

## Hepatitis C virus NS4A inhibits cap-dependent and the viral IRES-mediated translation through interacting with eukaryotic elongation factor 1A

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### Summary

The genomic RNA of hepatitis C virus (HCV) encodes the viral polyprotein precursor that undergoes proteolytic cleavage into structural and nonstructural proteins by cellular and the viral NS3 and NS2-3 proteases. Nonstructural protein 4A (NS4A) is a cofactor of the NS3 serine protease and has been demonstrated to inhibit protein synthesis. In this study, GST pull-down assay was performed to examine potential cellular factors that interact with the NS4A protein and are involved in the pathogenesis of HCV. A trypsin digestion followed by LC-MS/MS analysis revealed that one of the GST-NS4A-interacting proteins to be eukaryotic elongation factor 1A (eEF1A). Both the N-terminal domain of NS4A from amino acid residues 1–20, and the central domain from residues 21–34 interacted with eEF1A, but the central domain was the key player involved in the NS4A-mediated translation inhibition. NS4A(21–34) diminished both cap-dependent and HCV IRES-mediated translation in a dose-dependent manner. The translation inhibitory effect of NS4A(21–34) was relieved by the addition of purified recombinant eEF1A in an *in vitro* translation system. Taken together, NS4A inhibits host and viral translation through interacting with eEF1A, implying a possible mechanism by which NS4A is involved in the pathogenesis and chronic infection of HCV.

### Introduction

Hepatitis C virus (HCV) is the major causative agent of human chronic hepatitis and is closely associated with hepatocellular carcinoma [1]. It is an enveloped virus and is classified as a separate genus in the family *Flaviviridae* [2]. The genome of HCV is a single-stranded, positive sense RNA of approximately 9.6 kb that encodes a polyprotein of approximately 3000 amino acid residues [3–5]. The polyprotein precursor is processed cotranslationally and post-

translationally by cellular ER peptidase and viral proteases to generate mature structural and non-structural proteins [6–8]. Cleavage at the junction of NS2-NS3 occurs autoproteolytically by the NS2-3 protease [9], whereas cleavage of the downstream nonstructural proteins is performed by the viral NS3 serine protease [10–12].

Nonstructural protein 4A (NS4A) is a multifunctional protein with 54 amino acid residues. It acts as a cofactor of NS3 serine protease and plays an essential role in the NS4A-dependent cleavage at the NS3-NS4A and NS4B-NS5A junctions [13, 14]. Both NS4A and NS4B proteins were previously demonstrated to suppress translation in

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culture cells [15, 16]. However, neither NS4A nor NS4B affected the steady state level of canonical translational factors including eIF4G, eIF4E, PABP, and 4E-BP1 [16]. The mechanisms by which the HCV NS4A and NS4B involved in the translational inhibition remained unclear.

In this study, we have demonstrated that NS4A specifically interacted with eukaryotic elongation factor 1A (eEF1A) and inhibited both cap- and HCV IRES-dependent protein synthesis. The inhibitory effect was mainly mediated by the central domain of NS4A and could be restored by the addition of recombinant eEF1A in an *in vitro* translation system.

## Materials and methods

### Plasmids

- (i) Plasmids pCRII-Topo-NS(3c-5An), pCRII-Topo-NS4B, and pcDNA-HCV-SG. Plasmid pCRII-Topo-NS(3c-5An) encompasses cDNA sequences of the HCV genome (genotype 1b) from nucleotides 4458–6381 inserted into pCR<sup>®</sup>II-TOPO (Invitrogen). The cDNA fragment was generated from a serum sample of an HCV patient by reverse transcriptase–polymerase chain reaction (RT-PCR) with Advantage<sup>®</sup> One-Step RT-PCR kit (BD Biosciences Clontech) and the primer sets 5'-CTCGCAGCGGGCAGGCAGGACTGG-3' and 5'-CCCATCCACTTCCGTGAAGAA-3' for the initial amplification and 5'-GCGGCGAGGCGCGACTGGTAGG-3' and 5'-CCCATCCACTTCCGTGAAGAA-3' for a further amplification. An NS4B cDNA fragment was amplified from pCRII-Topo-NS(3c-5An) with primer set 5'-GGAATTCCATATGGCCTCACCTCCCTTACATCGAACAA-3' and 5'-CGGGATCCTCTAGATCAGCATGGCGTGGAGCAGTCTTCATT-3', and cloned into pCR<sup>®</sup>II-TOPO to generate plasmid pCRII-Topo-NS4B. Plasmid pcDNA-HCV-SG represents a subgenomic replicon of HCV genomic type 1b and consists of the HCV-IRES, the neo gene, and the EMCV-IRES fused to the HCV sequences from NS3 to NS5B and the 3' noncoding region. These plasmids were used to generate expression plasmids of HCV NS3, NS4A and NS4B proteins.
- (ii) Plasmids pCMV-Tag2C-NS4A and pGST-NS4A. For generation of pCMV-Tag2C-NS4A, a full-length NS4A cDNA was amplified from pCRII-Topo-NS(3c-5An) with the primer set 5'-CGGGATCCATATGAGCACCTGGGTGCTTGTA-3' and 5'-GGAATTCAGCACTCCTCCATTTTC-3', the PCR fragment was digested with *Bam*HI and *Eco*RI restriction endonucleases, and cloned into the *Bam*HI-*Eco*RI site of pCMV-Tag2C (Stratagene). For generation of plasmid pGST-NS4A, plasmid pCMV-Tag2C-NS4A was digested with *Bam*HI and treated with the Klenow fragment of DNA polymerase I prior to a further digestion with *Xho*I restriction endonuclease. The resulting fragment was inserted into the pGEX-6p-1 (GE Healthcare Bio-Sciences) from which the polylinker sequences between *Bam*HI and *Xho*I had been deleted and the *Bam*HI site blunted.
- (iii) Plasmids pcDNA-NS4A-V5HisTopo, pcDNA-NS3-V5HisTopo, and pcDNA-NS4B-V5HisTopo. For generation of plasmid pcDNA-NS4A-V5HisTopo, cDNA sequence of the full-length NS4A was amplified from pCMV-Tag2C-NS4A with the primer set 5'-CACCATGAGCACCTGGGTGCTTGTA3' and 5'-GCACTCCTCCATTTTCATC-3', and cloned into pcDNA<sup>TM</sup>3.1D/V5-His-TOPO<sup>®</sup> (Invitrogen). For generation of pcDNA-NS3-V5HisTopo and pcDNA-NS4B-V5HisTopo, full length cDNA fragments of the HCV NS3 and NS4B were amplified from pcDNA-HCV-SG with the primer set 5'-CACCATGGCGCCCATCACTGCCTACGCTCAACAGA-3' and 5'-CGTGACGACCTCCAGGTCAGCTGCCATGCATGT-3', and the primer set 5'-CATGGCCTGACACCTCCCTTACATCGAACAA-3' and 5'-GCATGGCGTGGAGCAGTCTTCATTA-3', respectively, the resulting PCR fragments were cloned into pcDNA<sup>TM</sup>3.1D/V5-His-TOPO<sup>®</sup>.
- (iv) Plasmids pcDNA-NS3-V5, pcDNA-NS4A-V5, and pcDNA-NS4B-V5. For construction of pcDNA-NS3-V5, plasmid pcDNA-NS3-V5HisTopo was digested with *Age*I restriction endonuclease, treated with the Klenow fragment of DNA polymerase I prior to a further digestion with *Hind*III restriction

