Nonstructural Protein 5A of Hepatitis C Virus Inhibits the Function of Karyopherin β3

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Hepatitis C virus (HCV) is the major etiologic agent of non-A, non-B hepatitis (1, 8, 38). Chronic infection with HCV results in liver cirrhosis and hepatocellular carcinoma (7, 45). HCV belongs to the family Flaviviridae, having a positive-sense RNA genome (32, 42, 47). The RNA encodes a polyprotein (~3,010 amino acids) with the following gene order: 5′-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-3′ (3, 010 amino acids) with the following gene order: 5′-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-3′. During and/or after translation, the polyprotein is processed into functional proteins by host- and virus-encoded proteases. Core (C) and envelope (E1 and E2) proteins are believed to compose the structural elements of the virion particle. Nonstructural protein 2 (NS2), NS3, and NS4A are involved in the proteolytic processing of the HCV polyprotein (4, 5, 15, 18, 25, 26, 27, 28, 30, 40, 50, 52). RNA-dependent RNA polymerase and RNA helicase activities are assigned to NS5B and the C-terminal two-thirds of NS3, respectively (6, 36). No function has yet been assigned to NS4B.

NS5A exists in two different forms (p56 and p58) in cells. The proteins differ in their phosphorylation status (2, 34, 51). NS4A or NS3-4A-4B augments hyperphosphorylation of NS5A (43, 51). The sequence around the middle part of NS5A (amino acids 2209 to 2248) is termed the interferon sensitivity-determining region, since it correlates with interferon sensitivity of the HCV genotype 1b (16, 17, 39). The sequence in the interferon sensitivity-determining region was shown to play a key role in the inhibition of the protein kinase PKR, a mediator of interferon-induced resistance, through protein-protein interaction (20, 21). NS5A was also shown to interact with a SNARE-like protein (53). The C-terminal region of NS5A contains a potential transcriptional activation domain, but the role of its activity in viral replication is not known (10, 35, 49). Recently, NS5A was shown to perturb Grb2-mediated signaling pathways by selectively targeting the growth factor receptor-bound protein 2 (Grb2) adapter protein (48), and the introduction of NS5A into murine fibroblasts (NIH 3T3) promoted anchorage-independent growth and tumor formation in nude mice (22). This suggests that NS5A may also have a role in cell growth regulation. However, no biochemical function has yet been assigned to the N terminus of HCV NS5A.

To investigate the various roles of HCV NS5A in viral replication, we searched for cellular proteins interacting with the NS5A protein by yeast two-hybrid screening of a human hepatocyte cDNA library. We identified karyopherin β3, a member of the karyopherin β family also known as RanBP5, as the cellular counterpart of HCV NS5A. Karyopherins are a group of proteins mediating transport of proteins and possibly RNAs (reference 44 and references therein). For instance, karyopherin β1 (importin β), in association with karyopherin α (importin α), facilitates nuclear import of proteins containing classical nuclear localization signals (24). Karyopherin β3, a 124-kDa protein, exhibits a significant level of similarity to karyopherin β1 (44.4% similarity and 17.6% identity). The similarity of karyopherin β3 to other members of the karyopherin β family, its localization in the cytoplasm and nuclear rim, and its binding to repeat-containing nucleoporins and to Ran-GTP strongly suggest that karyopherin β3 may play a role in nucleoplasmic transport (13, 55). Karyopherin β3 is highly homologous to Saccharomyces cerevisiae protein Pse1i (KAP121) (65.2% similarity and 28.3% identity) and to Kap123 (58.9% similarity and 23% identity). Functional relationships among these proteins are yet to be elucidated. Several potential activities of karyopherin β3 and Pse1i have been proposed. Karyopherin β3 facilitated nuclear import of ribosomal proteins in an in vitro transportation assay system (33). Overexpression of yeast Pse1i resulted in an increase in protein secretion and stimulated mitochondrial import of hydrophobic proteins in yeast cells (9, 12). In addition, the conditional loss of Pse1i in a strain lacking Kap123 resulted in a
specific blockage of mRNA export from the nucleus (46). The molecular bases of these phenomena remain obscure.

