[1,2]. The current combination therapy using pegylated interferon alpha plus the nucleotide analogue ribavirin achieved a sustained virological response in 40% to 50% of individuals infected with HCV genotypes 1a and 1b [3,4]. More than two-thirds of the HCV-positive population has been chronically infected with genotype 1 in Western countries and Japan [5]. Therefore, more effective therapeutic and preventative measures are needed for the treatment of hepatitis C patients who are not responsive to chemotherapy.

HCV is an enveloped virus possessing a single positive strand RNA genome that encodes a large polyprotein with approximately 3000 amino acid residues [1]. After translation that is dependent on the internal ribosomal entry site (IRES), the polyprotein is cleaved by the viral proteases NS2 and NS3 and host proteases. A capsid protein (core) and two envelope proteins (E1 and E2) are viral structural proteins that occupy the N-terminal third of the polyprotein, while nonstructural (NS) proteins make up the remaining region. NS3, NS4A, NS4B, NS5A and NS5B are predicted to form a replication complex with several host proteins and lipids, and are essential for autonomous replication of the genome within the hepatoma cell line [6]. NS3 has RNA helicase and protease activities required for RNA replication and for processing of downstream nonstructural proteins, respectively [7,8]. NS4A encourages NS3 to anchor on the endoplasmic reticulum (ER) membrane and to support its protease activity [9]. NS4B is a multi-spanning membrane protein that remodels the ER membrane to be suitable for HCV viral replication [10]. NS5A is a phosphoprotein regulating replication of the HCV genome [11], and mutations adaptive for efficient RNA replication in the cell culture system have been selectively introduced within the NS5A coding region [12]. NS5B is the active subunit of the replication complex known as the RNA-dependent RNA polymerase [13]. Several recent reports suggest that some host proteins attend to the formation of the HCV replication complex [14–17].

Several chemical compounds targeted to the viral and host proteins have been developed based on the *in vitro* viral replication and infection systems. The HCV replicon, which focuses on the viral replication deficient in production of infectious viral particles, is basically composed of an antibiotic gene for selection and HCV genomic RNA for autonomous replication in the intracellular compartments around ER [6]. To evaluate the early step of infection, pseudotype virus systems based on the vesicular stomatitis virus and retrovirus have been developed, and several candidates for HCV receptor and/or co-receptor have been reported [1]. Recently, an *in vitro* cell culture system for infectious HCV of genotype 2a, which is highly sensitive to interferon therapy [18,19], was developed [20–22]. However, development of a robust cell culture system for the HCV 1a and 1b genotypes, which are the most prevalent genotypes in the world and resistant to interferon therapy, has not yet been successful. Some intergenotype chimeric viruses have been reported to be less efficiently grown than the genotype 2a virus, as described above. Experimental animals for the study of HCV are limited only to chimpanzees and the mice transplanted with human hepatocytes. In this review, we summarize the recent progress of *in vitro* and *in vivo* systems for investigation of HCV and candidate compounds for the treatment of chronic hepatitis C.

2. *In vivo* and *in vitro* systems for the study of HCV

2.1. Experimental animals

The chimpanzee is the only animal model susceptible to HCV infection and exhibits hepatitis progression similar to that of humans, leading to establishment of persistent infection in 40% to 60% of chimpanzees experimentally infected with HCV [23,24]. Chimpanzees inoculated with HCV exhibit one to ten million genome copies per milliliter of serum at one week post-infection [25]. For both ethical and economic reasons, however, it is difficult to employ chimpanzees as an experimental model.

Several reports have suggested that HCV was infectious to Tree shrews (*Tupaia belangeri*) [26,27]. The animals were susceptible to infection with HCV genotypes 1a, 1b, and 3 [27], and The susceptibility was dependent on the SR-BI gene, which is highly homologous between chimpanzees and humans [25], but was independent of CD81 [26]. Further studies are required to clarify the susceptibility of Tree shrews to HCV infection.

The Alb-uPA mouse, which possesses the urokinase-type plasminogen activator (uPA) gene under the control of the albumin promoter, exhibits a decrease in liver cells after birth [28]. Hemizygous Alb-uPA mice have been crossed with homozygous SCID/bg mice to yield a strain immunodeficient for xenoplantation with human liver fragments [28]. Human liver cells were increased in these mice after xenoplantation. Transgenic mice in which half of all cells were replaced with human liver cells remained alive until at least 35 weeks and exhibited the ability to propagate HCV [28]. The antiviral effects of interferon alpha and BILN2061 NS3 protease inhibitor in these mice were similar to those in human clinical trials [29]. This system is applicable for the assessment of anti-HCV drugs *in vivo* but not for that of immunotherapeutics for HCV infection due to immunodeficiency.

2.2. Cell culture systems to evaluate HCV replication: replicon systems

The assessment of antiviral drugs for chronic hepatitis C patients had been hampered by the lack of a robust cell culture system. Lohmann et al. succeeded in the establishment of an HCV replicon system in which an artificial RNA comprised of the 5′-UTR, *neo* gene, EMCV IRES, a cDNA encoding HCV NS2 or NS3 up to NS5B, and 3′-UTR replicates autonomously in the Huh7 cell line [6]. The replicon system partially mimics the authentic replication cycle of HCV without production of infectious particles due to detection of positive and negative strands of RNAs and processing of the nonstructural proteins. Analysis of the replication efficiency revealed that the regions of 5′- and 3′-UTR are responsible for the replication [30,31]. Efficient replication of subgenomic replicons carrying the type 1b Con1 strain generally requires adaptive mutations introduced into the regions encoding the NS3, NS5A and NS5B proteins [6,12]. Adaptive mutations within NS5A were found to confer

efficient replication *in vitro* independently of IFN sensitivity [12,32].

Bukh et al. reported that adaptive mutations are not required for *in vivo* infection of HCV [33]. A viral RNA genome bearing three adaptive mutations was not infectious to chimpanzees, while a clone bearing one adaptive mutation was infectious, but this mutation reverted back to the wild-type sequence [33]. The HCV replicons described above are derived from the genomes of type 1b strains (Con1 and HCV-N), while replicons based on type 1a sequences exhibit lower sensitivity to IFN alpha than type 1b replicons [34], and adaptive mutations appear within NS3, NS5A, and NS5B [34,35]. A subgenomic replicon derived from a patient with fulminant hepatitis of HCV genotype 2a was efficiently replicated in the Huh7 cell line and employed as a powerful replication unit for an infectious clone [36]. Several drugs targeted to NS3 and NS5B have been identified using the replicon systems, as described below.

2.3. Pseudotype viruses

Vesicular stomatitis virus (VSV) is known as a member of the *Rhabdovirus* family bearing a non-segmented 11 kb genome of negative stranded RNA, which is transcribed in the cytoplasm and codes five structural proteins. VSV pseudotyped with other membrane proteins instead of the G envelope protein is an ideal system for the study of the mechanism of virus entry. Transient expression studies have suggested that HCV E1 and E2 proteins are retained in the endoplasmic reticulum (ER) by C-terminal retention signals [37–39]. Therefore, pseudotype VSV bearing the chimeric HCV envelope proteins consisting of the signal sequence and transmembrane region of the VSV G envelope protein and ectodomains of HCV E1 and E2 proteins was generated, and the virus was infectious to HepG2 cells in CD81-independent manner [39]. Human Fab antibody isolated from a chronic hepatitis C patient neutralized the infection of the pseudotype VSV to HepG2 cells, suggesting that infection of the VSV pseudotype mimics natural HCV infection [40]. However, a recombinant VSV encoding the chimeric HCV envelope proteins in place of the G protein was non-infectious [41]. Post-translational modifications of the chimeric envelope proteins that are dependent on the producer cells may affect the infectivity of VSV bearing HCV envelope proteins.

Although a previous study revealed that HCV envelope proteins are statically retained in the ER [37], unmodified HCV envelope proteins are partially sorted to the cell surface in 293 T cells and encapsulated with retrovirus nucleocapsid, resulting in the development of pseudotype retrovirus [42]. Development of a pseudotype retrovirus resulted in a virus that exhibited the highest infectivity to Huh7 cells among that to the tested cell lines and this infectivity was dependent on CD81 [42]. An HIV-based pseudotype virus was generated using the same strategy [43]. Although CD81, LDL receptor, human SB-R1 and DC-SIGN have been reported as candidates for HCV receptors, additional factors are required for HCV entry, because cell lines expressing all of the factors described above on the plasma membrane do not permit entry of the pseudotype retrovirus [43]. Very recently, claudin-1 was identified as a co-receptor candidate for a late step of HCV entry by using a retrovirus repackaging system [44]. A monoclonal antibody AP33 recognizes a linear epitope conserved among E2 proteins of HCV genotypes 1 to 6 and neutralizes infection of the pseudotype retroviruses bearing envelope proteins of the different genotypes [45]. Given that the linear epitope recognized by AP33 might be a new target for the development of vaccines and antibodies for prophylaxis and treatment of HCV infection. The pseudotype viruses are powerful tools for analysis of the initial steps of infection, including binding and entry, and for the development of new antivirals counteracting virus entry.

2.4. Cell culture system for infectious HCV production

The replicon system has been developed as an important tool in the investigation of HCV replication and it has served as a cell-based assay system for the evaluation of antiviral drugs. Cell culture systems for *in vitro* replication and for a productive infection were recently established based on an HCV genome of genotype 2a JFH1 strain [20–22]. However, no robust and reliable *in vitro* culture systems for the authentic viruses of the genotypes 1a and 1b, which are the most prevalent HCV genotypes in the world, have yet been established.

Wakita et al. characterized the subgenomic replicons of the HCV genotype 2a strains JFH1 isolated from a patient with fulminant hepatitis C. The JFH1 clone exhibited an efficient replication in Huh7 cells without any adaptive mutations [36,46,47]. A full-length viral genome prepared from a JFH1 clone was autonomously replicated in Huh7 cells, and its culture supernatant was infectious to Huh7 cells [21]. JFH1 virus inoculated into a chimpanzee exhibited a transient replication without any apparent hepatitis and immune response [21]. Lindenbach et al. constructed a chimeric HCV clone consisting of nonstructural proteins of JFH1 and structural proteins of the J6 strain of genotype 2a, and demonstrated that it is infectious to the Huh7.5 cell line in a CD81-dependent manner [20]. The chimeric genotype 2a clone was infectious to chimpanzees and the uPA-SCID mice and progeny viruses recovered from the infected animals were infectious to the cell line [48]. Zhong et al. also reported a robust replication model using a cell culture system based on the HCV JFH-1 molecular clone and an Huh7-derived cell line, Huh7.5.1 [22]. Among the chimeric HCV clones composed of structural proteins of genotype 1a, 1b, or 2a and nonstructural proteins of JFH1, viruses comprised of 2a strains have been shown to exhibit the highest replication [49]. Cell culture system for infectious HCV derived from JFH1 strain would contribute to the development of antivirals and vaccines.