- endonuclease, and cloned into the pcDNA3.1 (+) from which the polylinker sequences between *Xba*I and *Hind*III had been deleted and the *Xba*I end blunted. A similar approach was taken to generate pcDNA-NS4A-V5 from pcDNA-NS4A-V5HisTopo and pcDNA-NS4B-V5 from pcDNA-NS4B-V5HisTopo, except that *Bam*HI was used instead of *Hind*III.
- (v) Plasmids pcDNA-NS4A(1–34)-V5HisTopo and pcDNA-NS4A(35–54)-V5HisTopo. For construction of deletion mutants of pcDNA-NS4A-V5HisTopo, cDNA fragments representing NS4A(1–34) and NS4A(35–54) were amplified from pcDNA-NS4A-V5HisTopo with the primer set 5'-CGGGATCCACCA TGAGCACCTGG-3' and 5'-GCTCTAGA CTCTCCCGGACAAGATG-3' and primer set 5'-CGGGATCCACCATGCCGGCTG TCATTCCTGA-3' and 5'-TAGAAGGCA CAGTCGAGG-3' (BGH reverse primer), respectively. The PCR fragments were digested with *Bam*HI and *Xba*I restriction endonucleases and the resulting fragments were used independently to replace the cognate fragment of pcDNA-NS4A-V5HisTopo to generate pcDNA-NS4A(1–34)-V5HisTopo and pcDNA-NS4A(35–54)-V5HisTopo.
- (vi) Plasmids pcDNA-eEF1A-V5HisTopo, pcDNA-eEF1A(1–240)-V5HisTopo, pcDNA-eEF1A(201–462)-V5HisTopo, and pcDNA-eEF1A-HisTopo. For generation of plasmids pcDNA-eEF1A-V5HisTopo and its deletion mutants, RNA was isolated from Huh7 cells by a single step extraction method as described previously [17]. The RNA was used to perform RT-PCR with primers sets described below and the resulting cDNA fragments were cloned into pcDNA<sup>TM</sup>3.1D/V5-His-TOPO<sup>®</sup>. The primer sets used in the amplification were EF-K (5'-CAC CATGGGAAAGGAAAAGA CTC-3') and EF-R (5'-TTTAGCCTTCTGAGCTTTCT GG-3') for generating pcDNA-eEF1A-V5HisTopo that represents V5His-tagged full length eEF1A, EF-K and EF-NR (5'-ACGAGTTGGTGGTAGGAT3') for generating pcDNA-eEF1A(1–240)-V5HisTopo that represents V5His-tagged N-terminal eEF1A from amino acid residues 1–240, and EF-MK (5'-CACCATGCTGGAGCCAAG TGCTAA-3') and EF-R for generating pcDNA-eEF1A(201–462)-V5HisTopo that represents V5His-tagged C-terminal eEF1A from amino acid residues 201–462. For generation of plasmid pcDNA-eEF1A-HisTopo, the V5-epitope was removed from pcDNA-eEF1A-V5HisTopo following a digestion with *Xho*I and *Age*I restriction endonucleases and the ends were blunted with the Klenow fragment of DNA polymerase I prior to self-ligation.
- (vii) Plasmids pGST-NS3, pGST-NS4B, and pGST-eEF1A. For construction of pGST-NS3, plasmid pcDNA-NS3-V5HisTopo was digested with *Nco*I and treated with the Klenow fragment of DNA polymerase I prior to a further digestion with *Xho*I restriction endonuclease. The resulting fragment was inserted into the pGEX-6p-1 from which the polylinker sequences between *Eco*RI and *Xho*I had been deleted and the *Eco*RI site blunted. A similar approach was taken to generate pGST-eEF1A from pcDNA-eEF1A-V5HisTopo. For generation of plasmid pGST-NS4B, pCRII-Topo-NS4B described earlier was digested with *Eco*RI restriction endonuclease and the resulting NS4B-containing fragment was inserted into the *Eco*RI site of pGEX-6p-1.
- (viii) Deletion mutants of the plasmid pGST-NS4A. For construction of plasmids pGST-NS4A(1–34) and pGST-NS4A(21–54), cDNA fragments were obtained by PCR-amplification from pGST-NS4A with primer set 5'-CGGGATCCATATGAGCACCTGGGTGC TTGTA-3' and 5'-GGAATTCACCT CCCGGACAAGAT-3' and primer set 5'-GGAATTCATATGGGCAGCGTGGTCA TTGT-3' and 5'-GGAATTCAGCACTCCTC CATTTC-3', respectively, and the resultant fragments were digested with *Nde*I and *Eco*RI restriction endonucleases. For construction of plasmids pGST-NS4A(1–20), pGST-NS4A(21–34), and pGST-NS4A(35–54), cDNA fragments with *Nde*I and *Eco*RI recognition sequences at the 5' and 3' ends, respectively, were generated by annealing the following synthetic oligonucleotide sets: 5'-TATGAGCACCTGGGTGCTTGTAGGCG GGGTCCTTGCAGCTCTGGCCGCATAC TG-3' and 5'-AATTCACGTTGTCAGG

CAGTATGCGGCCAGAGCTGCAAGGAC  
 CCCGCCTACAAGCACCCAGGTGCTCA-  
 3' for pGST-NS4A(1–20), 5'-TATGGG  
 CAGCGTGGTCATTGTGGGCAGGATC  
 ATCTTGTC GGAAGTG-3' and 5'-  
 AATTCACCTCCCGGACAAGATGATC  
 CTGCCACAATGACCACGCTGCCCA-3'  
 for pGST-NS 4A(21–34), and 5'-TA  
 TGCCGGCTGTCATTCTGATAGGGA  
 GGTCTCTACCGGGAGTTCGATGAAA  
 TGGAGGAGTGCTG -3' and 5'-AATTMC  
 AGCACTCCTCCATTTTCATCGAACTCC  
 CGGTAGAGAACCTCCCTATCAGGAAT  
 GACAGCCGGCA-3' for pGST-NS4A(35–  
 54). These cDNA fragments were used to re-  
 place the cognate fragment of pGST-NS4A  
 to generate the deletion mutants of GST-  
 NS4A.

- (ix) Plasmids pCMV-Luc and pJSS12. Plasmid pCMV-Luc represents a cap-dependent monocistronic reporter of firefly luciferase that is driven by the promoter of cytomegalovirus. pJSS12 represents a bicistronic luciferase reporter containing the structure components of CMV promoter-T7 promoter-*Renilla* luciferase-stem loop-IRES(HCV1-371)-Firefly luciferase-poly A. Transcription of the bicistronic reporter can be driven by the promoter of cytomegalovirus in culture cells or by the promoter of T7 RNA polymerase *in vitro*. Expression of the *Renilla* luciferase and firefly luciferase genes are directed by the cap-dependent and HCV-IRES-mediated mechanism, respectively. In addition, the stem loop structure is derived from the sequence 5'-GTACCCCGGTACGGCAGTG CCGTACGACGAATTCGTTCGTACGGCA CTGCC GTACCGGGGTAC-3' and was inserted into the bicistronic structure to prevent leaky scanning of ribosome.