Here we show protein-protein interaction between NS5A and karyopherin β3 by an in vitro binding assay and an in vivo coimmunoprecipitation method. The effect of NS5A on the karyopherin β3 activity was investigated using a yeast cell line with mutations in both PSE1 (pse-1) and KAP123 (Δkap123), genes that are homologous to the human karyopherin β3 gene. Human karyopherin β3 complemented the loss of both PSE1 and KAP123 functions and supported growth of the double mutant cells at permissive temperature, but expression of NS5A hampered the growth of the double mutant cells supplemented with human karyopherin β3. On the other hand, expression of NS5A by itself had no effect on the growth of the double mutant expressing introduced wild-type yeast PSE1. This indicates that NS5A may inhibit karyopherin β3 function via protein-protein interaction. Therefore, it is likely that HCV NS5A modulates cellular activities by inhibiting the activity of karyopherin β3.

**MATERIALS AND METHODS**

**Plasmid construction.** For the yeast two-hybrid system, the plasmids pAS2 and pACT2 (Clontech, Inc.) were used as sources of the GAL4 DNA-binding domain (BD) and GAL4 transcriptional activation domain (AD), respectively. The plasmids pYBD-5A(1973-2419), pYBD-5A(1973-2302), pYBD-5A(1973-2204), pYBD-5A(1973-2191), and pYBD-5A(1973-2204) were constructed as described previously (10). For the construction of pYBD-5A(1973-2372), HCV cDNA corresponding to amino acids 1973 to 2172 was amplified by PCR using the DNA of pTHE1964-3011 as a template (27). Oligonucleotides 5′-TACCCATACCCGGGACCATGTACCCATACGATGTTCCAGATTACGCCTTC-3′ and 5′-AGGTATCCGGGGATCCATGCTCACC-3′ were used as plus- and minus-strand DNA primers, respectively. The PCR product was digested with XmaI and then inserted into the XmaI site of pAS2 (Clontech, Inc.) to construct pYBD-5A(1973-2172), which contains the GAL4 AD and the full-length HCV NS5A protein, was constructed by inserting the DNA insert of pYBD-5A(1973-2172) excised with XmaI into the XmaI site of pACT2. pYBD-karyopherin β3(1007-1097) was constructed by inserting the NcoI fragment of pYAD-karyopherin β3(1007-1097) into the NcoI site of pACT2. pTM-NS5A, used for in vitro translation of the HCV NS5A protein, was constructed by inserting the blunt-ended XmaI fragment from pYBD-NS5A(1973-2191) into the blunt-ended XmaI site of pTM-1. The plasmid pGEX-karyopherin β3, expressing a glutathione S-transferase (GST)-karyopherin β3 fusion protein in *Escherichia coli*, was constructed by ligating the blunt-ended Apal-Xhol fragment from pSK-karyopherin β3 to the blunt-ended XmaI-Xhol fragment of pGEX-KG. To generate a Myc epitope-tagged full-length protein of karyopherin β3 (pM-NS5A/myc), the NAT- and NotI sites of pYBD-5A(1973-2191) containing the NAT and NotI sites of karyopherin β3 using the 5′-ACCATACATCCCGGGACCATGACACAAACAACTCTATCTGCGAGAGGG-3′ and 5′-CCTCCAGAAGATCTGACCTGCGTG-3′ (GSP2). Myc- and Norl-Klenow-treated pGEX-PE1-N1 (Clontech, Inc.) was blunt-ended with a blunt-ended NotI fragment containing the PCR product of the BamHI-EcoRV fragment of pSK-karyopherin β3 were ligated to generate plasmid pCMV/myc-karyopherin β3. The plasmid pCMV/HA-NS5A, encoding HCV NS5A and a hemagglutinin (HA) epitope tag at the N terminus, was constructed by inserting an NcoI-digested PCR product generated with the primers 5′-TACTGTGC-3′ and 5′-GGCG-3′ into the SmaI site of pEGEP-N1 (Clontech, Inc.). The yeast expression vector pSS16/ADH-AD was constructed by inserting a blunt-ended SpeI fragment of pGAPD4 (Clontech, Inc.) into the blunt-ended XbaI-Xhol site of pPS1066 (46). pM84, a URA CEN plasmid containing an 817 fragment of the ADH1 promoter, was obtained by selection of *HindIII*-digested yeast expression plasmids pM84-karyopherin β3 and pM84-myc-karyopherin β3 were constructed by inserting the blunt-ended XbaI-Xhol fragment of pSK-karyopherin β3 or the blunt-ended SalI-Xhol fragment of pCMV-myc-karyopherin β3 into pM84 digested with *HindIII*. The yeast expression plasmid pM84-karyopherin β3 and pM84-myc-karyopherin β3 were constructed by inserting the blunt-ended SpeI fragment of pGAPD4 (Clontech, Inc.) into the blunt-ended NotI-Xhol site of pYES2 (Invitrogen). The plasmids pGal-NS5A(1973-2419) and pGal-NS5A(1973-2172), galactose-inducible vectors encoding full-length NS5A and the N-terminal region of NS5A, respectively, were constructed by inserting the blunt-ended XbaI fragment of pYBD-5A(1973-2419) or pYBD-5A(1973-2172), respectively, into the blunt-ended EcoRI site of pGal. pGal-NS5A(2173-2419), a galactose-dependent vector encoding the C-terminal region of NS5A, was constructed by inserting the blunt-ended XbaI fragment of pYBD-5A(1973-2419) or pYBD-5A(1973-2172), respectively, into the blunt-ended XbaI site of pGal-NS5A(1973-2419) or pGal-NS5A(1973-2172), respectively. The yeast expression plasmid pGal-NS5A was generated using an in vitro transcription-translation system (Promega) and [35S]S-methionine (DuPont NEN). Equal amounts of [35S]labeled translation products (as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] and a BAS Radioanalytic Imaging System) were incubated with 80 µl of GST beads (50% slurry) in 1 ml of GB buffer (final concentrations of 20 mM Tris-HCl [pH 8.0], 0.25% NP-40, 50 mM NaCl, and 1 mM EDTA). After 2 h of incubation at 4°C on a rotating mixer, the beads were washed three times with 1 ml of GB buffer and boiled for 5 min in 30 µl of 2× SDS sample buffer before analysis by SDS-PAGE. Gels were dried and exposed to X-ray film.

