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The Core Protein of Hepatitis C Virus Is Imported into the Nucleus by Transport Receptor Kap123p but Inhibits Kap121p-dependent Nuclear Import of Yeast AP1-like Transcription Factor in Yeast Cells*

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The core protein of hepatitis C virus (HCV) is a major component of the viral nucleocapsid. The HCV core protein includes nuclear localization signal-like sequences and has various effects on cellular metabolism, playing roles, for example, in the regulation of transcription, apoptosis, and transformation. To examine the possibility of an effect of the core protein on nucleocytoplasmic transport, we used the yeast *Saccharomyces cerevisiae* as a model system. The core protein (p23) is processed to p21 and is localized in both the cytoplasm and nucleus in yeast cells, similar to that observed in mammalian cells in several cases. The nuclear import of the core protein requires the activity of small GTPase Ran/Gsp1p and is mediated by Kap123p in yeast cells. When the core protein was expressed in yeast cells, the import of the yeast AP1-like transcription factor Yap1p into the nucleus was inhibited. Experiments *in vitro* involving Kap121p, also known as Pse1p, a receptor for the nuclear import of Yap1p, indicated that the amount of Yap1p bound to Kap121p was reduced in the presence of core protein. These results suggest that the HCV core protein affects cellular metabolism by disturbing transport of proteins to the nucleus.

Hepatitis C virus (HCV),[1] a major causative agent of non-A, non-B hepatitis (1), is classified in a separate genus of the Flaviviridae family. Persistent infection results in chronic active hepatitis, which can lead to liver cirrhosis and hepatocellular carcinoma. The genome of HCV is a single-stranded, positive sense RNA of ~9,500 nucleotides that encodes a single polyprotein of ~3,000 amino acids (2–4) which is processed by cellular and viral proteases into at least 10 different structural and nonstructural proteins (5–7).

The core protein of HCV, which is a major component of the viral nucleocapsid, is produced by processing of the viral polyprotein by a host signal peptidase on the membrane of the endoplasmic reticulum (8, 9). In addition to a possible role in the encapsidation of viral RNA (10), the core has multiple effects on cellular metabolism and it can regulate transcription from several viral and cellular promoters (11–14), apoptosis (15–19), and transformation (20–23). These properties of the core protein imply that it contributes to the pathogenesis of HCV by altering cellular metabolism. However, because of its complexity, the precise mechanisms responsible for the effects of the core protein (abbreviated as "core" hereafter) in the host cell remain to be elucidated.

The amino-terminal half of core contains some nuclear localization signal-like (NLS-like) sequences and is strongly hydrophilic, whereas the carboxyl-terminal region is hydrophobic. The carboxyl-terminal region of core is processed to yield at least three products of different molecular masses (9, 24, 25). Full-length core p23 (amino acid residues 1–191), as well as the truncated product p21, are localized both in the cytoplasm and nucleus. The ratio of their distribution depends on the strain of HCV (24, 26, 27). A minor product, p19, is concentrated in the nucleus (24, 25). Thus, the carboxyl-terminal hydrophobic region of core is a determinant of the distribution of the protein between the cytoplasmic and nuclear compartments. Recent studies indicate that core is predominantly localized in the cytoplasm in cultured cells (28, 29) and liver biopsy samples of HCV-infected patients (30). Regardless of whether the steady-state localization of core is in the cytoplasm or in the nucleus, the structural characteristics of core indicate that elucidation of molecular mechanism for nucleocytoplasmic transport of core, and whether core can affect nucleocytoplasmic transport of cellular protein, might provide some insight into the effects of core on metabolism.

In eukaryotic cells, many molecules are transported into or out of the nucleus through the nuclear pore complex (NPC), which is embedded in the nuclear envelope. Transport through the NPC is mediated by a family of soluble nuclear transport receptors (members of the importin β/karyopherin β family; Kaps), which have a binding domain for the GTP-loaded form of Ran (Ran-GTP) in the amino-terminal region, a carboxyl-terminal cargo-binding domain, and the ability to bind the components of the NPC. These receptors recognize their cargo by the presence of transport signals, such as the NLS or the nuclear export signal. Multiple nucleocytoplasmic transport pathways exist that exploit different transport receptors and

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[1] The abbreviations used are: HCV, hepatitis C virus; NLS, nuclear localization signal; NPC, nuclear pore complex; Ran-GTP, GTP-loaded form of Ran; Ran-GDP, GDP-loaded form of Ran; GST, glutathione *S*-transferase; GFP, green fluorescent protein; His, polyhistidine; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

transport signals (31, 32). In the budding yeast, *Saccharomyces cerevisiae*, 14 transport receptors (members of the importin β family) have been identified, on the basis of sequence homology, from the sequence of the entire genome (33, 34). These receptors include nine import receptors and five export receptors. Several mammalian orthologues of these receptors have been identified.

