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Systematic Identification of Hepatocellular Proteins Interacting with NS5A of the Hepatitis C Virus

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The hepatitis C virus is associated with the development of liver cirrhosis and hepatocellular carcinomas. Among the 10 polyproteins produced by the virus, no function has been clearly assigned to the non-structural 5A (NS5A) protein. This study was designed to identify the hepatocellular proteins that interact with NS5A of the HCV. Yeast two-hybrid experiments were performed with a human liver cDNA prey-library, using five different NS5A derivatives as baits, the full-length NS5A (NS5A-F, amino acid (aa) 1~447) and its four different derivatives, denoted as NS5A-A (aa 1~150), -B (aa 1~300), -C (aa 300~447) and D (aa 150~447). NS5A-F, NS5A-B and NS5A-C gave two, two and 10 candidate clones, respectively, including an AHNAK-related protein, the secreted frizzled-related protein 4 (SFRP4), the N-*myc* downstream regulated gene 1 (NDRG1), the cellular retinoic acid binding protein 1 (CRABP-1), ferritin heavy chain (FTH1), translokin, tumor-associated calcium signal transducer 2 (TACSTD2), phosphatidylinositol 4-kinase (PI4K) and centaurinδ 2 (CENTδ2). However, NS5A-A produced no candidates and NS5A-D was not suitable as bait due to transcriptional activity. Based on an *in vitro* binding assay, CRABP-1, PI4K, CENTδ2 and two unknown fusion proteins with maltose binding protein (MBP), were confirmed to interact with the glutathione *S*-transferase (GST)/NS5A fusion protein. Furthermore, the interactions of CRABP-1, PI4K and CENTδ2 were not related to the P*XX*P motif (class II), as judged by a domain analysis. While their biological relevance is under investigation, the results contribute to a better understanding of the possible role of NS5A in hepatocellular signaling pathways.

Keywords: HCV, NS5A, P*XX*P domain, Yeast two-hybrid

Introduction

The hepatitis C virus (HCV) is the major etiologic agent of non-A and non-B hepatitis (Kim, 2002). Chronic infection with HCV results in the development of liver cirrhosis and hepatocellular carcinomas (Tong *et al*., 1995). HCV belongs to the *Flaviviridae* family, having a 9.5-kb positive-sense RNA genome (Choo *et al*., 1989). The RNA encodes a polyprotein (3,010 amino acids) in the following gene order: NH_2-Core-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (Grakoui *et al*., 1993). Processing of the precursor polyprotein requires both host and viral proteases to produce the structural (core, E1, E2 and p7) and non-structural (NS2, NS3/4A, NS4B, NS5A and NS5B) proteins of the virion (Bartenschlager and Lohmann, 2000). The non-structural NS5A protein is generated in the form of a mature protein by the action of NS3/4A serine-protease.

NS5A has generated considerable interest because it interacts with a wide variety of host signaling proteins and, as such, may play a role in immune invasion and carcinogenesis (Street *et al*., 2004). One indication that NS5A may play a role in cell signaling is the presence of polyproline (P*XX*P) motifs, which are highly conserved throughout a range of HCV genotypes. Of particular interest is the observation that the highly conserved C-terminal P*XX*P polyproline motif in NS5A is able to interact with the Src-homology (SH) 3 domains of the adaptor protein, Grb2 (growth factor receptor-

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bound protein 2), and members of the Src family of tyrosine kinases (Tan *et al*., 1999; Macdonald *et al*., 2004). In addition to these interactions, it has been reported that NS5A interacts with the Core protein of the HCV (Goh *et al*., 2001), p53 (Majumder *et al*., 2001), CDK1 (Arima *et al*., 2001), the SNF-2 related CBP activator protein (SRCAP) (Ghosh *et al*., 2000), karyopherin β3 (Chung *et al*., 2000), P85 phosphatidylinositol 3-kinase (He *et al*., 2002), a SNARE-like protein (hVAP-33, a 33 kDa human vesicle-associated membrane protein-associated protein) (Tu *et al*., 1999), apolipoproteins (Shi *et al*., 2002) and the TNF receptor-associated factor (TRAF) 2 (Park *et al*., 2002). The implications of these interactions are unclear, although they suggest that NS5A may play a role in the modulation of mitogenic and replication-related signaling pathways in HCV-infected hepatocytes.

