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Usefulness of a New Immunoradiometric Assay of HCV Core Antigen to Predict Virological Response during PEG-IFN/RBV Combination Therapy for Chronic Hepatitis with High Viral Load of Serum HCV RNA Genotype 1b

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Key Words

Chronic hepatitis · HCV core antigen · HCV RNA genotype 1b · Immunoradiometric assay · PEG-IFN/RBV combination therapy · Prediction, virological response

Abstract

We investigated the clinical usefulness of a new immunoradiometric (IRM) assay of hepatitis C virus (HCV) core antigen in predicting virological response during pegylated interferon plus ribavirin (PEG-IFN/RBV) combination therapy for chronic hepatitis with high viral loads of serum HCV RNA genotype 1b. Thirty-nine patients received a regimen of PEG-IFNα-2b (1.5 μg/kg/week s.c.) in combination with RBV (600–1,000 mg/day). Of the 39 patients, 18 (46.2%) achieved sustained virological response (SVR), 11 (28.2%) attained partial response (PR) and 10 (25.6%) showed no response (NR). Four weeks after the start of therapy, 1- and 2-log reductions in the amount of HCV core antigen were observed in 20 (2/10) and 0% (0/10) showing NR, 91 (10/11) and 63.6% (7/11) with PRs, and 88.9 (16/18) and 55.6% (10/18) of patients with SVR, respectively. The 1- and 2-log reductions 4 weeks after the start of therapy were not a defining condition for PR and SVR. The amount of HCV core antigen was significantly different between SVR and PR patients on days 1 and 7, and between patients with NR and SVR at all points of time. In conclusion, this new IRM assay is useful in predicting virological response during PEG-IFN/RBV therapy.

Introduction

Recently, global consensus has been reached that a combination of interferon (IFN) or pegylated IFN plus ribavirin (PEG-IFN/RBV) is the treatment of choice for chronic hepatitis C. Even with this treatment regimen, however, sustained virological response (SVR) for those infected with the most resistant genotypes, hepatitis C virus (HCV)-1a and -1b, still hover at ~50% [1, 2]. Thus,

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it is worthy to identify the predictive factors that allow the selection of patients who would achieve the eradication of HCV RNA either before or during therapy, especially since IFN/RBV combination therapy is costly and has several side effects [3].

Predictors of IFN-based therapy can be classified into pre- and on-treatment factors. Pre-treatment factors comprise (1) host factors, such as age, gender, obesity, alcohol consumption, hepatic iron overload, fibrosis, immune responses and co-infection with other viruses, and (2) viral factors that mainly include viral genotypes, particular amino acid sequence variations in the NS5A region [4, 5] and in the core protein region of HCV [6] within a given genotype and the viral load. On-treatment factors are mainly related to viral kinetics within the first few weeks of treatment [7].

Although the detection of HCV RNA by reverse transcription-polymerase chain reaction (RT-PCR) represents the most sensitive method for determining persistent HCV infection, the assay is time-consuming, costly and technically demanding. In contrast, enzyme immunoassays (EIAs) for detecting HCV core antigen are simple and relatively inexpensive. A number of reports have demonstrated the utility of measuring HCV core antigen using EIAs [8–11]. Moreover, a new immunoradiometric (IRM) assay for detecting HCV core antigen has recently been developed [12].

In this study, we assessed the usefulness of the new IRM assay for HCV core antigen in efficiently predicting SVR, based on virological dynamics at 24 h, and 1, 2 and 4 weeks after the start of PEG-IFN/RBV combination therapy, in patients with HCV-1b ≥100 KIU/ml.

Patients and Methods

Between December 2004 and July 2006, 39 patients included in this study demonstrated high viral loads (>100 KIU/ml) of serum HCV RNA of genotype 1b; they had been diagnosed with chronic hepatitis C on the basis of abnormal serum alanine aminotransferase persisting for at least 6 months and positive HCV RNA assessed by RT-PCR. None of the patients was positive for hepatitis B surface antigen or other liver diseases (autoimmune hepatitis or alcoholic liver disease). All the patients received a regimen of PEG-IFNα-2b (Peg-Intron; Schering-Plough, Kenilworth, N.J., USA; 1.5 μg/kg/week, s.c.) in combination with RBV (Rebetol; Schering-Plough; 600–1,000 mg/day) for 48 weeks. RBV was administered at a dose of 600 mg/day (three capsules) to patients weighing <60 kg, 800 mg/day (four capsules) to those weighing <80 kg, and 1,000 mg/day (five capsules) to those weighing ≥80 kg.

The efficacy of the combination therapy was evaluated by HCV RNA negativity based on qualitative RT-PCR analysis at the end of therapy (end of therapy response) and 6 months after the completion of therapy (SVR). The amount of HCV RNA was measured quantitatively by RT-PCR (Amplicor HCV monitor; version 2.0; Roche, Basel, Switzerland) before therapy. The lower detection limit of the assay was 5 KIU/ml. Samples collected during and after therapy were also checked by qualitative RT-PCR (Amplicor, Roche), which has a higher sensitivity than quantitative analysis, and the results were labeled as positive or negative. The lower limit of the assay was 50 KIU/ml.

SVR was defined as undetectable serum HCV RNA 24 weeks after cessation of treatment, partial response (PR) as undetectable HCV RNA at the end of treatment, but positive 24 weeks after discontinuation of treatment, and no response (NR) as detectable HCV RNA at the end of treatment. Informed consent was obtained from all patients enrolled in the study after a thorough explanation of the aims, risks and benefits of the therapy.

The amount of HCV core antigen was assessed by the IRM assay (Ortho Clinical Diagnostics, Tokyo, Japan). The HCV core antigen assay has a detection limit of 20 fmol/l, as established by the manufacturer. HCV core antigen was measured on days 0, 1, 7 (1 week), 14 (2 weeks) and 28 (4 weeks).

Statistical Analysis

Differences between the groups were assessed by non-parametric tests (Mann-Whitney test, χ^2 test and Fisher's exact test). $p < 0.05$ was considered statistically significant.

Results

Of the 39 patients treated with combination therapy, 18 (46.2%) achieved SVR and 21 were still HCV RNA positive 6 months after therapy. Of the latter, 11 (28.2%) relapsed after the end of therapy (PR) and 10 (25.6%) showed NR. Patient characteristics (table 1) showed no significant differences among the three groups (NR, PR and SVR) except for the degree of fibrosis.

A good correlation was observed between the amount of HCV core antigen and the amount of HCV RNA in 39 samples at the start of therapy ($r^2 = 0.648$; fig. 1).

The time course of HCV RNA eradication during therapy showed no significant difference between PR and SVR (fig. 2). In the NR, PR and SVR groups, the amounts of HCV core antigen during the initial 4 weeks of therapy (fig. 3) were as follows: 12,781 ± 18,444, 7,875 ± 3,418 and 5,809 ± 5,919 fmol/l, at the start of therapy; 3,382 ± 4,903, 681 ± 721 and 426 ± 698 fmol/l, on day 1; 6,177 ± 6,682, 1,540 ± 2,376 and 393 ± 469 fmol/l, on day 7; 7,048 ± 10,323, 525 ± 953 and 135 ± 166 fmol/l, on day 14, and 3,543 ± 5,363, 168 ± 395 and 29 ± 19 fmol/l, on day 28, respectively. On days 1 and 7, there was a significant difference between SVR and PR ($p < 0.05$). At all points of time, the difference in the amount of HCV core antigen was significant between NR and SVR ($p < 0.05$), but not between PR and NR.

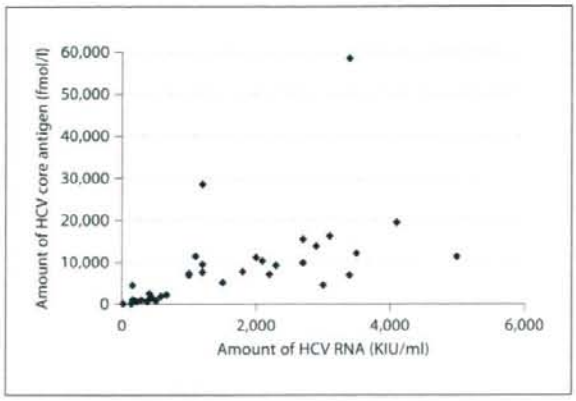

Fig. 1. Correlation between the amount of HCV core antigen and the amount of HCV RNA at the start of PEG-IFN/RBV combination therapy. There was a significant positive correlation (r^2 = 0.648, p = 0.0002. y = 4.48x + 1,470).