**Complementation.** *Cox-7* cells were transiently transfected with the indicated plasmids using an electroporation method described previously (37). After 48 h of cultivation, the cells were washed and resuspended in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.1% NP-40, 1 mM phenylmethylsulfonyl fluoride). Equal amounts of cleared cell lysates were subjected to immunoprecipitation with monoclonal anti-HA antibody (F-7; Santa Cruz), followed by adsorption to protein G agarose (Boehringer Mannheim). The washed beads were washed three times with washing buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.01% NP-40). The antibody-protein complexes were then resolved by SDS-PAGE, and the Myc-tagged protein was
identified by Western blotting with a monoclonal anti-Myc antibody (9E10; Santa Cruz) probe using an enhanced chemiluminescence system.

Complementation of a yeast \textit{PSE1} and \textit{KAP123} double mutation with human karyopherin \(b_3\). Yeast strain PSY1042 (\textit{MATa ura3-52 leu2-3,112 trp1-289 gal1-1 pse1 
\textit{D} kap123}), which contains the \textit{PSE1} temperature-sensitive allele (\textit{pse1-1}) and the \textit{KAP123} null mutation (\textit{D} \textit{kap123}), was used for the complementation test (46). These cells grow well at 25°C but do not grow at 36°C. Yeast strain PSY1042 was transformed with plasmid pKM84-karyopherin \(b_3\) or pKM84/myc-karyopherin \(b_3\) and then cultivated on a uracil-deficient SD plate at 25°C. The resulting transformants were streaked onto uracil-deficient SD plates and then cultivated at either 25 or 36°C. In order to investigate the effect of HCV NS5A on the human karyopherin \(b_3\) in the transformed yeast, yeast cells containing pKM84/myc-karyopherin \(b_3\) were subsequently transformed with plasmid pGal-NS5A(1973-2419), pGal-NS5A(1973-2172), or pGal-NS5A(2173-2419). The resulting transformants were selected on uracil- and tryptophan-deficient SD. The effect of NS5A was determined by cultivating the transformants on an SGAL plate at 36°C.