As a first step in a systematic search for cellular proteins that interact with NS5A, a yeast two-hybrid system was employed, using NS5A-derivatives as bait. In this study, for the first time, the interactions of 14 new hepatocellular proteins with NS5A are reported. Although their biological relevance is currently under investigation, the results will contribute to a better understanding of the possible role of NS5A in liver cirrhosis and hepatocellular carcinomas.

Materials and Methods

Yeast two-hybrid screening In search of host proteins that interact with HCV NS5A, a yeast two-hybrid screening was performed, where the entire or partial cDNA-coding region of NS5A (genotype 1b, generously donated by Dr. Soichiro Miura at National Defense Medical College in Japan) was fused in-frame with the Lex DNA binding domain into the pHybLex/Zeo vector from Invitrogen (San Diego, USA) as bait for screening a liver cDNA library. The yeast two-hybrid screening was carried out following the protocol recommended by the manufacturer (Invitrogen, Hybrid Hunter #K5000-01) (Ahn *et al*., 2003). For the construction of the bait plasmid containing the full-length or partial cDNA of NS5A (NS5A-F, aa 1~447; NS5A-A, aa 1~150); NS5A-B, aa 1~300; NS5A-C, aa 300~447; NS5A-D, aa 150~447), PCR was performed, with the primers indicated in Table 1, and cloned into pHybLex/Zeo using the linker sites at both ends, as shown in Fig. 1A. Before full-scale screening, autonomous activation of the reporter gene (β-galactosidase) by the bait was assessed by a colony-lift filter assay, as described in the next section. The liver cDNA library in pYESTrp (500 μg, #A203-01) and the NS5A derivatives in pHybLex/Zeo (each 500 μg) were cotransfected into the L40 yeast strain (*MATa, his3Δ200 trp1-901 leu2-3112 ade2 LYS2::(4lexAop-HIS3) URA3::(8lexAop-lacZ)GAL4*), using the standard

Table 1. PCR primers used in this study

Primer name	Primer sequence	Restriction site	Size (mer)
A. PCR primers used in the construction of pHybLex/Zeo-NS5A-F			
NS5A(Full-F)	5'-C**GTCGAC**TGCCTCCGGCTCGTG-3'	*Sal* I	22
NS5A(Full-R)	5'-**GTCGAC**TCAGCAGCAGACGAC-3'	*Sal* I	21
B. PCR primers used in the construction of pHybLex/Zeo-NS5A-A, -B, -C and -D			
NS5A (1-F)	5'-**GCGGCCGC**TTTCCGGCTCGTGGCTAAGG-3'	*Not* I	28
NS5A(150-F)	5'-**GCGGCCGC**TTTTCACAGAATTGGATGGGGTGC-3'	*Not* I	32
NS5A(300-F)	5'-**GCGGCCGC**TTGCGGAGATCCTGCGGAGAT-3'	*Not* I	29
NS5A(150-R)	5'-**GTCGAC**TCATTAGAAGAATTCAGGGGCCGGAA-3'	*Sal* I	32
NS5A(300-R)	5'-**GTCGAC**TCATTACGCCGGAACGGATACTTCCC-3'	*Sal* I	32
NS5A(447-R)	5'-**GTCGAC**TCATTATCAGCAGCAGACGACGTCC-3'	*Sal* I	31
C. PCR primers used in the construction of pMAL-cri/C14,- C18, -C39, -C45 and -C78			
C14-F	5'-G **GAATTC**ATGCCCAACTTCGCCGGCACCTG-3'	*EcoR*I	30
C14-R	5'-G **GGATCC**TCATTCCCGGACATAAATTCTGG-3'	*BamH*I	30
C18-F	5'-G **GAATTC**ATGCGGCGTCTGCGGCCGCACCT-3'	*EcoR*I	30
C18-R	5'-G **GGATCC**TCATGGCGCTTCAGGGTCTCGCG-3'	*BamH*I	30
C39-F	5'-G **GAATTC**ATGGATACACCGTTAAGGCGCAG-3'	*EcoR*I	30
C39-R	5'-G **GGATCC**TCAGATCTTGGCTGCCGGCTGCT-3'	*BamH*I	30
C45-F	5'-G **GAATTC**ATGCAGAGTGCTGCAAAAGCCCC-3'	*EcoR*I	30
C45-R	5'-G **GGATCC**TCAGTAGGGGATGTCATTCTGAT-3'	*BamH*I	30
C78-F	5'-G **GAATTC**ATGGGGGACACGCTGTCGGAGCA-3'	*EcoR*I	30
C78-R	5'-G **GGATCC**TCAGACGTTGCGCAGAAGAGACA-3'	*BamH*I	30
D. PCR primers used in the construction of pHybLex/Zeo-NS5A-F-mut and NS5A-C-mut			
NS5A -mut1	5'-ATCGTCCTCTTCCTCCGTGCGGCTGCTATCGCAGCGGCCTTGGTAGGTG-3'		49
NS5A mut2	5'-CACCTACCA AGGCCGCTGCGATAGCAGCCGCACGGAGGAAGAGGACGAT-3'		49

