Direct interaction between α-actinin and hepatitis C virus NS5B

Shuiyun Lan, Hua Wang, Hong Jiang, Hongxia Mao, Xiaoying Liu, Xiaonan Zhang, Yunwen Hu, Li Xiang, Zhenghong Yuan*

Key Laboratory of Medical Molecular Virology, Shanghai Medical College, Fudan University, Shanghai 200032, PR China

Received 27 May 2003; revised 22 August 2003; accepted 17 September 2003

First published online 20 October 2003

Edited by Hans-Dieter Klenk

Abstract **It has been suggested that cellular proteins are involved in hepatitis C virus (HCV) RNA replication. By using the yeast two-hybrid system, we isolated seven cDNA clones encoding proteins interacting with HCV RNA polymerase (NS5B) from a human liver cDNA library. For one of these, α-actinin, we confirmed the interaction by coimmunoprecipitation, immunofluorescent staining and confocal microscopic analysis. Experiments with deletion mutants showed that domains $NS5B_{84–95}$, $NS5B_{466–478}$, and $\alpha\text{-actinin}_{621–733}$ are responsible for the interaction. Studies of the HCV subgenomic replicon system with small interference RNA indicate that α-actinin is essential for HCV RNA replication. Our results suggest α-actinin may be a component of the HCV replication complex.**

Key words: Hepatitis C Virus; RNA polymerase; α-Actinin; Protein–protein interaction; Yeast two-hybrid system

1. Introduction

Hepatitis C virus (HCV) belongs to the family Flaviviridae, which comprises three genera of enveloped positive-strand RNA viruses. The 9.6 kb genome of HCV consists of a long open reading frame flanked by 5′ and 3′ non-translated regions (NTRs) [1,2]. The HCV 5′ NTR, 341 nucleotides in length, functions as an internal ribosome entry site for cap-independent translation initiation. The HCV polyprotein is cleaved co- and post-translationally into at least 10 individual polypeptides with the following gene order: 5′-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-3′ [1–4]. The structural proteins result from signal peptidase cleavages in the N-terminal portion of the polyprotein. Two viral proteases mediate downstream cleavages to produce non-structural proteins that function as components of the HCV RNA replicase. The NS2–3 protease spans the C-terminal half of NS2 and the N-terminal one-third of NS3 and catalyzes autocatalytic *cis*-cleavage at the two-thirds site. This same portion of NS3 also encodes the catalytic domain of the NS3–4A serine protease that cleaves at four downstream sites. The C-terminal two-thirds of NS3 is highly conserved among HCV isolates, with RNA-binding, RNA-stimulated NTPase and RNA-unwinding activities. Although NS4B and the NS5A phosphoprotein are also likely components of the replicase, their specific roles are unknown. The C-terminal polyprotein cleavage product, NS5B, is the elongation subunit of the HCV replicase possessing RNA-dependent RNA polymerase (RdRp) activity [5]. Following a translation stop codon, the HCV 3′ NTR consists of three subregions: (i) a 28–42-base sequence that varies among genotypes, (ii) an internal poly(U/UC) tract of variable length with rare A or G residues, and (iii) a highly conserved 3′ terminal 98-base sequence [6]. It is generally believed that conserved sequences and structures at the 3′ terminus of viral genomic RNA function as *cis*-acting signals that interact with viral and cellular proteins to initiate the synthesis of minus-strand RNA during viral replication.

The NS5B protein of HCV is a membrane-associated phosphoprotein that possesses the conserved GDD motif of RdRps [2]. NS5B RdRp activity has been demonstrated in vitro, and several amino acid motifs essential for its enzymatic activity have been identified [6]. It seemed likely that initiation of minus-strand RNA synthesis might be upon recognition and specific binding of the NS5B RNA polymerase or replicative complex to the 3′ terminus of the viral genomic RNA [7]. However, recent studies have indicated that HCV NS5B interacts with the 3′ conserved 98 nt of the viral genome with little specificity [5,7] and no clear preference for utilization of the 98-nt RNA as a template in RdRp activity assay [8–10]. Recently, NS5B was reported to bind to hVAP-33, a human vesicle-associated membrane protein, and to complex with NS3 and NS4A [11,12]. Although as yet untested, association of NS5B with other non-structural proteins and cellular proteins could be required to produce an RNA replicase with specificity for HCV template RNA.

To uncover possible components of the HCV replication complex, we employed the yeast two-hybrid system and a human hepatocyte cDNA library to search for cellular proteins that interact with HCV NS5B. α-Actinin, a member of a superfamily of actin cross-linking proteins, was among seven interactors identified. α-Actinin interacts with actin subdomain 2 and part of subdomain 1. We confirmed protein–protein interaction between NS5B and α-actinin by coimmunoprecipitation, immunofluorescent staining and confocal microscopy. The interaction domains of NS5B and α-actinin were mapped by deletion mutant analysis. Studies with the HCV subgenomic replicon system indicate α-actinin is essential for HCV RNA replication. These data suggest that α-actinin may be a component of the HCV RNA replication machinery.