Western blot analysis of NS5A in yeast cells. The yeast transformants with galactose-inducible expression plasmids were grown at 25°C in SGAL. Cells were harvested at an optical density at 600 nm of 0.7 and then lysed by vigorous sonication and vortexing together with glass beads. The yeast lysate was centrifuged at 12,000 rpm as previously described (46), and the supernatant was used for Western blot analysis. Equal amounts of proteins were resolved by SDS-PAGE, and NS5A and its derivatives were identified by Western blotting using a polyclonal antibody against NS5A that was kindly provided by R. Bartenschlager.

RESULTS

Identification of cellular proteins interacting with HCV NS5A in the yeast two-hybrid system. To identify cellular proteins interacting with HCV NS5A, a yeast two-hybrid system was employed to screen a human liver cDNA library (MATCHMAKER cDNA library from Clontech) using the C-terminally truncated HCV NS5A (amino acids 1973 to 2302) as bait. Nine positive clones were obtained from the screening of 2 \(\times\) 10\(^6\) independent yeast colonies. DNA sequence analysis showed that four of the nine positive clones encoded the C-terminal portion of karyopherin \(b_3\) encompassing amino acid residues 1007 to 1097 (Fig. 1). A full-length cDNA clone of karyopherin \(b_3\) was obtained from mRNA of HeLa cells as described in Materials and Methods. The entire cDNA clone of the karyopherin \(b_3\) gene was then sequenced to confirm the identity. Alignment of its deduced amino acid sequence with the yeast \textit{PSE1} (or \textit{KAP121}) amino acid sequence revealed 65.2% similarity and 28.3% identity over the entire length of the protein (13, 55), which suggested that karyopherin \(b_3\) may have functions equivalent to those of the yeast \textit{PSE1} product.

Furthermore, the human karyopherin \(b_3\) exhibited 58.9% homology and 23% identity to the yeast \textit{KAP123} product (55). The mammalian homologue of yeast \textit{KAP123} has not yet been identified.

The N-terminal region of NS5A is essential for interaction with karyopherin \(b_3\). In order to determine the region in HCV NS5A required for interaction with karyopherin \(b_3\), two-hybrid analyses were carried out using NS5A, NS5A derivatives, and truncated karyopherin \(b_3\) genes (Fig. 1). The yeast plasmids used for the two-hybrid system (Fig. 2A) were cotransformed into yeast strain HF7c, and the transformants were grown on a medium lacking histidine and plaqueline (Fig. 2B). In the two-hybrid system, a protein-protein interaction is indicated by viability of yeast cells on histidine-deficient plates (Fig. 2C) and by \(\beta\)-galactosidase activity in the yeast cells (Fig. 2D). As shown in Fig. 2C and D, yeast cells containing plasmid BD-NS5A(1973-2172) and plasmids encoding larger NS5A con-
structs [BD-NS5A(1973-2204), BD-NS5A(1973-2302), and BD-NS5A(1973-2419)] grew on histidine-deficient plates (Fig. 2C, sectors 1, 2, 3, and 4) and exhibited β-galactosidase activity (Fig. 2D, sectors 1, 2, 3, and 4). A further C-terminal deletion of NS5A [BD-NS5A(1973-2119)] and an N-terminal deletion of NS5A [BD-5A(2120-2204)] abolished the protein-protein interaction (Fig. 2C and D, sectors 5 and 6). This indicates that the amino acid residues 2120 to 2172 of HCV NS5A include an essential part for the interaction with karyopherin b3 and that residues 1973 to 2172 are sufficient for the interaction. The protein-protein interaction in the two-hybrid system was also detected when the bait and the prey plasmids were exchanged reciprocally (Fig. 2C and D, sector 7). On the other hand, the full-length karyopherin b3 did not give a positive signal in the yeast two-hybrid system (data not shown). Misfolding of the fusion protein and/or exclusion of the protein from the nucleus is a possible reason for this phenomenon. Nevertheless, the full-length karyopherin b3 did bind to HCV NS5A in in vitro and in vivo assay systems (see below).

