Ribosomal Protein S5 Interacts with the Internal Ribosomal Entry Site of Hepatitis C Virus

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Translational initiation of hepatitis C virus (HCV) genome RNA occurs via its highly structured 5′ noncoding region called the internal ribosome entry site (IRES). Recent studies indicate that HCV IRES and 40 S ribosomal subunit form a stable binary complex that is believed to be important for the subsequent assembly of the 48 S initiation complex. Ribosomal protein (rp) S9 has been suggested as the prime candidate protein for binding of the HCV IRES to the 40 S subunit. RpS9 has a molecular mass of ~25 kDa in UV cross-linking experiments. In the present study, we examined the ~25-kDa proteins of the 40 S ribosome that form complexes with the HCV IRES upon UV cross-linking. Immunoprecipitation with specific antibodies against two 25-kDa 40 S proteins, rpS5 and rpS9, clearly identified rpS5 as the protein bound to the IRES. Thus, our results support rpS5 as the critical element in positioning the HCV RNA on the 40 S ribosomal subunit during translation initiation.

Translational initiation of most eukaryotic messenger RNAs is mediated by the binding of elongation initiation factor eIF-4 to the modified nucleotide cap on the 5′ end of the mRNA and the binding of the 40 S ribosomal subunit and other initiation factors (1). However, in hepatitis C virus (HCV) as well as in other picornaviruses, translation initiation, because its addition to La-deficient rabbit reticulocyte lysates stimulates and corrects PV translation (8). In addition to these two proteins, other cellular factors (e.g. the 97-kDa protein and poly(rC)-binding protein-2) are likely to stimulate translation initiation directed by the picornavirus IRES (9, 10).

Identification and characterization of IRES-binding proteins are important to the understanding of the mechanisms of internal initiation and, ultimately, to the development of novel therapies for HCV. The binding PTB and La are required for translation by HCV IRES (11, 12). However, Kaminski et al. (13) reported that recombinant PTB did not stimulate HCV IRES function, suggesting that PTB may not be necessary during HCV translation initiation under certain experimental conditions. Despite a considerably lower level of La protein in rabbit reticulocyte lysate, HCV IRES promotes efficient translation activity (14–16). This clearly differs from the case of translation initiation on the PV genome. Thus, general models proposed for the process of 40 S ribosome entry to IRES elements on the picornavirus genome do not seem to apply to the HCV genome. In this respect, the formation of a stable binary complex between HCV IRES and purified 40 S ribosomal subunit is of great interest. It differs fundamentally from the initiation process on picornavirus IRES, which depends absolutely on one or more initiation factors, and direct ribosome binding to IRES may be an important step in internal initiation of the HCV genome (17).

Although other ribosomal proteins may interact with HCV IRES, the binding of ribosomal protein S9 (rpS9) to the IRES has been assumed to be the initial step in translation initiation (17). RpS9, with a molecular mass of 25 kDa in UV cross-linking experiments, is a primary target protein of binary IRES-40 S subunit complex. This protein is a primary target protein of binary IRES-40 S subunit complex. (17, 18). It remains unclear whether rpS9 binding is essential for function of HCV IRES, and unambiguous identification of ribosomal protein cross-linked to HCV IRES is still missing.

We have previously shown that the binding of a 25-kDa cellular protein (p25) to the HCV IRES is most likely an important step for efficient translation of HCV (19). This p25 protein interacts specifically with the HCV IRES and is cross-linked to the IRES when purified 40 S subunits are being used as a binding material (20). Binding of p25 to HCV IRES, therefore, seems to be crucial for the interaction between 40 S subunit and IRES and to be a unique feature in the translation mechanism of HCV. In this study, we purified p25 from cultured HeLa cells and characterized it by with specific antibodies. As the main result we present here the first evidence that the HCV-IRES-binding protein of the 40 S ribosomal subunit is ribosomal protein S5 (rpS5) and not rpS9 as suggested earlier.