2.5. Inhibitors for viral protease and polymerase

BILN2061 was selected from the macrocyclic inhibitor series optimized for inhibition of protease activity of HCV NS3 [50]. This compound exhibited reversible and non-covalent competitive inhibition against NS3 proteases of genotype 1a and 1b with inhibition constants of 0.30 and 0.66 nM, respectively, and showed a high specificity to NS3 but not to human elastase and

cathepsin B, which are representative serine and cystein proteases, respectively [50]. The ability of BILN2061 to decrease the plasma viral load of HCV was sufficiently potent to reach 3 $\log_{10}$ but remained transient in clinical trials, suggesting that combination therapy is necessary for effective therapy [50,51]. BILN2061 has a low affinity to NS3 proteases of genotypes 2 and 3 at several tens times lower than that of genotype 1 [52]. This may be the reason that the antiviral efficacy of BILN2061 is rather weak and unsteady in patients infected with genotypes 2 and 3 [53]. Unfortunately, cardiac toxicity was histologically detected in rhesus monkeys challenged with a high dose of BILN2061 over 4 weeks [51,53].

VX-950, which was designed on the basis of the structure of NS3, is a covalent but reversible peptidomimetic inhibitor of NS3 protease and had an inhibition constant of 7 nM [54]. An *in vitro* study using replicon revealed that BILN2061-resistant mutants and VX-950-resistant mutants remain sensitive to VX-950 and BILN2061, respectively [54]. Substitution of Ala^{156} to Val or Thr was shown to reduce the susceptibility to both BILN2061 and VX-950 in a study using purified enzymes and replicons [55]. Treatment with VX-950 and interferon alphasynergistically suppressed the viral RNA level by 4 $\log_{10}$ without any cytotoxicity [56]. A phase I, placebo-controlled, double-blind study showed that the viral loads dropped more than 3 $\log_{10}$ and 2 $\log_{10}$ in 93% and 100%, respectively, of patients administered VX-950 [57]. All adverse events in patients administered VX-950 were of mild intensity and there were no clinically significant changes in the electrocardiograms [57].

A novel ketoamide inhibitor, SCH 503034, that was recently made available for oral administration exhibited potent antiviral activity [58]. Analyses of variant replicons with regard to their efficiency in producing colonies suggest that emergence of the highly resistant viruses is moderate or rare in naïve patients in a clinical setting [59]. Combination therapy with interferon alpha and SCH 503034 may potently suppress the emergence of resistant viruses *in vivo*, because the replicons resistant to SCH 503034 were shown to be susceptible to treatment with IFN-alpha [59]. SCH 503034 has been advanced to phase 1 clinical trials for the treatment of hepatitis C patients [58].

NM283 is a nucleotide inhibitor that is an orally bioavailable pro-drug of 2′-*C*-methylcytidine and it is processed into phase IIb clinical trial [60]. This compound inhibits viral RNA-dependent RNA polymerase NS5B by incorporating into growing strands of viral RNA to terminate the extension of viral RNA [61]. The mutational resistance to this nucleotide analogue was found in examination using the replicon but reduced viral fitness in terms of polymerase activity, suggesting that emergence of a resistant virus is rare *in vivo* [61]. Although an increased amount of viral load was reported during monotherapy [61], combination therapy with interferon alpha may exhibit potent efficacy.

2.6. Immunophilins and chaperones

The peptide bond *cis/trans* isomerases, which have the ability to catalyze conversion between *cis* and *trans* peptide bonds for correct folding of the protein substrate, include peptidyl prolyl *cis/trans* isomerase (PPIase). PPIase is composed of the families of cyclophilins [62], FK506-binding proteins (FKBP) [63,64], and parvulins [65]. Cyclophilin and FKBP are classified as immunophilins capable of binding to the immunosuppressants cyclosporine and FK506, respectively [66]. Based on their amino acid sequences, substrate specificities, and inhibitor sensitivities, the family members do not share a homologous domain. Recently, immunophilins were reported as target proteins for HCV therapy.

We screened the human fetal brain and liver libraries by using a yeast two-hybrid system and identified FKBP8 as an HCV NS5A-interacting protein [15]. FKBP8 is a member of the FKBP family, but does not share amino acid residues essential for FK506 binding or PPIase activity [67]. The *in vitro* biochemical data suggest that FKBP8 has a weak activity of PPIase and a low affinity to FK506 [68,69]. The splicing variants with expected sizes of 44 and 46 kDa were reported in mice but not in humans, while human FKBP8 is one form with a molecular mass of 45 kDa corresponding to 44 kDa of murine protein [70]. The physiological function of FKBP8 may be associated with the intracellular events that suppress the morphogen known as the Sonic hedgehog (Shh) protein [71]. FKBP8-deficient mice have been reported to exhibit the phenotypes observed on abnormality in brain of the mouse expressing excessive Shh signaling [71]. Shh- and FKBP8-double knockout embryos showed partial rescue of cyclopia and holoprosencephaly except for a limb outgrowth defect [71], suggesting that FKBP8-controlled signaling overlaps Shh signaling in brain. FKBP8 is expected to be the inherent phosphatase inhibitor and retains Bcl-2 on the mitochondria membrane to inhibit apoptosis [72]. However, physiological apoptosis may be not controlled by FKBP8, at least in the brain, because no difference between the wild type and FKBP8-deficient mice was found with respect to apoptosis [71]. FKBP8 may modulate phosphatases such as carcineurin to enhance the phosphorylation required for suppression of Shh signaling.

Our previous study suggests that NS5A can bind to FKBP8 but not to either the homolog FKBP52 or cyclophilin D in cells [15]. A combination of immunoprecipitation analysis and proteomics analysis revealed that FKBP8 forms a complex with Hsp90 [15]. The amino acid residues of the carboxylate clamp position in the TPR domain of FKBP8 grasps the C-terminal MEEVD motif of Hsp90 [15,73]. Mutations of these residues of the carboxylate clamp in the TPR domain suppress the interaction between FKBP8 and Hsp90 but not the interaction between NS5A and FKBP8, suggesting that FKBP8 interacts with NS5A and Hsp90 at different sites within the TPR domain [15]. Knockdown of FKBP8 and the Hsp90 inhibitor geldanamycin could down-regulate the HCV replication but not affect the cell viability [15]. These data suggest that HCV NS5A is correctly folded by formation of a multicomplex with Hsp90 and FKBP8. Another hypothesis is that NS5A modulates the unknown phosphatase activity by interaction with FKBP8 to facilitate replication of the HCV genome. Although Hsp90 was shown to be involved in the cleavage between NS2 and NS3 [74], NS2 is not required for the replication of the HCV genome [6].

Heat shock protein 90 (Hsp90) seems to be involved in the functions of several viral polymerases and other categories of viral enzyme. Hsp90 was shown to bind to a viral polymerase subunit of influenza virus to facilitate the replication complex formation and the nuclear localization of the viral polymerase subunit [75, 76]. Hsp90 is also responsible for nuclear localization of the DNA polymerase of herpes simplex virus type 1 [77] and the complex formation of the RNA-dependent RNA polymerase of alphanodavirus on the intracellular membrane [78]. Hsp90 inhibitors could potently reduce the replication efficiency of poliovirus without the emergence of drug-resistant mutants [79]. Hsp90 activity is important for the rapid growth of negative strand RNA viruses [80] and for the activity of the hepatitis B reverse transcriptase [81, 82]. Geldanamycin and its derivatives may become multiviral drug. Hsp90 generally requires the cochaperone protein to acquire the substrate specificity. Several cochaperones such as FKBP8 may be involved in the mechanism of the folding of the viral proteins.

Cyclophilin A is incorporated into virions of HIV, although the involvement of cyclophilin A in the viral cycle has not been completely clarified yet [83]. The interaction between cyclophilin A and HIV capsid renders the capsid proteins capable of binding to the host protein TRIM5 alpha, which is known to be a potent antiretroviral restriction factor [84,85]. Cyclophilin A modulates the conformation of the gag capsid protein to facilitate the interaction with TRIM5 alpha in macaque cells, thereby leading to the establishment of host range restriction [84,85]. Cyclophilin B is an immunophilin with a molecular mass of 20 kDa and is secreted as a neurotrophic factor for spinal cord cells of chick embryos [86]. Cyclophilin B is also found in human milk and blood [87,88]. Secreted cyclophilin B could improve the nuclear retrotranslocation of prolactin [89], has been implicated in presynaptic function, and suppresses the folding of prion protein, leading to the accumulation of prion protein in aggresomes [90]. Cyclophilin B may regulate the correct folding and translocation of host proteins under extracellular and intracellular conditions. Cyclosporin A and its derivatives lacking an immunosuppressive effect could suppress HCV genome replication and could be utilized for HCV therapy [17,91,92]. The ability to inhibit cyclophilin is responsible for the suppressive effect of cyclosporin derivatives on HCV genome replication [17]. Watashi et al. reported that cyclophilin B specifically interacts with HCV NS5B on the ER of the replicon cells and promotes association of NS5B with the genomic RNA [17]. Treatment with cyclosporin A and knockdown of cyclophilin B suppressed the replication of HCV, suggesting that cyclophilin B plays an important role in HCV genome replication by enhancing the interaction between NS5B and viral RNA [17].

Immunophilins and Hsp90 may be involved in HCV replication through the correct folding of the replication complex required for efficient enzymatic activity. In addition, cyclophilin B may also participate in the translocation of NS5B, as seen in the polymerase subunits of influenza virus, to facilitate binding to viral RNA. Elucidation of the HCV replication complex may lead to the development of new therapeutics for chronic hepatitis C.

