないことにあった。近年、HCV JFH-1株を用いた培養細胞感染増殖系が開発され、培養細胞での抗ウイルス剤の評価が可能となった。しかし現行の方法では、抗ウイルス剤の活性を評価するために感染細胞の免疫染色が必要であり操作が煩雑であるため、多くの薬剤のスクリーニングには不向きである。さらに感染細胞同定のためには細胞の固定が必要となり、感染増殖が可能な細胞と不可能な細胞の宿主因子等の違いを評価することが難しい。そこで、簡便なHCVの培養細胞へ感染検出系を構築するため、GFP遺伝子をHCVのゲノムに挿入し、細胞感染後にGFPを発現するレポーターウイルスシステムの構築を試みた。このシステムでは、HCV感染後に生細胞中でGFPが発現するため、HCVの感染増殖が可能な細胞が生きたまま分離でき、宿主側の因子も同定可能であると考えられる。

B. 研究方法

1．サブジェノミックレプリコンシステムによるGFP 挿入可能部位の同定

既報の HCV 遺伝子型 1b の GFP 挿入レプリコンを参考に JFH-1 株のレポーターレプリコンシステムの NS5a 領域 C 末端のいくつかの部位に GFP を挿入したコンストラクトを作製し、JFH-1 株の細胞内増殖に影響を与えない挿入部位を同定する。

2．JFH-1 株感染クローンを用いた GFP 挿入ウイルスの作製

レポーターレプリコンシステムで増殖可能であった GFP 挿入レプリコンから、GFP 挿入部位を

JFH-1 株感染性クローンに移植し GFP 挿入ウイルスの増殖と感染性を検討する。

3．効率の良い GFP 挿入ウイルス作製のためのコンストラクトの検討

GFP 挿入 JFH-1 ウイルスは、本来の JFH-1 株の感染クローンから作製されたウイルスに比べ増殖能、ウイルス生成能に劣るため、細胞からのウイルス分泌が良くなる J6/JFH-1 キメラウイルスや他の方法で GFP を挿入したウイルスを用い、どの GFP 挿入ウイルスが最も効率的に感染ウイルスを作製できるかを検討する。

（倫理面への配慮）

本研究で使用するヒト由来試料はすでに樹立された細胞株であり倫理面での問題はないと考えられる。各種組換え DNA を用いた感染ウイルス生成および感染実験は、大臣確認申請を行い承認を受けた。

C. 研究結果

1．サブジェノミックレプリコンシステムによる GFP 挿入可能部位の同定

NS5a 領域 C 末端の遺伝子型 1b のレプリコンシステムで報告されている GFP 挿入可能部位二カ所と、さらにその C 末側一カ所の計三カ所に GFP を挿入したレポーターレプリコンを作製し、細胞内での増殖を検討した。その結果、最も N 末側に GFP を挿入したレプリコンではほとんど増殖が認められなかった。他の二つのコンストラクトでは増殖が可能であったが、その増殖能は通常のレプリコンの約 1/10 であった。

2．JFH-1 株感染クローンを用いた GFP 挿入ウイルスの作製

レポーターレプリコンシステムで増殖可能であった二カ所の部位に GFP を挿入した JFH-1 株感染性クローンを作製し、Huh7.5.1 細胞に遺伝子導入し、GFP 挿入ウイルスの生成を検討した。その結果、遺伝子導入細胞中で GFP の発現を認め、GFP を持った HCV が増殖していると考えられた。さらにその培養上清を新たな Huh7.5.1 細胞に感染させたところ、感染細胞で HCV コア蛋白と GFP の発現を認め、GFP 遺伝子を持った HCV が培養上清中に分泌されていると考えられた。しかし、GFP 遺伝子を持った JFH-1 ウイルスは通常の JFH-1 ウイルスに比べ、ウイルスの生成効率が悪く、感染阻止実験を行うための十分な量のウイルスが得られなかった。

3．効率の良い GFP 挿入ウイルス作製のためのコンストラクトの検討

まず、細胞からのウイルス分泌が良くなると報告されている J6/JFH-1 キメラウイルスを用い、GFP 挿入ウイルスの生成効率を JFH-1 ウイルスと比較した。その結果、J6/JFH-1 キメラウイルスを用いることで、HCV RNA 量は遺伝子導入細胞で約 20 倍、培養上清で 2 - 5 倍高値となり、ウイルス生成効率の改善が認められた。さらに、NS5a 以外の領域に GFP を挿入したレポーターウイルスと比較するため、コア領域の N 端に FMDV 2a 遺伝子、ユビキチン遺伝子とともに挿入したコンストラクト、ネオマイシン耐性遺伝子の代わりに GFP を挿入した全長レプリコンコンストラクトを作製し、ウイルス生成効率を比較検討した。その結果、遺伝子導入細胞中では全長レプリコンで約 2 倍、コア領域 N 端挿入コンストラクトでは約 20 倍の HCV RNA 量を

示した。また培養上清中の HCV RNA は、全長レプリコンで約 4 倍、コア領域 N 端挿入コンストラクトでは約 1.5 倍であった。それぞれの培養上清を新たな Huh7.5.1 細胞に感染させ、感染細胞で HCV コア蛋白と GFP の発現を確認したところ、全長レプリコンではコア蛋白と GFP の両者の発現を認めたが、コア領域 N 端挿入コンストラクトでは、多くの細胞で HCV コア蛋白のみの発現を認め、GFP は発現していなかった。従って、このコンストラクトを導入した細胞中で HCV RNA が高値になっていたのは、GFP が排除されたクローンが強く増殖していたためと考えられた。