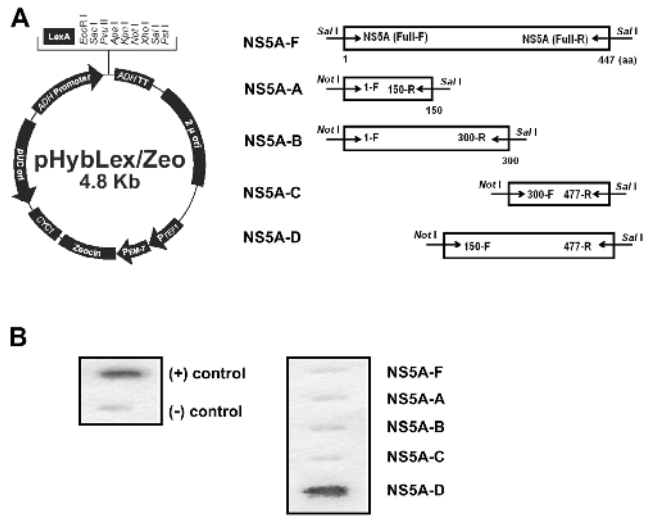

Fig. 1. Schematic drawings of the five different NS5A derivatives and a self-activation test. (A) The DNA fragments of the NS5A derivatives were PCR-amplified and subcloned into pHybLex/Zeo, using the enzyme sites indicated at the flaking ends of the PCR products. (B) The five NS5A derivatives were tested to determine their suitability as bait for full-scale yeast two-hybrid screening by a colony-lift filter assay. For the (+) control, pHybLex/Zeo-Fos2 and pYESTrp-Jun were contransfected into the L40 yeast strain, and for the (-) control pHybLex/Zeo-Lamin was transfected alone. The NS5A-D type was eliminated from screening because it showed self-activated transcriptional activity.

lithium acetate method. At least 5×10^6 transformed cells were screened for growth on SD/Zeocin agar plates (0.67% yeast nitrogen base without amino acid, 2% dextrose, 2% agar powder, 10X dropout solution without uracil, tryptophan, and histidine, supplements and 300 μg/ml Zeocin) over a 7-day period. Positive yeast transformants growing in the His-deficient medium were collected and replated for the β-galactosidase assay using the colony-lift filter procedure. For plasmid isolation, $His^+/LacZ^+$ positive colonies were grown, harvested and ruptured by vortexing with glass beads (mini-beadbeater). The pYESTrp plasmids containing the potential NS5A interacting cDNA inserts were transformed into *E. coli* DH5α (*SupE44ΔlacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1)* and selected on LB/ampicillin (50 μg/ml) plates. The plasmids were back-transformed into the L40 yeast strain and confirmed as $His^+/LacZ^+$ positive. Positive interacting cDNA clones were analyzed by nucleotide sequencing and compared with known DNA or protein sequences deposited in GenBank using a BLAST search (http://www.ncbi.nlm.nih.gov/ blast/).

Colony-lift filter assay For the colony-lift filter assay, yeast cells were grown on SD minimal plates, and transferred to a filter paper (Whatman no #1). The filter was placed in liquid nitrogen for 30 sec, and incubated at room temperature in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl and 1 mM $MgSO_4$), containing 0.82 mM 5-bromo-4-chloro-3-indolyl-β-*D*-galactopyranoside (X-Gal). The filters were placed at 30°C and monitored for color change as an indicator of the expressed β-galactosidase activity.