2.7. Host proteins are modified by lipid and related to lipid biosynthesis

Host lipid components are required for the assembly, budding, and replication of several viruses [93–98]. Additional administration of saturated and monounsaturated fatty acids enhanced HCV RNA replication, although polyunsaturated fatty acids suppressed it [99]. Saturated or monosaturated fatty acid may be essential components for formation of the HCV replication complex [99]. Sterol regulatory element-binding protein-1c (SREBP-1c) up-regulates the transcription of acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase, leading to the production of saturated and monounsaturated fatty acids [100]. HCV core protein has the ability to induce liver steatosis and enhances the production of lipid droplets [101,102]. Our recent study suggests that HCV core protein up-regulates SREBP-1c through an LXRalpha/RXRalpha-dependent pathway, leading to induction of fatty liver [103]. The up-regulation of SREBP-1c by HCV core protein requires PA28gamma, which regulates the nuclear proteasome activity to degrade the HCV core protein by directly interacting with it [104,105]. A lipophilic long-chain compound derived from microbial metabolites, an inhibitor of sphingolipid biosynthesis, was shown to inhibit HCV replication [98]. HCV NS proteins are known to be localized in the lipid raft for formation of the viral replication complex [106–108]. Therefore, compounds disrupting sphingolipid biosynthesis may inhibit the replication of HCV by modifying the lipid raft.

HCV replication was disrupted with an inhibitor of geranylgeranyl transferase I or lovastatin, which is an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase in the upstream region of the mevalonate pathway, but not with an inhibitor of farnesyl transferase, suggesting that geranylgeranylation of viral or host protein controls the efficiency of HCV replication [109]. Ikeda et al. examined several statins, including atorvastatin, fluvastatin, lovastatin, pravastatin, and simvastatin, for their potential use in the development of anti-HCV drugs [110]. Geranylgeranylate is an intermediate of the mevalonate pathway and is attached to various cellular proteins for anchoring to the plasma or intracellular membrane [100]. Wang et al. reported that the geranylgeranylated F-box protein, FBL2, is required for efficient replication of HCV genomic RNA [111]. The CAAX motif (CVIL) of FBL2 is suggested to be modified by geranylgeranylation [111]. FBL2 lacking a CAAX motif was not modified by geranylgeranylation and could not bind to NS5A [111]. The F-box motif is generally required for formation of the ubiquitin ligase complex [112], suggesting that FBL2 regulates the ubiquitination of host or viral proteins through interaction with NS5A to up-regulate the efficiency of HCV replication.

3. Conclusion

Recent progress in the establishment of *in vitro* and *in vivo* systems for the study of HCV has accelerated our comprehension of the mechanisms of infection, replication, and pathogenesis of HCV. Promising candidates for therapeutics for chronic hepatitis C targeting the viral proteins, especially proteases and

polymerases, have been developed using these surrogate systems. However, the compounds targeted to viral enzymes may induce the emergence of drug-resistant mutants, as seen in the case of HIV infection. Host proteins essential for HCV replication might be ideal targets for the development of compounds for the treatment of chronic hepatitis C patients with a low rate of emergence of escape mutants. Further studies on the host proteins related to the viral replication as well as host factors related to the mechanisms by which HCV infection induces the carcinogenesis will be necessary for the development of efficient antiviral drugs for chronic hepatitis C.

Acknowledgments

We gratefully thank H. Murase for her secretarial work. This work was supported partly by grants-in-aid from the Ministry of Health, Labor, and Welfare; the Ministry of Education, Culture, Sports, Science, and Technology; the 21st Century Center of Excellence Program; and the Foundation for Biomedical Research and Innovation.

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JOURNAL OF VIROLOGY, Feb. 2007, p. 1727–1735 Vol. 81, No. 4
0022-538X/07/$08.00+0 doi:10.1128/JVI.01683-06

Involvement of the PA28γ-Dependent Pathway in Insulin Resistance Induced by Hepatitis C Virus Core Protein[▽]

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Received 4 August 2006/Accepted 16 November 2006

The hepatitis C virus (HCV) core protein is a component of nucleocapsids and a pathogenic factor for hepatitis C. Several epidemiological and experimental studies have suggested that HCV infection is associated with insulin resistance, leading to type 2 diabetes. We have previously reported that HCV core gene-transgenic ($PA28\gamma^{+/+}$CoreTg) mice develop marked insulin resistance and that the HCV core protein is degraded in the nucleus through a PA28γ-dependent pathway. In this study, we examined whether PA28γ is required for HCV core-induced insulin resistance in vivo. HCV core gene-transgenic mice lacking the PA28γ gene ($PA28\gamma^{-/-}$CoreTg) were prepared by mating of $PA28\gamma^{+/+}$CoreTg with PA28γ-knockout mice. Although there was no significant difference in the glucose tolerance test results among the mice, the insulin sensitivity in $PA28\gamma^{-/-}$CoreTg mice was recovered to a normal level in the insulin tolerance test. Tyrosine phosphorylation of insulin receptor substrate 1 (IRS1), production of IRS2, and phosphorylation of Akt were suppressed in the livers of $PA28\gamma^{+/+}$CoreTg mice in response to insulin stimulation, whereas they were restored in the livers of $PA28\gamma^{-/-}$CoreTg mice. Furthermore, activation of the tumor necrosis factor alpha promoter in human liver cell lines or mice by the HCV core protein was suppressed by the knockdown or knockout of the PA28γ gene. These results suggest that the HCV core protein suppresses insulin signaling through a PA28γ-dependent pathway.

Hepatitis C virus (HCV) is the causative agent in most cases of acute and chronic non-A, non-B hepatitis (15). Over one-half of patients with the acute infection evolve into a persistent carrier state (24). Chronic infection with HCV frequently induces hepatic steatosis, cirrhosis, and eventually hepatocellular carcinoma (22) and is known to be associated with diseases of extrahepatic organs, including an essential mixed cryoglobulinemia, porphyria cutanea tarda, membranoproliferative glomerulonephritis, and type 2 diabetes (13).

HCV is classified into the genus *Hepacivirus* of the family *Flaviviridae* and possesses a viral genome consisting of a single positive-strand RNA with a nucleotide length of about 9.5 kb. This viral genome encodes a single polyprotein composed of approximately 3,000 amino acids (9). The polyprotein is post-translationally cleaved by host cellular peptidases and viral proteases, resulting in 10 viral proteins (6, 10, 12). The HCV core protein is known to interact with viral-sense RNA of HCV to form the viral nucleocapsid (44). The HCV core protein is cleaved off at residue 191 by the host signal peptidase to release it from the E1 envelope protein and then by the host signal peptide peptidase at around amino acid residues 177 to 179 within the C-terminal transmembrane region (30, 39, 40). The mature core protein is retained mainly on the endoplasmic reticulum, although a portion moves to the nucleus and mitochondria (11, 51).

Recent epidemiological studies have indicated that type 2 diabetes is an HCV-associated disease (7, 29). However, it remains unclear how insulin resistance is induced in patients chronically infected with HCV, since there is no suitable model for investigating HCV pathogenesis. Type 2 diabetes is a complex, multisystemic disease with pathophysiology that includes a high level of hepatic glucose production and insulin resistance, which contribute to the development of hyperglycemia (8, 18). Although the precise mechanism by which these factors contribute to the induction of insulin resistance is difficult to understand, a high level of insulin production by pancreatic β cells under a state of insulin resistance is common in the development of type 2 diabetes. The hyperinsulinemia in the fasting state that is observed relatively early in type 2 diabetes is considered to be a secondary response that compensates for the insulin resistance (8, 18).

The HCV core protein is also known as a pathogenic factor that induces steatosis and hepatocellular carcinoma in mice (33, 35). Previously, we reported that insulin resistance occurs in HCV core gene-transgenic mice due at least partly to an increase in tumor necrosis factor alpha (TNF-α) secretion (47) and that the HCV core protein is degraded through a PA28γ/REGγ (11S regulator)-dependent pathway in the nucleus (32). It is well known that PA28γ enhances latent proteasome activity, although the biological significance of PA28γ is largely unknown, with the exception that PA28γ is known to regulate steroid receptor coactivator 3 (28). Although several reports suggested that the degradation of insulin receptor substrate (IRS) proteins by a ubiquitin-dependent proteasome activity contributes to insulin resistance (43, 50), the involvement of the HCV core protein in cooperation with PA28γ in the stability of IRS proteins and in the development of insulin resis-

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▽ Published ahead of print on 29 November 2006.

tance is not known. In this study, we examined the involvement of PA28γ in the induction of insulin resistance by the HCV core protein in vivo.

MATERIALS AND METHODS

Preparation of PA28γ-knockout HCV core gene-transgenic mice. C57BL/6 mice carrying the gene encoding HCV core protein genotype 1b ($PA28\gamma^{+/+}$ CoreTg) line C49 and $PA28\gamma^{-/-}$ mice have been described previously (35, 36). These two genotypes were crossbred to create $PA28\gamma^{+/-}$CoreTg mice. $PA28\gamma^{+/-}$CoreTg mice were bred to generate $PA28\gamma^{-/-}$CoreTg mice (35, 36). The HCV core gene and the target sequence to knock out the PA28γ gene were identified by PCR. The mice were given ordinary feed (CRF-1; Charles River Laboratories, Yokohama, Japan) and were maintained under specific-pathogen-free conditions.

Glucose tolerance test. The mice were fasted for more than 16 h before glucose administration. D-Glucose (1 g/kg body weight) was intraperitoneally administered to the mice. Blood samples were taken from the orbital sinus at the indicated time points. The plasma glucose concentration was measured by means of a MEDI-SAFE Mini blood glucose monitor (TERUMO, Tokyo, Japan). The serum insulin level was determined by a Mercodia (Uppsala, Sweden) ultrasensitive mouse insulin enzyme-linked immunosorbent assay (ELISA).

Insulin tolerance test. The mice were fed freely and then fasted during the study period. Human insulin (2 U/kg body weight) (Humulin; Eli Lilly, Indianapolis, IN) was intraperitoneally administered to the mice. The plasma glucose concentration was measured at the indicated time and was normalized based on the glucose concentration at the time just before insulin administration.

Histological analysis of pancreatic islets. Pancreas tissues were fixed with paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The relative islet area and islet number were determined with Image-Pro PLUS image analyzing software (NIPPON ROPER, Tokyo, Japan).