**EXPERIMENTAL PROCEDURES**

*Plasmid Construction*—Plasmid pUC5END-nc containing a cDNA of full-length HCV 5′ NCR located just downstream of the T7 promoter was described earlier (19). The cDNA for human rpS5 was amplified from a HeLa cDNA library with polymerase chain reaction primers HS51 (5′-GGATCCGATGACGATGACAAAATGACCGAGTGGGAGAC-3′) and HS52 (5′-GAAGCTTTCAGCGGTTGGACTTGGCCAC-3′), digested with BamHI and HindIII, and inserted into the pQE30 vector (Qiagen) to yield pQE-RSS. Plasmid pQE-RS9, which contains a cDNA for human rpS9, was constructed with the primers HS91 (5′-G-
GATCCGATGACGAGACAAAAACGTTGCGCCGAGAGCTGGT3′) and HS92 (5′-GAAGCTTATCCCTCTCCTGTCGTC-3′) by the same strategy as used for pQE-RS5.

**Purification and Characterization of p25—Cytoplasmic S10 extracts were prepared from HeLa S3 cells cultured in suspension. Purification of p25 protein was monitored by UV cross-linking of [32P]UTP-labeled HCV RNA probe to protein chromatographic fractions. A HeLa S10 cytoplasmic extract containing ~650 mg of protein was fractionated by gel filtration on Sephacryl S-300 HR (Amersham Pharmacia Biotech) pre-equilibrated with buffer A (10 mM HEPES-KOH, pH 7.5, 1.5 mM MgCl2, 1 mM dithiothreitol, 5% glycerol) containing 120 mM KCl.

The affinity of p25 to the HCV 5′ NCR RNA probe was measured in the void fractions. The void fractions were diluted with buffer A and loaded onto a HiTrap heparin column (Amersham Pharmacia Biotech) pre-equilibrated with buffer A. Fractions were collected by stepwise elutions with 0, 150, 300, and 500 mM KCl in buffer A. Fractions containing binding activity to the HCV 5′ NCR RNA were eluted with 300 and 500 mM KCl in buffer A. The 500 mM KCl fraction was 5-fold diluted with buffer A and loaded onto a S 5′/5′ column (Amersham Pharmacia Biotech) pre-equilibrated with buffer A. Fractions were eluted by a gradient of 100–500 mM KCl in buffer A. The fractions with the highest HCV 5′ NCR RNA binding activities were eluted by 250–340 mM KCl and pooled. Approximately 38 mg of the partially purified protein was obtained.

**Amino Acid Sequence Analysis—**Partially purified protein was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 14% gel. After electrophoretic transfer to a polyvinylidene difluoride membrane, the proteins were visualized with the stain Ponceau S.

**In Vitro Transcription and UV Cross-linking—**A [32P]UTP-labeled probe of high specific activity and corresponding to nucleotides 1–347 of the HCV 5′ NCR was transcribed from ScI-digested pUC5END-nc with the Riboprobe T7 system (Promega). Analyses of protein-RNA interactions after UV-induced cross-linking were performed as described (19).

**Antibodies—**Preparation of rabbit antisera against rat 40 S ribosomal proteins, their purification by immunosorption, and their monospecificity were as described (21, 22). Preparation of polyclonal antibodies directed against PTB was as described (23).

**Immunoblotting and Immunoprecipitation—**JM109 cells were transformed with pQE-RS5 and pQE-RS9, and recombinant rpS5 and rpS9 were isolated by Ni-NTA-agarose chromatography according to the manufacturer’s instructions (Qiagen). Recombinant ribosomal proteins were separated by gradient SDS-PAGE on a 5–20% gel and used as reference proteins in Western immunoblotting according to the method of Towbin et al. (24).

Immunoprecipitation was performed with antibodies against rat ribosomal proteins and PTB. HeLa S10 extracts cross-linked with [32P]UTP-labeled HCV IRES RNA were heat-denatured in cross-linking buffer containing 0.5% SDS and centrifuged. The supernatants were diluted 5-fold in sample buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, and 1 mM dithiothreitol). After pre-incubation with uncoated protein G-Sepharose for 60 min, the samples were centrifuged briefly, and the supernatants were incubated with antibody-coated protein G-Sepharose. The precipitates were washed with sample buffer six times and analyzed by SDS-PAGE and autoradiography.