*Corresponding author. Fax: (86)-21-64227201.
E-mail address: zhyuan@shmu.edu.cn (Z. Yuan).

Abbreviations: HCV, hepatitis C virus; RdRp, RNA-dependent RNA polymerase; GFP, green fluorescent protein; mAb, monoclonal antibody; siRNA, small interference RNA; aa, amino acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

0014-5793/03/$22.00 © 2003 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)01163-3

2. Materials and methods

2.1. Plasmids

For the yeast two-hybrid system, the plasmids pLexA and pB42AD (Clontech) were used as sources of the LexA DNA-binding domain and LexA transcriptional activation domain (AD), respectively. To generate the bait plasmids (pLexA-NS5B and pLexA-NS5BΔ21), pLexA was inserted with the *Bam*HI fragment from the prokaryotic expression vector pET32c-NS5B or pET32c-NS5BΔ21, which express the full-length or truncated HCV NS5B protein (without the C-terminal 21 amino acids to ensure translocation to the nucleus). A human liver cDNA library cloned into pB42AD was obtained from Clontech. The plasmid pcDNA3/HA-actinin was constructed by insertion of the *Eco*RI-*Xho*I fragment of plasmid pB42AD-actinin. The plasmid pCMV/HA-actinin, encoding α-actinin and a hemagglutinin (HA) epitope tag at the N-terminus, was also derived from pB42AD-actinin. To generate a myc epitope-tagged NS5B protein, mammalian recombinant vector pcDNA3.1/myc-HisB-NS5B was constructed by inserting the *Bam*HI-*Xho*I fragment of plasmid pET28a-NS5B into vector pcDNA3.1/myc-HisB excised by the same two enzymes. The NS5B fragment excised from expression vector pET32c-NS5B by *Bam*HI digestion was inserted into pEGFPC2 to generate pEGFPC2-NS5B that encodes the green fluorescent protein (GFP)-NS5B fusion protein. Deletion mutants of NS5B and α-actinin were generated by polymerase chain reaction (PCR) and then cloned into pLexA and pB42AD plasmids.

2.2. Two-hybrid screening

The yeast reporter strain EGY48 [p8op-lacZ] obtained from Clontech was used for the two-hybrid selection. Either plasmid pLexA-NS5B or pLexA-NS5BΔ21 was used as bait. The pB42AD cDNA library (Clontech) from human liver was used as the source of prey genes. Yeast cells were grown on YPD (1% yeast extract, 2% peptone, 2% dextrose, 2% agar [for plates]) or on synthetic minimal medium (0.67% yeast nitrogen base, the appropriate auxotrophic supplements, 2% agar [for plates]) containing 2% dextrose or 2% galactose and 1% raffinose. Yeast was transformed with appropriate plasmids by the lithium acetate method. The transformants were selected on the appropriate synthetic minimal medium. The bait plasmid and the pB42AD cDNA library were introduced into the yeast strain EGY48 [p8op-lacZ]. The two-hybrid screen and interaction assays were performed essentially as described in the protocol (Clontech) in the presence of 2% galactose and 80 mg of 5-bromo-4-chloro-3-indolyl-D-galactopyranoside per liter. The prey plasmids were selected from yeast colonies giving a positive signal according to the manufacturer's protocol. False positives were eliminated by retransforming the host EGY48 [p8op-lacZ] strain with pB42AD-cDNA plus bait plasmid or pLexA control plasmid. The positive clones that contained cDNAs encoding NS5B-interacting proteins were sequenced and subjected to BLAST analysis.

2.3. Coimmunoprecipitation

For in vitro coimmunoprecipitation, plasmids pcDNA3/HA-actinin and pcDNA3.1/myc-HisB-NS5B were used. [^{35}S]Methionine-labeled HA-actinin and NS5B-myc were translated in vitro using the TNT Quick Coupled Transcription/Translation System (Promega). HA-actinin and NS5B-myc were incubated with monoclonal anti-HA antibody or anti-myc antibody in lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40, 1 mM phenylmethylsulfonyl fluoride), followed by adsorption to bovine serum albumin-blocked protein A/G-agarose (Boehringer Mannheim). The beads were washed thrice with lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40). The antibody–protein complexes were then resolved in 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and subjected to autoradiography.

For in vivo coimmunoprecipitation, 1×10^7 Huh-7 cells containing HCV replicons were washed three times with ice-cold phosphate-buffered saline (PBS) and solubilized with 1 ml lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 5 mM EDTA, 5 mM EGTA, 15 mM $MgCl_2$, 60 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1× proteinase inhibitor cocktail [Roche]). Detergent-insoluble materials were removed by centrifugation at $15\,000\times g$ for 10 min at 4°C. The whole cell lysates were incubated with monoclonal antibody (mAb) against α-actinin (Santa Cruz, sc-17829) or mouse normal IgG at 4°C for 2 h. Pre-equilibrated protein G-agarose beads were then added, and after 2 h of incubation, they were collected by centrifugation and then gently washed three times with the lysis buffer. The bound proteins were eluted by boiling in SDS sample buffer and resolved on a 10% SDS–PAGE gel. The proteins were transferred onto a polyvinylidene difluoride membrane and probed with a polyclonal antibody against HCV NS5B (Santa Cruz, sc-17532). Proteins were detected using 3,3′,5,5′-tetramethylbenzidine (TMB) substrate.