Construction of expression plasmids and protein expression For the construction of an expression plasmid producing the GST/NS5A-F fusion protein, pGEX-4T-2, from Amersham Bioscience (Buckinghamshire, UK), was used as the parent plasmid. The DNA fragment of NS5A-F in pHybLex/Zeo was digested with *Sal* I, and transferred into the *Sal* I linker site of pGEX-4T-2 in frame. For the construction of the expression plasmids producing the MBP/C14, C18, C39, C45 and C78 fusion proteins, pMAL-cri (New England Biolabs, Beverly, USA) was used as the parent plasmid. The partial or entire cDNA of the five C-series clones were PCR-amplified with the primers (Bioneer, Daejeon, Korea) indicated in Table 1, digested with *Eco*R I and *Bam*H I, and ligated into the *Eco*R I/*Bam*H I site of pMAL-cri. The resulting plasmids were transformed into *E. coli* DH5α for expression. The expression of the GST/NS5A and five MBP/C-series fusion proteins were induced by 0.5 mM isopropyl-β-*D*-thiogalactopyranoside (IPTG) and 1 mM maltose, respectively.

For the preparation of the fusion proteins, 100 ml (MBP/C-series fusion proteins) or 1 liter (GST/NS5A-F fusion protein) volume cultures were harvested and resuspended in NETN buffer (20 mM Tris/Cl pH 8.0, 1 mM EDTA, 100 mM NaCl and 0.5% (v/v) nonidet P (NP)-40), containing 10% glycerol, 0.2% Tween-20, 1 mM β-mercaptoethanol and protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μM pepstatin, 1 μM leupeptin and 10 nM aprotinin). The cells were lysed by sonication, and the supernatants clarified by centrifugation at 4°C. The expression of the GST/NS5A-F or MBP/C-series fusion proteins was analyzed by SDS-PAGE and Western blot analysis.

***In vitro* binding assay** For the detection of C-series proteins interacting with NS5A-F, a GST pull-down experiment was performed. Briefly, the supernatants, containing the GST/NS5A-F fusion protein, were immobilized on glutathione-Sepharose-4B beads (#17-0756-01, Amersham Bioscience, Richmond, USA) for 1 h at 4°C on a rotating mixer, and the unbound GST/NS5A-F washed out with 10 volumes of NETN buffer by brief centrifugation. The supernatants containing the MBP/C-series fusion proteins were then allowed to interact with the immobilized GST/NS5A-F for 12 h at 4°C. After 10 consecutive washes with 1 ml of NETN buffer containing 1% Triton X-100 and 0.5% NP-40, the beads were collected. Bound proteins were released by boiling in SDS sample buffer (62.5 mM Tris/Cl pH 6.8, 2% SDS and 10% glycerol), resolved by SDS-PAGE and transferred to membranes for Western blot analysis. A MBP pull-down experiment was performed *vice versa,* using amylose resin (#800-21C) (New England Biolabs).

Western blot analysis Each sample was resolved by 10% SDS-PAGE, transferred to PVDF membranes (#66543, Pall, East Hills, USA), analyzed with antibodies, according to the suppliers protocol, and visualized with peroxidase and an enhanced-chemiluminescence system (ECL Western blotting detection reagents #RPN2106) from Amersham Bioscience (Hebert *et al*., 2003). The rabbit polyclonal GST (Z-5, 1 : 2,000) and mouse MBP antibodies (MCA1412R, 1 : 2,000) were purchased from Santa Cruz Biotechnology (Santa Cruz, USA) and SeroTec (Oxford, UK), respectively. The rabbit or mouse secondary antibodies, conjugated with horseradish peroxidase (NA934 or

NA931, each 1 : 5,000 dilution), were purchased from Amersham Biosciences.

Mutation analysis For the construction of the pHybLex/Zeo-NS5A-F-mut, where five proline amino acids of the P*XX*P motif (class II) were all mutated into alanine, two PCR reactions were performed with the pair of PCR primers (Bioneer); NS5A-mut1/NS5A(447-R) and NS5A(1-F)/NS5A-mut2 (for sequences, refer to Table 1), using pHybLex/Zeo-NS5A-F as a template. A bloc PCR was then performed with the NS5A(1-F) and NS5A(447-R) primers, using the two PCR fragments as a template. The resulting PCR fragment was treated with *Not* I and *Sal* I, and ligated into the *Not* I/*Sal* I site of pHybLex/Zeo. For the construction of pHybLex/Zeo-NS5A-C-mut, PCR was performed with the NS5A(300-F) and NS5A(477-R) primers. The PCR fragment was treated with *Not* I and *Sal* I, and ligated into the *Not* I/*Sal* I site of pHybLex/Zeo. For the interaction analysis, pHybLex/Zeo-NS5A-F-mut or pHybLex/Zeo-NS5A-C-mut was cotransfected with pYESTrp-C45 and -C78, and analyzed by a colony-lift filter assay.