D. 考察

今回の一連の検討から、J6/JFH-1 キメラウイルスの NS5a 領域に GFP を挿入したコンストラクトを用いることで、JFH-1 株に挿入したものよりも効率的に GFP 遺伝子を持ったウイルスの生成が可能であることがわかった。しかし、その生成効率は全長のレプリコンには及ばず、現状ではこの NS5a 領域に GFP を挿入したコンストラクトの優位な点は無いと考えられた。同時に検討した、コア領域の N 端に GFP を挿入したコンストラクトは細胞内の増殖能は強いものの GFP の排除が起こるためレポーターウイルスには不適と考えられた。

E. 結論

JFH-1 レポーターレプリコンシステムを用いることで、NS5A 領域に GFP 遺伝子の挿入が可能な領域を二カ所同定した。さらに、J6/JFH-1 キメラウイルスを使用することで、効率的に GFP 遺伝子を持った HCV を作製することが可能であった。しかし、その生成効率は現状では全長レプリコンに及ばず、今後は生成効率を良くする適応変異の導入等さらに検討が必要であると考えられた。

F. 研究発表

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D. 知的所有権の出願・登録状況

なし

厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業）

分担研究報告書

B型肝炎ウイルスレセプターのクローニングと in vitro, in vivoにおける感染系の構築及び応用

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研究要旨　B型肝炎ウイルス（HBV）はウイルスの発見から既に40余が経とうとしているが未だにレセプターも同定されず感染系モデルの構築ができずにいる。このことは肝炎や肝硬変、肝がんを発症する本ウイルスの病原性や疾患発生機構そのもの、治療法の開発にとって極めて不利な状況をもたらしている。そこで本提案ではpseudotype HBV（HBVの皮を被ったレトロウイルス）の作製からHBVレセプターの分離・同定を試み、さらにそれを利用した感染系の構築を構築し病態発症機構の解析や治療法開発へ応用する。

A. 研究目的

前述の如くHBV）はその発見から40年余経ったが未だにその有効な感染系は存在せず､HBVレセプターはおろかHBV感染病態発症（急性肝炎、慢性肝炎、肝がん）機構の詳細は不明のままである。HBV感染に対してはワクチンが開発され大部分の新規の感染は防ぐことが可能になっているが、本ワクチンを回避するHBVサブタイプの報告もあり決して完璧なワクチンではない。日本においても20歳以上では未だ多くのHBVキャリアーや慢性肝炎を発症している患者さんたちが多く存在し、また世界では約2～3億人の患者が存在する世界的にも有数の巨大感染症である。B型慢性肝炎に対しては核酸アナログを中心とした治療がされているが、相次ぐ変異体の出現により治療そのものも極めて困難をきたしている難治性の感染症である。このようなウイルス感染症を克服し新たな治療戦略を考案するにはHBVの *in vitro*（培養細胞感染系という意味で）、*in vivo*（動物感染モデル）を樹立し、病態発症機構を詳細に解明することが不可欠であり、そのためにはHBVの感染に際して機能するレセプターの分離・同定が欠かせないと考えられる。そこで本研究ではpseudotype HBV（HBVの皮を被ったレトロウイルス）の作製を試み、それを利用したHBVレセプターの分離・同定から先に述べたHBVの *in vitro*、*in vivo*感染系構築を目指す。

B. 研究方法（倫理面への配慮）

HBVレセプターの分離･同定に成功しマウスを用いたin vivo感染系の構築の際には動物実験に関する法令或は本学学内規定に基づいて実験を進行するものとする。またヒト検体を使用する事態に当たっては本学倫理規定に従い許可を受けた上で進めるものとする。

C. 研究結果

1) レトロウイルスゲノム供給ベクター；LTR-hygR-CMVep-EGFP-LTR を構築し、肝がん由来培養細胞 Huh7 及びヒト腎実質細胞 293GP2(Clontech 社製、本細胞はレトロウイルス gag、pol 遺伝子を発現している）で安定発現株 GFP/H7、GFP/GP2 をそれぞれ樹立した。

2) GFP/H7 細胞にレトロウイルス gag-pol (gp) を導入し gp/GFP/H7 細胞を樹立した。VSV-G pseudotype の実験から gp/GFP/H7 細胞、GFP/GP2 細胞がともに packaging 細胞として機能することを確認した。

3) HBVのlarge S (LS)、middle S (MS)、small S（SS) の三つの膜蛋白を発現する発現ベクター、pCEP4-LS、pREP8-LS、pHBgを構築した。

4) pCEP4-LS或はpREP8-LS或はpHBgをgp/GFP/H7細胞、GFP/GP2へトランスフェクションし培養上清中に目的のpseudotype HBVが形成されているかを、粒子を分離し、粒子内ゲノムを標的としたRT-PCRにより検討中である。

D. 考察

pseudotype HBV 作製準備は整っているので、pseudotype HBV 産生確認後速やかに HBV レセプタークローニングのスクリーニングを進める必要がある。

E. 結論

pseudotype HBV 作製準備は整った。

G. 研究発表

なし

H. 知的財産の出願・登録状況

なし

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

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Ⅳ. 研究成果の刊行物・別冊

Hepatitis C virus non-structural proteins responsible for suppression of the RIG-I/Cardif-induced interferon response

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Viral infections activate cellular expression of type I interferons (IFNs). These responses are partly triggered by RIG-I and mediated by Cardif, TBK1, IKKε and IRF-3. This study analysed the mechanisms of dsRNA-induced IFN responses in various cell lines that supported subgenomic hepatitis C virus (HCV) replication. Transfection of dsRNA into Huh7, HeLa and HEK293 cells induced an IFN expression response as shown by IRF-3 dimerization, whilst these responses were abolished in corresponding cell lines that expressed HCV replicons. Similarly, RIG-I-dependent activation of the IFN-stimulated response element (ISRE) was significantly suppressed by cells expressing the HCV replicon and restored in replicon-eliminated cells. Overexpression analyses of individual HCV non-structural proteins revealed that NS4B, as well as NS34A, significantly inhibited RIG-I-triggered ISRE activation. Taken together, HCV replication and protein expression substantially blocked the dsRNA-triggered, RIG-I-mediated IFN expression response and this blockade was partly mediated by HCV NS4B, as well as NS34A. These mechanisms may contribute to the clinical persistence of HCV infection and could constitute a novel antiviral therapeutic target.