2.4. Indirect immunofluorescence and confocal microscopic analysis

COS7 cells seeded in six-well chamber slides were transfected with plasmids pCMV/HA-actinin and either pEGFPC2-NS5B or pEGFPC2 (5 μg) by the calcium phosphate method. At 48 h post transfection, the cells were fixed with 2% formaldehyde in PBS. Permeabilization of the cells was carried out with 0.2% Triton X-100 in PBS containing 1% fetal calf serum. Samples were then incubated with mouse anti-HA mAb (Babco) at 4°C overnight. After being washed thrice with PBS containing 1% fetal calf serum, samples were incubated at 37°C for 1 h with a second antibody, rhodamine-conjugated anti-mouse immunoglobulin. After further washing, coverslips were finally mounted on glass plates and cells were observed by confocal laser scanning microscope (Leica, TCS-NT, Heidelberg, Germany)

2.5. Generation of stable HCV subgenomic replicon cell line

The BB7 HCV subgenomic replicon (genotype 1b) was obtained from Charles Rice (The Rockefeller University, New York, NY, USA). Huh-7 cells were transfected with HCV subgenomic replicon RNA and maintained as described by Blight et al. [13]. Huh-7 clone R cells harboring autonomously replicating HCV replicon RNA were used in related experiments.

2.6. Construction of plasmid expressing interfering RNA targeting on α-actinin

According to the small interference RNA (siRNA) finder (Ambion, http://www.ambion.com/techlib/misc/siRNA_finder.html), the α-actinin-targeting sequence 5′-TCGCATCTGCAAGGTGTTG-3′ was selected. Sense and antisense strands of siRNA oligonucleotides against α-actinin RNA were synthesized and were then annealed at 95°C for 1 min followed by slow cooling in PBS, pH 6.8, containing 2 mM $MgCl_2$. They were inserted immediately downstream of the U6 promoter in pSilence1.0 U6 siRNA Expression vector (Ambion). Control plasmid pSi-EGFP, which can produce siRNAs targeting EGFP RNA, was constructed similarly. Transfections of the plasmids were performed in six-well plates using Fugen6 (Roche) in accordance with the manufacturer's instructions. 2 μg plasmid was transfected in each well.

2.7. RNA analysis

Total cellular RNA was isolated using TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. After treatment with DNase I, one-tenth of each RNA sample from a 36-mm well was reverse-transcribed with random primers (dN_6) by Superscript II (Invitrogen). Real-time PCR (LightCycler, Roche) amplifications were performed using the CYBR I reagents (Roche) according to the manufacturer's protocol. Cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA from the same cell lysate was used as an internal control for cell number and metabolic status. The primers specific for the HCV 5′ NTR are: 5′-CCCTGTGAGGAACTA/TCTGTCTTCACGC-3′ (sense), 5′-TCCAGAGCATCTGGCACG-TA/GGTACTCG-3′ (antisense). The primers specific for GAPDH are: 5′-GGTATCGTGGAAGGACTCATGAC-3′ (sense), 5′-ATGC-CAGTGAGCTTCCCGTTCAGC-3′ (antisense). Forty cycles of PCR were performed with cycling conditions of 15 s at 95°C, 20 s at 55°C, 25 s at 72°C. The real-time PCR signals were analyzed using LightCycler 3 software. Each experiment was repeated at least three times.

3. Results

3.1. Identification of cellular proteins interacting with HCV NS5B

To identify cellular proteins interacting with HCV NS5B, the LexA yeast two-hybrid system was employed to screen a human liver cDNA library (MATCHMAKER cDNA library

from Clontech). We screened the hepatocellular cDNA library with full-length NS5B protein and obtained 24 candidates; after cotransformation to eliminate false positive phenotypes, none were retained (data not shown). However, when using the truncated HCV NS5B protein (lacking the C-terminal 21 amino acids) as bait, 43 clones were detected as positive candidates during the first round screening of 2×10^6 independent yeast colonies and seven clones remained consistently positive after rigorous testing. The cDNA clones were sequenced and subjected to BLAST analysis. As supported by NCBI database (USA), the following genes showed 100% homology to identified cDNA clones: α-actinin, protease 26S subunit, proteasome subunit β4, HeLa cyclin-dependent kinase 2 interacting protein and tetratricopeptide repeat protein 4 as well as hydroxyacid oxidase 1.