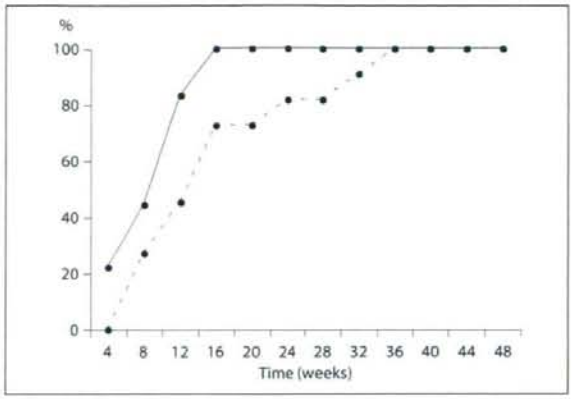

Fig. 2. Time course of HCV RNA eradication during PEG-IFN/RBV combination therapy. No significant difference was observed between SVR (——) and PR (– – –).

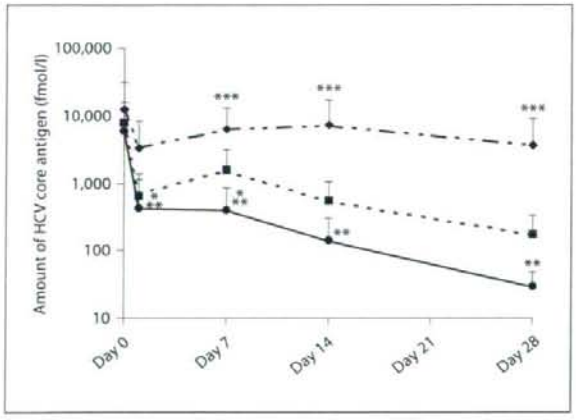

Fig. 3. Amount of HCV core antigen during PEG-IFN/RBV combination therapy. —— = SVR; – – – = PR; – – – – – = NR. * $p < 0.05$, SVR vs. PR; ** $p < 0.05$, SVR vs. NR, and *** $p < 0.05$, NR vs. PR.

Four weeks after the start of therapy, the following reductions in the amount of HCV core antigen were observed: a 1-log reduction in 20% (2/10) of the NR group, in 91% (10/11) of the PR group and in 88.9% (16/18) of the SVR group, and a 2-log reduction in 0% (0/10) of the NR group, in 63.6% (7/11) of the PR group and in 55.6% (10/18) of the SVR group (table 2).

Discussion

HCV core antigen, first detected in the circulation of HCV-infected hosts by EIA-based methods [13], had some limitations, in that levels under 20 fmol/l of HCV RNA could not be detected. Consequently, the methods were limited to the monitoring of late events during and after antiviral treatment. A modified version of the EIA developed for HCV core antigen [11] easily exposes the epitope of HCV core antigen, and the binding by anti-HCV core antibody in the serum can be reduced by incubation with three types of detergents. Since the modified EIA requires only one pretreatment step, it is simpler than the first-generation versions; moreover, it is 100-fold more sensitive. The second-generation EIA for HCV core antigen is useful in the diagnosis of acute and chronic hepatitis C and in predicting and monitoring the effect of IFN treatment [14].

Recently, a new IRM assay-based test for detecting HCV core antigen, a further modification of the EIA method of Aoyagi et al. [11], overcomes the effects of the serological HCV genotype group at the level of HCV core antigen detectable by EIA in serum. The sensitivity and specificity of the IRM assay are 96.4 and 100%, respectively. The sensitivity is similar between HCV serotype I (HCV genotypes 1a and 1b; 97.6%) and HCV serotype II (HCV genotypes 2a and 2b; 94.0%) [12].

Furthermore, the cost of the IRM assay kit is less than one third of the RT-PCR assay. Thus, this new IRM assay

Table 1. Host-dependent, virus-related profile

	NR	PR	SVR	p
Gender, males/females	6/4	7/4	9/9	NS
Age, years	59.8 ± 9.9	63.5 ± 7.3	55.5 ± 9.4	NS
HCV RNA level, KIU/ml	1,185 ± 1,154	2,093 ± 1,355	1,328 ± 1,321	NS
HCV core antigen, fmol/l	12,781 ± 18,444	7,875 ± 3,418	5,809 ± 5,919	NS
Body weight, kg	61.2 ± 11.2	60.8 ± 14.5	57.5 ± 9.6	NS
Treatment history (retreatment/naïve)	6/4	4/7	7/11	NS
Body mass index	23.7 ± 3.7	22.7 ± 3.7	22.4 ± 9.6	NS
F0–1/F2–3	3/6	1/6	14/3	0.003

is an economically valuable option for monitoring the amount of HCV in patients with chronic HCV infection. Indeed, in our study, there was a strong correlation between the amount of HCV core antigen in serum by the IRM assay and the amount of HCV RNA in serum measured by quantitation RT-PCR ($r^2 = 0.648$).

Although some studies have suggested that quantitative HCV RNA determinations allow earlier assessment of treatment response, the assays were not generally available commercially, and they were not standardized. Therefore, we used the IRM assay to predict virological response during PEG-IFN/RBV therapy in patients with HCV-1b ≥100 KIU/ml.

To be able to assess whether long-term response is attainable as early as possible during the treatment course, and to have the option of discontinuing treatment in cases where virological response is not expected, is desirable. This strategy has the potential of making a trial more appealing to patients by providing a limited 'test' period of treatment before committing to a full course of therapy [6].

The accuracy of the degree of viral inhibition, during the early weeks of treatment (early virological response: EVR) with PEG-IFNα-2b/RBV, has been examined to identify patients who would not respond to therapy. The best definition of EVR is a reduction in HCV RNA by at least 2 log after the first 12 weeks of treatment compared with baseline. Depending on the treatment regimen, between 69 and 76% of patients have achieved this threshold, with SVR attained in 67–80% [15].

The importance of EVR has been emphasized in predicting SVR and non-SVR: patients who do not reach EVR are not responsive to further therapy. Discontinuation of treatment in patients not reaching EVR would reduce drug costs by >20%; consequently, early confirmation of viral reduction after initiating antiviral therapy for chronic hepatitis C is worthwhile [16].

Table 2. Reduction in the amount of HCV RNA 4 weeks after the start of PEG-IFN/RBV therapy

Reduction	NR	PR	SVR
1 log	20% (2/10)	91% (10/11)	88.9% (16/18)
2 log	0% (0/10)	63.6% (7/11)	55.6% (10/18)

Treatment with IFN results in a decline in HCV RNA levels, which can be resolved mathematically into two phases. The first-phase decline is usually measured at 24 or 48 h, and probably reflects direct inhibition of intracellular HCV production and release [17], with IFN efficacy ranging from about 70% (approximately 0.7 log units) for standard IFN given three times a week to more than 90% (1 log units) for high daily doses of standard IFN or PEG-IFN once a week [18, 19]. The second-phase decline begins after 24–48 h, is slower and more variable than the first phase, and is thought to reflect continued inhibition of replication and the gradual elimination of virus-infected cells [17]. The decay correlates less with the IFN dose than the first phase, but is more rapid with PEG-IFN as compared with standard IFN preparations [16].

Lowering HCV RNA during the first phase is essential for efficient elimination of HCV during the second phase. Decreases in HCV RNA titers within the first 24–48 h after the start of IFN, therefore, would be dependable estimates of antiviral efficacy [18, 19].

As observed with the first phase, RBV does not appear to influence second-phase kinetics [16]. Four weeks after

the start of therapy, a 1-log reduction was observed in 20% (2/10) of NR patients, 91% (10/11) of PR and 88.9% (16/18) of SVR, and a 2-log reduction in 0% (0/10) of NR, 63.6% (7/11) of PR and 55.6% (10/18) of SVR patients. These results indicate that the reduction of 1 and 2 log in the amount of HCV core antigen 4 weeks after therapy is not a defining condition for PR and SVR. There are two kinds of non-SVR (PR and NR): PR is defined as HCV RNA undetectable at the end of treatment and positive 24 weeks after the discontinuation of therapy; NR is defined as HCV RNA detectable at the end of treatment.