#### *Expression and purification of GST fusion proteins*

GST fusion plasmids were transformed into *Escherichia coli* BL21, or BL21(DE3) where indicated. The bacterial cells were grown in LB or 2X YT medium containing 50 µg ampicillin/ml to a density of 0.6–0.8 at 600 nm. Following an induction of the expression of GST fusion proteins with isopropyl-β-D-thiogalactopyranoside

(IPTG), the bacterial cells were harvested and resuspended in lysis buffer consisting of 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1.5% N-laurylsarcosine, and 2% Triton X-100. After two freeze-thaw cycles and sonication, cell lysates were separated into soluble and insoluble fractions. For partial purification of GST fusion proteins, the soluble fractions were incubated with glutathione-Sepharose 4B beads (GE Healthcare Bio-Sciences) at 4 °C for 2 h. Following a centrifugation in a microcentrifuge for 5 min, GST fusion proteins immobilized on the beads were spun-down and washed for five times with PBS. As a control, plasmid pGEX-6p-1 was transformed into the bacterial cells, and the expression and purification of GST protein were performed in parallel. In the *in vitro* translation inhibition assay, GST fusion proteins of the HCV nonstructural proteins were recovered from the beads with 10 mM glutathione in 50 mM Tris-HCl (pH 8.0). In addition, eEF1A was recovered from beads-immobilized GST-eEF1A with PreScission™ Protease (GE Healthcare Bio-Sciences).

#### *Cell line and expression of recombinant proteins in culture cells*

Huh7 cells (a human hepatoma cell line) were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum plus 100 units of penicillin, and 100 µg of streptomycin/ml. Expression of recombinant proteins in Huh7 cells was performed by DNA transfection with cationic liposomes (Invitrogen) or by infecting cells with recombinant vaccinia virus (vTF7-3) harboring T7 RNA polymerase gene followed by DNA transfection as described previously [18]. Two days posttransfection, the transfected cells were harvested for further analysis.

#### *Preparation of Huh7 cell lysates, in vitro translation products, and the NS4A(21–34) peptide*

To perform GST pull-down assay, cell lysates were prepared from Huh7 cells grown to confluency with a lysis buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% sodium deoxycholate, 1% NP-40, and 0.1% SDS. Alternatively, *in vitro*



translation products were used. *In vitro* translation was performed in the presence of [<sup>35</sup>S]methionine (NEN) by the T<sub>N</sub>T<sup>®</sup> Quick Coupled Transcription/Translation System (Promega). In addition, the NS4A(21–34) peptide used in the *in vitro* translation inhibition assay was synthesized and purified through HPLC to > 85% purity.

#### *GST pull-down assay*

To perform GST pull-down assay, GST fusion proteins immobilized on glutathione-Sepharose 4B beads were incubated independently with the Huh7 cell lysates or *in vitro* translation products at 4 °C for 2 h. The protein-bound glutathione beads were then washed for five times with PBS and boiled for 5 min prior to SDS-PAGE. Proteins that copurified with GST fusion proteins were visualized by Coomassie blue staining, silver staining, or autoradiography. Identities of the copurified proteins were further examined by Western blot analysis.

#### *Coimmunoprecipitation and Western blot analysis*

To perform coimmunoprecipitation experiments, transfected cells were washed twice with PBS and lysed in a RIPA buffer consisting of 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% sodium deoxycholate, 1% NP-40, 0.1% SDS, and Complete<sup>™</sup> protease inhibitors (Roche). Equivalent amounts of the cell lysates were incubated with specific antibodies for 16 h at 4 °C and then with protein A Sepharose CL-4B (GE Healthcare Bio-Sciences) for additional 4 h. The immunoprecipitates were washed five times with RIPA buffer and resuspended in a lysis buffer consisting of 1.7 mM urea, 3.3% SDS, and 0.65 M β-mercaptoethanol. The resultant supernatants were resolved on polyacrylamide gel and subjected to Western blot analysis. Western blot analysis was performed as described previously [19]. The specific interactions between antigens and antibodies were detected by the enhanced chemiluminescence system (GE Healthcare Bio-Sciences).

#### *Antibodies*

Mouse monoclonal antibody against V5 epitope (GKPIPPLLGLDST) was purchased from Invitrogen. Rabbit polyclonal antibody against His-tag

was purchased from Santa Cruz. Mouse monoclonal antibody against eEF1A was purchased from Upstate. Goat polyclonal antibody against luciferase was purchased from Promega.

#### *RT-PCR of luciferase mRNA*

The level of luciferase mRNA was examined by RT-PCR. In brief, total RNA was isolated from culture cells by using TRIzol<sup>®</sup> reagent (Invitrogen). Reverse transcription was performed with the RNA template, AMV reverse transcriptase (Roche), and oligo-dT primer. The products were subjected to polymerase chain reaction with the primer set 5'-CAGAGGACCTATGATTATGTC 3' and 5'-CGGTACTTCGTCCACAAACACAA C-3'. In addition, primers 5'-GAAGGTGAAGG TCGGACTC-3' and 5'-TTTAGCCTTCTGA GCTTTCTGG-3' were used in parallel to analyze the level of GAPDH mRNA as an internal control.

#### *Translation inhibition assay in cell culture system*

To perform translation inhibition assay in culture cells, plasmids encoding V5-tagged HCV nonstructure proteins NS3, NS4A, NS4B, and NS4A mutants were independently cotransfected with the cap-dependent monocistronic luciferase reporter pCMV-Luc or the bicistronic luciferase reporter pJSS12 into Huh7 cells. The cells were harvested 2 days posttransfection. Luciferase activities were analyzed followed the procedures as described by the manufacturer (Promega) and measured with a luminometer (Orion II, Berthold). In addition, the levels of luciferase protein and luciferase mRNA were examined by Western blot analysis and RT-PCR, respectively.