3.2. Coimmunoprecipitation of NS5B with α-actinin

For in vitro coimmunoprecipitation, tagged proteins HA-α-actinin and NS5B-myc were translated in vitro using the TNT Quick Coupled Transcription/Translation System in the presence of [^{35}S]methionine. Radiolabeled HA-actinin and NS5B-myc were incubated with an anti-HA mAb and immune complexes were captured on protein A/G-agarose. The antibody–protein complexes were then resolved in 10% SDS–PAGE and subjected to autoradiography. NS5B-myc was coimmunoprecipitated with HA-α-actinin by the anti-HA antibody (Fig. 1A, lane 3). In contrast, NS5B-myc was not precipitated by the anti-HA antibody when HA-α-actinin was absent from the reaction mix (Fig. 1A, lane 4). Similarly, HA-α-actinin could be coimmunoprecipitated with NS5B-myc by the anti-myc antibody (Fig. 1A, lane 5) and it was not immunoprecipitated by the anti-myc antibody when NS5B-myc was absent from the reaction mix (Fig. 1A, lane 6). These results indicated that NS5B interacted with α-actinin in vitro.

For in vivo coimmunoprecipitation, lysates of Huh-7 cells harboring HCV replicons were incubated with mAb against α-actinin. The antibody–protein complexes were probed with a polyclonal antibody against HCV NS5B. Results showed that NS5B could be precipitated from Huh-7 HCV replicon cells with antibody against α-actinin (Fig. 1B, lane 3) but not by mouse normal serum (Fig. 1B, lane 4). No NS5B could be detected from Huh-7 cells by immunoprecipitation with mAb against α-actinin (Fig. 1B, lane 2). These results indicated that endogenous α-actinin could interact with NS5B in Huh-7 cells harboring HCV replicons.

3.3. NS5B colocalizes with α-actinin

In COS7 cells transiently transfected with plasmids pCMV/HA-actinin and pEGFPC2-NS5B, NS5B was found to colocalize with α-actinin by confocal laser scanning microscopy (Fig. 2A). As a negative control, COS7 cells were cotransfected with plasmids pCMV/HA-actinin and pEGFPC2. Results showed that α-actinin and EGFP were distributed independently and α-actinin was distributed in the whole cell when GFP alone was overexpressed, and the GFP signals were spread diffusely in both the nucleus and cytoplasm (Fig. 2B). However, GFP-NS5B was exclusively distributed in the cytoplasm and α-actinin was redistributed to the cytoplasm when coexpressed with GFP-NS5B protein (Fig. 2A). These results indicated that the binding of α-actinin to NS5B resulted in a change in distribution of α-actinin. The strong cytoplasmic distribution of α-actinin in the NS5B-expressing

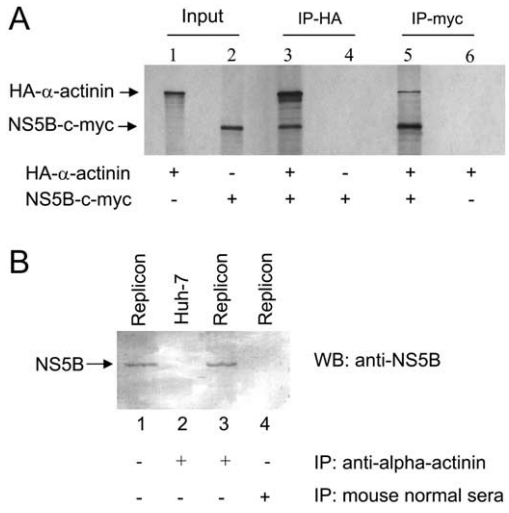

Fig. 1. A: In vitro coimmunoprecipitation of NS5B with α-actinin. Tagged proteins HA-α-actinin and NS5B-myc were translated in vitro with [^{35}S]methionine incorporation (lanes 1 and 2). After HA-α-actinin and NS5B-myc were immunoprecipitated with monoclonal anti-HA antibody or anti-myc antibody, the antibody–protein complexes were resolved in 10% SDS–PAGE and subjected to autoradiography. NS5B-myc was coimmunoprecipitated with HA-α-actinin by the anti-HA antibody (lane 3) and it was not immunoprecipitated by the anti-HA antibody when HA-α-actinin was absent from the reaction mix (lane 4). Similarly, HA-α-actinin was coimmunoprecipitated with NS5B-myc by the anti-myc antibody (lane 5) and it was not immunoprecipitated by the anti-myc antibody when NS5B-myc was absent from the reaction mix (lane 6). B: In vivo coimmunoprecipitation of NS5B. Lysates of Huh-7 cells harboring HCV replicons were incubated with an mAb against α-actinin. The antibody–protein complexes were probed with a polyclonal antibody against HCV NS5B. Proteins were detected using TMB substrate. Lane 1, input of the lysate of Huh-7 cells harboring HCV replicons without immunoprecipitation; lane 2, immunoprecipitation of lysate of Huh-7 cells with mAb against α-actinin; lane 3, immunoprecipitation of lysate of Huh-7 cells harboring HCV replicons with mAb against α-actinin; lane 4, immunoprecipitation of lysate of Huh-7 cells harboring HCV replicons with mouse normal sera.

cells indicates the binding of α-actinin to NS5B may play an important role in NS5B function and HCV RNA replication.