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INTRODUCTION

Type I interferon (IFN) plays a central role in eliminating virus, not only following clinical therapeutic application but also as a cellular immune response (Samuel, 2001; Taniguchi & Takaoka, 2002). Hepatitis C virus (HCV) infection is characterized by persistence and replication of the virus in the liver, despite an intact host immune system (Alter, 1997). Indeed, even after administration of the currently most potent IFN reagents, as many as half of the patients are refractory to the treatment and fail to eradicate the virus (Fried *et al.*, 2002). These features have led to speculation that HCV escapes from or attenuates the host antiviral response (Katze *et al.*, 2002).

Cellular antiviral responses are primarily mediated by IFN and IFN-stimulated genes (ISGs), including 2,5-oligoadenylate synthetase, dsRNA-dependent protein kinase R (PKR) and MxA proteins, as well as by as yet uncharacterized genes (Itsui *et al.*, 2006; Stark *et al.*, 1998). A study of experimental chimpanzee HCV infection has shown that various cytokines and chemokines are induced in the liver during the course of acute HCV infection and its clearance, and that a considerable proportion of the genes is induced by type I IFN (Bigger *et al.*, 2001).

Control of expression of ISGs is mediated by binding of type I IFNs to their receptors. Following receptor binding, STAT1 and STAT2 are phosphorylated to form ISGF-3, which translocates to the nucleus and binds the IFN-stimulated response element (ISRE), located in the promoter/enhancer region of ISGs, and activates transcription of ISGs (Samuel,

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2001; Taniguchi *et al.*, 2001; Taniguchi & Takaoka, 2002). ISRE-dependent gene expression is also mediated by binding of the ISRE by molecules such as IRF-1, IRF-3 and IRF-7 (Kanazawa *et al.*, 2004). IRF-3 is a transducer of virus-mediated signalling and plays a critical role in the induction of cellular antiviral responses (Lin *et al.*, 1998; Sato *et al.*, 2000; Taniguchi *et al.*, 2001; Yoneyama *et al.*, 1998). Transcriptional activation and suppression of IRF-3 are inversely correlated with the level of HCV replication *in vitro* (Yamashiro *et al.*, 2006). Following virus infection, IRF-3 is phosphorylated by two cytoplasmic kinases, TBK1 and IKKε (Fitzgerald *et al.*, 2003; Sharma *et al.*, 2003). The phosphorylated IRF-3 forms a homodimer, translocates to the nucleus and predominantly activates expression of the IFN-β gene and certain ISGs (Doyle *et al.*, 2002; Nakaya *et al.*, 2001; Taniguchi & Takaoka, 2002).

RIG-I is a recently identified cytoplasmic DExD/H box RNA helicase that participates in recognition of virus-related dsRNA as a pathogen-related molecular pattern (Yoneyama *et al.*, 2005). RIG-I contains two caspase-recruitment domains (CARDs) in the N terminus and a DExD/H box RNA helicase in the C terminus. MDA5 has been identified as another CARD-containing DExD/H box RNA helicase (Andrejeva *et al.*, 2004). More recently, an adaptor molecule of RIG-I and MDA5, Cardif (also known as IPS-I, MAVS and VISA), has been identified by four independent groups (Kawai *et al.*, 2005; Meylan *et al.*, 2005; Seth *et al.*, 2005; Xu *et al.*, 2005). On association with dsRNA, RIG-I or MDA5 causes conformational changes and homo-oligomerization, and binds the CARD of Cardif (Saito *et al.*, 2007). Cardif subsequently recruits the kinases TBK1 and IKKε, which catalyse phosphorylation and activation of IRF-3 (Yoneyama *et al.*, 1998).

The IRF-3-mediated IFN-β induction pathway could be a target for viruses to counteract antiviral responses and promote their replication in host cells. Ebola virus, bovine viral diarrhea virus (BVDV) and influenza A virus interfere with the activation of IRF-3 through interactions of their virus-encoded proteins (Basler *et al.*, 2003; Schweizer & Peterhans, 2001; Talon *et al.*, 2000). There are several reports that HCV proteins interact with IFN-mediated antiviral systems. The NS5A and E2 proteins have been reported to interfere with the action of IFN by inhibiting the activity of PKR (He & Katze, 2002). It was reported recently that the HCV NS34A protease blocks virus-induced activation of IRF-3, possibly by proteolytic cleavage of Cardif (Foy *et al.*, 2003; Meylan *et al.*, 2005).

The HCV subgenomic replicon is an *in vitro* model that simulates autonomous cellular replication of HCV genomic RNA (Lohmann *et al.*, 1999). Expression of the HCV replicon can be abolished by treatment with small amounts of type I and type II IFNs (Blight *et al.*, 2000; Frese *et al.*, 2002; Guo *et al.*, 2001), suggesting intact IFN receptor-mediated cellular responses. In contrast, viral expression persists in the absence of the exogenous IFN. Baseline expression levels of ISG were substantially decreased in cells expressing the HCV replicon compared with parental Huh7 cells (Kanazawa *et al.*, 2004). These findings led us to speculate that intracellular virus-induced antiviral responses are attenuated or caused to malfunction by the expression of viral proteins.