#### *Translation inhibition assay and restore experiment in in-vitro-translation system*

*In-vitro*-translation system was applied to study the inhibitory effects of NS4A on both cap-dependent and HCV IRES-mediated translation. To perform cap-dependent *in-vitro*-translation inhibition assay, GST and GST-NS4A fusion proteins that were recovered from the Sepharose 4B beads as described earlier were preincubated with 5 μl of rabbit reticulocyte lysate (RRL, Promega) at 4 °C for 1 h. Translation reaction

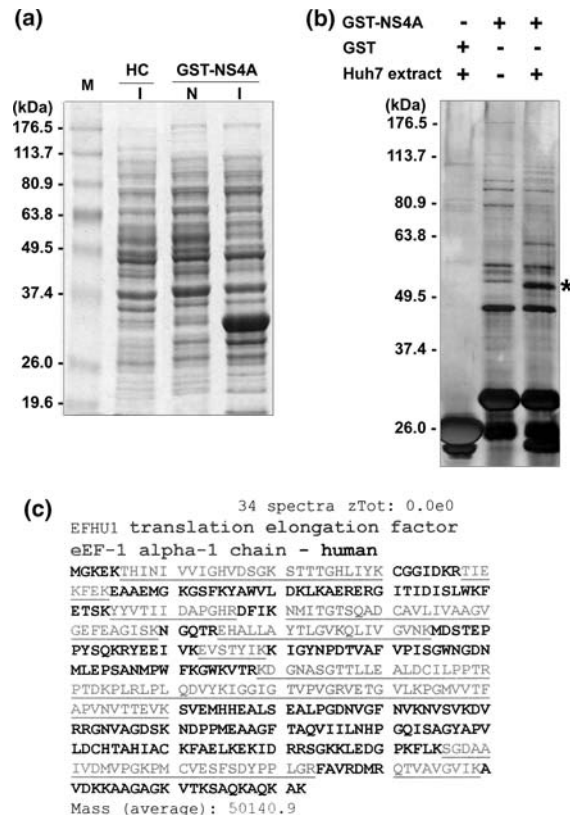
was then carried out at 30 °C for 90 min after addition of 250 ng of *in vitro*-transcribed monocistronic luciferase mRNA, amino acid mixtures, and RNase inhibitor, followed by luciferase activity assay. Alternatively, the translation reaction was performed in the presence of [<sup>35</sup>S]methionine (NEN) and the inhibitory effects of NS4A on translation were analyzed by autoradiography following SDS-PAGE. On the other hand, *in vitro*-translation inhibition assay was performed with synthetic NS4A(21–34) peptide and 500 ng of the bicistronic luciferase mRNA *in vitro*-transcribed from plasmid pJSS12. Luciferase activities of *Renilla* and firefly that represent the cap-dependent and HCV IRES-mediated translation, respectively, of the bicistronic reporter were analyzed using the Dual-Glo™ Luciferase Assay System (Promega). In addition, to examine the ability of eEF1A to restore the translation inhibition, eEF1A was released from GST-eEF1A by PreScission™ Protease, dialyzed to 50 mM Tris-HCl (pH 8.0), and incubated for 30 min with the reaction mixture of GST-NS4A protein and RRL in the cap-dependent *in vitro*-translation inhibition assay. Luciferase activity was analyzed as described earlier.

## Results

### Identification of cellular proteins specifically interact with the NS4A protein of HCV

To learn the possible association of host factors with the HCV NS4A protein that may render NS4A pathogenic to the host, GST pull-down assay was performed to identify NS4A-interacting proteins. GST-NS4A protein was expressed in *E. coli* BL21 in the presence of IPTG (Figure 1a). Following purification with glutathione-Sepharose 4B beads, the GST-NS4A protein was subjected to GST pull-down assay with Huh7 cell extracts. Silver staining identified several proteins that specifically pulled down by the GST-NS4A protein (Figure 1b). The most abundant NS4A-interacting candidate protein was subjected to trypsin digestion and LC-MS/MS analysis. Thirty-four spectra that represent 19 independent tryptic fragments of 7–29 amino acid residues all identified the protein as human translation elongation factor 1 alpha-1 (eEF1A)

(Figure 1c). Cellular factors that participate in protein synthesis are now well characterized. It is clear that eEF1A interacts with GTP and is responsible for binding aminoacyl-tRNA to the ribosome during polypeptide elongation [20].



**Figure 1.** Identification of cellular factors that specifically interact with HCV NS4A protein by GST pull-down assay and LC-MS/MS analysis. (a) Expression of GST-NS4A fusion protein. Expression of GST-NS4A fusion protein was induced in the presence of 1 mM IPTG for 3 h at 37 °C in *E. coli* BL21. The soluble fractions of cell extracts were separated on SDS-8 % polyacrylamide gel. Coomassie blue staining is shown. N, noninduction; I, induction; HC, host control. (b) GST pull-down assay. Huh7 cell extracts were incubated with the soluble fractions of bacterial lysates prepared from BL21 that expressed the GST and GST-NS4A fusion protein as indicated, followed by GST pull-down analysis. Silver staining is shown. The protein that specifically interacted with GST-NS4A as indicated by asterisk was sliced out from the gel and subjected to LC-MS/MS analysis. (c) LC-MS/MS analysis. Underlines show the peptide sequences of 19 independent tryptic fragments of the NS4A-interacting protein marked in panel (b). Sequence comparison with data banks indicated that the NS4A-interacting protein to be eEF1A.

*HCV NS4A protein specifically interacts with eEF1A and inhibits both cap-dependent and HCV IRES-mediated protein synthesis in culture cells*

Previous studies have demonstrated that both NS4A and NS4B inhibit protein synthesis [15, 16], but the molecular basis involved in the inhibition was not clear. By performing cotransfection experiments with an HCV NS3-, NS4A-, or NS4B-encoding plasmid and a cap-dependent monocistronic luciferase reporter into Huh7 cells, specific inhibitory effects of NS4A and NS4B on the cap-dependent translation were detected in this study. The luciferase activity was significantly reduced when the reporter plasmid was coexpressed with NS4A or NS4B protein (Figure 2a). In addition, the effects correlated very well with the protein levels of luciferase (Figure 2b), whereas the luciferase mRNA level was not affected by the HCV nonstructural proteins (Figure 2c). Possible effects of the viral nonstructural proteins on HCV IRES-mediated translation were further examined by cotransfecting into Huh7 cells a bicistronic reporter that consists of the *Renilla* luciferase, the HCV IRES (genotype 1b), and the firefly luciferase genes. Two days posttransfection, Dual-Glo™ luciferase assay (Promega) was performed. *Renilla* luciferase activity represents the cap-dependent translation and firefly luciferase activity represents the HCV IRES-mediated translation. The results demonstrated that both NS4A and NS4B inhibited HCV IRES-mediated translation to levels similar to those of the cap-dependent translation, whereas no effect was detected with the viral NS3 protein (Figure 2d). We proposed that through interacting with eEF1A, HCV NS4A protein may deplete or interfere eEF1A in forming functional complexes involved in both cap-dependent and IRES-mediated translation. To test this hypothesis, we first examined the specificity of the interaction between NS4A and eEF1A. GST fusion proteins of the viral NS3, NS4A, and NS4B were expressed in *E. coli* BL21(DE3) in the presence of IPTG, and immobilized on glutathione-Sepharose 4B beads (Figure 3a). GST pull-down assay was performed with Huh7 cell extracts followed by Western blot analysis with anti-eEF1A antibody. The results demonstrated that NS4A but neither NS3 nor NS4B interacted with eEF1A in the GST pull-down system (Figure 3b). To examine the interaction in culture cells, cotransfection experiments