3.4. Mapping the interacting domains of NS5B and α-actinin

To examine the regions of HCV NS5B protein and α-actinin responsible for their binding to each other, a series of deletion mutants were constructed (Fig. 3) and the interactions were assayed using the yeast two-hybrid system. The results showed that mutants $NS5B_{1-96}$, $NS5B_{83-150}$ and $NS5B_{428-478}$ could interact with α-actinin while $NS5B_{1-83}$, $NS5B_{96-150}$ and $NS5B_{377-465}$ could not. So the interaction for HCV NS5B on α-actinin maps to two independent domains: aa 84–95 and aa 466–478. For α-actinin, the C-terminal fragments aa 499–892 and aa 621–892 could interact with NS5BΔ21. Further deletion from aa 621 to aa 733 abolished the NS5B interaction. Therefore the minimum region necessary for binding NS5B is within aa 621–733, which harbors spectrin domain 4.

3.5. α-Actinin is essential for HCV RNA replication

The direct binding of α-actinin and NS5B may affect the synthesis of HCV RNA. To test this hypothesis, we undertook siRNA strategy in the recently developed HCV subgenomic

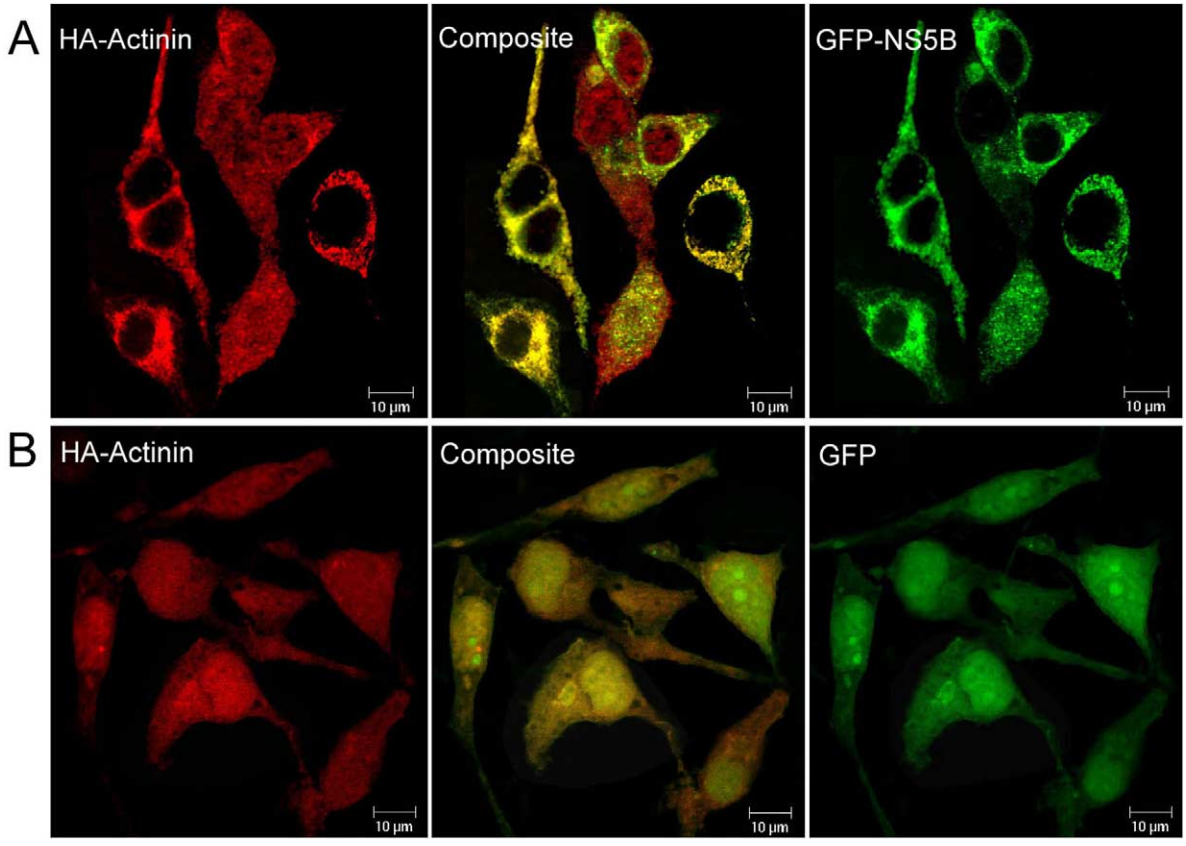

Fig. 2. NS5B was colocalized with α-actinin in COS7 cells. COS7 cells on coverslips were transfected with plasmids pCMV/HA-actinin and either pEGFPC2-NS5B or pEGFPC2. At 48 h post transfection, the cells were fixed and immunostained with mouse anti-HA monoclonal antibody and rhodamine-conjugated anti-mouse immunoglobulin. Coverslips were finally mounted on glass plates and cells were observed in a confocal laser scanning microscope (Leica, TCS-NT, Heidelberg, Germany). Images recorded in red (rhodamine) and green (GFP) channels are presented separately on the left and on the right, respectively, and composite images are shown in the middle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