In this study, we investigated cell lines that support subgenomic HCV replication and HCV cell culture for the dsRNA-induced cellular IFN expression pathway. Here, we report that RIG-I- and Cardif-mediated IFN gene activation is uniformly attenuated in several replicon-expressing cell lines of different lineages and, more importantly, that the HCV NS4B protein is involved in the suppression of antiviral IFN responses.

METHODS

Plasmids. Plasmids pEF-flagRIG-I and ΔRIG-I expressed full-length and C-terminally truncated RIG-I protein, respectively (Yoneyama *et al.*, 2004). The plasmid pER-flagRIG-IKA (RIG-IKA) has a point mutation in the putative ATP-binding site of the RIG-I helicase domain and was used as a negative control for ΔRIG-I and RIG-I full transfection assays. Expression plasmids for full-length Cardif (Cardif), Cardif CARD (CARD) and CARD-truncated Cardif (ΔCARD) were provided by Dr J. Tschopp (University of Lausanne, Switzerland) (Meylan *et al.*, 2005). Expression plasmids for toll-like receptor 3 (TLR3) and TIR domain-containing adaptor inducing IFN-β (TRIF), the transmembrane receptor of dsRNA and the adaptor molecule of TLR3, respectively, were provided by Dr S. Akira (Osaka University, Japan). Plasmids expressing HCV NS345, NS3, NS34A, NS4A, NS4B, NS5A and NS5B were amplified from HCV pCV-J4-L4S (Yanagi *et al.*, 1997) by PCR and subcloned. The DNA fragments were inserted into the vector pcDNA4/TO/*myc*-His (Invitrogen). Nucleotide sequences were confirmed by sequencing. Plasmids TOPO-NS34A (HCV N), TOPO-NS4B (HCV N) and pcDNA-NS4B (HCV JFH1) expressed Myc-tagged NS34A and NS4B proteins derived from the HCV N (Beard *et al.*, 1999) and HCV JFH1 (Wakita *et al.*, 2005) strains, as indicated. Plasmid pISRE-TA-Luc (Invitrogen) contained five copies of consensus ISRE motifs upstream of the firefly luciferase gene. Plasmid pIFNβ-Fluc was constructed by cloning the human IFN-β promoter region, spanning nt −110 to −36, upstream of the firefly luciferase gene of pGL3 Basic (Promega). Plasmid pcDNA3.1 (Invitrogen) was used as an empty vector for mock transfection. pRL-CMV (Promega), which expressed the *Renilla* luciferase protein, was used for correction of transfection efficiency.

Cell culture. HCV strain JFH1-infected Huh7.5.1, Huh7, Huh7.5.1 (kindly provided by Dr F. Chisari, The Scripps Institute, CA, USA; Zhong *et al.*, 2005), HeLa and HEK293 cells were maintained in Dulbecco's modified minimal essential medium (Sigma) supplemented with 2 mM L-glutamine and 10 % fetal calf serum at 37 °C with 5 % CO_2. Cells expressing the HCV replicon were cultured in medium containing 100 μg G418 (Wako) ml^{-1}.

HCV replicon constructs and transfected cell lines. An HCV subgenomic replicon plasmid, pHCVIbneo-delS (designated pRep-N), was derived from an HCV clone of strain N, genotype 1b, and pSGR-JFH1 was derived from HCV JFH1, genotype 2a (Guo *et al.*, 2001; Wakita *et al.*, 2005). These replicons were reconstructed by substituting the neomycin phosphotransferase gene with a fusion gene comprising *Renilla* luciferase and neomycin phosphotransferase to construct pRep-Reo-1b and pRep-Reo-2a, respectively (Tanabe *et al.*, 2004; Yokota *et al.*, 2003). RNA was synthesized from the replicons using T7 polymerase (Promega) and transfected into Huh7,

HeLa and HEK293 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established (Huh7/1bReo, Huh7/2aReo, HeLa/2aReo and 293/2aReo). We have previously reported that firefly luciferase activities of Feo-replicon-expressing cells correlate well with HCV NS3, NS4A and NS5A protein expression levels and with the levels of replicon RNA (Yokota *et al.*, 2003).

Transient transfection. Transient DNA transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. ISRE reporter assays were carried out as previously described (Nakagawa et al., 2004). To analyse IFN expression in HCV JFH1 cell cultures, a total of 1×10^5 Huh7.5.1, JFH-1 infected Huh7.5.1 and IFN-treated Huh7.5.1 cells were seeded into 24-well plates the day before transfection. Plasmids pISRE-TA-Luc and ΔRIG-I (200 ng each) were transfected using 1 μl Lipofectamine 2000. RIG-IKA was used as a control. Luciferase assays were performed on day 3 post-transfection.

For further study, 400 ng of each non-structural protein was added to 1×10^4 Huh7 or HEK293 cells that had been seeded into 96-well plates the day before transfection. pISRE-TA-Luc and ΔRIG-I (40 ng each) were transfected using 0.5 μl Lipofectamine 2000. RIG-IKA was used as a control.

Western blotting. Preparation of the cytoplasmic and nuclear fractions of cell lysates was carried out as described previously (Tanabe *et al.*, 2004). Protein (20 μg) was separated using NuPAGE 4–12 % Bis/Tris gels (Invitrogen) and blotted onto an Immobilon PVDF membrane (Roche). The membrane was immunoblotted with anti-IRF-3 (Santa Cruz) and detected by chemiluminescence (BM Chemiluminescence Blotting Substrate; Roche).