At all points of time, a significant difference was observed in the amount of HCV core antigen between SVR and NR. Accordingly, SVR can easily be differentiated from NR by IRM assay during therapy. With regard to the time course of HCV RNA eradication, however, there is no difference between PR and SVR. The prediction of SVR and PR by the earliest possible use of the IRM assay is desirable.

In our study, 1 and 7 days after the start of therapy, there was a significant difference in the amount of HCV core antigen between SVR and PR. Our finding that SVR and PR could be differentiated by the IRM assay during the first and second phases is very useful for clinicians engaged in the treatment of C-type hepatitis, because PR patients should be treated for 72 weeks in order to maximize the probability of SVR [20, 21].

Taken together, our results demonstrate that early viral dynamics, such as changes in the amount of HCV core antigen detected by the IRM assay in the first and second phases during PEG-IFNα-2b/RBV therapy, predict outcome not only between SVR and NR but also between SVR and PR. Since the number of patients was small in our study, further studies including larger patient cohorts are needed to confirm the promising potential of the IRM assay.

In conclusion, this new IRM assay is useful in predicting virological response during PEG-IFN/RBV combination therapy administered for chronic hepatitis C with high viral loads of HCV RNA genotype 1b.

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Disclosure Statements

The authors have no disclosures to make.

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Hepatitis C virus NS5A protein interacts with and negatively regulates the non-receptor protein tyrosine kinase Syk

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Hepatitis C virus (HCV) is the major causative agent of hepatocellular carcinoma. However, the precise mechanism underlying the carcinogenesis is yet to be elucidated. It has recently been reported that Syk, a non-receptor protein tyrosine kinase, functions as a potent tumour suppressor in human breast carcinoma. This study first examined the possible effect of HCV infection on expression of Syk *in vivo*. Immunohistochemical analysis revealed that endogenous Syk, which otherwise was expressed diffusely in the cytoplasm of normal hepatocytes, was localized near the cell membrane with a patchy pattern in HCV-infected hepatocytes. The possible interaction between HCV proteins and Syk in human hepatoma-derived Huh-7 cells was then examined. Immunoprecipitation analysis revealed that NS5A interacted strongly with Syk. Deletion-mutation analysis revealed that an N-terminal portion of NS5A (aa 1–175) was involved in the physical interaction with Syk. An *in vitro* kinase assay demonstrated that NS5A inhibited the enzymic activity of Syk and that, in addition to the N-terminal 175 residues, a central portion of NS5A (aa 237–302) was required for inhibition of Syk. Moreover, Syk-mediated phosphorylation of phospholipase C-γ1 was downregulated by NS5A. An interaction of NS5A with Syk was also detected in Huh-7.5 cells harbouring an HCV RNA replicon or infected with HCV. In conclusion, these results demonstrated that NS5A interacts with Syk resulting in negative regulation of its kinase activity. The results indicate that NS5A may be involved in the carcinogenesis of hepatocytes through the suppression of Syk kinase activities.

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INTRODUCTION

Hepatitis C virus (HCV) is the major aetiological agent of viral hepatitis worldwide after hepatitis A and B viruses (Choo *et al.*, 1989), with about 170 million people being infected. The majority of HCV-infected individuals develop chronic infection, which may progress to liver cirrhosis and hepatocellular carcinoma (HCC). HCV is a member of the family *Flaviviridae* and its genome consists of a single-stranded, positive-sense RNA of approximately 9600 nt, which encodes a polyprotein precursor of about 3010 aa. Currently, clinical HCV isolates are classified into six genotypes and more than 60 subtypes (Doi *et al.*, 1996; Mellor *et al.*, 1995; Robertson *et al.*, 1998). The polyprotein is cleaved by signal peptidase, signal peptide peptidase and two virally encoded proteases to generate at least ten mature proteins: core, envelope glycoprotein 1 (E1), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B (Okamoto *et al.*, 2004; Reed & Rice, 2000).

HCV NS5A is part of the replication complex that catalyses replication of the viral genome. NS5A takes two forms, p56 and p58, with different degrees of phosphorylation, which may play distinct roles in the virus replication cycle (Evans

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et al., 2004; Song *et al.*, 1999). The SNARE-like membrane fusion proteins VAP-A and VAP-B have been reported to interact with NS5A, and the binding capacity is inversely correlated to the degree of NS5A phosphorylation (Evans *et al.*, 2004; Gao *et al.*, 2004; Hamamoto *et al.*, 2005). NS5A binds to and inhibits double-stranded RNA-dependent protein kinase (PKR) (Gale *et al.*, 1998) and 2′,5′-oligoadenylate synthetase (Taguchi *et al.*, 2004). NS5A seems to have the potential to regulate not only interferon responses but also many other cellular functions, such as mitogenic signalling, apoptosis, the cell cycle and reactive oxygen species signalling, by interacting with a variety of host proteins (Macdonald *et al.*, 2004). These NS5A-interacting proteins include SRCAP (Ghosh *et al.*, 2000), Grb2 (He *et al.*, 2002; Tan *et al.*,1999), p53 (Majumder *et al.*, 2001; Qadri *et al.*, 2002), phosphatidylinositol 3-kinase p85 subunit (He *et al.*, 2002; Street *et al.*, 2004), karyopherin β3 (Chung *et al.*, 2000), apolipoprotein A1 (Shi *et al.*, 2002), amphiphysin II (Zech *et al.*, 2003) and Src family protein tyrosine kinases (Macdonald & Harris, 2004; Macdonald *et al.*, 2004).

The non-receptor protein tyrosine kinase Syk is widely expressed in cells of the haematopoietic lineage, endothelium, epithelium and hepatocytes (Coopman *et al.*, 2000; Sada *et al.*, 2001; Tsuchida *et al.*, 2000; Turner *et al.*, 2000; Yanagi *et al.*, 1995, 2001). Syk contains tandem SH2 and kinase domains that are connected by an inter-SH2 domain and a linker region (Taniguchi *et al.*, 1991). The tandem SH2 domains of Syk bind to diphosphorylated immunoreceptor tyrosine-based activation motifs [ITAMs: $YXX(L/I)X_{6-8}YXX(L/I)$] in the cytoplasmic tail of the Fc receptor γ-chain or B-cell receptor subunit Igα to be activated after the engagement of immune receptors (Kurosaki *et al.*, 1995; Sada *et al.*, 2001; Shiue *et al.*, 1995; Turner *et al.*, 1995; Weiss & Littman, 1994). Autophosphorylation of Syk on Tyr^{525} and Tyr^{526} in the activation loop of the kinase domain results in an increase in its intrinsic kinase activity to phosphorylate its downstream signalling molecules, such as phospholipase C (PLC)-γ (Kurosaki *et al.*, 1995). Autophosphorylation on Tyr^{352} in the linker region is required for tyrosine phosphorylation of PLC-γ1 (Law *et al.*, 1996). Genetic studies have demonstrated that Syk is required for the development and maturation of B cells, mast-cell activation and platelet aggregation (Cheng *et al.*, 1995; Costello *et al.*, 1996; Poole *et al.*, 1997; Turner *et al.*, 1995, 2000). Furthermore, it has been reported that Syk functions as a tumour suppressor in breast cancers and that loss of Syk expression appears to be associated with malignant phenotypes (Coopman *et al.*, 2000).

In the present study, we demonstrated that HCV NS5A interacts physically with Syk to inhibit its kinase activity in human hepatoma-derived Huh-7 cells. Our results indicate that NS5A-induced downregulation of the possible tumour suppressor Syk may play a role in malignant transformation of HCV-infected hepatocytes.

METHODS

Expression plasmids. Mammalian expression plasmids for each of the Myc-tagged HCV proteins were constructed by amplifying and subcloning the corresponding cDNA fragments of pFK5B/2884Gly (Lohmann *et al.*, 2001) in frame to the pEF1/Myc-His(−) vector (Invitrogen). pFK5B/2884Gly was a kind gift from Dr R. Bartenschlager (University of Heidelberg, Germany). An expression plasmid for a polyprotein consisting of NS3–NS5B was amplified from pFK5B/2884Gly and subcloned into pEF1/Myc-His(−). Deletion mutants of NS5A were also amplified by PCR and subcloned into pEF1/Myc-His(−). Point mutations in NS5A [Tyr^{118} to Phe (Y118F), Val^{121} to Ala (V121A)] were introduced into pEF1/NS5A-Myc-His(−) by site-directed mutagenesis. Human Syk cDNA was a gift from Dr B. Müller-Hilke (University of Rostock, Germany). cDNA fragments for FLAG-tagged truncated forms and the kinase-inactive form of Syk were generated by PCR. All mutant forms of FLAG-tagged Syk were subcloned into pcDNA3.1/Hygro(+) (Invitrogen).