Estimation of tumor necrosis factor alpha and HCV core protein. Mouse TNF-α was measured by using a mouse TNF-α ELISA kit (Pierce, Rockford, IL) and normalized based on the amount of total protein in each sample. The protein concentration was estimated by using a BCA protein assay kit (Pierce). The amount of HCV core protein in the liver tissues was determined by using an ELISA system as described previously (4).

In vivo insulin stimulation and immunoblot analysis. Mice were fasted for more than 16 h before insulin stimulation and then anesthetized with ketamine and xylazine. Five units of insulin were injected into the mice via the interior vena cava. Livers of the mice were collected 5 min after the insulin injection and frozen in liquid nitrogen. Immunoblot analyses of the HCV core protein, PA28γ, and each of the insulin-signaling molecules were carried out with the liver tissue homogenates prepared in the homogenizing buffer containing 25 mM Tris-HCl (pH 7.4), 10 mM Na_3VO_4, 100 mM NaF, 50 mM $Na_4P_2O_7$, 10 mM EGTA, 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P40 supplemented with Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany) (53). Tissue lysates were subjected to sodium dodecyl sulfate–2% to 15% gradient polyacrylamide gel electrophoresis (PAG Mini DAIICHI 2/15 13W; Daiichi Diagnostics, Tokyo, Japan) and electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). The protein transferred onto the membrane was reacted with rabbit anti-HCV core (32), rabbit anti-Akt (Cell Signaling, Danvers, MA), rabbit anti-phospho-Ser473-Akt (Cell Signaling), rabbit anti-IRS1 (Upstate, Lake Placid, NY), rabbit anti-phospho-Tyr608 mouse insulin receptor substrate 1 (Sigma, St. Louis, MO), or rabbit anti-IRS2 (Upstate) polyclonal antibody and then incubated with horseradish peroxidase-conjugated anti-rabbit antibody. Blotted protein was visualized using Super Signal Femto (Pierce) and an LAS3000 imaging system (Fuji Photo Film, Tokyo, Japan).

Quantitative reverse transcription-PCR (RT-PCR). Total RNA was isolated from mouse liver using an RNeasy kit (QIAGEN, Valencia, CA). The RNA preparation was treated with a TURBO DNA-free kit (Ambion, Austin, TX) to remove DNA contamination in the samples. The first-strand cDNAs were synthesized by a first-strand cDNA synthesis kit (Amersham Biosciences, Franklin Lakes, NJ). The targeted cDNA was estimated by using Platinum SYBR Green qPCR Super Mix UDC (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The fluorescent signal was measured by using an ABI Prism 7000 (Applied Biosystems, Foster City, CA). The genes encoding mouse TNF-α, IRS1, IRS2, and hypoxanthine phosphoribosyl transferase were amplified with the following primer pairs: 5′-GGTACAACCCATCGGCTGGCA-3′ (forward) and 5′-GCGACGTGGAACTGGCAGAAG-3′ (reverse) for TNF-α, 5′-ATAG CTCTGAGACCTTCTCAGCACCTAC-3′ (forward) and 5′-GGAGTTGCCCT CATTGCTGCCTAA-3′ (reverse) for IRS1, 5′-AGCCTGGGGATAATGGTG ACTATACCGA-3′ (forward) and 5′-TTGTGGGCAAAGGATGGGGACAC T-3′ (reverse) for IRS2, and 5′-CCAGCAAGCTTGCAACCTTAACCA-3′ (forward) and 5′-GTAATGATCAGTCAACGGGGGAC-3′ (reverse) for hypoxanthine phosphoribosyl transferase. Each PCR product was found as a single band with the correct size by agarose gel electrophoresis (data not shown).

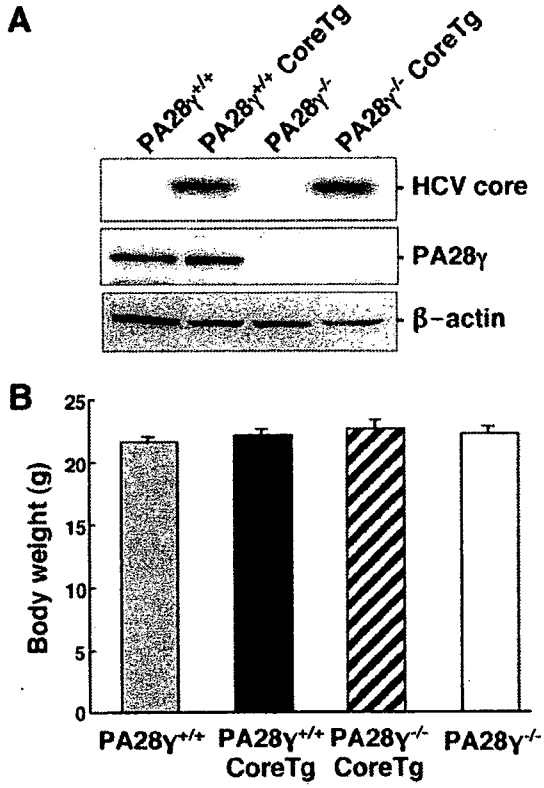

FIG. 1. Characterization of HCV core gene-transgenic mice deficient in the PA28γ gene. (A) Expression of the HCV core protein and PA28γ in the livers of $PA28\gamma^{+/+}$, $PA28\gamma^{+/+}$CoreTg, $PA28\gamma^{-/-}$, and $PA28\gamma^{-/-}$CoreTg mice. Lysates obtained from liver tissues of the mice (100 μg protein/lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting using antibodies to the HCV core protein, PA28γ, and β-actin. (B) Body weights of the mice. Body weights of 2-month-old mice were measured ($n = 7$ in each group). There were no statistically significant differences in body weights among the mice ($P > 0.05$).

Reporter assay for TNF-α promoter activity. The promoter region of the TNF-α gene (located from residues −1260 to +140) was amplified from mouse genomic DNA and was then introduced into the KpnI and BglII sites of pGL3-Basic (Promega, Madison, WI) (25). The resulting plasmid was designated as pGL3-tnf-aPro. The gene encoding the HCV core protein was amplified from HCV strain J1 (genotype 1b) and cloned into pCAG-GS (1, 38). To avoid contamination with endotoxin from *Escherichia coli*, the plasmid DNA was purified by using an EndoFree Plasmid Maxi kit (QIAGEN). The total amount of transfected DNA was normalized by the addition of empty plasmids. Plasmid vector was transfected into hepatoma cell lines by lipofection using Lipofectamine 2000 (Invitrogen). Cells were harvested at 24 h posttransfection. Luciferase activity was determined by using the Dual-Luciferase Reporter Assay system (Promega). Firefly luciferase activity was normalized to coexpressed *Renilla* luciferase activity. The amount of firefly luciferase activity was presented as the increase (n-fold) relative to the value for the sample lacking the HCV core protein, which was taken to be 1.0. PA28γ-knockdown cell lines were established by using pSilencer 2.1 U6 Hygro (Ambion) according to the manufacturer's protocol.

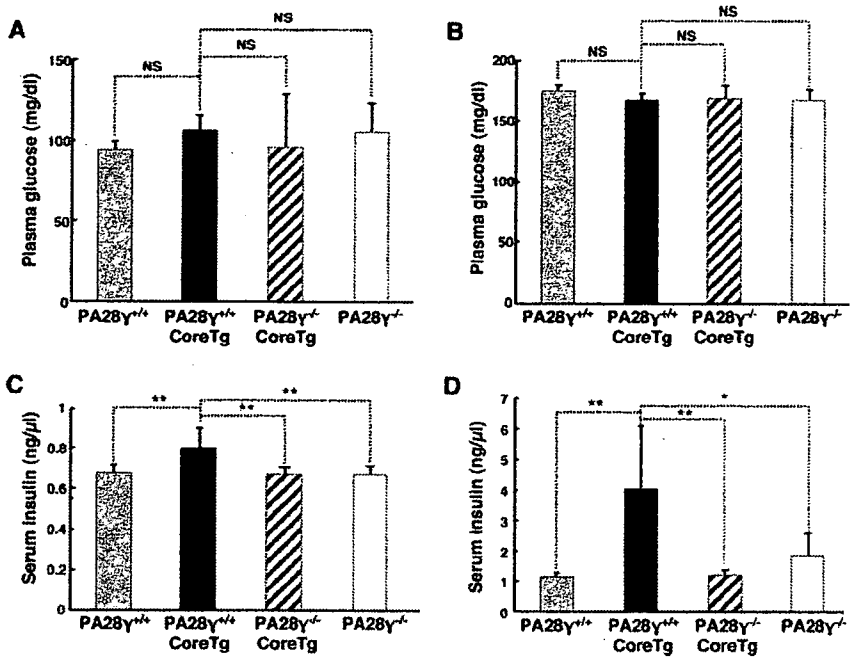

FIG. 2. Knockout of the PA28γ gene inhibited the hyperinsulinemia induced by HCV core protein. Plasma glucose levels of PA28$\gamma^{+/+}$, PA28$\gamma^{+/+}$CoreTg, PA28$\gamma^{-/-}$CoreTg, and PA28$\gamma^{-/-}$ mice under fasting (A) or fed (B) conditions (n = 7 in each group) are shown. Serum insulin levels in fasting (C) or fed (D) mice (n = 7 in each group) are also shown. Values are represented as means ± standard deviations. *$P < 0.05$; **$P < 0.01$. NS, not statistically significant.

Statistical analysis. The results are presented as means ± standard deviations. The significance of the differences was determined by Student's t test. P values of <0.05 were considered statistically significant.