RT-PCR. Interleukin (IL)-8 mRNA was detected by RT-PCR as described previously (Itsui *et al.*, 2006). The primers used were IL8-S (5′-GCACAAACTTTCAGAGACAGCAGAGCACAC-3′) and IL8-AS (5′-CAGAGCTGCAGAAATCAGGAAGGCTGCCAA-3′).

Indirect immunofluorescence assay. Cells seeded onto tissue culture chamber slides were fixed with cold acetone. The cells were incubated with anti-protein disulphide isomerase (PDI) or anti-Myc antibodies and subsequently with Alexa 488- or Alexa 568-labelled secondary antibodies. Cells were mounted with VECTA SHIELD Mounting Medium and DAPI (Vector Laboratories) and visualized by fluorescence microscopy (BZ-8000; Keyence).

Luciferase reporter assays. Luciferase activity was measured using a 1420 Multilabel Counter (ARVO MX; PerkinElmer) using a Bright-Glo Luciferase Assay System (Promega) or a Dual Luciferase Assay System (Promega). Assays were carried out in triplicate and the results expressed as means ± SD.

MTS assay. To evaluate cell viabilities, dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assays were performed using a CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega) according to manufacturer's instructions.

Statistical analyses. Statistical analyses were performed using an unpaired, two-tailed Student's *t*-test. *P* values of less than 0.05 were considered to be statistically significant.

RESULTS

IRF-3 dimer formation is attenuated in cells expressing the HCV replicon

In the HCV replicon-expressing cell lines Huh7/Rep-Reo-2a, Hela/Rep-Reo-2a and 293/Rep-Reo-2a, replicon expression levels corresponded well to internal *Renilla* luciferase activities. Expression of the HCV replicon was suppressed by IFN in a dose-dependent manner (data not shown).

Activation of RIG-I or MDA5 induces phosphorylation and homodimerization of IRF-3. Following transfection of poly(I : C) into Huh7, HeLa or HEK293 cells, IRF-3 dimers were detected (Fig. 1). However, in cells supporting HCV replicons, IRF-3 dimer formation was almost completely abolished. These findings showed that expression of HCV proteins blocked activation of dsRNA-mediated IFN expression and that these effects were consistently found in several cell lines of different origin.

The HCV replicon suppresses RIG-I/Cardif-induced IFN responses

ISRE reporter activities did not increase in naïve Huh7, HeLa or HEK293 cells following transfection of poly(I : C), whilst overexpression of full-length RIG-I increased poly(I : C)-mediated ISRE reporter activity in Huh7 and HEK293 cells (data not shown). In RIG-I-overexpressing Huh7 cells, transduction with an HCV replicon abolished the poly(I : C)-induced ISRE activation, and elimination of the replicon by IFN treatment restored these ISRE responses (Fig. 2a). Consistent results were obtained by overexpression of ΔRIG-I, a constitutively active form. Transfection of ΔRIG-I in Huh7 and HEK293 cells induced ISRE activation, whilst these responses were abolished or significantly suppressed in cell lines expressing HCV replicons and were recovered by eliminating the replicon by IFN treatment (data not shown). Similarly, ISRE activation by overexpression of Cardif, an adaptor molecule of RIG-I, was almost completely blocked in replicon-expressing cells and was recovered by eliminating the replicon from the cells (data not shown). The RIG-I-mediated IFN response was

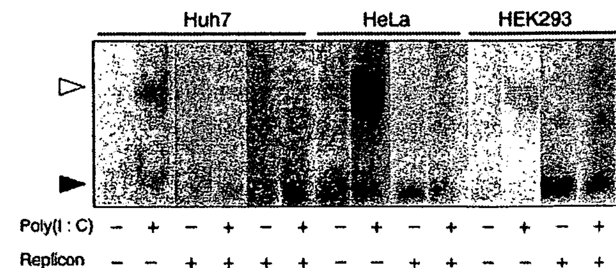

Fig. 1. Double-stranded RNA-induced IRF-3 dimer formation in cell lines that support HCV subgenomic replication. Poly(I : C) was transfected into naïve Huh7, HeLa and HEK293 cells, and into corresponding cell lines expressing the HCV replicon. Six hours after transfection, cell lysates were prepared, separated in polyacrylamide gels and blotted onto PVDF membrane. The membrane was immunoblotted with anti-IRF-3 and visualized by chemiluminescence (see Methods). The positions of the IRF-3 dimer (open arrowhead) and monomer (closed arrowhead) are indicated.