Cells, HCV RNA replicon and virus. Huh-7 human hepatoma-derived cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (FCS). Huh-7.5 cells (Blight *et al.*, 2002) were kindly provided by Dr C. M. Rice (The Rockefeller University, USA). BJAB cells, a human B-cell line expressing endogenous Syk, were cultured in RPMI 1640 supplemented with 10% FCS.

Huh-7.5 cells stably harbouring an HCV subgenomic RNA replicon were prepared by using pFK5B/2884Gly, as described previously (Hidajat *et al.*, 2005; Lohmann *et al.*, 2001; Taguchi *et al.*, 2004; Takigawa *et al.*, 2004).

The plasmid pFL-J6/JFH1 encoding the entire genome of the HCV J6/JFH-1 strain was kindly provided by Dr C. M. Rice, and cell-free virus was propagated in Huh-7.5 cell cultures, as described previously (Lindenbach *et al.*, 2005).

Protein expression. Protein expression was performed using a recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3), as described previously (Deng *et al.*, 2006; Muramatsu *et al.*, 1997). In some experiments, protein expression was performed using a plasmid-based expression system without vTF7-3. For BJAB cells, we used an electroporation method (Schneider & Kieser, 2004). In brief, 3×10^6 cells were washed once with PBS and incubated for 10 min with 15 μg plasmid DNA in 250 μl RPMI 1640. Electroporation was carried out in a 4 mm cuvette using a Bio-Rad Gene Pulser II with a capacity of 975 μF and a voltage of 180 V. Immediately after electroporation, 500 μl FCS was added to the cells, which were then transferred to 4.5 ml RPMI 1640.

To activate Syk under hyperosmolarity conditions, cells were incubated with serum-free medium containing 400 mM sorbitol for 30 min at 37 °C, as described previously (Miah *et al.*, 2004). In addition, cells were treated with sodium pervanadate (generated by mixing 0.1 mM Na_3VO_4 with 1 mM H_2O_2) for 30 min to activate Syk (Wienands *et al.*, 1996).

Immunohistochemistry. Human normal adult liver autopsy materials and surgically resected liver tissue of patients with HCV-associated HCC were obtained with written informed consent. The tissues were fixed with 10% buffered formalin, embedded in paraffin and sectioned. Immunohistochemical staining was performed with a Dako EnVision+ kit, according to the manufacturer's instructions. In brief, fixed sections were depleted of paraffin by treatment with xylene, dehydrated in ethanol and incubated with 3% H_2O_2 to quench endogenous peroxidase activity. After being autoclaved at 121 °C for 20 min, the sections were incubated with a blocking

solution and then with anti-Syk rabbit polyclonal antibody (N-19; Santa Cruz Biotech). Normal rabbit IgG served as a control. The sections were then incubated with horseradish peroxidase-labelled polymer-conjugated secondary antibody. The sections were counterstained with haematoxylin and examined under a light microscope. To confirm the specificity of immunostaining, anti-Syk antibody was pre-incubated with a 1000-fold excess of blocking peptide (Santa Cruz Biotech) for 2 h at room temperature prior to staining.

Detection of HCV RNA by *in situ* RT-PCR. *In situ* RT-PCR was performed as described previously (Maeda *et al.*, 2004) with some modifications. Briefly, OCT-embedded frozen liver biopsy sections were fixed with 10% formaldehyde and treated with proteinase K. The samples were subjected to *in situ* reverse transcription using Moloney murine leukemia virus reverse transcriptase with an antisense primer for HCV (nt 290–272; 5′-AGTACCACAA GGCCTTTCG-3′), followed by *in situ* PCR using an *in situ* PCR System 1000 (Applied Biosystems) in the reaction mixture containing the antisense and a sense primer (nt 129–147; 5′-CCGGGAGAG CCATAGTGGT-3′). After being fixed in 4% paraformaldehyde, the PCR products were detected by *in situ* hybridization using a digoxigenin (DIG)-labelled oligonucleotide probe, 5′-(DIG)-ATTTGGGCTGTGCCCCCGCGAGACTGCTAGCCGAGTAGTGTT-GGGT-(DIG)$_n$-3′ (nt 225–270). Anti-DIG antibody conjugated with alkaline phosphatase (Roche) was used to detect the probe. The slides were incubated in a dye solution containing nitro blue tetrazolium, 5-bromo-4-chloro-3-indolylphosphate and levamizole to yield a purplish-blue precipitate.

Immunoprecipitation and Western blotting. Cultured cells were lysed with a buffer containing 1% Triton X-100, 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 10 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4 and 1 mM PMSF. The lysate was centrifuged at 12 000 *g* for 20 min at 4 °C and the supernatant was immunoprecipitated with appropriate antibodies. In the case of liver tissue, each tissue sample was placed in a tube containing glass beads (1 mm diameter; BioSpec Products) to which 1 ml lysis buffer was added. The tube was then shaken at 4 °C for 3 min using a Mini-BeadBeater (BioSpec Products) to homogenize the tissues. After centrifugation at 80 *g* for 3 min, the supernatant was collected for immunoprecipitation analysis.

Immunoprecipitation and Western blot analyses were performed as described previously (Deng *et al.*, 2006). In brief, the supernatants of the lysates were pre-cleared with control IgG and protein A–Sepharose 4 Fast Flow (GE Healthcare) and incubated with appropriate antibodies at 4 °C for 1 h, followed by incubation with protein A–Sepharose 4 Fast Flow for another 1 h. After six washes with lysis buffer, the immunoprecipitates were analysed by Western blotting.

Antibodies used were as follows: anti-FLAG rabbit polyclonal antibody (Sigma); anti-Myc polyclonal and monoclonal antibodies (Santa Cruz Biotech); anti-Syk monoclonal antibody (4D10; Santa Cruz Biotech); anti-phospho Syk(Tyr^{352}) and Syk(Tyr$^{525/526}$) rabbit polyclonal antibodies (Cell Signaling Technology); anti-PLC-γ1 monoclonal antibody (BD Biosciences); mouse monoclonal antibodies against core (Yasui *et al.*, 1998), NS3, NS4A and NS5A (kind gifts from Dr I. Fuke, Osaka University, Japan); anti-NS5A rabbit polyclonal antibody (NS5ACL1; a kind gift from Dr K. Shimotohno, Kyoto University, Japan; Miyanari *et al.*, 2007); and anti-NS5B goat polyclonal antibody (sc-17532; Santa Cruz Biotech). Normal IgG served as a control.

***In vitro* protein kinase assay.** An *in vitro* protein kinase assay was performed as reported previously (Miah *et al.*, 2004; Sada *et al.*, 2000, 2001). In brief, immunoprecipitates obtained with anti-Syk antibody from differentially transfected cells were incubated with 10 μg H2B histone (Sigma) as substrate in 20 μl kinase buffer, composed of 30 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 2 mM $MnCl_2$, 4 μM ATP and 4 μCi (148 kBq) [γ-^{32}P]ATP, for 30 min at room temperature. Reactions were terminated by boiling for 5 min in 2× sample buffer.

