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.

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
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)X6–8YXX(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 & Litman, 1994). Autophosphorylation of Syk on Tyr525 and Tyr526 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 Tyr522 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 [Tyr118 to Phe (Y118F), Val121 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 (T7F-7-3), 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 T7F-7-3. For BJAB cells, we used an electroporation method (Schneider & Kieser, 2004). In brief, 5 × 10⁶ 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 Na₃VO₄ with 1 mM H₂O₂) 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 % H₂O₂ quench endogenous peroxidase activity. After being autoclaved at 121 °C for 20 min, the sections were incubated with a blocking
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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′-AGTACCCACAAA GGCCTTGGC-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′-CCCGGAGAG 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- GGTT-(DIG)-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 levamisole 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 Na3VO4 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-BeadBater (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 (Tyr522) and Syk(Tyr255/256) 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 MgCl2, 2 mM MnCl2, 4 µM ATP and 4 µCi (148 kBq) [γ-32P]ATP, for 30 min at room temperature. Reactions were terminated by boiling for 5 min in 2× sample buffer.
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-32P 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 FLK4, are negative for 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
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\textit{Y}118VE\textit{Y}121TRVGDF\textit{Y}129VTG) 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
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 Tyr118 (Y118F) or double mutations of Tyr118 and Val121 (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). Thus, it is unlikely that NS5A binds to Syk through its ITAM-related sequence in the same manner as that observed for immune receptors.

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**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
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 Tyr352 and/or Tyr525/526 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).

**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 (Taniuchi et al., 1991). It is also expressed in human mammary...
(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
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 Tyr348 and Tyr352 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 Ca²⁺ 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 Tyr352 of Syk (Fig. 6b), which correlated with the inhibition of Syk kinase activity. The phosphorylation state of Tyr352 also correlated well with the tyrosine phosphorylation state of PLC-γ1. This suggests the possibility that Ca²⁺ mobilization is affected in HCV-infected hepatocytes through the NS5A-mediated downregulation of Tyr352 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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