replicon system. RNA interference was used to reduce intracellular α-actinin expression. To determine whether siRNA specific to α-actinin could inhibit the expression of α-actinin in cells, Western blot analysis was performed. Compared with mock-transfected cells, the α-actinin level declined to 37% in cells transfected with plasmid pSi-Act (Fig. 4), while transfection of plasmid pcDNA3/HA-actinin resulted in the overexpression of α-actinin. HCV RNA quantitative analysis revealed that the HCV RNA levels in subgenomic replicon cells decreased significantly after transfection with plasmid pSi-Act, which produced siRNA against α-actinin RNA ($P < 0.05$), but were not affected by control plasmid pSi-EGFP, which produced siRNAs unrelated to the α-actinin or HCV RNA sequence (Fig. 5). These data indicate that α-actinin may be a component of the HCV replication complex and play a role in the replication of the subgenomic replicon via the interaction with NS5B.

4. Discussion

HCV replication consists of minus-strand RNA replication and plus-strand RNA replication and has been regarded as a target for intervention to block chronic infection. Both processes seem to happen at membranous structures where nonstructural proteins dynamically assemble as a replication complex with HCV RNA. Recently it was proposed that NS5B RdRp activity could be regulated at the level of protein–protein interactions, homomeric oligomerization and heteromeric interaction between NS5B and NS5A [14–16].

Here, using the yeast two-hybrid system, we identified seven cellular proteins that interact with HCV NS5B. The interaction between NS5B and α-actinin was further confirmed by an in vitro coimmunoprecipitation assay and by immunofluorescent staining and confocal microscopy. Two independent domains of NS5B, aa 84–95 and aa 466–478, and domain 621–733 of α-actinin are responsible for the interaction (Fig. 3). Primary functional study showed that α-actinin could modulate NS5B activity. Our study suggests that α-actinin (and perhaps other cellular proteins) may be involved in control of HCV RNA replication.

We first used full-length NS5B for two-hybrid screening. With this bait, no positive clones were obtained. These negative results may have resulted from the amphipathic helix at the C-terminus of NS5B. Mammalian expression studies suggest that this 21-residue hydrophobic segment serves as a membrane anchor [17]. Deletion of this domain during *Escherichia coli* expression facilitates NS5B solubility and was an important step towards determining the crystal structure of this enzyme [8]. In the LexA yeast two-hybrid system, reporter genes are efficiently activated when both fusion proteins can be imported into the nucleus. Consistent with this idea, deletion of the carboxy-terminal 21 aa leads to nuclear redistribution of NS5B [17]. Use of this carboxy-terminally truncated NS5B protein as bait yielded seven positive cDNA clones. It

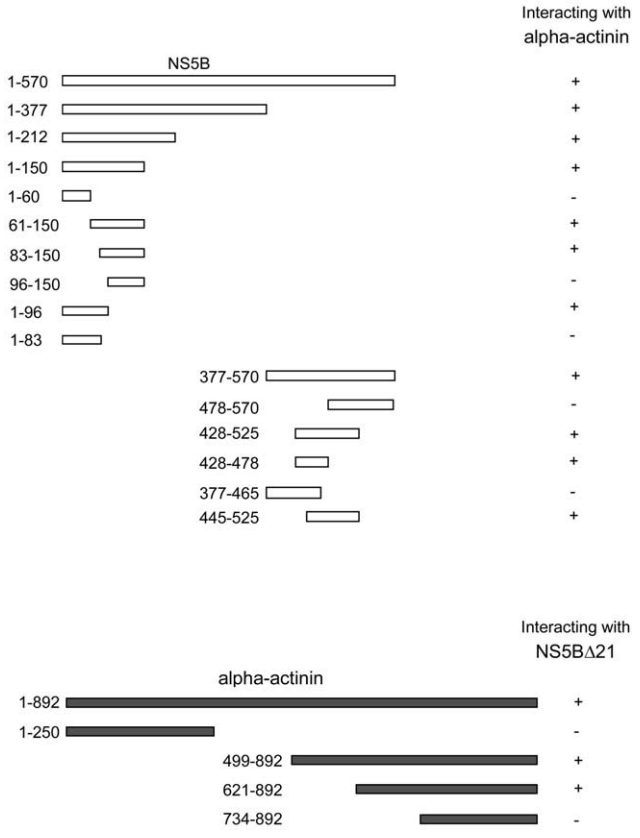

Fig. 3. Schematic representation of the amino- or carboxy-terminally truncated NS5B and α-actinin proteins used for the two-hybrid system assay to map the interaction domain. The open columns indicate NS5B and its deletion mutants and the closed ones indicate α-actinin and its deletion mutants. The numbers on the left of columns indicate the amino acid sites. The results of the yeast two-hybrid assay are summarized on the right.

should be noted, however, that full-length NS5B was used in subsequent experiments to confirm protein–protein interactions.