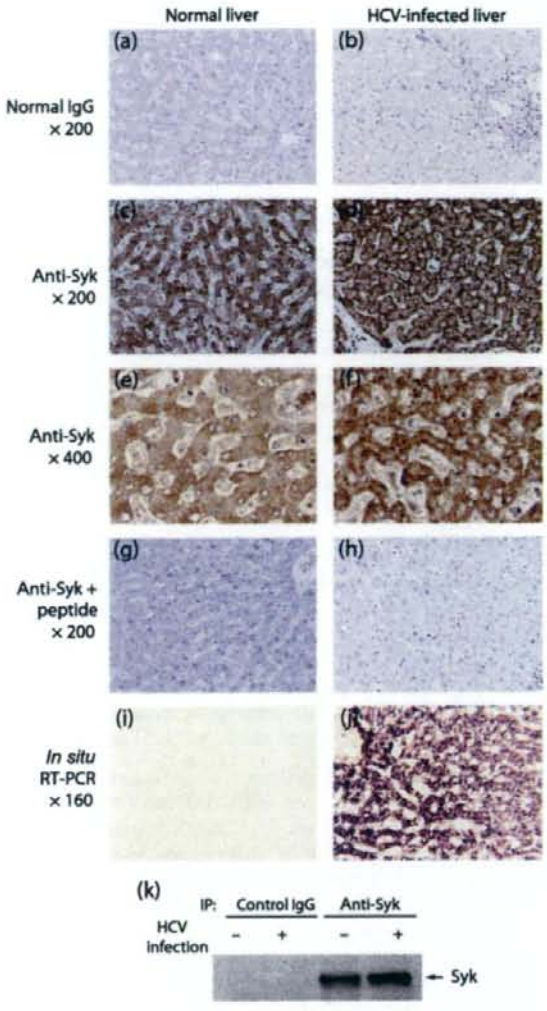

Fig. 1. Endogenous Syk expression in human liver tissues *in vivo*. Normal liver tissues (a, c, e, g, i) and HCV-infected non-cancerous liver tissues (b, d, f, h, j) were analysed. Formalin-fixed samples were stained with control IgG (a, b) or anti-Syk polyclonal antibody without (c–f) or with (g, h) pre-incubation with an excess amount of the immunogenic peptides. Frozen tissues were sectioned and examined for the presence of HCV RNA by *in situ* RT-PCR (i, j). Representative results are shown from four normal livers and ten HCV-infected livers. (k) Western blot analysis of normal human liver and HCV-infected non-cancerous liver. Supernatants of liver tissue homogenates (1.75 mg protein equivalent) were immunoprecipitated with anti-Syk monoclonal antibody (4D10) and probed with the same antibody or with control IgG.

Proteins were separated by SDS-PAGE. The gels were treated with 1 M KOH for 1 h at 56 °C to remove phosphoserine and most of the phosphothreonine. After gel drying, radiolabelled proteins were visualized by autoradiography. For quantitative analysis, γ-^{32}P incorporation was measured using a PhosphorImager (BAS2000; Fuji) and protein amounts with an LAS1000 image analyser (Fuji).

RESULTS

Different expression patterns of endogenous Syk in normal and HCV-infected liver tissues

We first examined whether Syk was expressed in human liver tissues. Immunohistochemical analysis revealed that Syk was indeed expressed and rather diffusely distributed throughout the cytoplasm of normal adult hepatocytes (Fig. 1c, e). This pattern was observed with four out of four normal liver tissues (100 %; data not shown). The specificity of the staining was verified by pre-incubating the antibody with an excess amount of the immunogenic peptides (Fig. 1g, h). We then examined Syk expression in non-cancerous liver tissue obtained from patients with HCV-associated HCC. Interestingly, Syk was detected near the plasma membrane with a patchy pattern in hepatocytes of eight out of ten HCV-infected patients (80 %; Fig. 1d, f, and data not shown). All of the specimens stained with normal rabbit IgG were negative (Fig. 1a, b). We confirmed that almost all of the hepatocytes in the tissue samples were infected with HCV using *in situ* RT-PCR (Fig. 1i, j).

Western blot analysis confirmed Syk expression in human liver tissue, irrespective of HCV infection (Fig. 1k). It should be noted, however, that the Syk expression was rather weak, as we could achieve successful Western blotting only after the tissue lysates were concentrated by immunoprecipitation with specific antibody. Also, possibly due to the low level of expression and comparatively low sensitivity of the antibodies used for Western blotting, we could not detect the phosphorylated forms of Syk in the liver tissue (data not shown).

Identification of Syk as a novel NS5A-interacting protein

We then examined the possible interaction between HCV proteins and Syk in cultured cells. For this purpose, various HCV proteins and Syk were expressed ectopically in Huh-7 cells, as these cells do not express endogenous Syk. Co-immunoprecipitation analysis revealed that NS5A associated with Syk, whereas the other HCV proteins associated with Syk very weakly or not at all (Fig. 2a, b). A specific interaction of NS5A with Syk was also observed when NS5A was expressed as part of an NS3–NS5B polyprotein (Fig. 2c). These results collectively suggested that NS5A interacts specifically with Syk.

Next, we examined the possible interaction of NS5A with endogenously expressed Syk. As human hepatoma-derived cell lines, such as Huh-7, HepG2 and FLC4, are negative for

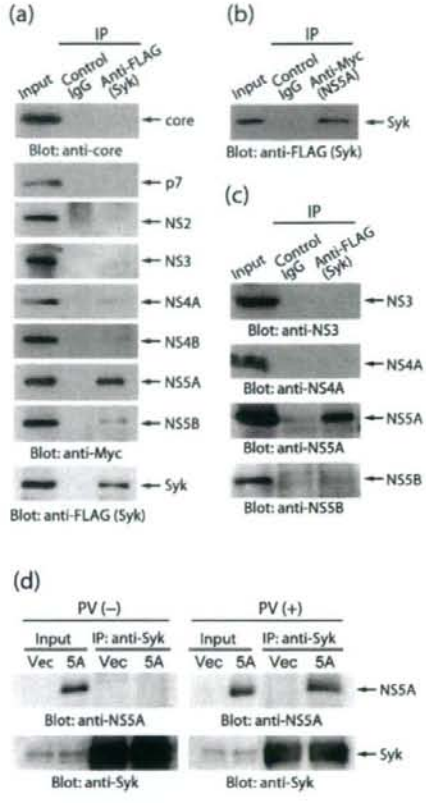

Fig. 2. NS5A specifically interacts with Syk in Huh-7 cells. (a) Each of the Myc-tagged HCV proteins was expressed with FLAG-tagged full-length Syk. Cell lysates were immunoprecipitated using anti-FLAG antibody or control IgG. Cell lysates (input) and the immunoprecipitates were probed with anti-core or anti-Myc antibodies. A representative result verifying efficient immunoprecipitation is shown at the bottom. (b) Myc-tagged NS5A was expressed with FLAG-tagged full-length Syk. Cell lysates were immunoprecipitated using anti-Myc antibody or control IgG, and probed with anti-FLAG antibody. (c) A polyprotein consisting of NS3–NS5B was expressed with FLAG-tagged Syk. Cell lysates were immunoprecipitated with anti-FLAG antibody or control IgG, and probed with the indicated antibodies. (d) NS5A was expressed in BJAB cells expressing endogenous Syk. The cells were treated with pervanadate (PV) or left untreated. Cell lysates were immunoprecipitated with anti-Syk monoclonal antibody and probed with anti-NS5A or anti-Syk monoclonal antibody. Vec, control using empty vector.

endogenous Syk expression, we used BJAB cells endogenously expressing Syk. Unlike ectopically expressed Syk, endogenous Syk in BJAB cells is not tyrosine phosphorylated. Therefore, we treated the cells with pervanadate to induce tyrosine phosphorylation of Syk. Co-immunoprecipitation experiments clearly demonstrated that NS5A