**RESULTS AND DISCUSSION**

We used a HeLa S10 cytoplasmic extract as the starting material in our efforts to purify and characterize p25 cross-linked to HCV IRES. Purification of p25 was monitored by UV-induced cross-linking of the protein fractions to a HCV probe. During subsequent gel filtration on a Sephacryl S-300 column, p25 bound to the HCV probe and migrated close to the void fractions, suggesting that p25 exists as a component of a macromolecular complex rather than as a single cytoplasmic molecule with an affinity for HCV (20).

The initial void fraction was subjected to heparin-Sepharose and cation exchange chromatography. Maximum activities of HCV 5′ NCR RNA binding were eluted from cation exchange column by 250–340 mM KCl (Fig. 1A). The proteins for this fraction were separated by SDS-PAGE, and silver staining revealed two closely migrating proteins of ~25 kDa and a peptide corresponding to p25 just between them (Fig. 1B). The gel sections containing each of the two proteins were cut out (Fig. 1C), and the Lys-C-digested fragments were subjected to amino acid sequencing. Nine of 10 amino acids of the slower migrating band (a) matched amino acids 155–163 of human rpS9 (Fig. 1D). Ten amino acids of the faster migrating band (b) were identical to amino acids 23–32 of human rpS5. To identify which protein corresponded to p25, we analyzed the fractions by immunoprecipitation with rabbit anti-rpS5 and anti-rpS9 antibodies. The monospecificities of these antibodies had been established previously (21), and we reconfirmed those by immunoblot analysis. The anti-rpS5 and anti-rpS9 antibodies specifically recognized recombinant human rpS5 and rpS9, respectively (Fig. 2A). These antibodies were mixed with HeLa cytoplasmic extracts that had been UV cross-linked to [32P]UTP-labeled HCV IRES. Immunoprecipitation was carried out under highly stringent conditions to avoid nonspecific precipitation of aggregated protein during incubation with antibodies (see “Experimental Procedures”). Analysis by SDS-PAGE and auto-
radiography demonstrated that the cross-linked p25 was immunoprecipitated by anti-rpS5 antibodies but not by anti-rpS9 antibodies (Fig. 2B). Furthermore, p25 was not precipitated with a different rabbit anti-rpS9 preparation raised against recombinant human rpS9 (data not shown). Among the control antibodies, anti-PTB precipitated a single protein of 58 kDa, a size corresponding to that of PTB. Anti-ribosomal protein S26 did not precipitate any protein, indicating that nonspecific precipitation did not exist under our experimental conditions. Thus, we conclude that the p25 protein cross-linked to HCV IRES was rpS5.

In contrast, a previous report (17) indicated that rpS9 was the 25-kDa protein cross-linked to HCV RNA. We cannot explain the discrepancy between their results and ours. However, it is obviously difficult to clearly identify two ribosomal proteins of similar molecular mass and electrophoretic mobility, particularly when they are cross-linked to the HCV IRES. Although the radioactive signal of p25 shown on the autoradiogram is closer to rpS9 than to rpS5, special caution is required particularly when they are cross-linked to the HCV IRES. It is obviously difficult to clearly identify two ribosomal proteins when binding to HCV IRES and the 25-kDa cellular protein (18, 19), suggesting that the binding of rpS5 is dependent on domain II. In this way, rpS5 may be involved in positioning the IRES in the ribosomal decoding center, a process accompanied by pronounced conformational changes in the structure of the 40 S subunit (22).

In summary, we have shown that the rpS5 protein interacts specifically with the IRES. Additional studies of this interaction may reveal insights into potential target for the specific inhibition of HCV translation in the infected cells. Further understanding of these mechanisms may provide new strategies for development of novel antiviral drugs.

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