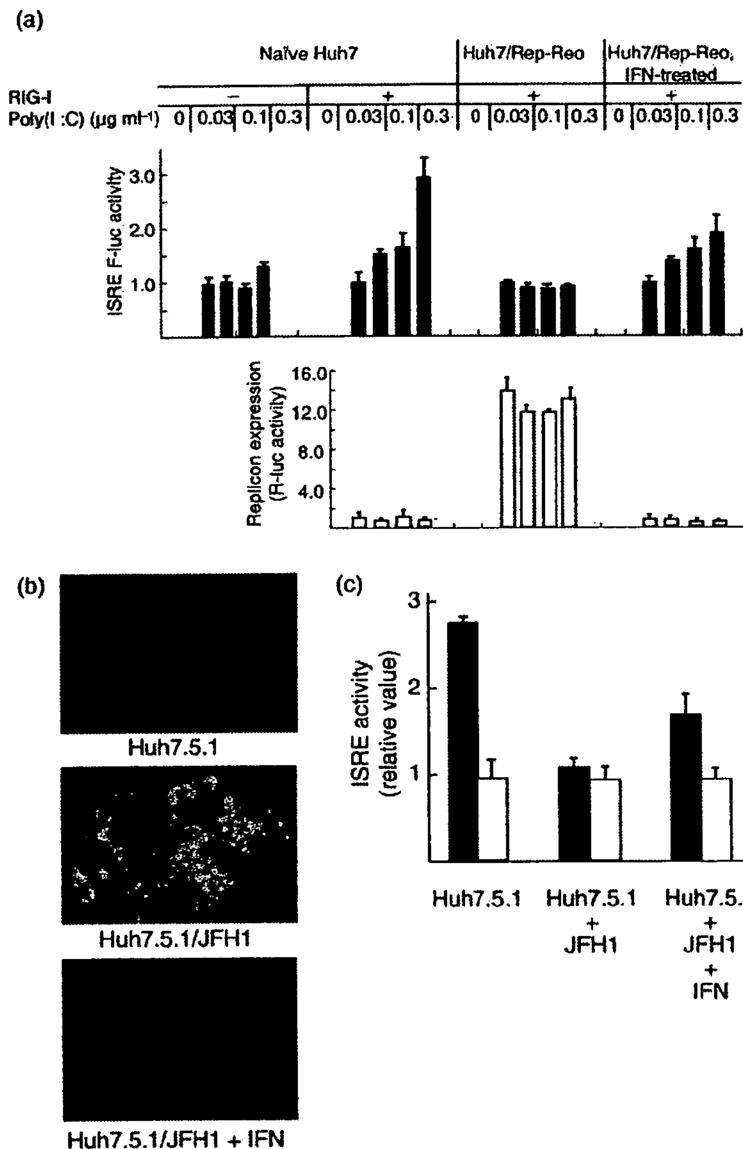

Fig. 2. Suppression of dsRNA-induced, RIG-I-mediated ISRE activation by HCV replication. (a) The HCV replicon suppresses transcriptional activation after poly(I : C) stimulation. The RIG-I expression plasmid and pISRE-TA-Luc were transiently transfected into the cell lines indicated. The following day, the amounts of poly(I : C) indicated were transfected into the corresponding cell lines and dual luciferase assays were carried out 8 h after transfection. Filled bars indicate ISRE-regulated firefly luciferase (F-luc) activities and open bars indicate *Renilla* luciferase (R-luc) activities representing replicon expression levels. In both graphs, scales for the *y*-axis are shown as relative values. Assays were carried out in triplicate and results are shown as means ± SD. (b) Immunofluorescence microscopy results. Huh7.5.1 cells infected with HCV JFH1 (Huh7.5.1/JFH1) and JFH1-infected cells from which the virus had been eliminated by IFN treatment (Huh7.5.1/JFH1 + IFN) were incubated with anti-core primary antibodies followed by Alexa Fluor-conjugated secondary antibody (green). Nuclei were stained with DAPI (blue). (c) ISRE activation by ΔRIG-I overexpression. The plasmid pISRE-TA-Luc was co-transfected with ΔRIG-I (filled bars) or RIG-IKA (empty bars) into naïve Huh7.5.1, Huh7.5.1/JFH1 or Huh7.5.1/JFH1 + IFN cells. Luciferase assays were carried out 8 h after transfection. The *y*-axis indicates ISRE-regulated luciferase activity shown as relative values. Assays were carried out in triplicate and results are shown as means ± SD.

also suppressed in HCV JFH1 virus cell culture. In JFH1-infected Huh7.5.1 cells, ΔRIG-I-induced ISRE reporter activation was significantly suppressed, but was recovered in IFN-treated, virus-eliminated cells (Fig. 2b and c). These results demonstrated that RIG-I- and Cardif-mediated antiviral responses were substantially suppressed by both subgenomic and genomic viral replication in both hepatocyte- and non-hepatocyte-derived host cells.

NS34A and NS4B are responsible for suppressing RIG-I-mediated IFN responses

We next sought to define which HCV proteins were responsible for inhibition of the RIG-I- and IRF-3-mediated IFN induction pathway. We constructed expression plasmids that expressed the non-structural proteins NS345, NS3, NS34A, NS4A, NS4B, NS5A and NS5B (Fig. 3a). We transfected each expression plasmid with simultaneous activation of the RIG-I pathway by overexpression of ΔRIG-I, Cardif, TBK1 and IKKε (Fig. 3b–e). Expression of full-length non-structural (NS345) and NS34A proteins inhibited ISRE activation mediated by expression of RIG-I and Cardif but not that mediated by TBK1 and IKKε. Interestingly, it was found that NS4B also inhibited ISRE activation mediated by expression of RIG-I and Cardif, but not by TBK1 and IKKε. Consistent with Fig. 3(b), overexpression of NS4B significantly suppressed ΔRIG-I-induced activation of the authentic IFN-β promoter (Fig. 3f).

Another group has studied IFN antagonism of flavivirus non-structural proteins and has reported that HCV NS4B did not affect IFN responses (Muñoz-Jordán *et al.*, 2005).