RESULTS

HCV core gene-transgenic mice deficient in the PA28γ gene. To investigate the role of PA28γ in the development of insulin resistance in HCV core gene-transgenic (PA28$\gamma^{+/+}$CoreTg) mice, we generated HCV core gene-transgenic mice deficient in the PA28γ gene (PA28$\gamma^{-/-}$CoreTg). A PA28$\gamma^{+/+}$CoreTg mouse expressing an amount of PA28γ equal to that of its normal littermates (Fig. 1A) was crossbred with a PA28$\gamma^{-/-}$ mouse to generate a PA28$\gamma^{+/-}$ CoreTg mouse. PA28$\gamma^{+/-}$ CoreTg mice were bred with each other, and a PA28$\gamma^{-/-}$ CoreTg mouse was selected by PCR. The HCV core protein was expressed in PA28$\gamma^{+/+}$CoreTg and PA28$\gamma^{-/-}$CoreTg

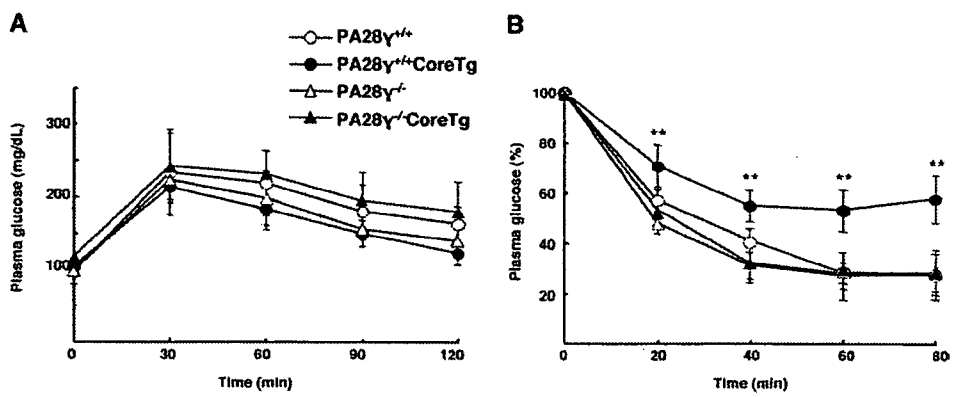

FIG. 3. Knockout of the PA28γ gene inhibits the insulin resistance induced by the HCV core protein. (A) Glucose tolerance test. D-Glucose was intraperitoneally administered to mice fasted for more than 16 h at 1 g/kg of body weight. Plasma glucose levels were estimated at the indicated times (n = 5 in each group). There were no significant differences in glucose levels among the mice ($P > 0.05$). (B) Insulin tolerance test. Human insulin (2 units/kg body weight) was intraperitoneally administered to the mice, and the plasma glucose levels were estimated at the indicated times. Values were normalized to the baseline glucose concentration at the time of insulin administration (n = 5 in each group). The values for the PA28$\gamma^{+/+}$ (open circles), PA28$\gamma^{+/+}$CoreTg (closed circles), PA28$\gamma^{-/-}$ (open triangles), and PA28$\gamma^{-/-}$CoreTg (closed triangles) mice are represented as means and ± standard deviations. Significant differences in insulin sensitivity ($P < 0.01$) in PA28$\gamma^{+/+}$CoreTg mice compared to that in PA28$\gamma^{+/+}$, PA28$\gamma^{-/-}$, or PA28$\gamma^{-/-}$CoreTg mice are indicated by double asterisks (**). There were no significant differences among PA28$\gamma^{+/+}$, PA28$\gamma^{-/-}$, and PA28$\gamma^{-/-}$CoreTg mice ($P > 0.05$).

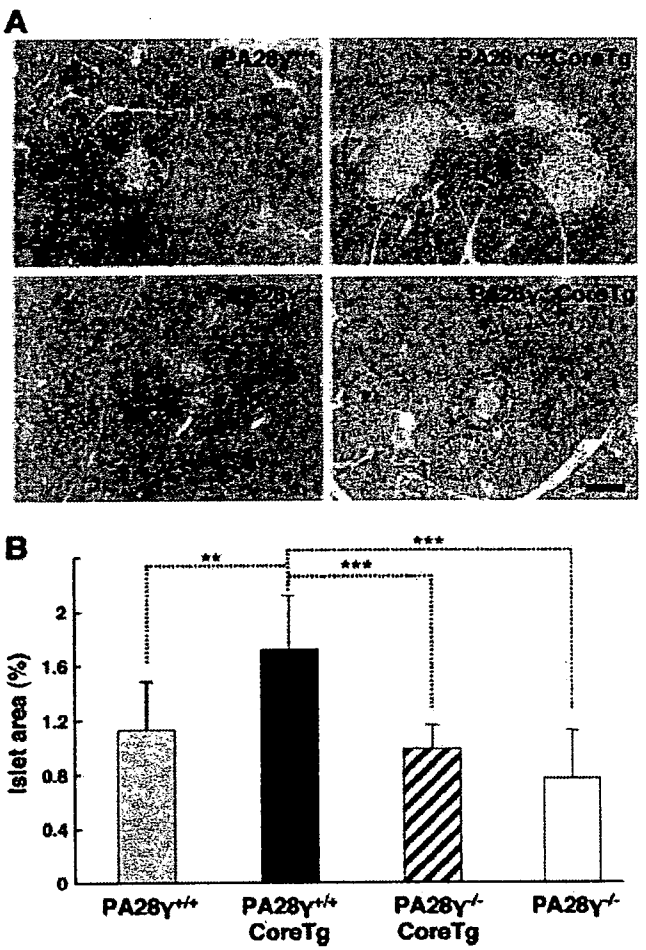

FIG. 4. PA28γ participated in the enlargement of pancreatic islets induced by the HCV core protein. (A) Histological sections prepared from pancreas tissues of PA28$\gamma^{+/+}$, PA28$\gamma^{+/+}$CoreTg, PA28$\gamma^{-/-}$, and PA28$\gamma^{-/-}$CoreTg mice were stained with hematoxylin and eosin. Dotted circles indicate pancreatic islets. (B) The area occupied by pancreatic islets was measured by computer software in three different fields of every six randomly selected sections of 10 mice per genotype and is represented as a percentage of the total pancreatic area. $**P < 0.01$; $***P < 0.001$. The scale bar indicates 100 μm.

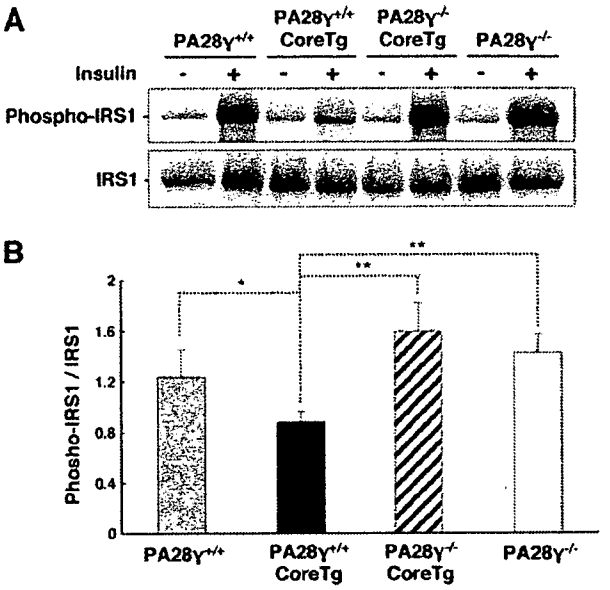

FIG. 5. PA28γ participated in the inhibition of the tyrosine phosphorylation of IRS1 induced by the HCV core protein. Liver tissues from PA28$\gamma^{+/+}$, PA28$\gamma^{+/+}$CoreTg, PA28$\gamma^{-/-}$, and PA28$\gamma^{-/-}$CoreTg mice were prepared after administration of insulin (+) or phosphate-buffered saline (−). The samples (100 μg of total protein) were examined by immunoblotting with antibodies against IRS1 and phospho-Tyr608 of mouse IRS1 (A). Phosphorylated IRS1 was estimated from the density on the immunoblotted membrane by using computer software (B) ($n = 5$ in each group). The data presented are representative of three independent experiments. $*P < 0.05$; $**P < 0.01$.

mice but not in PA28$\gamma^{+/+}$ (normal littermates) or PA28$\gamma^{-/-}$ mice. PA28γ was found at a similar level in PA28$\gamma^{+/+}$CoreTg and PA28$\gamma^{+/+}$ mice but was not present in either PA28$\gamma^{-/-}$ or PA28$\gamma^{-/-}$CoreTg mice (Fig. 1A). The expression of the HCV core protein in the livers of 2-month-old male mice was slightly higher in PA28$\gamma^{-/-}$CoreTg (1.36 ± 0.44 ng/mg of total protein; $n = 7$) than in PA28$\gamma^{+/+}$CoreTg (1.23 ± 0.22 ng/mg of total protein; $n = 7$) mice, but these values were not significantly different ($P > 0.05$). Insulin sensitivity is dependent on several conditions such as body weight, obesity, and liver steatosis (26). PA28$\gamma^{-/-}$ mice were slightly smaller than their normal littermates (PA28$\gamma^{+/+}$) at more than 3 months old, as described previously (36), but this was not significantly different in 2-month-old mice (Fig. 1B). PA28$\gamma^{+/+}$CoreTg mice exhibited severe hepatic steatosis from 4 months of age (35). To avoid the influence of hepatic steatosis and body weight on the examination of insulin resistance, 2-month-old mice were used in this study. Figure 1B shows the body weights of 2-month-old mice. There were no significant differences in body weight among PA28$\gamma^{+/+}$CoreTg, PA28$\gamma^{-/-}$CoreTg, PA28$\gamma^{-/-}$, and PA28$\gamma^{+/+}$ mice. Steatosis was not detected in the livers of the 2-month-old mice (data not shown).

PA28γ is involved in the development of hyperinsulinemia and insulin resistance in PA28$\gamma^{+/+}$CoreTg mice. In our previous study, we found a significant difference in serum insulin levels, but not in plasma glucose levels, between PA28$\gamma^{+/+}$CoreTg mice and normal littermates (47). To determine the involvement of PA28γ in the development of insulin resistance in PA28$\gamma^{+/+}$CoreTg mice, we examined here the plasma glucose and insulin levels in the mice under fasting and fed conditions. Although no significant difference in plasma glucose levels was observed in the mice under either fasting (Fig. 2A) or fed (Fig. 2B) conditions, serum insulin levels were significantly higher in PA28$\gamma^{+/+}$CoreTg mice than in PA28$\gamma^{+/+}$ mice under both conditions (Fig. 2C and D), as described previously (47). In contrast, the serum insulin concentration in PA28$\gamma^{-/-}$CoreTg mice was recovered to a normal level similar to that of PA28$\gamma^{+/+}$ and PA28$\gamma^{-/-}$ mice under either fasting (Fig. 2C) or fed (Fig. 2D) conditions.