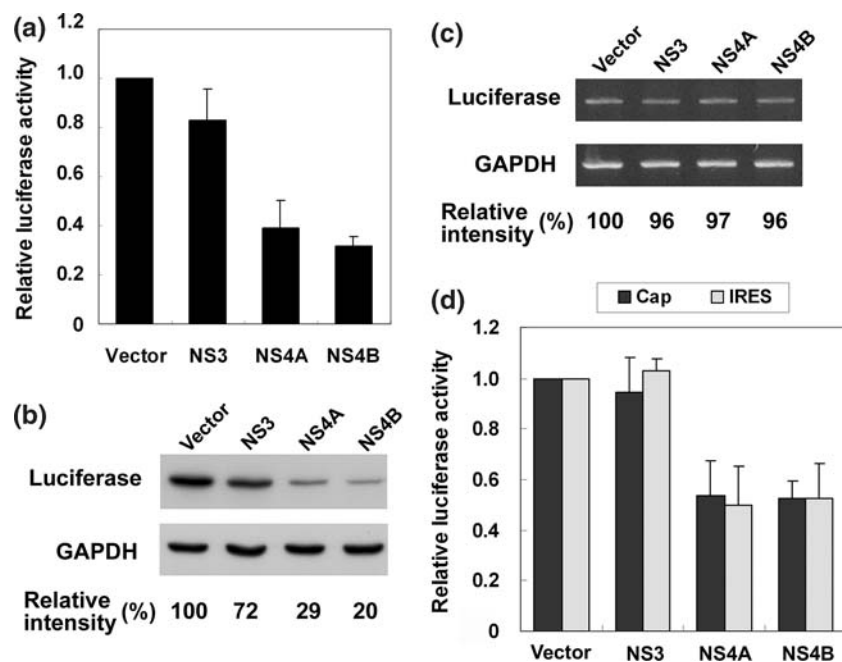
were performed in Huh7 cells with plasmids encoding V5-tagged NS4A (pcDNA-NS4A-V5) or NS4B (pcDNA-NS4B-V5) and a His-tagged eEF1A (pcDNA-eEF1A-HisTopo). Two days posttransfection, cell extracts were immunoprecipitated with anti-His antibody followed by Western blot analysis with anti-V5 antibody. As shown in Figure 3c, NS4A but not NS4B was coimmunoprecipitated with the eEF1A. These results indicate that, although both NS4A and NS4B are capable of inhibiting protein synthesis, binding to eEF1A is a unique characteristic of NS4A. It is likely that NS4A and NS4B inhibit protein translation through different mechanisms.

*NS4A interacts with eEF1A through its N-terminal and central domains*

To identify the subdomains of NS4A responsible for interacting with eEF1A, deletion plasmids representing GST fusion proteins of various domains of the NS4A were generated and expressed in *E. coli* BL21. Following a partial purification, the GST-NS4A mutant proteins were subjected to GST pull-down assay with Huh7 cell extracts. As shown in Figure 4a, eEF1A was copurified with the GST-NS4A(1–34) bound on the glutathione-Sepharose 4B beads, but not with the GST-NS4A(35–54) fusion protein. Nevertheless, GST-NS4A(21–54) showed an eEF1A-binding activity. Further deletion analysis indicated that the central domain of NS4A from amino acid residues 21–34 [GST-NS4A(21–34)] is sufficient for the binding of eEF1A. In addition, NS4A(1–20) represents a second domain that, independent of NS4A(21–34), is capable of interacting with eEF1A.

*NS4A interacts with the C terminus of eEF1A*

The functional domain of eEF1A involved in the binding of NS4A was examined. Full-length eEF1A, its N terminus from amino acid residues 1–240 [eEF1A(1–240)], and the C terminus from amino acid residues 201–462 [eEF1A(201–462)] were synthesized *in vitro* by the T<sub>N</sub>T Quick Coupled Transcription/Translation system. The translation products were subjected to GST pull-down assay with partially purified GST and GST-NS4A fusion proteins. As shown in Figure 4b, both the full-length eEF1A and the



**Figure 2.** Translation inhibition of luciferase reporters by the HCV NS4A and NS4B proteins in culture cells. (a)–(c) The inhibitory effects of NS4A and NS4B proteins on cap-dependent translation. Plasmids pcDNA-NS3-V5, pcDNA-NS4A-V5, and pcDNA-NS4B-V5 encoding HCV NS3, NS4A, and NS4B, respectively, were independently cotransfected into Huh7 cells with the monocistronic luciferase reporter plasmid pCMV-Luc. Two days posttransfection, cell lysates were harvested and equal amounts of the proteins were used to perform luciferase assay (panel a) and Western blot analysis (panel b). Meanwhile, total RNA was isolated to perform RT-PCR (panel c). Luciferase activities derived from the transfected cells were normalized by the activity of the cells cotransfected with pCMV-Luc and pcDNA3.1(+) control vector. The results represent the average of four independent experiments. Western blot analysis was performed with antibodies specific to luciferase and GAPDH as indicated. Intensities of the luciferase proteins were normalized in each set against the intensity of GAPDH and compared to the control. Relative intensities of the luciferase protein are shown. RT-PCR of luciferase mRNA was performed with oligo-dT and primers as described in the materials and methods for 20 amplification cycles. Intensities of the luciferase cDNA fragment were normalized in each set against the intensity of GAPDH and relative intensities as compared to the vector control are shown. (d) The inhibitory effects of NS4A and NS4B proteins on HCV IRES-mediated translation. The bicistronic luciferase reporter plasmid pJSS12 was cotransfected independently with the HCV NS3-, NS4A-, and NS4B-encoding plasmid. The activities of *Renilla* luciferase and firefly luciferase that representing cap-dependent and HCV IRES-mediated translation, respectively, were analyzed 2 days posttransfection with Dual-Glo™ Luciferase Assay System as described in the Materials and methods. Relative luciferase activities in the presence of the HCV nonstructural proteins were calculated by normalization of the activities in each set to that of the cells cotransfected with the reporter plasmid and pcDNA3.1(+) control vector.

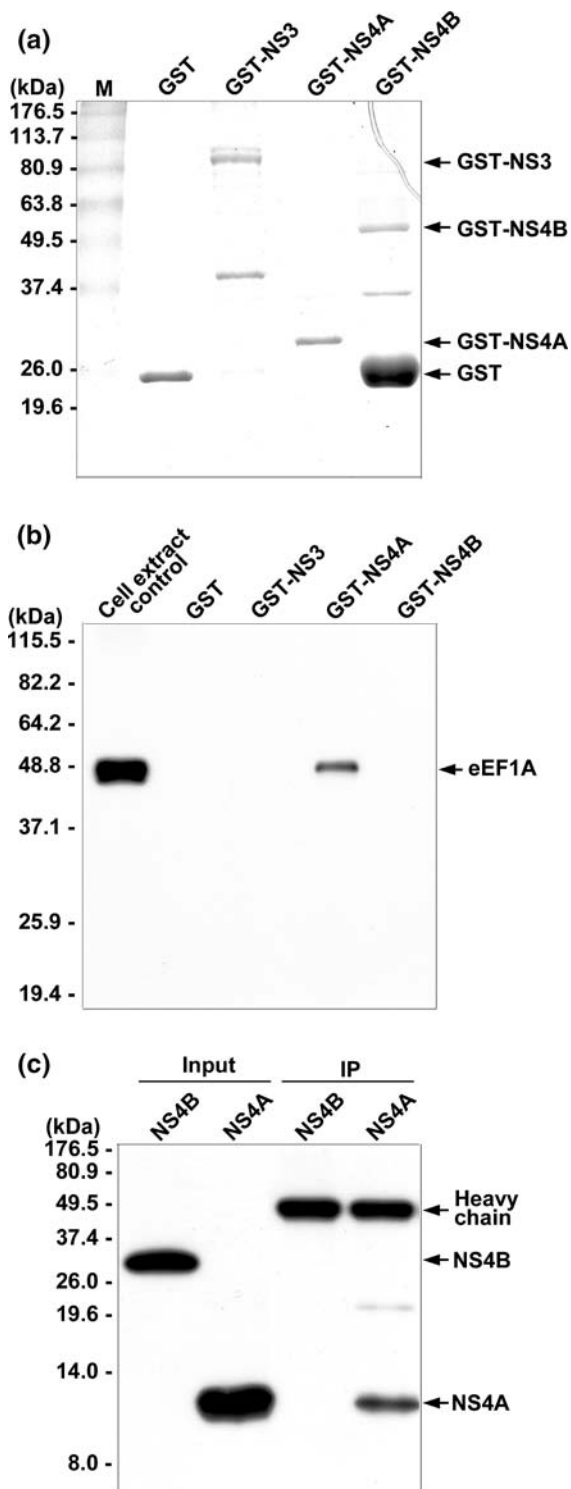
eEF1A(201–462) were pulled-down by the GST-NS4A fusion protein, but the eEF1A(1–240) could not. These results indicate a specific interaction between NS4A and the C-terminal domain of eEF1A.