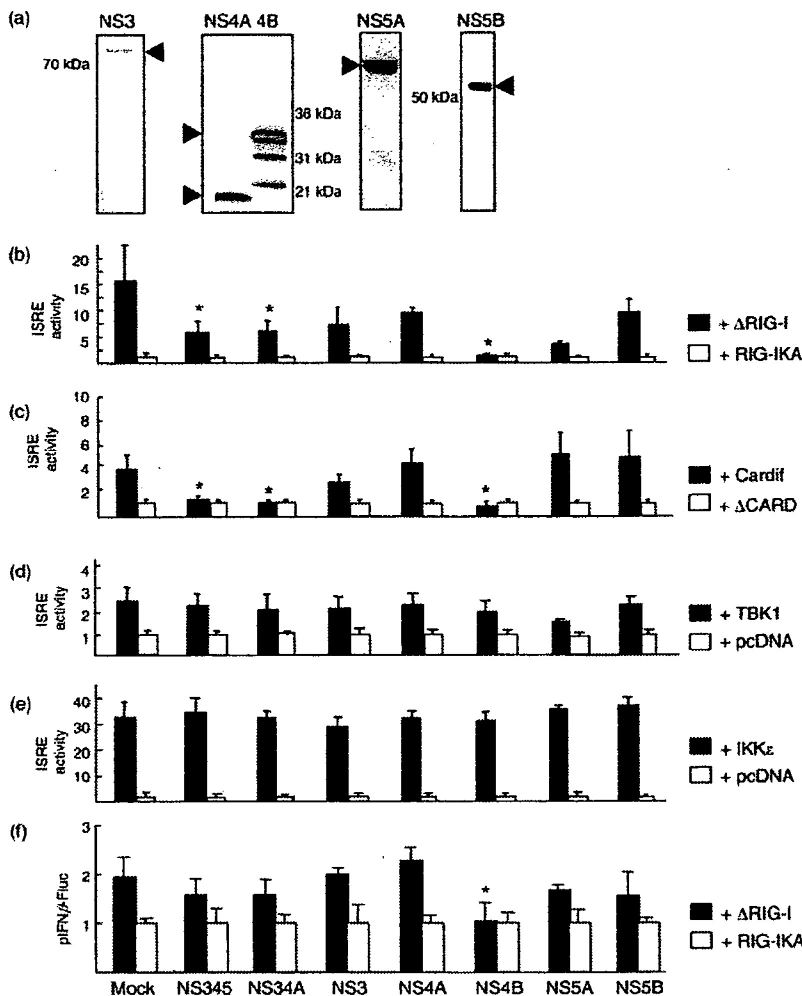

Fig. 3. Co-transfection analyses using plasmids that express individual HCV non-structural proteins. (a) Western blotting. Plasmids expressing the indicated Myc-tagged HCV proteins were transfected into Huh7 cells. Western blotting was carried out using anti-Myc antibody. (b–e) The following plasmids were co-transfected into Huh7 cells: pISRE-TA-Luc, pRL-CMV, the indicated plasmids expressing ΔRIG-I (b), Cardif (c), TBK1 (d) and IKKε (e), and the indicated plasmids expressing individual HCV non-structural proteins. Plasmids RIG-IKA, ΔCARD or pcDNA were used as negative controls as indicated. Twenty-four hours after transfection, luciferase activities were measured. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD. *, $P<0.05$. (f) pIFN-β and pRL-CMV were co-transfected into Huh7 cells, with plasmids expressing individual HCV non-structural proteins and plasmid expressing ΔRIG-I. Luciferase activities were measured 24 h after transfection. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD. *, $P<0.05$. Plasmid RIG-IKA was used as a negative control.

To investigate strain-specific differences in the characteristics of NS4B proteins, we performed co-transfection assays using NS4B expression constructs from HCV N (Beard *et al.*, 1999) and JFH1 (Wakita *et al.*, 2005) strains, as well as HCV strain J4 (Fig. 4a and b). All NS4B constructs suppressed ΔRIG-I- or Cardif-mediated ISRE activation. These results suggested that the above-described effects of NS4B were independent of HCV strain.

NS4B has been reported to induce an unfolded protein response or endoplasmic reticulum (ER) stress through ATF6 or IRE1-X box protein (XBP1) pathways (Zheng *et al.*, 2005). The ER stress induces production of IL-8, which has been reported to interfere with the IFN system (Polyak *et al.*, 2001). Therefore, we detected expression of IL-8 using RT-PCR in cells with and without overexpression of NS4B. As shown in Fig. 4(c), no significant difference was observed in IL-8 mRNA levels among mock-, NS34A- and NS4B-transfected cells. These results showed that NS4B overexpression in the present study did not induce expression of IL-8 and that the IFN-antagonizing effects of NS4B were independent of IL-8.

It has been reported that NS34A suppresses the TLR3-mediated IFN response (Breiman *et al.*, 2005; Ferreon *et al.*, 2005). However, overexpression of HCV non-structural proteins did not suppress ISRE activation that was induced by overexpression of TLR-3 or TRIF (Fig. 5a and b), nor did NS34A from two different HCV strains, J4 and N, show significant suppression of TRIF-mediated ISRE activation (Fig. 5c). Although strain-specific differences might be involved, these data suggest that neither NS34A nor NS4B affect the TLR3-triggered, TRIF-mediated IFN expression signalling pathway.

The NS4B N terminus is involved in inhibition of the RIG-I-mediated pathway

Given the result that NS4B suppressed the RIG-I-mediated IFN expression pathway, we next investigated which domain of NS4B was responsible. We constructed plasmids that expressed truncated NS4B in which the protein-coding frame was truncated at four positions corresponding to the five transmembrane domains (Lundin *et al.*, 2003) (Fig. 6a).