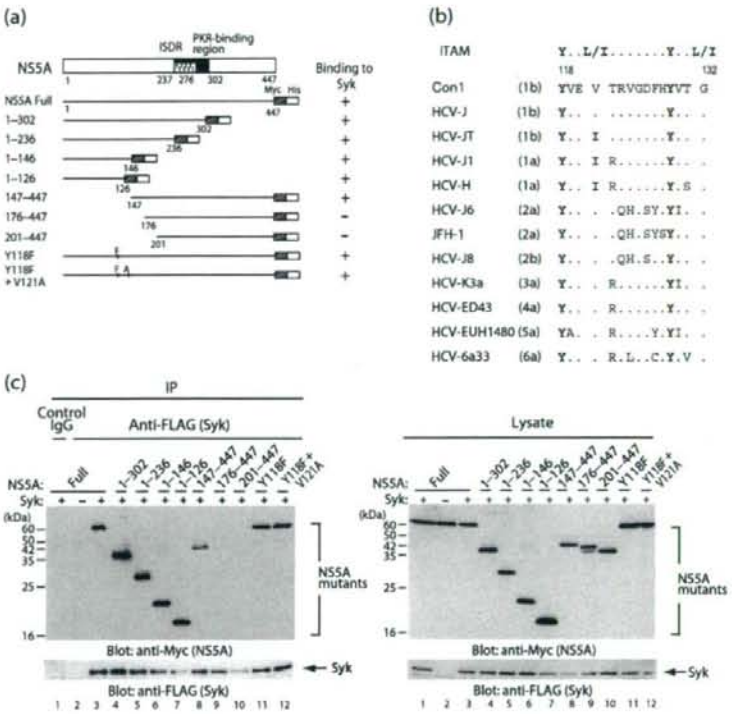

Fig. 3. Determination of the Syk-binding region(s) of NS5A. (a) Schematic diagram of various deletion mutants of NS5A and their Syk-binding capacity. (b) Alignment of amino acid sequences surrounding the ITAM-related sequence in NS5A of various HCV strains. The genotype is indicated in parentheses. Residues identical to those of HCV strain Con1 are shown by a dot. Residues identical to ITAM are shown in bold. (c) Full-length (Full) and a series of deletion mutants of Myc-tagged NS5A were expressed in Huh-7 cells with or without FLAG-tagged full-length Syk. Cell lysates were immunoprecipitated using anti-FLAG antibody and probed with anti-Myc antibody (left panel). Efficient immunoprecipitation was verified (bottom). Cell lysates were probed directly with anti-Myc and anti-FLAG antibodies to verify comparable expression levels of the NS5A mutants and Syk, respectively (right panels).

interacted with endogenous Syk when the cells were treated with pervanadate, but not when the cells were left untreated (Fig. 2d).

The N-terminal region of NS5A is required for interaction with Syk

To map a Syk-interacting region(s) of NS5A, interaction between various deletion mutants of NS5A and Syk was tested. C-terminally deleted mutants of NS5A up to aa 126, as well as the full-length NS5A, were co-immunoprecipitated with Syk (Fig. 3a, c). This result suggested that neither the PKR-binding region nor the interferon sensitivity-determining region (ISDR) of NS5A was required for the interaction with Syk. A proline-rich region of NS5A (aa 343–356), which is reported to bind to the Src family kinases (Macdonald & Harris, 2004; Macdonald *et al.*, 2004), was not involved in the Syk interaction either. In contrast, the N-terminally truncated mutant of NS5A(147–447), but not the further truncated mutants NS5A(176–447) or NS5A(201–447), was co-immunoprecipitated with Syk, suggesting that a region of NS5A between aa 147 and 175 is also involved in the interaction with Syk. We also observed that NS5A(1–126) and NS5A(174–447), but not NS5A(201–447), interacted with Syk(1–261) or Syk(379–635) (data not shown). These results collectively suggested that NS5A interacts with Syk through two independent regions of NS5A (aa 1–126 and 147–175).

Syk is activated by interaction with a diphosphorylated ITAM of immune receptors (Sada *et al.*, 2001; Turner *et al.*, 2000; Weiss & Littman, 1994). NS5A from HCV strain Con1 possesses a sequence (AEE**Y**^{118}**V**E**V**^{121}-TRVGDFH**Y**^{129}VTG) that resembles an ITAM (Fig. 3b). We found that the two tyrosine residues at positions 118 and 129 are highly conserved across different genotypes and subtypes. The tyrosine at position 118 is exposed on

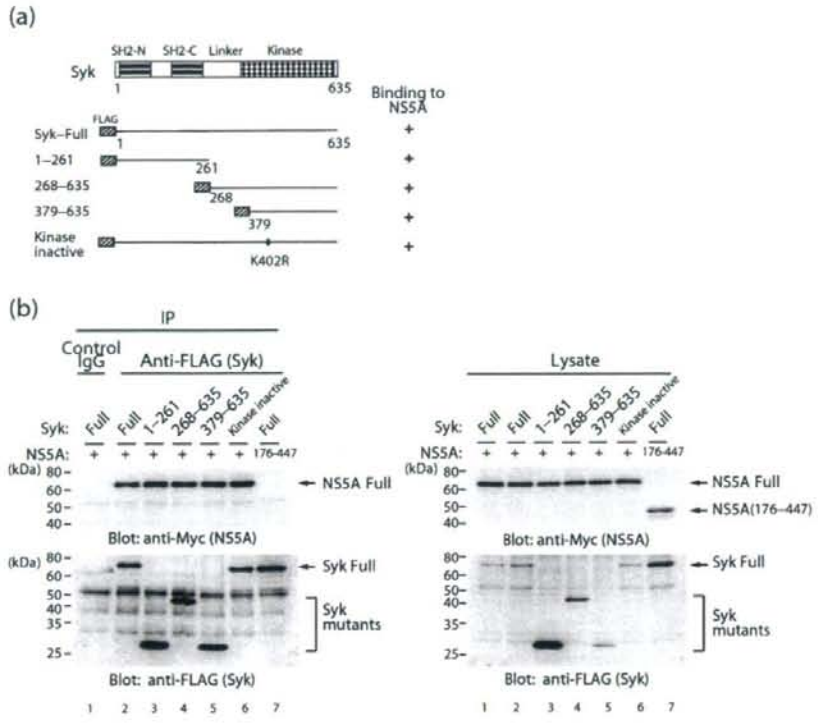

Fig. 4. NS5A interacts with both N-terminal and C-terminal regions of Syk. (a) Schematic diagram of the deletion mutants of Syk and their NS5A-binding capacity. (b) Full-length (Full) and a series of domain-deletion mutants of FLAG-tagged Syk was expressed in Huh-7 cells with Myc-tagged full-length NS5A (lanes 1–6) or NS5A(176–447) (lane 7). Cell lysates were immunoprecipitated using anti-FLAG antibody and probed with anti-Myc antibody (left upper panel). Efficient immunoprecipitation of Syk deletion mutants was verified (bottom). Cell lysates were probed directly with anti-Myc and anti-FLAG antibodies to verify comparable expression levels of the NS5A and Syk mutants, respectively (right panels).

the surface of the NS5A molecule (Tellinghuisen *et al.*, 2005). We examined whether this sequence motif was involved in the interaction with Syk. A single point mutation of Tyr^{118} (Y118F) or double mutations of Tyr^{118} and Val^{121} (Y118F and V121A) in NS5A did not affect the interaction with Syk (Fig. 3c, lanes 11 and 12). Thus, it is unlikely that NS5A binds to Syk through its ITAM-related sequence in the same manner as that observed for immune receptors.

To map the NS5A-binding region in Syk, a series of domain-deleted mutants of Syk was examined. The results obtained revealed that both N-terminal (tandem SH2 domains) and C-terminal halves (linker and the kinase domain) interacted with NS5A (Fig. 4). The kinase domain alone and a kinase-inactive form of Syk were also co-immunoprecipitated with NS5A. These results suggested that the NS5A–Syk interaction occurs through the N- and C-terminal regions of Syk and that the catalytic activity of Syk is not necessary for the interaction.

NS5A expression downregulates the kinase activity of Syk

Next, we tested the possible effect of NS5A expression on Syk kinase activity. An *in vitro* kinase assay revealed that full-length NS5A and a C-terminally deleted NS5A(1–302) mutant significantly inhibited Syk kinase activity (Fig. 5, lanes 2–4). In contrast, NS5A(1–236), which lacked both the PKR-binding region (aa 237–302) and ISDR (aa 237–276), failed to inhibit Syk kinase activity, although it could interact with Syk. NS5A(176–447), which contained the PKR-binding region and ISDR but lacked the Syk-binding region, did not affect Syk kinase activity. These results collectively suggested that NS5A requires both N-terminal (aa 1–175) and central (aa 237–302) regions for the downregulation of Syk kinase activity (Table 1).