Results

Identification of hepatocellular proteins interacting with NS5A derivatives To identify the hepatocellular proteins that interacted with the HCV NS5A, a yeast two-hybrid system was exploited, with the human liver cDNA prey-library, using NS5A derivatives as bait. As shown in Fig. 1A, five different NS5A derivatives were constructed as bait; the full-length NS5A (NS5A-F, aa 1~447) and its four different derivatives, denoted as NS5A-A (aa 1~150), -B (aa 1~300), -C (aa 300~447) and D (aa 150~447). Of these, NS5A-D was not suitable as bait protein as it produced a blue color in the absence of a prey protein due to transcriptional activity (Fig. 1B) (Kato *et al*., 1997; Tanimoto *et al*., 1997).

At least 5×10^6 independent yeast colonies were screened for the four different NS5A derivatives, with the exception of NS5A-D. Primary selection for the $His^+/LacZ^+$ phenotype resulted in 89, 3, 10 and 82 positive clones for NS5A-F, NS5A-A, NS5A-B and NS5A-C, respectively. To further eliminate out-of-frame false positives, the cDNA inserts of the $His^+/LacZ^+$ liver cDNA clones were sequenced and analyzed by the BLAST search program. As shown in Table 2, two, two and 10 candidate clones were finally identified as interacting with NS5A-F, NS5A-B and NS5A-C, respectively. Their cDNA inserts (aa) are represented, along with their entire open reading frame (ORF), in Table 2. Intriguingly, NS5A-A produced no candidate clones, even though it contained the proline-rich P*XX*P (class I) motif (Wientjes and Segal, 2003).

***In vitro* binding of the five C-series cellular proteins with the full-length NS5A protein** Out of the 14 new cellular proteins that interacted with NS5A, five C-series proteins (C14, C18, C39, C45 and C78) were selected and analyzed for their *in vitro* binding by means of a pull-down assay. The DNA fragments from NS5A-F and the five C-series clones were subcloned into pGEX-4T-2 and pMAL-cri in frame, as indicated in Figs. 2A and 2B, respectively. The NS5A and C-series proteins were produced as GST and MBP fusion proteins in *E. coli*, and partially purified by single-step batch binding to glutathione-Sepharose4B beads and a maltose-amylose resin, respectively. The quality and quantity of the partially purified fusion proteins were assessed by Coomasie blue staining (Fig. 2C and 2D), and used for the next *in vitro* binding assay. The results showed that the fusion proteins had been successfully produced in a bacterial expression system.

For a pull-down experiment against the GST beads, bound proteins, through pull-down with GST beads, were eluted by

Table 2. List of cellular proteins interacting with NS5A-derivatives

Clone No.	Gene	Identified region / entire ORF (aa)	GI	Accession No.
F 82	AHNAK-related protein	71~293 / 533	535176	CAA52817
F 88	SFRP4 (Secreted frizzled-related protein 4)	69~315 / 346	4506895	NP_003005
B 01	NDRG1 (N-myc downstream regulated gene 1 protein)	123~261 / 394	13112001	AAH03174
B 03	MGP (matrix Gla protein)	1~103 / 103	13528965	BC005272
C 14	CRABP-I (Cellular Retinoic Acid Binding Protein 1)	45~137 / 137	4758051	NM_004378
C 18	Human Chromosome 19 Clone CTD-2537I9	1~280 / 315	21743742	AC008735
C 22	FTH1 (Ferritin Heavy Chain)	31~183 / 183	16877183	BC016857
C 26	Translokin	57~500 / 500	29469443	AY225092
C 39	Unknown (Protein for MGC 2574)	4~294 / 360	12655056	BC001378
C 44	TACSTD2 (Tumor-associated calcium signal transducer 2)	1~323 / 323	14495611	AAH09409
C 45	PI4K (Phosphatidylinositol 4-kinase)	1799~1916 / 2044	2326227	AAD13352
C 66	PTMA (Prothymosin α, gene sequence 28)	1~110 / 110	18490876	BC022433
C 78	CENTδ2 (Centaurin δ2, Rho-GAP)	850~1181 / 1209	21264595	NM_015242
C 80	C9 ORF6 (Human chromosome 9 open reading frame 6)	26~181 / 181	12002684	AAG43367