#### *The central domain of NS4A plays a critical role in the translation inhibition*

In Figure 4a, we have demonstrated that NS4A interacted with eEF1A through two independent domains within the N-terminal 34 amino acid residues. To link the interaction between NS4A and eEF1A to the effect of NS4A on protein

synthesis, translation inhibition assay was performed. Huh7 cells were cotransfected with the monocistronic luciferase reporter and a plasmid representing NS4A(1–34) or NS4A(35–54). Two days posttransfection, cells were harvested and analyzed for the luciferase activity and the levels of luciferase protein and mRNA. As shown in Figure 5, NS4A(1–34) inhibited both the luciferase activity and luciferase protein to levels compatible to the full-length NS4A protein did but had little effect on the level of luciferase mRNA. These results indicated that the N-terminal 34 amino acid residues are responsible for the inhibitory effect of NS4A on translation. To determine which of the



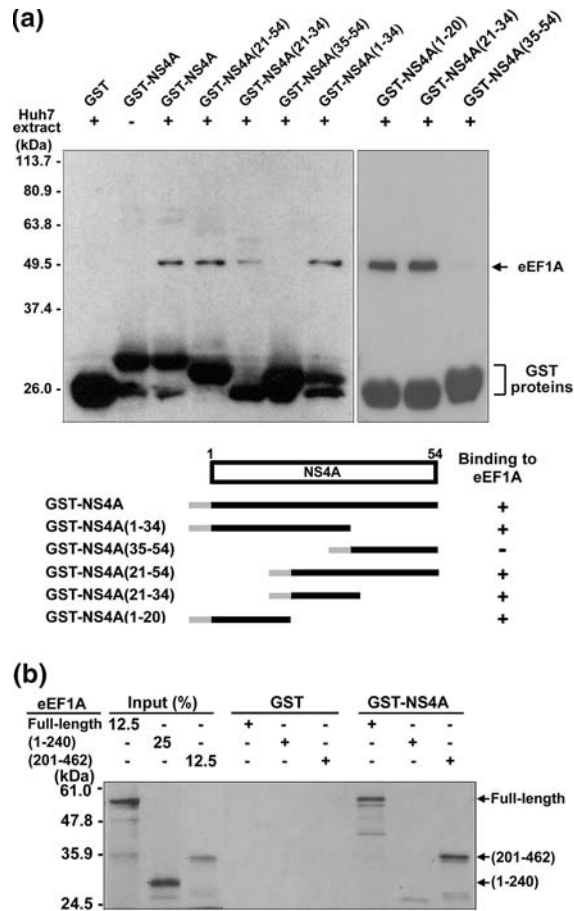


two eEF1A-interacting domains, NS4A(1–20) and NS4A(21–34), is essential for the translation inhibition, GST-NS4A and its deletion mutant

←

**Figure 3.** Specific interaction between HCV NS4A and eEF1A. (a) Expression and partial purification of GST fusion proteins. Expression of GST, GST-NS3, GST-NS4A, and GST-NS4B proteins were induced in the presence of 0.5 mM IPTG at 37 °C for 5 h in *E. coli* BL21(DE3). Following a partial purification, the GST fusion proteins immobilized on the glutathione-Sepharose 4B beads were analyzed by SDS-PAGE. Coomassie blue staining is shown. (b) eEF1A specifically copurified with GST-NS4A. Huh7 cell extracts were incubated with partially purified GST-fusion proteins as indicated, followed by GST pull-down assay and Western blot analysis with antibody specific to eEF1A. Cell extract control represents Huh7 cell extract that was loaded directly onto the gel to serve as a control. (c) Interaction between NS4A and eEF1A in transfected culture cells. Plasmids pcDNA-NS4A-V5 and pcDNA-NS4B-V5 that encode V5 epitope-tagged NS4A and NS4B proteins, respectively, were independently cotransfected into Huh7 cells with plasmid pcDNA-eEF1A-HisTopo that encodes a His-tagged fusion protein of the full-length eEF1A (eEF1A-His). Cell lysates were prepared 2 days posttransfection to perform coimmunoprecipitation assay with antibodies against the His-tag of eEF1A-His protein and Western blot analysis with antibodies specific to the V5-epitope to detect V5-tagged NS4A and NS4B proteins. Input lanes represent 5 % of the cell lysates used in the coimmunoprecipitation assay and were loaded directly onto the gel to serve as positive controls of Western blot analysis.