To determine the glucose intolerance among the mice, glucose was administered to the mice after fasting, and the plasma glucose level was then determined. There was no significant difference among the genotypes at any time point in the glucose tolerance test (Fig. 3A), suggesting that the volume of glucose was maintained at a normal level by the higher concentration of insulin in PA28$\gamma^{+/+}$CoreTg mice. In our previ-

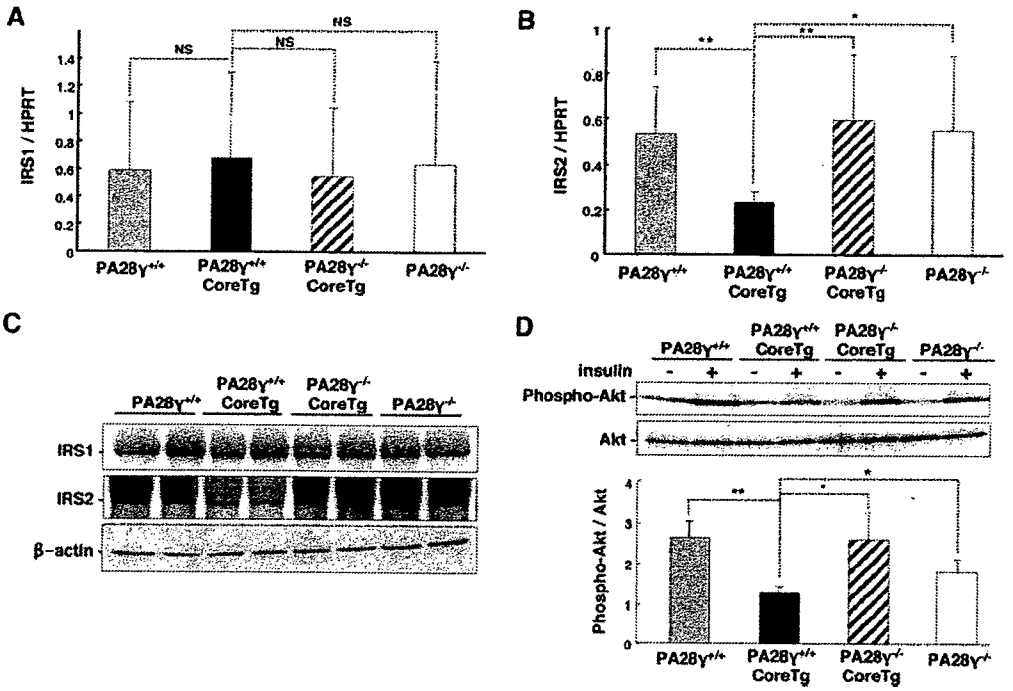

FIG. 6. PA28γ participated in the inhibition of the IRS2 expression and Akt phosphorylation induced by HCV core protein. The transcription of IRS1 (A) and IRS2 (B) was estimated by quantitative RT-PCR ($n = 5$ in each group). (C) The expression levels of IRS1 and IRS2 in the livers of the mice were determined by immunoblotting with specific antibodies. (D) Phosphorylation of Akt in the livers of the mice was examined by immunoblotting with antibodies against Akt and phosphorylated Akt. The ratio of Akt phosphorylation was determined by computer software based on the densities of phosphorylated Akt and a total amount of Akt ($n = 3$ in each group). The data presented are representative of three independent experiments. $*P < 0.05$; $**P < 0.01$. NS, not statistically significant; HPRT, hypoxanthine phosphoribosyl transferase.

ous study, the reduction in the plasma glucose concentration after insulin administration was impaired in PA28γ$^{+/+}$CoreTg mice (47). In this study, PA28γ$^{-/-}$CoreTg mice exhibited a normal insulin level comparable to those of PA28γ$^{+/+}$ and PA28γ$^{-/-}$ mice by an insulin tolerance test, in contrast to PA28γ$^{+/+}$CoreTg mice, in which a high concentration of plasma glucose was detected at all time points, as previously reported (Fig. 3B). These data suggest that hyperinsulinemia was induced in PA28γ$^{+/+}$CoreTg mice to compensate for insulin resistance and retain a physiological level of plasma glucose and that PA28γ participates in the development of hyperinsulinemia and insulin resistance in PA28γ$^{+/+}$CoreTg mice.

Morphology of pancreatic islets. Hyperinsulinemia and insulin resistance are expected to enlarge the pancreatic islet mass due to the overexpression of insulin. Our previous report showed the enlargement of the pancreatic islets in PA28γ$^{+/+}$ CoreTg mice. To clarify whether a knockout of the PA28γ gene restores the enlarged pancreatic islets to their normal size, the morphology of the pancreatic islets of the mice was evaluated by histologic examination (Fig. 4A). The relative islet area in the pancreatic cells of the PA28γ$^{-/-}$CoreTg mice was smaller than that of PA28γ$^{+/+}$CoreTg mice and comparable to that of PA28γ$^{+/+}$ and PA28γ$^{-/-}$ mice (Fig. 4B). Infiltration of inflammatory cells within or surrounding the islets was not found in all genotypes of mice. These results suggest that PA28γ also participates in the enlargement of pancreatic islets induced in PA28γ$^{+/+}$CoreTg mice.

PA28γ impairs the insulin-signaling pathway through the suppression of both tyrosine phosphorylation of IRS1 and expression of IRS2. Insulin binds to insulin receptors, resulting in the activation of downstream signaling (26). The activated insulin receptors phosphorylate themselves, IRS1, and IRS2. Phosphorylated IRS1 and IRS2 can activate phosphatidylinositol 3 (PI3)-kinase signaling, leading to the activation of glucose metabolism and cell growth. Our previous report showed that tyrosine phosphorylation of IRS1 is suppressed in the livers of PA28γ$^{+/+}$CoreTg mice and that the administration of anti-TNF-α antibody restores insulin sensitivity (47). We examined whether a knockout of the PA28γ gene could restore the tyrosine phosphorylation of IRS1. Tyrosine phosphorylation of IRS1 was suppressed in the livers of PA28γ$^{+/+}$ CoreTg mice in response to insulin stimulation, whereas it was recovered in PA28γ$^{-/-}$CoreTg mice to levels comparable to those in PA28γ$^{+/+}$and PA28γ$^{-/-}$ mice (Fig. 5).

Chronic hyperinsulinemia downregulates the expression of IRS2, which is one of the essential components of the insulin-signaling pathway in the liver (46). However, in our previous study, we showed that there was no significant difference in the phosphorylation of IRS2 between PA28γ$^{+/+}$CoreTg mice and their normal littermates (47). To gain more insight into the mechanisms of regulation of IRS expression, we determined the transcription and translation of IRS1 and IRS2 in the livers of the mice by real-time PCR and Western blotting, respectively. Although there was no significant difference in IRS1 expression at either the transcriptional or translational level among the mice

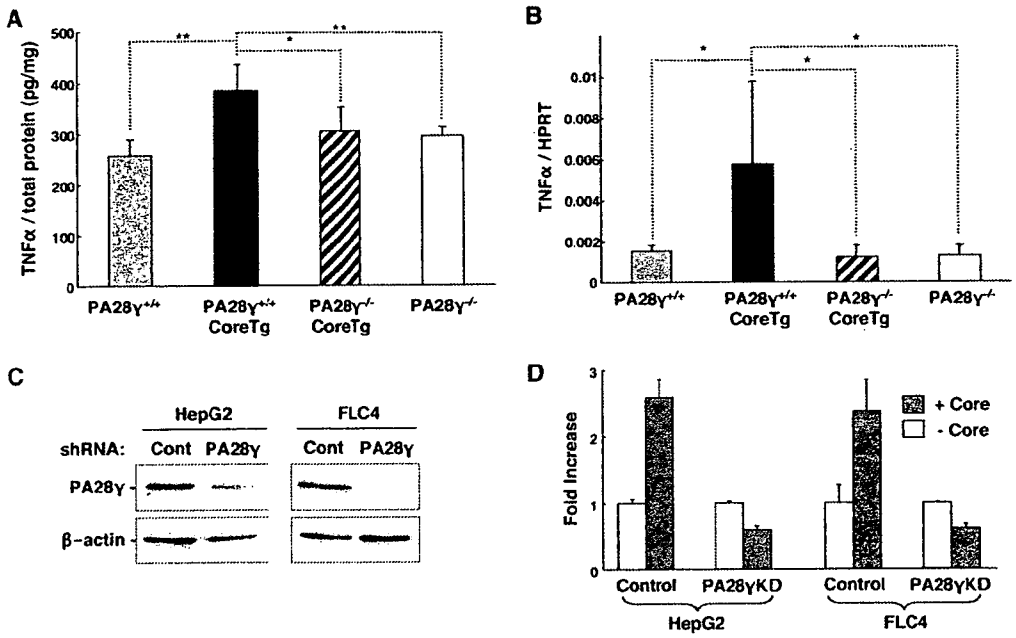

FIG. 7. PA28γ was required for activation of the TNF-α promoter by the HCV core protein. (A) Expression of TNF-α in the livers of mice was determined by ELISA (n = 5 in each group). (B) TNF-α mRNA in the livers of mice was examined by quantitative RT-PCR (n = 5 in each group). (C) Knockdown of the expression of PA28γ in the HepG2 and FLC-4 cell lines by the introduction of a plasmid encoding a short hairpin RNA (shRNA) targeted to the PA28γ gene. The expression levels of PA28γ and β-actin were determined by immunoblotting with specific antibodies. (D) Promoter activity of TNF-α in the presence or absence of the HCV core protein was determined by luciferase assay in the PA28γ-knockdown and control cell lines. The data presented are representative of three independent experiments. HPRT, hypoxanthine phosphoribosyl transferase.

(Fig. 6A and C), the expression of IRS2 was clearly impaired in PA28γ$^{+/+}$CoreTg mice at both the transcriptional and translational levels compared with that in other mice (Fig. 6B and C). The serine/threonine protein kinase Akt is phosphorylated by phosphoinositide-dependent kinase 1 (PDK1) under the activated condition of IRS family proteins (26). The insulin-induced phosphorylation of Akt was suppressed in the livers of PA28γ$^{+/+}$ CoreTg mice but not in those of PA28γ$^{+/+}$, PA28γ$^{-/-}$, or PA28γ$^{-/-}$CoreTg mice (Fig. 6D). These results suggest that the expression of the HCV core protein in the livers of mice in the presence of PA28γ impairs the insulin-signaling pathway through the suppression of both the tyrosine phosphorylation of IRS1 and the expression of IRS2.