Formation of a membrane-associated replication complex is a characteristic feature of positive-strand RNA viruses [18]. The non-structural proteins of dengue virus, Japanese encephalitis virus and HCV (NS3 to NS5B) are able to assemble into membrane-associated replication complexes, competent for authentic RNA genome replication [19,20]. Although the

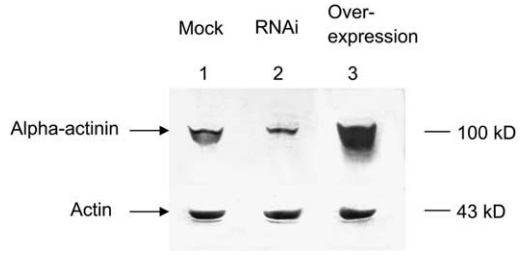

Fig. 4. Inhibition of α-actinin expression by siRNA. Western blot analysis was performed on total cell lysates harvested from mock-, pSi-Act-, or pcDNA3/HA-actinin-transfected replicon-bearing cells at 48 h post transfection using α-actinin-specific or actin-specific antibodies. Molecular mass markers (in kDa) are shown on the right of each gel. Densitometry readings (as percentages of the mock-transfected control) for lanes 1–3 were 100, 37 and 931, respectively, for α-actinin, 100, 99 and 106 for actin.

determinants of membrane association are beginning to be defined [17], protein–protein interactions involved in formation of the HCV replicase are poorly understood. Regarding a possible role of α-actinin in formation and/or function of the HCV replicase, host cytoskeletal proteins have been implicated in HCV and other viral systems. It has been demonstrated that microtubule and actin polymerization were required for HCV RNA synthesis in the HCV replicon cell culture system [21]. For the paramyxoviruses, measles virus and Sendai virus, mRNA synthesis is stimulated in vitro by tubulin, the major structural component of microtubules, and inhibited by anti-β-tubulin antibodies [22–24]. mRNA synthesis of human parainfluenza virus type 3 and respiratory syncytial virus requires cellular actin, a major component of microfilaments in the host cytoskeletal network [25,26]. Actin and tubulin might serve as anchoring sites on the cytoskeleton for viral RNA polymerase. Assembling the RNA polymerase on the cytoskeleton or on cell membranes may ensure appropriate concentrations of replication components and hence control the rates or efficiencies of replication reactions.

α-Actinin is a member of a superfamily of actin cross-linking proteins and interacts with actin subdomain 2 and a part of subdomain 1 through its 27-kDa actin-binding domain [27]. While the NS5B-binding site on α-actinin maps to the C-terminus, the N-terminus of α-actinin is responsible for binding to actin. So NS5B may interact with actin through α-actinin. It has been reported that NS5B can be oligomerized which is a prerequisite for RdRp activity and the residues E18 in the long loop and H502 in the thick thumb are critical for oligomerization [14]. As E18 and H502 are located in the vicinity of the two independent domains which are essential for α-actinin binding NS5B, there is a possibility that, like nucleolin [28], actinin may affect the oligomerization of NS5B. Furthermore, the two binding domains of NS5B, aa 84–95 and aa 466–478, are located in the regions that surround the template strand according to structure analysis [29]. So the RdRp activity of NS5B could possibly be affected by the interaction with α-actinin. This finding may lead to the elucidation of the detailed protein–protein interactions and their involvement in formation of the membrane-associated replication complex and may provide new insights into fundamen-

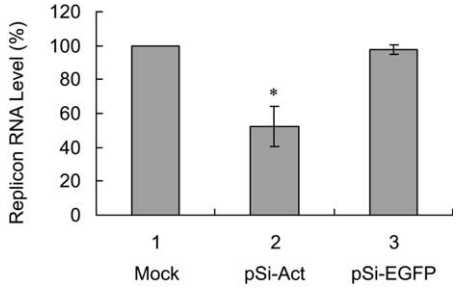

Fig. 5. Effects of siRNA targeting α-actinin on HCV RNA replication in HCV subgenomic replicon cells. In a six-well plate, Huh-7 cells harboring HCV replicons were transfected with 2 μg plasmid pSi-Act or pSi-EGFP. At 48 h post transfection the cells were harvested for RNA extraction. Real-time PCR amplifications were performed after reverse transcription. The signals were analyzed using Light-Cycler 3 software. The levels of HCV replicon RNA, after normalization against GAPDH, are shown as percentages of the mock-transfected control. Representative data (mean ± S.D.) from three independent experiments are shown. Asterisk indicates $P <$ 0.05.

tal cellular processes and identify novel targets for antiviral intervention.

Acknowledgements: This work was supported by State Key Basic Research Program (Grant G1999054105), Chinese National Natural Scientific Grant (Grants 39870010, 39825501), Shanghai Scientific Development Foundation (Grant 00QMH1404) and a Young Scientist Grant from Fudan University (2001). The authors are grateful to Dr. Jane Wu (Washington University School of Medicine) and Charles M. Rice (The Rockefeller University) for their kind discussion and suggestions.