**HCV NS5A binds to karyopherin b3 in vitro.** In vitro binding assays were performed to confirm the interaction between HCV NS5A and human karyopherin b3. The full-length karyopherin b3 cDNA was connected in frame to the C-terminal end of the GST gene in a bacterial expression vector to produce a GST-karyopherin b3 fusion protein. The protein was expressed in E. coli and then partially purified. Direct in vitro binding assays were carried out using the purified GST-karyopherin b3 and 53S-labeled NS5A generated by in vitro translocation. The radiolabeled NS5A efficiently coprecipitated with the GST-karyopherin b3 but not with the GST negative control protein (Fig. 3, lanes 4 and 6). Luciferase, another negative control protein, did not bind to either GST or GST-karyopherin b3 (Fig. 3, lanes 3 and 5). This indicates that NS5A directly interacted with karyopherin b3.

**HCV NS5A interacts with karyopherin b3 in mammalian cells.** To determine whether HCV NS5A is capable of binding to karyopherin b3 inside cells, coimmunoprecipitation was performed using cells that expressed both of the proteins. Cos-7 cells were transfected with vectors encoding both the HA-tagged HCV NS5A (HA-NS5A) and the Myc-tagged karyopherin b3 (myc-karyopherin b3). The cell lysates were then subjected to immunoprecipitation using an anti-HA monoclonal antibody (Fig. 4, lanes 2 and 3). Immunoprecipitates were resolved by SDS-PAGE and transferred to a nitrocellulose membrane for Western blot analysis using the anti-Myc monoclonal antibody. The myc-karyopherin b3 was coimmunoprecipitated with HA-NS5A by the anti-HA antibody (Fig. 4, lane 5). In contrast, myc-karyopherin b3 was not precipitated by the anti-HA antibody when NS5A was absent from the cell (Fig. 4, lane 4). This indicates that NS5A directly interacted with karyopherin b3 in vivo.

**Human karyopherin b3 complements the double mutation of yeast PSE1 and KAP123.** Since the sequence of human karyopherin b3 is highly homologous to the sequences of yeast PSE1 and KAP123, we investigated whether human karyopherin b3 might be functionally homologous to yeast PSE1
KAP123 products. The null mutation of KAPI23 (Δkap123) does not exhibit any phenotypic difference from the wild type yeast (46). The temperature-sensitive mutation of PSE1 (pse1-1) caused delayed cell growth at the nonpermissive temperature (46). On the other hand, the PSE1 and KAP123 double mutant (PSY1042 [pse1-1 Δkap123]) grew at the permissive temperature (25°C) but not at the nonpermissive temperature (36°C) (Fig. 5, vector) (46). However, the double mutant could grow at the nonpermissive temperature (36°C) when the yeast cell contained plasmids encoding either yeast PSE1, human karyopherin b3, or Myc-tagged karyopherin b3 (Fig. 5, PSE1, karyopherin b3, and myc-karyopherin b3, respectively). This indicates that human karyopherin b3 can complement the function of the yeast PSE1 and KAP123 products.