PA28γ is required for activation of the TNF-α promoter by HCV core protein. TNF-α is an adipokine (54) and suppresses the signaling pathway of IRS1 and IRS2 (14, 42). Several reports suggested that the serum TNF-α level is higher in HCV patients than in healthy individuals (19, 37). Elevations of TNF-α levels have also been demonstrated in the livers of PA28γ$^{+/+}$CoreTg mice (47). To determine the involvement of PA28γ in the enhancement of TNF-α expression, the expression of TNF-α in the livers of each genotype was determined by ELISA and real-time PCR (Fig. 7A and B). Transcription and translation of TNF-α were increased in the livers of PA28γ$^{+/+}$ CoreTg mice but were restored in the livers of PA28γ$^{-/-}$ CoreTg mice to levels comparable to those of PA28γ$^{+/+}$ and PA28γ$^{-/-}$ mice. To determine the effect of PA28γ expression on the promoter activity of TNF-α in human liver cells, PA28γ-knockdown human hepatoma cell lines HepG2 and FLC4 were established by the introduction of a plasmid encoding a short hairpin RNA targeting the PA28γ gene in the cell lines. The expression of PA28γ was clearly suppressed in the cell lines (Fig. 7C). The expression of HCV core protein in the hepatoma cell lines potentiated TNF-α promoter activity, whereas the promoter activation by the HCV core protein was suppressed in the PA28γ-knockdown cell lines (Fig. 7D). These results suggest that PA28γ is required for the activation of the TNF-α promoter induced by the expression of the HCV core protein in human hepatoma cell lines.

DISCUSSION

HCV infection has a close association with type 2 diabetes, which is a polygenic disease with a pathophysiology that includes a defect in insulin secretion, increased hepatic glucose production, and resistance to the action of insulin (2, 8, 18). Insulin binds to insulin receptors, which exhibit tyrosine kinase activity, leading to the autophosphorylation and phosphorylation of IRS (56). Tyrosine phosphorylation in IRS proteins leads to the interaction between IRS proteins and the regulatory subunit p85 of PI3-kinase, which enhances glucose uptake and inhibits lypolysis (21). Activated PI3-kinase phosphorylates phosphatidylinositol 4,5-biphosphate to produce phosphatidylinositol 3,4,5-triphosphate, which contributes to the activation of PDK1 (55). Activated PDK1 phosphorylates downstream substrates including Akt and other kinases (55). A diabetic phenotype that included insulin resistance was found in IRS2-knockout mice with normal growth (57), although a

knockout of the IRS1 gene has been shown to lead to growth retardation and insulin resistance but not overt diabetes (5, 52). The double knockdown of IRS1 and IRS2 genes in the liver induces hyperinsulinemia and insulin resistance in mice (53). The reduction of both IRS1 and IRS2 under conditions of insulin resistance and hyperinsulinemia (3) and in the livers of *ob/ob* mice, an obese diabetic mouse model (20), has been reported previously. In the present study, the expression of the HCV core protein reduced the phosphorylation of tyrosine on IRS1 and the production of IRS2 in the livers of mice but did not completely abolish the activities of these genes, suggesting that residual activities of IRS transfer a faint signal to the downstream region of IRS. Therefore, PA28γ$^{+/+}$CoreTg mice may exhibit a milder phenotype than IRS1- and/or IRS2-knockout mice. In this study, knockout of the PA28γ gene restored the insulin sensitivity and signaling of IRS1 and IRS2 in PA28γ$^{+/+}$CoreTg mice, suggesting that the expression of the HCV core protein leads to the dysfunction of both IRS1 and IRS2 through a PA28γ-dependent pathway.

Our previous study suggested that the induction of TNF-α by the HCV core protein plays a role in insulin resistance (47). An increase in TNF-α levels has been correlated with obesity and insulin resistance in animal models and humans (14, 42). However, the mechanism by which TNF-α induces insulin resistance is not completely known. The expression of TNF-α has been shown to be increased in PA28γ$^{+/+}$CoreTg mice, resulting in the suppression of phosphorylation of IRS1, and insulin sensitivity in PA28γ$^{+/+}$CoreTg was improved by the administration of an anti-TNF-α antibody (47). In the present study, the expression level of TNF-α in PA28γ$^{-/-}$CoreTg mice was similar to that in PA28γ$^{-/-}$ mice or their normal littermates. The expression of the HCV core protein enhanced the promoter activity of the TNF-α gene in human liver cell lines but not in those with a knockdown of the PA28γ gene by RNA interference (Fig. 7D). These data suggest that PA28γ plays a crucial role in HCV core-induced expression of TNF-α. Sterol regulatory element-binding proteins (SREBPs) were shown to be increased at the stage of viremia in HCV-infected chimpanzees (49). SREBPs are known to regulate not only the biosynthesis of lipid but also the transcription of IRS2 and TNF-α (17, 45). Therefore, it might be feasible to speculate that the HCV core protein may cooperate with PA28γ to regulate the expression of SREBPs.

Houstis et al. previously reported that reactive oxygen species (ROS) are increased in both cellular and mouse models of insulin resistance induced by treatment with TNF-α or dexamethasone and that insulin sensitivity was restored by treatment with small antioxidant molecules (16). The HCV core protein potentiates ROS production in hepatoma cells and HCV core gene-transgenic mice (23, 34, 41). Accelerated production of ROS results in mitochondrion dysfunction, which contributes to a decrease in fatty acid oxidation. Defects in mitochondrial fatty acid oxidation enhance the production of intracellular fatty acyl coenzyme A (CoA) and diacylglycerol (48, 58). Mitochondrion dysfunction and accumulation of lipid droplets in mice expressing the HCV core or the full-length HCV polyprotein have been reported (27, 34). An increase in lipid droplets also leads to the accumulation of fatty acid CoA and diacylglycerol (48, 58). Fatty acyl CoA and diacylglycerol nonspecifically activate the Ser/Thr kinase cascade, leading to the enhancement of the serine phosphorylation of IRS1 (26). Serine phosphorylation on IRS1 blocks the tyrosine phosphorylation of IRS1 by insulin receptors (26). In the present study, however, serine phosphorylation of IRS1 in PA28γ$^{+/+}$ CoreTg mice was similar to that in PA28γ$^{-/-}$CoreTg mice (data not shown). TNF-α signaling pathways other than the accumulation of ROS and fatty acid intermediates may also participate in the inhibition of tyrosine phosphorylation on IRS1 in PA28γ$^{+/+}$ CoreTg mice.

How does the HCV core protein induce TNF-α production? Our previous report suggests that the HCV core protein is degraded through a PA28γ-dependent pathway (32). Recently, PA28γ has been shown to participate in the proteasome-dependent degradation of steroid receptor coactivator 3 (28). Degradation products of the HCV core protein via the PA28γ-dependent pathway may regulate the promoter activity of the TNF-α gene. PA28 proteins are necessary and sufficient to fully reconstitute Hsp90-initiated refolding together with Hsc70 and Hsp40 (31). Therefore, it might also be feasible to speculate that the HCV core protein refolded by an Hsp90/PA28γ-dependent pathway activates the promoter of the TNF-α gene together with an unknown transcription factor(s) or regulator(s).

In conclusion, the data obtained in this study suggest that the expression of the HCV core protein enhances the production of TNF-α and suppresses the phosphorylation of tyrosine on IRS1 and the production of IRS2 through a PA28γ-dependent pathway, thereby leading to insulin resistance. PA28γ may be a novel target for the treatment of HCV-induced diabetes.

ACKNOWLEDGMENTS

We gratefully thank H. Murase for secretarial work.

This study was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare; the Ministry of Education, Culture, Sports, Science, and Technology; the Program for the Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO); the 21st Century Center of Excellence Program; and the Foundation for Biomedical Research and Innovation.

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Journal of Peptide Science
J. Pept. Sci. (2008)
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.1021

JOURNAL OF PEPTIDE SCIENCE

Short Communication

Facile detection of specific RNA-polypeptide interactions by MALDI-TOF mass spectrometry

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Received 19 September 2007; Revised 20 December 2007; Accepted 21 December 2007

Abstract: A simple method for the detection of specific RNA-polypeptide interactions using MALDI-TOF mass spectroscopy is described. Instead of direct observation of the RNA-polypeptide complex, we attempted the indirect observation of the binding event by focusing on the disappearance of the free polypeptide signal upon interaction with RNA. As a result, specific binding of the Rev-response element (RRE) RNA of the HIV with two RRE-binding peptide aptamers, DLA and RLA peptides, as well as the bacteriophage λ boxB RNA with the λ N peptide was observed. We also show that specific RNA-binding peptides can be identified from a mixture of peptides with varying RNA-binding affinity, showing that the method could be applied to high-throughput screening from simple peptide libraries. The method described in this study provides a quick and simple method for detecting specific RNA–polypeptide interactions that avoids difficulties associated with direct observation of RNA and RNA–polypeptide complexes, which may find various applications in the analysis of RNA–polypeptide interactions and in the identification of novel RNA-binding polypeptides. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Supplementary electronic material for this paper is available in Wiley InterScience at http://www.interscience.wiley.com/jpages/1075-2617/suppmat/

Keywords: RNA–polypeptide interactions; MALDI-TOF mass spectrometry; arginine-rich peptide; HIV RRE; lambda boxB; peptide library

INTRODUCTION

RNA–polypeptide interactions are intimately involved in various steps of gene regulation, and various methods for the detection and analysis of RNA–polypeptide interactions have been developed. However, *in vitro* methods such as calorimetry, gel mobility shift assays, surface plasmon resonance (SPR) require large quantities of substrate or radioactive/fluorescent labeling of either the RNA or polypeptide. Genetic methods for detecting RNA–polypeptide interactions such as the three-hybrid system [1] and the antitermination system [2] require cloning of sequences encoding the RNA and polypeptide of interest into plasmid vectors and yeast/bacterial culture. The development of simple methods for the detection of RNA–polypeptide complexes would be expected to facilitate the analysis of RNA–polypeptide interactions, and the identification of novel RNA-binding polypeptides.