To address the relevance of the interaction between NS5A and Syk, the possible effect(s) of NS5A on Syk-mediated cellular signalling in Huh-7 cells was examined. Ectopic

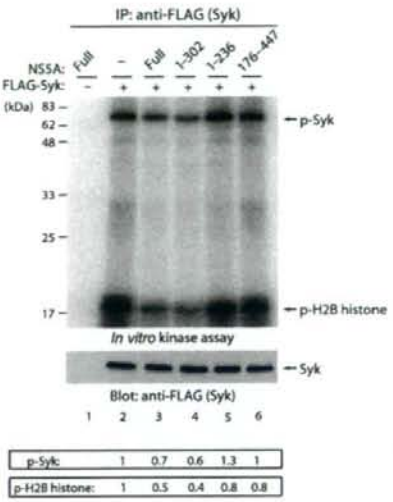

Fig. 5. NS5A downregulates Syk kinase activity. Myc-tagged NS5A and FLAG-tagged Syk were expressed in Huh-7 cells. Cell lysates were immunoprecipitated with anti-FLAG antibody and the immunoprecipitates were subjected to an *in vitro* kinase assay using H2B histone as substrate. Phosphorylation of Syk (p-Syk) and H2B histone (p-H2B histone) was visualized by autoradiography (upper panel). Efficient immunoprecipitation of Syk was verified (lower panel). Arbitrary units of Syk kinase activities, represented by the phosphorylation values of p-Syk and p-H2B histone normalized to the amounts of immunoprecipitated Syk, are shown at the bottom.

expression of Syk alone mediated signal transduction to induce tyrosine phosphorylation of a wide variety of cellular proteins, either directly or indirectly (Fig. 6a, lanes 1 and 3). Hyperosmolarity stress (400 mM sorbitol treatment) enhanced Syk-mediated tyrosine phosphorylation of cellular proteins (Fig. 6a, lanes 3 and 4), with the result being consistent with the previous observation (Miah *et al.*, 2004). Interestingly, co-expression of NS5A decreased Syk-mediated tyrosine phosphorylation of cellular proteins both in the absence and presence of hyperosmolarity stress (Fig. 6a, lanes 7 and 8). The phosphorylation of Syk on Tyr^{352} and/or $Tyr^{525/526}$ is a marker for Syk activation. Using these parameters, we confirmed that co-expression of NS5A inhibited Syk activation both in the absence and presence of hyperosmolarity stress (Fig. 6b).

PLC-γ1 has been reported to be a downstream molecule of Syk-mediated signal transduction (Law *et al.*, 1996). Our results demonstrated that NS5A inhibited PLC-γ1 phosphorylation, probably through downregulation of Syk kinase activity, both in the absence and presence of hyperosmolarity stress (Fig. 6c).

Table 1. Summary of NS5A deletion mutational analysis of the interaction with Syk and inhibition of Syk kinase activity

NS5A mutant	Interaction with Syk	Inhibition of Syk
NS5A(1–447; full)	+	+
NS5A(1–302)	+	+
NS5A(1–236)	+	–
NS5A(176–447)	–	–

NS5A expressed in the context of HCV RNA replication interacts with Syk in Huh-7.5 cells

The interaction of NS5A with Syk was examined further using Huh-7.5 cells harbouring an HCV subgenomic RNA replicon. The results obtained clearly demonstrated that NS5A expressed in the context of HCV RNA replication interacted with Syk (Fig. 7a). It is well known that NS5A takes two forms, p56 and p58, with the former being the basally phosphorylated form and the latter the hyperphosphorylated form (Kaneko *et al.*, 1994; Song *et al.*, 1999). It is noteworthy that Syk interacted with p56 more efficiently than with p58.

We also examined the interaction of NS5A with Syk in Huh-7.5 cells infected with the J6/JFH-1 strain of HCV. The results demonstrated that NS5A interacted with Syk in HCV-infected cells (Fig. 7b). These results collectively suggested that the NS5A–Syk interaction occurs in the context of virus replication, where NS5A is primarily utilized to form the viral replication complex. In this connection, HCV J6/JFH-1 replication was not affected significantly by ectopically expressed Syk in Huh-7.5 cells (data not shown). This observation, however, does not necessarily exclude the possibility that the NS5A interaction with Syk exerts certain biological effect(s) on the host cell's fate.

Syk kinase activity is suppressed in the context of HCV RNA replication

We then examined Syk kinase activity in the HCV subgenomic RNA-harbouring Huh-7.5 cells. An *in vitro* kinase assay demonstrated that Syk kinase activities, represented by autophosphorylation of Syk (p-Syk) and phosphorylation of a substrate (p-H2B histone), were significantly suppressed in HCV RNA-replicating cells compared with the control (Fig. 7c). These results suggested the possibility that Syk kinase activity is downregulated through an NS5A–Syk interaction in HCV-infected hepatocytes as well.

DISCUSSION

The non-receptor protein tyrosine kinase Syk is expressed in a wide variety of haematopoietic cell lineages (Taniguchi *et al.*, 1991). It is also expressed in human mammary

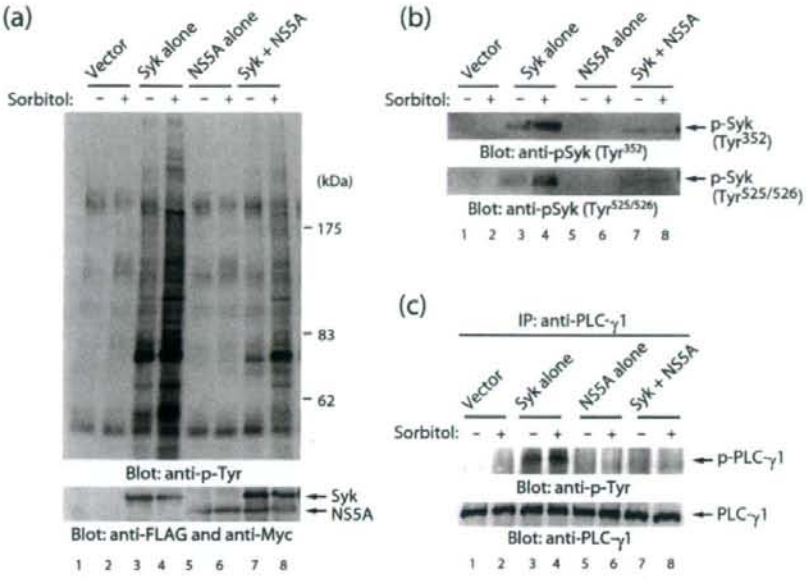

Fig. 6. NS5A suppresses hyperosmolarity stress-induced Syk-mediated tyrosine phosphorylation of cellular proteins. (a) Myc-tagged NS5A was expressed in Huh-7 cells with or without FLAG-tagged Syk. The cells were incubated with or without 400 mM sorbitol for 30 min and then lysed in lysis buffer. Half of the cell lysate was probed with anti-phosphotyrosine (p-Tyr) antibody (upper panel) and the remaining half with anti-FLAG and anti-Myc antibodies (bottom). (b) Cell lysates were probed with anti-p-Syk(Tyr^{352}) (upper panel) or anti-p-Syk($Tyr^{525/526}$) antibody (lower panel). (c) Cell lysates were immunoprecipitated using anti-PLC-γ1 antibody and probed with anti-p-Tyr antibody (upper panel). Efficient immunoprecipitation of PLC-γ1 was verified (lower panel).

(Coopman *et al.*, 2000) and airway epithelial cells (Ulanova *et al.*, 2005), nasal fibroblasts (Yamada *et al.*, 2001) and hepatocytes (Tsuchida *et al.*, 2000). These results suggest that Syk plays a general physiological role in non-haematopoietic cells as well. The first report of Syk having a role in cancer was a study of mammary epithelial cells (Coopman *et al.*, 2000). Since then, there have been several reports that Syk functions as a tumour suppressor in the process of malignant tumour development, such as gastric cancer (Wang *et al.*, 2004) and leukaemia (Goodman *et al.*, 2001). To look into the possible relevance of Syk in HCV-infected hepatocytes and also the possible involvement of Syk in HCC development, we first examined Syk expression in hepatocytes obtained from HCV-infected and uninfected subjects. We found that Syk was expressed near the plasma membrane of hepatocytes of HCV-infected patients, with a patchy pattern, whereas it was expressed rather diffusely in the cytoplasm of normal, uninfected hepatocytes (Fig. 1).