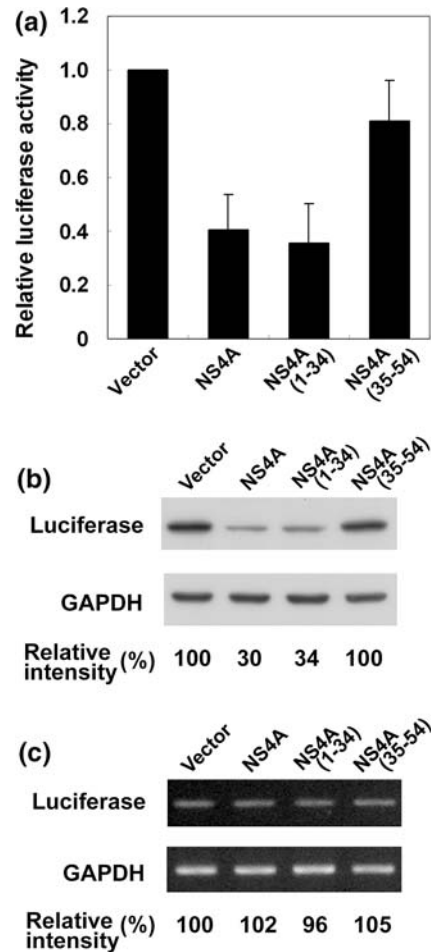
proteins were purified (Figure 6a) and used to perform a translation inhibition assay in a cell-free system. Increasing amounts of the purified GST fusion proteins were pre-incubated with RRL followed by *in vitro* translation reaction with the monocistronic luciferase mRNA as the reporter. The results demonstrated that both the GST-NS4A and GST-NS4A(21–34) significantly reduced the luciferase activity, whereas the effects of GST-NS4A(1–20) and GST-NS4A(35–54) proteins were negligible (Figure 6b). In addition, the GST-NS4A(21–34) protein had a stronger effect on the translation inhibition than that of the wild type. It required less than half amount of GST-NS4A(21–34) protein to reach a 50%-inhibition when compared with the full-length NS4A protein ( $4.29 \times 10^{13}$  moles for full-length and  $1.81 \times 10^{13}$  moles for the subdomain 21–34). The effects of NS4A subdomains on the translation inhibition were also examined by analyzing the level of luciferase protein following an *in-vitro*-translation reaction in the presence of [ $^{35}$ S]methionine and  $2 \times 10^{13}$  moles of the NS4A proteins. As shown in Figure 6c, GST-NS4A(21–34) had a greater effect than the GST-NS4A(1–20) whereas GST-NS4A(35–54) had no effect on the translation inhibition.



**Figure 4.** Mapping of the interacting domains of HCV NS4A and eEF1A by GST pull-down assay. (a) Both GST-NS4A(1-20) and GST-NS4A(21-34) are capable of interacting with eEF1A. GST-NS4A fusion protein and its deletion mutants were expressed in *E. coli* BL21 following an induction with 0.05 mM IPTG for 5 h at 25 °C. Soluble fractions of the bacterial lysates were subjected to GST pull-down assay with Huh7 cell extracts. The interactions between eEF1A and the NS4A deletion mutants were examined by Western blot analysis with antibodies specific to eEF1A and GST fusion proteins as indicated. Characteristics of the NS4A deletion mutants to bind eEF1A are summarized. Grey bars indicate the GST fusion domains. (b) The C-terminal domain of eEF1A is involved in forming complex with HCV NS4A protein *in vitro*. GST pull-down assay was performed with partially purified GST fusion proteins and the [<sup>35</sup>S]methionine-labeled *in vitro* translation products of the full-length eEF1A, the N-terminal eEF1A(1-240), and the C-terminal eEF1A(201-462) as indicated. Autoradiography is shown.

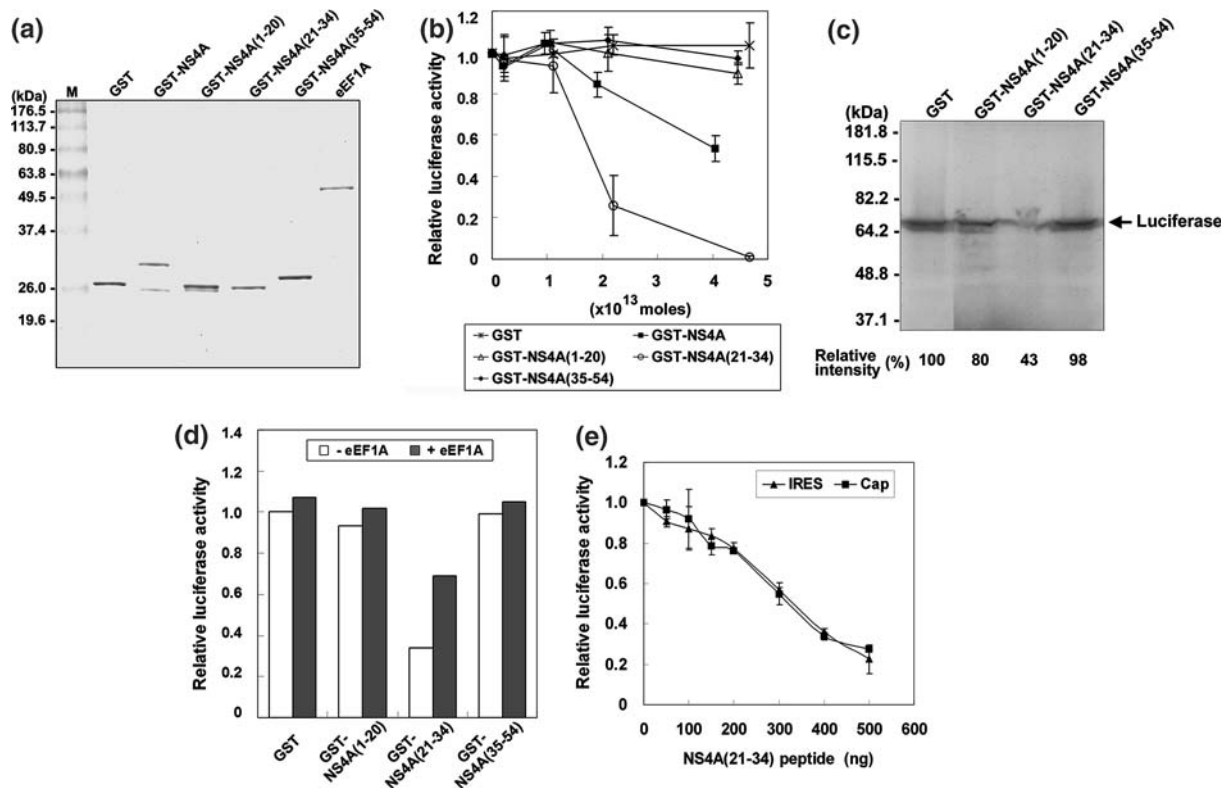
*Addition of purified recombinant eEF1A restores the translation defect caused by the NS4A(21-34) protein*

The effect of NS4A on the translation inhibition may be resulted from a competition



**Figure 5.** Translation inhibition activity of the NS4A(1-34) protein. (a) Inhibitory effect of NS4A(1-34) on luciferase activity. Plasmids representing the full-length NS4A and its deletion mutants NS4A(1-34) and NS4A(35-54) were independently co-transfected with the monocistronic luciferase reporter plasmid into Huh7 cells as described in the legend to Figure 2. Relative luciferase activity was analyzed 2 days posttransfection. The results represent the average of five independent experiments. (b) Inhibitory effect of NS4A(1-34) on the expression level of luciferase protein. The cell lysates isolated 2 days posttransfection were subjected to Western blot analysis with antibodies specific to luciferase and GAPDH as indicated. The intensities of luciferase protein were normalized in each set against the intensity of GAPDH and compared to the vector control. Relative intensities of the luciferase protein are shown. (c) NS4A(1-34) protein has no inhibitory effect on the level of luciferase mRNA. Total RNA was isolated 2 days posttransfection and RT-PCR was performed as described in the legend to Figure 2. Intensities of the luciferase cDNA fragment were normalized in each set against the intensity of GAPDH and relative intensities as compared to the vector control are shown.

between NS4A and translation factors that are involved in forming functional translation complexes with eEF1A. We therefore examined