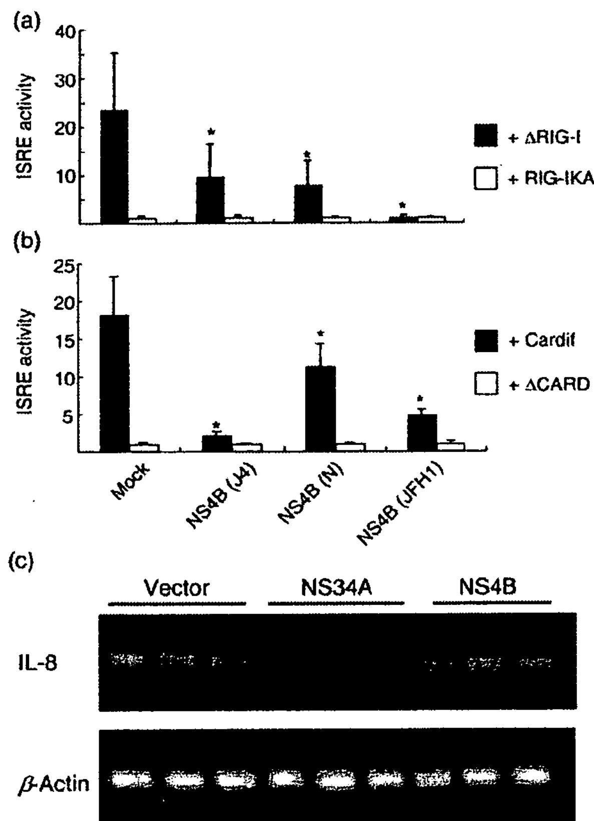

Fig. 4. Co-transfection analyses of HCV NS4B proteins of different origins. NS4B (J4), NS4B (N) and NS4B (JFH1) denote HCV NS4B proteins that were cloned from HCV strains J4, N and JFH1, respectively. The NS4B plasmids indicated were co-transfected with plasmids expressing ΔRIG-I (a) or Cardif (b). Luciferase activities were measured 24 h after transfection. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD. *, $P<0.05$. (c) Semi-quantitative detection of IL-8 mRNA by RT-PCR. cDNA was prepared from Huh7 cells transfected with empty vector or with NS34A or NS4B expression plasmid.

Expression and subcellular localization of NS4B truncated proteins were visualized by indirect immunofluorescence assays (Fig. 7). Each of the NS4B truncated proteins was localized predominantly to the perinuclear rim as dense spots. Some of the spots were similar to the staining of the ER-resident host protein PDI, consistent with previous reports (Lindström *et al.*, 2006; Lundin *et al.*, 2006). These truncated expression plasmids were co-transfected with Cardif expression plasmids into Huh7 cells. As shown in Fig. 6(b), Cardif-mediated ISRE activation was significantly suppressed by co-transfection of NS4Bt1–156 and NS4Bt1–186, as well as full-length NS4B, whilst transfection of NS4Bt90–260 and NS4Bt110–260 did not significantly suppress Cardif-mediated ISRE activation. The shortest construct, NS4Bt131–260, partially retained the ability to reduce ISRE activity. These results suggested that the N-terminal domain of NS4B, which includes aa 1–110, might function directly to suppress RIG-I-mediated IFN expression responses.

DISCUSSION

The recent discovery of cytoplasmic dsRNA sensor molecules has resulted in rapid expansion of knowledge about the IFN-mediated virus defence pathway (Yoneyama *et al.*, 2004). Several reports suggest that viruses target the IFN system to establish replication in the host cells (Kato *et al.*, 2006). We have confirmed that the dsRNA-triggered, IRF-3-mediated IFN activation pathway was blocked in several replicon-supporting cell lines (Fig. 1). Similarly, the dsRNA responses were substantially suppressed in HCV JFH-1 cell culture compared with parental Huh7 cells (Fig. 2b and c). Overexpression analyses showed that RIG-I- and Cardif-mediated ISRE activation was significantly suppressed in HCV replicon-expressing cells, which recovered after elimination of the replicon by IFN treatment (Fig. 2a). In contrast, TBK1- or IKKε-mediated ISRE activation was not suppressed in replicon-expressing cells. Overexpression of individual HCV non-structural proteins revealed that not only NS34A but also NS4B inhibited the ISRE activation signal (Figs 3, 4 and 5). These results suggested that HCV non-structural proteins suppress the IFN induction pathway and that the target host molecule could be Cardif or an unknown adaptor molecule acting between Cardif and TBK1/IKKε.

NS4B protein is a 27 kDa hydrophobic integral membrane protein that is localized in the ER with other non-structural proteins. Studies on other flaviviruses such as Kunjin virus and BVDV support the notion that NS4B may indeed be an essential part of the replication mechanism (Grassmann *et al.*, 2001; Khromykh *et al.*, 2000; Li & McNally, 2001; Qu *et al.*, 2001). These systems have demonstrated that intact NS4B is necessary in a *cis* configuration in the polyprotein for maintaining viral replication (Grassmann *et al.*, 2001; Khromykh *et al.*, 2000). Furthermore, single mutations in NS4B alter the cytopathic effects of BVDV and even mediate changes in the cellular tropism of Dengue virus (Hanley *et al.*, 2003; Qu *et al.*, 2001). In HCV, the search for cell-culture-adaptive mutations in HCV subgenomic replicons has led to the generation of mutations in the NS4B region that confer higher replication levels and resistance to IFNs, as well as broadening the tropism for different cell lines (Lohmann *et al.*, 2003; Sumpter *et al.*, 2004; Zhu *et al.*, 2003). These pieces of evidence may imply that NS4B is not only part of the replication machinery but may also have other functions that enable establishment of viral replication.

NS4B truncation assays showed that RIG-I/Cardif-mediated ISRE activation was significantly suppressed by expression of N terminus-containing constructs (Fig. 6). These results imply that the N-terminal domain of NS4B, which is located between positions 1 and 110, may be essential for suppressing IFN expression responses in host