We also demonstrated that NS5A interacted with Syk and inhibited its kinase activity when expressed ectopically in Huh-7 cells (Figs 2, 5 and 6). The NS5A interaction with Syk was observed even in the context of HCV RNA replication (Fig. 7a, b) and Syk kinase activity was inhibited in HCV RNA replicon-harbouring cells (Fig. 7c). It is likely, therefore, that Syk is a binding partner of NS5A and is functionally inhibited in HCV-infected hepatocytes as well. Whilst an N-terminal portion of NS5A (aa 1–175) was responsible for the binding to Syk, a central portion (aa 237–302) was also required for the inhibition of Syk kinase activity (Figs 3 and 5). It has been reported that NS5A associates with the non-receptor protein tyrosine kinases Lyn and Fyn, members of the Src family kinases, through the proline-rich region of NS5A (aa 343–356) and the SH3 domain of the kinases, thereby inhibiting and activating the kinase activities of Lyn and Fyn, respectively (Macdonald & Harris, 2004; Macdonald *et al.*, 2004). In contrast, Syk does not possess an SH3 domain but has two tandem SH2 domains. These SH2 domains are known to interact with diphosphorylated ITAM of immune receptors, resulting in activation of Syk in an autocrine or paracrine manner (Sada *et al.*, 2001; Yanagi *et al.*, 1995). However, it is unlikely that the NS5A–Syk interaction occurs through its ITAM-related sequence in the same manner as that observed for immune receptors, as NS5A mutants with a mutated ITAM-like sequence still interacted with Syk (Fig. 3). Also, the SH2 domains of Syk are not the only binding sites for NS5A (Fig. 4). These results suggest that the mechanism

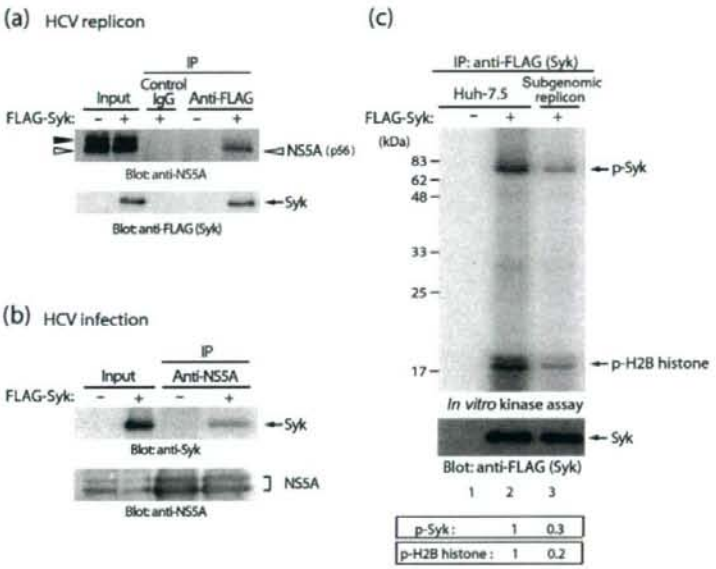

Fig. 7. NS5A expressed in the context of HCV RNA replication interacts with Syk and inhibits its kinase activity. (a) FLAG-tagged Syk was expressed in HCV RNA replicon-harbouring Huh-7.5 cells. Cell lysates were immunoprecipitated with anti-FLAG antibody or control IgG and probed with anti-NS5A (upper panel) or anti-FLAG antibody (lower panel). Filled and open arrowheads indicate the hyperphosphorylated (p58) and hypophosphorylated forms of NS5A (p56), respectively. (b) FLAG-tagged Syk was expressed in HCV J6/JFH-1-infected Huh-7.5 cells. Cell lysates were immunoprecipitated with anti-NS5A polyclonal antibody and probed with anti-Syk monoclonal antibody. (c) FLAG-tagged Syk was expressed in HCV RNA replicon-harbouring Huh-7.5 cells. Cell lysates were immunoprecipitated with anti-FLAG antibody and the immunoprecipitates were subjected to an *in vitro* kinase assay using H2B histone as substrate. Phosphorylation of Syk (p-Syk) and H2B histone (p-H2B histone) was visualized by autoradiography (upper panel). Efficient immunoprecipitation of Syk was verified (lower panel). Arbitrary units of Syk kinase activities, represented by the phosphorylation values of p-Syk and p-H2B histone normalized to the amounts of immunoprecipitated Syk, are shown at the bottom.

underlying the NS5A–Syk interaction differs from what has been observed for Syk and its interacting proteins in immune cells. It is possible that multiple regions of NS5A are involved in the interaction with Syk. Alternatively, NS5A may interact with Syk indirectly through the other host protein(s) that binds directly to Syk.

Syk is activated by cytokine stimulation, hyperosmolarity shock, oxidative stress and engagement with integrin (Corey *et al.*, 1994; Gao *et al.*, 1997; Miah *et al.*, 2004). However, the biological relevance of Syk in hepatocytes has not yet been demonstrated. We have shown in the present study that hyperosmolarity stress-induced activation of Syk resulted in increased tyrosine phosphorylation of endogenous PLC-γ1 (Fig. 6c). This result suggests that activated Syk sends signals to PLC-γ1 in hepatocytes, as observed in immune cells (Law *et al.*, 1996). Our findings that NS5A associates with Syk strongly suggest that NS5A affects the Syk signalosome to alter the signal transduction elicited by the Syk–PLC-γ1 interaction.

Phosphorylation of tyrosine residues in the linker region of Syk is required for immune receptor signalling. Genetic studies have demonstrated that phosphorylation of Tyr^{348} and Tyr^{352} in the linker region of Syk is involved in regulating tyrosine phosphorylation of LAT (linker for activating T cells), SLP-76 and PLC-γ1 and -γ2, and affects Ca^{2+} mobilization triggered by aggregation of the high-affinity IgE receptor (Simon *et al.*, 2005; Zhang *et al.*, 2002). We observed that NS5A downregulated phosphorylation of Tyr^{352} of Syk (Fig. 6b), which correlated with the inhibition of Syk kinase activity. The phosphorylation state of Tyr^{352} also correlated well with the tyrosine phosphorylation state of PLC-γ1. This suggests the possibility that Ca^{2+} mobilization is affected in HCV-infected hepatocytes through the NS5A-mediated downregulation of Tyr^{352} phosphorylation on Syk.

Unlike ectopically expressed Syk, endogenously expressed Syk in B cells under normal conditions is not tyrosine phosphorylated (Wienands *et al.*, 1996). Pervanadate stimulation is known to induce tyrosine phosphorylation of endogenous Syk. We examined the possible interaction of endogenous Syk and NS5A. Our results demonstrated that NS5A interacted with endogenous Syk when the cells were treated with pervanadate, but not when the cells were left untreated (Fig. 2d). These results suggest that NS5A interacts with the tyrosine-phosphorylated, active form of Syk.

Whilst Syk is commonly expressed in normal human breast tissues, benign breast lesions and low-tumorigenic breast

cancer cell lines, only a minimal or even an undetectable level of Syk expression has been demonstrated in invasive breast carcinoma tissues and cell lines (Coopman *et al.*, 2000). DNA methylation of the CpG sites in the *syk* gene promoter has been reported to be responsible for the loss or marked reduction of Syk expression in breast cancer (Yuan *et al.*, 2001). Moreover, Yuan *et al.* (2006) reported that DNA methylation of the *syk* gene in hepatitis B virus-associated HCC cancerous tissue was highly correlated with Syk expression and that the patients with a methylated *syk* gene had a significantly lower overall survival rate after hepatectomy than those with an unmethylated *syk* gene. In contrast, our results revealed that the expression levels of Syk did not differ between normal and HCV-infected hepatocytes (Fig. 1k) or between cancerous and non-cancerous hepatocytes (data not shown). At the functional level, however, NS5A downregulated Syk kinase activity in Huh-7 cells (Fig. 6). Moreover, Syk kinase activity was downregulated in cells harbouring an HCV RNA replicon (Fig. 7c). These results collectively suggest that NS5A is involved, at least partly, in the suppression of Syk kinase activity in HCV-infected cells. It is also interesting to assume that the NS5A-mediated Syk inhibition plays an important role in the development of HCC, although the precise molecular mechanism(s) is yet to be determined. Recently, a possible mechanism by which breast cancer cells become invasive was proposed: human breast cancer cells express and secrete a group of chemokines called growth-related oncogene (GRO)-α, GRO-β and GRO-γ, and their production is regulated by Syk (Li & Sidell, 2005). It would be interesting to examine the possible effects of NS5A and HCV RNA replication on the levels of GRO expression and secretion.

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1b型高ウイルス量高齢者C型慢性肝炎に対するPEG IFNα-2b/リバビリン治療（併用療法）の検討

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