The development of soft ionizing methods in mass spectrometry has made possible the simple and rapid detection of biological samples including RNA. MALDI-TOF mass spectrometry is the easiest and the most widely available method for the analysis of RNA [3]. However, in addition to the generally inefficient desorption of RNA, a number of difficulties that are common for oligonucleotides exist for the analysis of RNA. First, sample preparation is a key step in RNA analysis, as impurities can result in reduced sensitivity [4]. The choice of the proper combination of matrix material has been shown to be crucial for successful analysis, and 3-hydroxypicolinic acid (3-HPA) [5] and 2,4,6-trihydroxyacetophenone (2,4,6-THAP) [6] in combination with organic ammonium salts were found to be useful matrices. Organic ammonium salts such as ammonium citrate and ammonium tartrate were shown to suppress addition of alkali metals, which lead to the division of the molecular ion into multiple peaks ([M + Na] and [M + K]) and a reduction in resolution [6,7]. Ammonium salts have also been shown to suppress fragmentation of the oligonucleotides [7]. The 5′- and 3′-heterogeneity of T7 RNA transcripts has also been to implicated as a source of peak widening and low resolution [8]. Despite these advances, however, the sensitivity of RNA detection is much reduced compared to analysis of polypeptides of

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comparable size. While there has been a report that an enzymatically synthesized RNA of 461 nucleotides could be detected [8], there are few examples of the analysis of oligomers longer than 20 nucleotides.

In this study, the development of a novel method for the facile detection of specific RNA-polypeptide interactions by MALDI-TOF MS was attempted. While the detection of non-covalently linked complexes of short RNAs (9 to 19 nucleotides) and peptides (10 to 18 amino acids) has already been reported [9], simple and convenient analysis of such complexes was expected to be hampered by the difficulties associated with RNA as described above. We therefore focused on the disappearance of the signal for the more readily detectable polypeptide upon specific binding to RNA. In other words, it was expected that the signal for the RNA-binding peptide would not be detected when bound specifically to the RNA, while those peptides that do not bind specifically to the RNA would be observed, resulting in the indirect observation of specific RNA-polypeptide complex formation.

A 34-nucleotide RNA stem-loop from the Rev-response element (RRE) of HIV and RRE-binding peptides (DLA and RLA peptides) with differing affinities for the RRE [10], as well as the 19-mer boxB RNA from phage λ and the λ N peptide [11] were used as model RNA–polypeptide complexes (Figure 1). Using MALDI-TOF MS, it was shown, as expected, that specific RNA-binding by RRE-binding peptides and the λ N peptide could be observed by the disappearance of the peptide signal upon mixing with RNA. It was also shown that the identification of RNA-binding peptides from mixtures of peptides with differing RNA-binding affinity is possible.

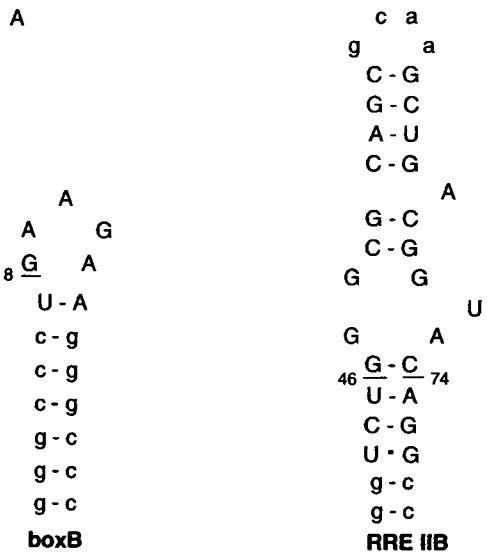

Figure 1 RNAs and RNA-binding peptide. (A) Secondary structures of the boxB RNA and the RRE IIB RNA. The underlined nucleotides are substituted in mutant RNAs. (B) RNA-binding peptides. The DLA and RLA peptides bind to RRE, the N peptide binds to boxB RNA. The underlined amino acids were not included in the synthetic peptide.

RESULTS AND DISCUSSION

Confirmation of RNA-peptide Interactions Using a Bacterial Reporter System

The interactions of the RNAs and peptides used in the present study were confirmed using a bacterial reporter system that utilizes the bacteriophage λ N protein mediated antitermination to monitor RNA–polypeptide interaction (Table 1) [2]. The pBR plasmids encoding the DLA, RLA or λ N peptides were transformed into pAC LacZ reporter cells encoding either the RRE or boxB RNA (Figure 1), and antitermination activity was assayed on tryptone plates containing X-gal. The intensity of colony color represented as plusses has been shown to correlate with binding affinity, where the interaction between the RRE RNA and the DLA peptide (Kd = 0.5 nM) reports an antitermination activity of 6+ [10], while that of the RRE and the RLA peptide (Kd = 30 nM) was 3.5+ [10], and that of the λ boxB RNA and the λ N peptide (Kd = 20 nM) was 8+ [11]. On

Table 1 The *in vivo* activities (X-gal assay) and Kd values (nM) of the DLA, RLA, λ N peptides with the boxB and RRE IIB

	X-gal (Kd, nM)[a]					
	DLA		RLA		λ N	
RRE	++++++	(0.5)	+++(+)	(30)	—	
RRE C46G74						
boxB	—		—		++++++++	(20)
boxB G8A						(>2560)

[a] The number of plusses indicates blue color scored by the colony color (X-gal) assay.

DOI: 10.1002/psc

the other hand, the interaction between the RRE and the λ N peptide, as well as the interaction between the boxB with both the DLA and RLA peptides did not show antiterrmination activity, and could not be detected.

Optimization of the Matrix Material for RNA and Polypeptide Detection

A number of different matrices, α-cyano-4-hydroxy-cinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), 3-HPA, 2,3,4-THAP and 2,4,6-THAP, were tested for the analysis of the above mentioned RNAs, peptides and RNA–peptide complexes in both the negative and positive-ion mode. CHCA is widely used for the analysis of various materials [12], and DHB is used for DNA and sugar analysis [13].

All measurements were repeated three times and contained a synthetic peptide, P14R peptide (MW 1534) or angiotensin II (MW 1046), as an internal standard. Ammonium citrate was included to suppress addition of alkali metals. Only peaks of the peptides were observed clearly with CHCA, DHB and 2,4,6-THAP, and were similar in both ion modes. The signals of the RNAs were weak with all matrices, and the RNA–peptide complex, RRE–DLA complex, could only be detected with 2,4,6-THAP in positive-ion mode. Therefore, all of the following mass analyses were performed with 2,4,6-THAP in the positive-ion mode.

The Detection of RNA–polypeptide Complex Formation by the Disappearance of the Free-Peptide Signal

We tried to detect RNA–peptide complex formation by monitoring the decrease of the signal corresponding to the peptide, using the P14R peptide as an internal standard. When ammonium citrate and 2,4,6-THAP were added immediately after mixing the RRE RNA and the DLA peptide to form a solid solution, the peak of the free peptide was observed (Figure 2(A)). When ammonium citrate and 2,4,6-THAP were added after a 10-min incubation of the RNA and peptide at room temperature or on ice, followed by solid solution formation, the peak of the RNA-binding peptide was not detected (Figure 2(B)). In addition, the intensity of the peak corresponding to the DLA peptide decreased as the molar ratio of RRE RNA added was gradually increased in 0.25 molar equivalent increments (Figure S1, supporting data). This suggested that, as expected, upon binding of the DLA peptide with the RRE RNA, the DLA peptide was no longer released upon laser desorption, thereby providing a means to indirectly detect the RNA–peptide binding event. Therefore, all of the following mass analyses were performed after a ten-minute incubation of the mixture of RNA and peptide at room temperature.

The Detection of Specific RNA–peptide Interactions

First, the interaction of HIV RRE RNA and RRE-binding peptides was examined. When a mixture of the DLA peptide, which binds to RRE with high affinity and high specificity (Kd = 0.5 nM, Table 1), was analyzed, the DLA peptide showed a mass peak with an intensity comparable to that of P14R, the internal standard (Figure 3(A)). In comparison, upon addition of the RRE RNA to this mixture, a complete disappearance of the signal corresponding to the DLA peptide was observed, while the peak of the P14R peptide remained unchanged (Figure 3(B)), as also observed in Figure 2(B). On the other hand, when an RRE mutant (C46G74) that does not bind to the DLA peptide was added, no effect on the DLA signal was observed (Figure 3(C)). This suggested that the disappearance of the DLA peak upon addition of the wild-type RRE represented a specific binding event, and was not due to non-specific binding. Next, when a mixture of the RRE RNA and the RLA peptide, which binds to the RRE with a Kd of 30 nM (Table 1), was analyzed, the peak corresponding to the RLA peptide disappeared (Figure 3(D), (E)). Furthermore, as in the case of the DLA peptide described above, the signal corresponding

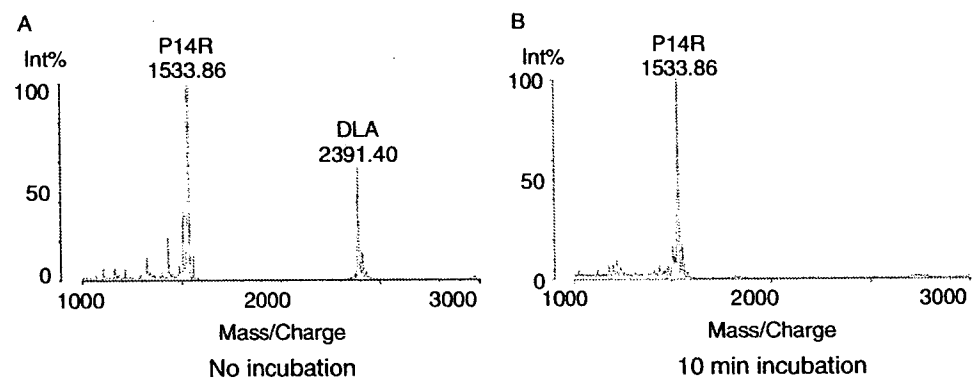

Figure 2 MALDI-TOF mass spectra of DLA peptide-RRE RNA. (A) No incubation (B) 10-min incubation. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

J. Pept. Sci. (2008)
DOI: 10.1002/psc