HCV NS5A inhibits the function of karyopherin b3. In order to evaluate the biological importance of the interaction between HCV NS5A and karyopherin b3, we investigated the effect of HCV NS5A on the function of karyopherin b3. We constructed yeast plasmids encoding HCV NS5A and its derivatives under the control of the GAL1 promoter and tested the effect of the proteins on yeast cells complemented with human karyopherin b3 (yeast strain PSY1042 containing plasmid pKM84/myc-karyopherin b3). Without the induction of NS5A and its derivatives, yeast cells complemented with human karyopherin b3 grew well at the nonpermissive temperature (Fig. 6A). On the other hand, upon the induction of the full-length HCV NS5A and the N-terminal domain of NS5A that binds to karyopherin b3, yeast cells complemented with human karyopherin b3 could not grow at the nonpermissive temperature (Fig. 6B). This indicates that NS5A inhibits the function of karyopherin b3.
temperature [Fig. 6B, NS5A(1973-2419) and NS5A(1973-2172)]. Under the same conditions, the C-terminal domain of NS5A, which does not bind to karyopherin β3, did not affect the growth of the yeast cells [Fig. 6B, NS5A(2173-2419)]. We also tested the effect of full-length HCV NS5A on yeast cells supplemented with yeast PSE1 instead of human karyopherin β3. The yeast cells producing yeast PSE1 grew well with or without expression of HCV NS5A [Fig. 6C and D, NS5A(1973-2419)]. The expression of the full-length HCV NS5A and of the deletion mutants of NS5A was confirmed by Western blot analysis using anti-NS5A antibody, which was kindly provided by R. Bartenschlager (Fig. 6E). Almost the same amounts of full-length NS5A were detected in yeast cells transformed with either the yeast PSE1- or human karyopherin β3-expressing plasmid was cotransformed with vector (lanes 1 and 2), full-length NS5A (lanes 3 and 4), N-terminal NS5A (lane 5), or C-terminal NS5A (lane 6). The positions of marker proteins are indicated. Bands of NS5A and its derivatives are indicated by arrowheads.

DISCUSSION

We found that HCV NS5A specifically interacted with karyopherin β3, blocking its activity in vivo. The N-terminal part of HCV NS5A (amino acids 1973 to 2172) was required for the protein-protein interaction and the inhibition of karyopherin β3. On the other side, the C-terminal end of karyopherin β3 was sufficient for the interaction with HCV NS5A. It has been seen before that the C-terminal regions of proteins of the karyopherin β family are required for direct interaction
with target molecules or with adapter molecules binding to substrates (24). Thus, it is possible that HCV NS5A may compete with substrates that naturally bind to karyopherin β3. The N-terminal parts of karyopherin β3, which are the most conserved regions among the karyopherin β family genes, compose interfaces that interact with components of the translocation apparatus. In the case of karyopherin β1, this region is required for binding to the nuclear pore complex and to Ran (54). Likewise, the N-terminal portion of karyopherin β3 has been shown to bind to Ran (55).

How would NS5A contribute to viral proliferation by interacting with karyopherin β3? It is hard to formulate a conclusive hypothesis with such limited studies of the functions of NS5A and karyopherin β3. Nevertheless, we can speculate about possible physiological roles of the protein-protein interaction by referring to previous reports about functions of karyopherin β3. Karyopherin β3 is a member of the karyopherin β family, which facilitates transportation of proteins and/or RNAs between different compartments of the cell. The cytoplasmic and nuclear rim localization of karyopherin β3 and its binding to the repeat sequence of nucleoporins and to Ran-GTP strongly suggest that karyopherin β3 is involved in nucleocytoplasmic transport (13, 55). The high homology of karyopherin β3 to the yeast PSE1 and KAP123 products and the results of our complementation experiments using the yeast double mutant strain (PSY1042 [pse1Δ/kap123Δ]) lead us to conclude that the human karyopherin β3 can replace the function(s) of yeast PSE1 and KAP123.

Several functions of karyopherin β3, PSE1, and/or KAP123 have been reported. First, karyopherin β3 may function in the nuclear import of macromolecules. In vitro nuclear import experiments have shown that karyopherin β3, importin beta, transportin, and RanBP7 facilitated ribosomal protein transport into the nucleus (33). Second, yeast PSE1 and/or KAP123 may be involved in mRNA export (46). Third, yeast PSE1 may enhance secretion of proteins (9). Fourth, yeast PSE1 may augment mitochondrial import of hydrophobic mitochondrial proteins (12). It is, however, not clear whether these effects of karyopherin β3, PSE1, and/or KAP123 are direct or indirect ones. We should consider all of these possible functions of karyopherin β3 when we think about a biological role(s) for the interaction between NS5A and karyopherin β3.