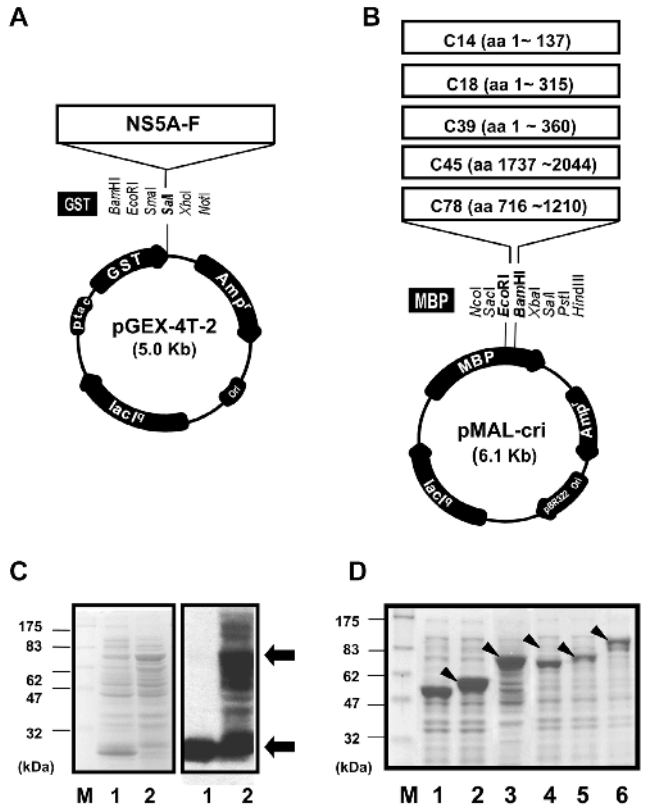

Fig. 2. Expression patterns of GST/NS5A-F and the five MBP/C-series fusion proteins. (A and B) NS5A-F and the five proteins that interact with NS5A-C were fused with GST and MBP, as illustrated. (C) The GST/NS5A-F fusion protein was produced in *E. coli*, resolved by SDS-PAGE, and subjected to Western blot analysis. Note: the GST/NS5A-F shows a band pattern consistent with degradation. The upper and lower arrows indicate the size of full-length GST/NS5A-F and GST only, respectively. Lane M: protein size marker; lane 1: GST only; lane 2: GST/NS5A-F. (D) The five C-series proteins were fused with the MBP produced in *E. coli* and resolved by SDS-PAGE. The arrowheads indicate the expected molecular weight of each fusion protein. Lane M: protein size marker; lane 1: MBP only; lanes 2-6: MBP/C-14, C-18, C-39, C-45 and C-78.

boiling, resolved by PAGE and analyzed by anti-GST and anti-MBP antibodies, as shown in Fig. 3. Similarly, a *vice versa* experiment against the MBP beads was performed. The results showed that NS5A directly interacted with the five different C-series clones, C14, C18, C39, C45 and C78.

Identification of the interacting domain The NS5A-C (aa 300~477), containing the P*XX*P motif (class II), functions as a potential region for interacting with many cellular proteins. Our results also showed that NS5A-C interacted with 10 different cellular proteins. Therefore, the issue of whether the P*XX*P motif was also important for their interactions with NS5A was also assessed. The proline amino acids of the P*XX*P motif (class II) were mutated to alanine by a PCR method, as illustrated in Fig. 4A, and whether the mutation has an influence on the interactions was verified by a colony filter-lift assay. Two different clones, C45 (PI4K) and C78 (CENTδ2), were examined in relation to their possible interactions with the mutated NS5A-F and NS5A-C, as shown in Fig. 4B. According to the results, the mutation in the P*XX*P motifs did not affect their interactions with C45 and C78, suggesting they interacted with NS5A in a P*XX*P motif-independent manner. This finding indicates that the NS5A-C region has a novel domain, other than the P*XX*P motif, which is responsible for the interactions.