**Figure 6.** The translation defect caused by the NS4A(21–34) protein could be significantly restored by the addition of purified recombinant eEF1A. (a) Partially purified GST-NS4A proteins and eEF1A. GST and GST fusion proteins of the wild-type NS4A and its deletion mutants were eluted out from the glutathione-Sepharose 4B beads. eEF1A was purified from bead-bound GST-eEF1A following a cleavage with PreScission<sup>TM</sup> Protease and dialyzed into 50 mM Tris-HCl (pH 8.0) as described in the Materials and methods. The purified proteins were analyzed by SDS-PAGE. Coomassie blue staining is shown. (b)–(c) Cap-dependent *in-vitro*-translation inhibition assay. Various amounts of the purified GST fusion proteins as indicated in panel b or  $2 \times 10^{13}$  moles of the GST fusion proteins as indicated in panel c were pre-incubated with RRL for 1 h at 4 °C. Translation reaction was then carried out at 30 °C following addition of the monocistronic luciferase mRNA, amino acid mixtures, and RNase inhibitor in the absence (panel b) or presence (panel c) of [<sup>35</sup>S]methionine. The translation products were subjected to luciferase activity assay (panel b) or SDS-PAGE followed by autoradiography (panel c). Relative luciferase activities were calculated by normalization of the luciferase activities in the presence of the GST and GST-NS4A proteins to that without any GST proteins. (d) Translation restore experiments. GST fusion proteins of  $2 \times 10^{13}$  moles were preincubated with RRL for 1 h at 4 °C followed by an additional incubation for 30 min in the presence of 0.5  $\mu$ g purified recombinant eEF1A. *In-vitro*-translation reaction was then performed. The ability of the purified eEF1A to restore the translation inhibition was analyzed by luciferase activity assay. Relative luciferase activities as compared to that of GST in the absence of exogenous eEF1A are shown. The result represents the average of two independent experiments. (e) HCV IRES-mediated *in-vitro*-translation inhibition assay. Various amounts of the NS4A(21–34) peptide as indicated were preincubated with RRL for 30 min at 4 °C prior to the *in-vitro*-translation reaction. *In-vitro*-translation reaction was performed as described in the legend to panel b except that the bicistronic luciferase reporter mRNA and NS4A(21–34) peptide were used. Luciferase activities of *Renilla* and firefly that represent cap-dependent and HCV IRES-mediated translation, respectively, were analyzed and normalized in each set against the luciferase activity of the reaction without addition of the NS4A(21–34) peptide.

whether exogenous eEF1A would restore the translation defect caused by the NS4A(21–34). Recombinant eEF1A was released from GST-eEF1A with PreScission<sup>TM</sup> protease (Figure 6a), added to the pre-incubation mixtures of RRL and GST-NS4A fusion proteins and subjected

to *in vitro* translation reaction with the monocistronic luciferase mRNA. The results from luciferase assay demonstrated that the addition of purified eEF1A significantly reduced the inhibitory effect caused by the GST-NS4A(21–34) protein (Figure 6d).

*Dose-dependent inhibition of both cap-dependent and HCV IRES-mediated translation by the synthetic NS4A(21–34) peptide*

To eliminate the possibility that the translation inhibition effect of GST-NS4A(21–34) is resulted from a trace contamination of inhibitors present in the protein preparation, a synthetic NS4A(21–34) peptide purified by HPLC to >85% purity was applied. Meanwhile, the bicistronic luciferase reporter mRNA was used in the *in vitro* translation system to examine the inhibitory effect of the NS4A(21–34) peptide on both cap- and IRES-dependent translation. The cap-dependent translation of Renilla luciferase and HCV IRES-mediated translation of firefly luciferase were analyzed by Dual-Glo™ luciferase assay. The results clearly demonstrated a dose-dependent inhibitory effect of the NS4A(21–34) peptide on both cap-dependent and HCV IRES-mediated translation (Figure 6e).

## Discussion

Upon virus infection, the translation machinery of host cells is known to be down-regulated. Viral proteins derive ability to modify or regulate the expression and function of translation factors that results in an inhibition of host protein synthesis [21]. Poliovirus-encoded proteases 2A and 3C cleave eIF4G (eIF4GI and eIF4GII) and PABP, respectively, of host cells [22, 23]. The NSP3 of rotavirus substitutes the function of cellular PABP. It binds to the 3' end of the nonpolyadenylated viral mRNA and competes with PABP in binding to eIF4G [24]. In both cases, the viral proteins selectively inhibit translation of capped host mRNA but not the viral RNA.

HCV NS4A was previously demonstrated to inhibit protein synthesis through its N-terminal 40 amino acid residues [15]. But the steady state levels of eIF4G, eIF4E, PABP, and 4E-BP1 were not affected [16]. The molecular basis involved in the inhibitory effect of NS4A was not clear. In this study, we found that HCV NS4A protein specifically interacted with the translation elongation factor, eEF1A (Figures 1 and 3). The N-terminal and the central domains of the NS4A were involved independently in the interaction (Figure 4), but the central domain encompassing amino acid residues 21–34 played a major role in the inhibition

of the cap-dependent and HCV IRES-mediated translation (Figures 6b, c, and e). The translation inhibitory effect caused by the NS4A(21–34) could be relieved by the addition of purified recombinant eEF1A into the translation system (Figure 6d). Nevertheless, the translation inhibition was not fully restored, suggesting that binding of NS4A to the eEF1A may simultaneously deplete other translation factors that are associated with eEF1A. Alternatively, other mechanisms may also involve in the inhibitory effect of NS4A. In addition, although both HCV NS4A and NS4B proteins were shown to inhibit translation (Figure 2, and [15, 16]), no interaction was detected between eEF1A and HCV NS4B protein (Figure 3). These indicate that NS4B inhibits translation through a mechanism different from that of the NS4A protein. Recently, the interaction between eEF1A and HCV NS4A protein was also identified by yeast-two hybrid screening [25].

Viral proteins including the NS5A protein of bovine viral diarrhea virus (BVDV) [26], the RNA-dependent RNA polymerase of vesicular stomatitis virus (VSV) [27], and the Gag protein of human immunodeficiency virus (HIV) have been demonstrated to interact with eEF1A [28]. Functional roles of the interactions are not completely understood, but the interactions were proposed to be involved in the regulation of viral replication, translation, and assembly. eEF1A is a G protein that recruits aminoacyl-tRNA to the elongating ribosomes. However, accumulated information leads us to believe that components of the translational apparatus also play important roles beyond protein synthesis. eEF1A couples the pathways of protein synthesis and degradation. It interacts with ubiquitinated proteins and proteasome following ATP depletion and is involved in the proteasome-mediated cotranslational protein degradation [29]. It was also demonstrated that eEF1A interacts with actin and is essential for the regulation of actin cytoskeleton and cell morphology [30–33]. In addition, overexpression of eEF1A is associated with oncogenic transformation and metastasis [34–36]. In this study, we found that HCV NS4A protein interacted with eEF1A and inhibited protein synthesis. The interaction may result in a reduction of viral translation and replication leading to the escape of immune responses and establishment of chronic infection. On the other hand, the interaction may also link



the un-conventional roles of eEF1A to the pathogenesis of HCV NS4A protein.

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