References

[1] Clarke, B. (1997) J. Gen. Virol. 78, 2397–2410.
[2] Rosenberg, S. (2001) J. Mol. Biol. 313, 451–464.
[3] Hijikata, M., Kato, N., Ootsuyama, Y., Nakagawa, M. and Shimotohno, K. (1991) Proc. Natl. Acad. Sci. USA 88, 5547–5551.
[4] Grakoui, A., Wychowski Lin, C.C., Feinstone, S.M. and Rice, C.M. (1993) J. Virol. 67, 1385–1395.
[5] Lohmann, V., Körner, F., Herian, U. and Bartenschlager, R. (1997) J. Virol. 71, 8416–8428.
[6] Kolykhalov, A.A., Mihalik, K., Feinstone, S.M. and Rice, C.M. (2000) J. Virol. 74, 2046–2051.
[7] Cheng, J.C., Chang, M.F. and Chang, S.C. (1999) J. Virol. 73, 7044–7049.
[8] Yamashita, T., Kaneko, S., Shirota, Y., Qin, W., Nomura, T., Kobayashi, K. and Murakami, S. (1998) J. Biol. Chem. 273, 15479–15486.
[9] Oh, J.W., Ito, T. and Lai, M.M. (1999) J. Virol. 73, 7694–7702.
[10] Zhong, W., Ferrari, E., Lesburg, C.A., Maag, D., Ghosh, S.K.B., Cameron, C.E., Lau, J.Y.N. and Hong, Z. (2000) J. Virol. 74, 9134–9143.
[11] Tu, H., Gao, L., Shi, S.T., Taylor, D.R., Yang, T., Mircheff, A.K., Wen, Y., Gorbalenya, A.E., Hwang, S.B. and Lai, M.M. (1999) Virology 263, 30–41.
[12] Ishido, S., Fujita, T. and Hotta, H. (1998) Biochem. Biophys. Res. Commun. 244, 35–40.
[13] Blight, K.J., McKeating, J.A. and Rice, C.M. (2002) J. Virol. 76, 13001–13014.
[14] Qin, W., Luo, H., Nomura, T., Hayashi, N., Yamashita, T. and Murakami, S. (2002) J. Biol. Chem. 277, 2132–2137.
[15] Wang, Q.M., Hockman, M.A., Staschke, K., Johnson, R.B., Case, K.A., Lu, J., Parsons, S., Zhang, F., Rathnachalam, R., Kirkegaard, K. and Colacino, J.M. (2002) J. Virol. 76, 3865–3872.
[16] Shirota, Y., Luo, H., Qin, W., Kaneko, S., Yamashita, T., Kobayashi, K. and Murakami, S. (2002) J. Virol. 277, 11149–11155.
[17] Schmidt-Mende, J., Bieck, E., Hugle, T., Penin, F., Rice, C.M., Blum, H.E. and Moradpour, D. (2001) J. Biol. Chem. 276, 44052–44063.
[18] Waggoner, S. and Sarnow, P. (1998) J. Virol. 72, 6699–6709.
[19] Chen, C.J., Kuo, M.D., Chien, L.J., Hsu, S.L., Wang, Y.M. and Lin, J.H. (1997) J. Virol. 71, 3466–3473.
[20] Lemm, J.A., Rumenapf, T., Strauss, E.G., Strauss, J.H. and Rice, C.M. (1994) EMBO J. 13, 2925–2934.
[21] Bost, A.G., Venable, D., Liu, L. and Heinz, B.A. (2003) J. Virol. 77, 4401–4408.
[22] Gupta, S., De, B.P., Drazba, J.A. and Banerjee, A.K. (1998) J. Virol. 72, 2655–2662.
[23] Moyer, S.A., Baker, S.C. and Horikami, S.M. (1990) J. Gen. Virol. 71, 775–783.
[24] Moyer, S.A., Baker, S.C. and Lessard,.J.L. (1986) Proc. Natl. Acad. Sci. USA 83, 5405–5409.
[25] De, B.P., Lesoon, A. and Banerjee, A.K. (1991) J. Virol. 65, 3268–3275.
[26] De, B.P., Burdsall, A.L. and Banerjee, A.K. (1993) J. Biol. Chem. 268, 5703–5710.
[27] Matsudaira, P. (1994) J. Cell Biol. 126, 285–287.
[28] Hirano, M., Kaneko, S., Yamashita, T., Luo, H., Qin, W., Shirota, Y., Nomura, T., Kobayashi, K. and Murakami, S. (2003) J. Biol. Chem. 278, 5109–5115.
[29] Bressanelli, S., Tomei, L., Roussel, A., Incitti, I., Vitale, R.L., Mathieu, M., Francesco, R.D. and Rey, F.A. (1999) Proc. Natl. Acad. Sci. USA 96, 13034–13039.