Discussion

The lack of a robust *in vitro* replication system for HCV has meant the exact role of the NS5A protein in HCV infections remains unknown. To date, information concerning the potential NS5A function has derived, almost exclusively, from the use of a yeast two-hybrid system followed by an *in vitro* transient expression assay. A potential clue that NS5A may perturb other cellular signaling functions is the presence of several proline-rich P*XX*P motifs. Although NS5A contains multiple potential P*XX*P sites, only two are highly preserved among HCV genotypes, including both class I (aa 26~32) and class II (aa 350~-356) motifs. Such P*XX*P motifs bind to the SH3 domain found in a diverse group of signal-transducing molecules (Pawson, 1995). It has been well documented that the latter is necessary for NS5A to interact with Grb2, an adaptor protein consisting of one SH2 domain flanked by two SH3 domains. In accordance with these findings, NS5A-A (aa 1~150), which contains the class I P*XX*P motif, resulted in no candidate clones when used as bait protein in the yeast two-hybrid system. In contrast, NS5A-C (aa 300~447), containing the class II P*XX*P motif, resulted in 10 interacting clones. However, these results do not mean that all these interactions arise from the P*XX*P motif, as judged by the results of the domain analysis showing that two of the tested clones interacted with NS5A through a novel domain(s) (Fig. 4B) and not the P*XX*P motif. Recent data from another group also suggests that the NS5A-B region has a novel binding site for interacting with the SH3 domain of PI3K p85 as well as the canonical P*XX*P motifs (Street *et al*., 2004).

It is conceivable that the role of the N-terminal region of NS5A is related with the regulation of NS5A itself, which masks the functional nuclear localization signal (aa 354~362), the class II P*XX*P motif (aa 350~356) and the transcriptional activating domain (aa 163~359) (Kato *et al*., 1997; Song *et al*., 2000). Consistent with this concept, NS5A-A (aa 1~150) resulted in no interacting candidates, with NS5A-D (aa 151~477) itself showing a blue color in the absence of a prey protein. According to previous reports, the N-terminally truncated form of NS5A functions as a potent transcriptional activator in both yeast and mammalian cells when fused to the DNA-binding domain of GAL4 (Kato *et al*., 1997; Tanimoto *et al*., 1997). Furthermore, the N-terminal deletion of NS5A,

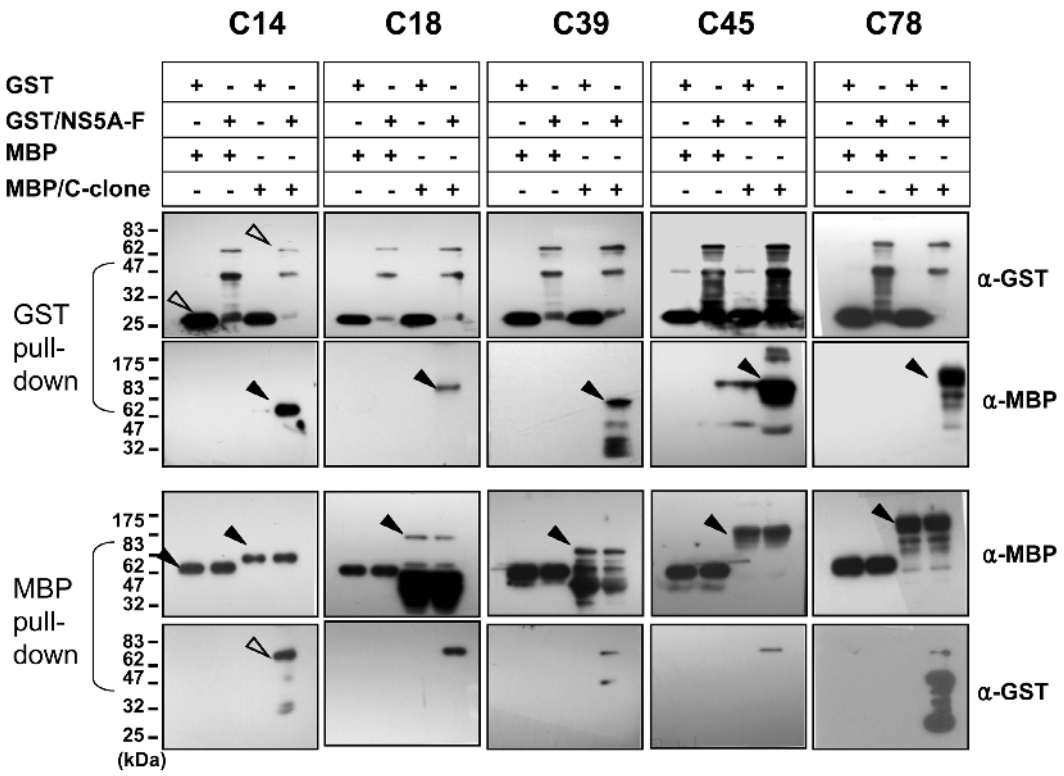

Fig. 3. *In vitro* interactions of NS5A-F with the five C-series proteins, C14, C18, C39, C45 and C78. GST/NS5A-F and each of the indicated MBP/C-series protein were mixed together and pulled-down by GST beads. The proteins were then resolved by SDS-PAGE and subjected to Western blot analyses with antibodies against GST and MBP (GST pull-down). The mixed proteins were pulled-down by MBP beads, and a *vice versa* Western blot analysis performed with antibodies against GST and MBP (MBP pull-down). The upper and lower arrowheads indicate the size of full-length GST/NS5A-F and GST only, respectively. The upper and lower filled arrowheads indicate the size of the MBP/C-series and MBP only, respectively.