For proliferation, differentiation, and changes in metabolism, cells respond to intra- and extracellular signals, including virus infection. The transmission of cellular signals is often executed by signal-transducing molecules shuttling between different subcellular compartments. It is therefore not surprising that some viruses use strategies that block the transport of cellular signaling molecules in order to incapacitate a host antiviral defense system and/or to perturb cellular homeostasis. For instance, the matrix protein (M protein) of vesicular stomatitis virus blocks transportation of RNAs and proteins between the nucleus and the cytoplasm by inhibiting Ran guanosine triphosphatase-dependent nuclear transport (29). HCV NS5A might function in a similar way as the M protein of vesicular stomatitis virus. The subcellular localization patterns of NS5A and karyopherin β3 support this possibility. HCV NS5A is localized in the cytoplasm and enriched in the perinuclear space region (31, 37, 51), which is similar to the distribution pattern of karyopherin β3 (13, 55). Therefore, it is plausible to consider that karyopherin β3 might be sequestered from its normal active sites by binding to NS5A.

It is also possible that the export of RNAs from the nucleus could be impeded by HCV NS5A, as suggested by the phenotype of the yeast PSE1 and KAP123 double mutant (46). The blockage of RNA export in turn may inhibit expression of genes exerting antiviral activities that normally would be induced by viral infection. Alternatively, NS5A may inhibit the protein secretion-enhancing activity of karyopherin β3, as was shown by overexpression of PSE1 in yeast cells (9). In these respects, NS5A might block production and/or secretion of cytokines from HCV-infected cells. For instance, NS5A may inhibit secretion of alpha interferon, one of the first cytokines produced in response to virus infection, from HCV-infected cells, thus preventing the initiation of antiviral activities of neighboring cells. Since alpha interferon activates NK cell cytotoxicity and induces lysis of virus-infected cells, the inhibition of the protein secretion apparatus in the virus-infected cells would be advantageous to virus proliferation. In addition, the blockage of the protein secretion pathway may also hamper presentation of viral antigens in association with major histocompatibility complex class I molecules, which is required to make cytokotoxic T lymphocytes recognize the virus-infected cell. In fact, suppression of antiviral activities through blockage of protein secretion has been discovered for several viruses. The poliovirus proteins 2B and 3A and the Epstein-Barr virus BARF1 inhibit secretion of cellular proteins (14) and alpha interferon (11), respectively, which modulates innate host responses to the viruses. In this respect, it is worth noting that the release of tumor necrosis factor alpha and interleukin-1 beta by phorbol myristate acetate was reduced for peripheral blood monocytes that had been collected from patients chronically infected with HCV (41). The inhibition of protein secretion by HCV NS5A, therefore, may be related to the correlation that exists between the nucleotide sequence of NS5A and the sensitivity of HCV to interferon treatment, even though the interferon sensitivity-determining region identified by Enomoto et al. (17) lies outside of the segment required for the interaction with karyopherin β3.

Analysis of yeast overexpressing PSE1 led to the conclusion that karyopherin β3 may also play a role in mitochondrial protein import (12). It is tempting to speculate that NS5A binding to karyopherin β3 could prevent normal mitochondrial protein import, resulting in alterations of mitochondrial functions. Intriguingly, frequent ultrastructural alterations of the mitochondria have been observed in patients’ hepatocytes infected with HCV genotype 1b (3). The increased production of free radicals in the damaged mitochondria might contribute to the development of the severe liver diseases caused by HCV.

Due to the lack of a reliable in vitro cultivation system for HCV, it is difficult to confirm the interaction between NS5A and karyopherin β3 in the presence of all other viral proteins and to investigate all of these possible roles for HCV NS5A in HCV-infected cells. Instead, we used yeast cells to investigate the roles of karyopherin β3 and HCV NS5A in vivo. A study of the effect of HCV NS5A on the transport of macromolecules in mammalian cells, utilizing an NS5A-expressing cell line, is in progress.

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