by 66 aa or more, causes conformational alteration and exposes a cleavage site in the NS5A sequence around aa 150 (Song *et al*., 2000). Similarly, the bacterially produced GST/NS5A-F fusion protein also caused the degradation of numerous discrete band patterns when observed by PAGE, suggesting that the N-terminal GST fusion resulted in the possible exposure of multiple cleavage sites and conformational alteration in the NS5A sequence (Fig. 2A). Therefore, it is granted that the number of clones interacting with NS5A-F or NS5A-B is less than that with NS5A-C, because the elimination of the masking N-terminal region of NS5A exposures the highly interacting C-terminal region. Therefore, the possibility of non-specific cellular interactions with the N-terminally-deleted NS5A-C is not excluded. However, this is not the case because our results showed that the cellular interacting clones with NS5A-C also interact with NS5A-F, but with a slightly decreased affinity (data not shown).

The identified clones suggest a potential association with diverse cellular signaling pathways. For example, translokin (C26) has been reported to serve as an intracellular mediator of FGF-2 trafficking (Bossard *et al*., 2003), and PI4K (C45) is a well-known regulatory molecule of bioactive lipids in the regulation of cell motility, the Ras pathway, vesicle trafficking, secretion and apoptosis (Gehrmann and Heilmeyer, 1998). Furthermore, CENTδ2 (C78) is a GTPase-activating protein for ARF-dependent and Rho-like GTPase, which may modulate G-protein-dependent signaling pathways for membrane trafficking and cytoskeleton dynamics (Santy and Casanova, 2002). Taken together, it is likely that NS5A perturbs diverse cellular signaling pathways through interacting with those cellular proteins, as in the previously well reported case of ERK signaling (Macdonald *et al*., 2004). In addition, the interaction of NS5A with those cellular proteins might disturb the membrane dynamics favoring virus replication. In summary, our experiments led to the identification of many hepatocellular molecules that may be related to cellular signaling pathways. Therefore, these results contribute to a better understanding of the possible role of NS5A in hepatocellular signaling pathways.

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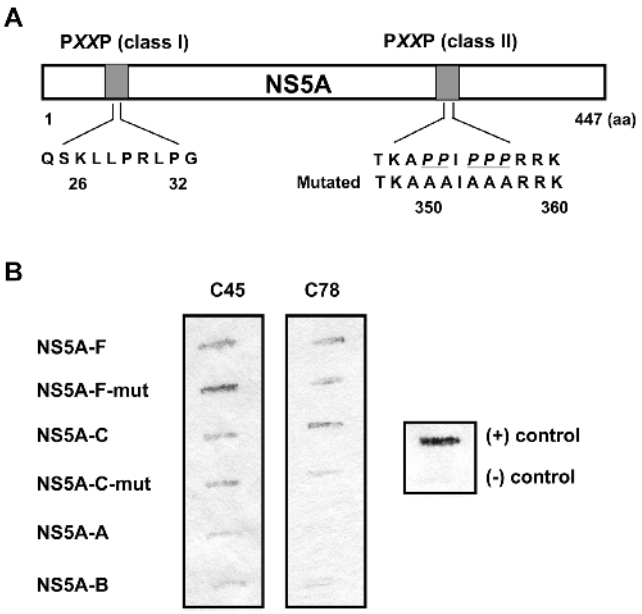

Fig. 4. Domain analysis for the interaction of NS5A-C with C-45 and C-78. (A) The class II P*XX*P domain of NS5A-F or NS5A-C was mutated using a PCR method, as illustrated. (B) A Colony-lift filter assay was performed to detect whether pYES/Trp-C-45 and -C-78 interact with the NS5A-F and NS5A-C mutants, where the proline amino acids of the P*XX*P motif are mutated to alanine. For the (+) control, pHybLex/Zeo-Fos2 and pYESTrp-Jun were contransfected into the L40 yeast strain, and for the (-) control pHybLex/Zeo-Lamin was transfected alone.

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