The directionality of nucleocytoplasmic transport is thought to be related to the activity of the small GTPase Ran (35, 36). It appears that Ran-GTP is localized in the nucleus, whereas the GDP-loaded form of Ran (Ran-GDP) is found in the cytoplasm. In the nucleus, the binding of Ran-GTP to an import receptor facilitates the dissociation of the import receptor-cargo complex and the release of the cargo (36–38). By contrast, the binding of Ran-GTP to an export receptor is required for the formation of an export receptor-cargo-Ran-GTP complex in the nucleus (39–41). In the presence of Ran-GDP, no export receptor-cargo complex is formed. Thus, the Ran-GTP gradient is a key determinant of the direction of transport between the nuclear and cytoplasmic compartments.

In this study, we examined the possible effects of core on nucleocytoplasmic transport using *S. cerevisiae* as a model system. Numerous genetic and cell biological studies using these yeast cells have contributed extensively to our current understanding of the mechanism of nucleocytoplasmic transport, which appears to be shortly conserved in yeast and mammalian cells (42). Our data indicate that the subcellular localization of core in yeast cells is similar to that in mammalian cells reported by a number of groups (24–27). We found that the nuclear import of core required Ran/Gsp1p and was mediated by the import receptor Kap123p in yeast cells. Moreover, core inhibited the Kap121p-dependent import of the yeast AP-1-like transcription factor Yap1p into yeast nuclei. Our experiments *in vitro* suggested that core reduced the amount of Yap1p that bound to Kap121p. These results imply that core affects metabolism by disturbing the nuclear transport of cellular proteins.

MATERIALS AND METHODS

Yeast Strains and Culture—The yeast strains used in this study are listed in Table I. Yeast cells were grown in YPAD (1% yeast extract, 2% peptone, 0.004% adenine sulfate, and 2% glucose) or in synthetic medium supplemented with amino acids (43). Yeast cells were transformed by the lithium acetate method essentially as described by Ito *et al.* (44). Nuclear export step of Yap1p was inhibited by the addition to the cell culture medium of diamide, a thiol oxidant, to a final concentration of 1.5 mM, as described previously (45).

Construction of Plasmid—Plasmid pK1b, which contains the 5′-untranslated region and the region that encodes core, E1, and the amino-terminal part of E2 of HCV genotype 1b was described elsewhere (46). A fragment encoding the entire core (p23; amino acid residues 1–191; core 191) was amplified from pK1b by the polymerase chain reaction (PCR) with 5′-GTCAGAATTCACCATGAGCACAAATCCTAAACCTC-3′ and 5′-GTCACTCGAGTTAAGCGGAAGCTGGGATGGTCA-3′ as sense and antisense primers, respectively; the *Eco*RI and *Xho*I sites are underlined. To express core in yeast cells, the product of PCR was digested with *Eco*RI and *Xho*I, and inserted with the corresponding restriction sites between promoter and terminator of a gene for glyceraldehyde-3-phosphate dehydrogenase in pKT10 (*URA3* 2μ ori) (47). The resultant plasmid was designated as pKT10-191. The construction of plasmids for expression of carboxyl-terminal deletion mutants of core was performed similarly with 5′-GTCACTCGAGTCAAGAGCAACCGGGCAAATTCC-3′ or 5′-GTCACTCGAGTCACAGGGCCCTGGCAGCAC-3′ as the antisense primer; the *Xho*I sites are underlined. The resultant plasmids, designated as pKT10-173 and pKT10-151, encoded for core p21 (amino acid residues 1–173; core 173) and core p19 (amino acid residues 1–151; core 151), respectively. For expression of glutathione *S*-transferase (GST) in yeast cells, the *GST* gene, isolated from pGEX-6P-2 (Amersham Biosciences), was inserted into pKT10. The resultant plasmid was designated as pKT10-GST.

The plasmid for expression of the NLS of SV40 T-antigen, fused to green fluorescent protein (GFP), in yeast cells was generated as follows. For construction of pGAD-GFP, a expression vector for GFP tagged with classical NLS, pGFP536 (48) that encode variant of GFP with enhanced fluorescence, was digested with *Eco*RI and *Sal*I, and the appropriate fragment was inserted at the corresponding sites of pGAD424 (Clontech) that included the NLS of SV40 T antigen at the amino terminus of Gal4 activation domain.

To generate polyhistidine-tagged (His-tagged) full-length core, we amplified a fragment that encoded the entire core protein from pK1b by PCR using 5′-GTCAGGATCCGATGAGCACAAATCCTAAACCTC-3′ and 5′-GTCACTCGAGTTAAGCGGAAGCTGGGATGGTCA-3′ as sense and antisense primers, respectively; *Bam*HI and *Xho*I sites are underlined. The product of PCR was digested with *Bam*HI and *Xho*I and then inserted at the corresponding sites in pET28b(+) (Novagen). The resultant plasmid was designated as pET-191.

Preparation of Yeast Extracts and Immunoblotting Analysis—Yeast cells were grown to mid-logarithmic phase, harvested by centrifugation, and washed with breakage buffer (200 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 500 μM *N*-tosyl-L-phenylalanine chloromethyl ketone, 25 μM *N*-α-tosyl-L-lysine chloromethyl ketone). The cells were resuspended in breakage buffer and disrupted with acid-washed glass beads at 4 °C. The yeast extract was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then proteins were subjected to immunoblotting. Core was detected with mouse core-specific monoclonal antibody 515S (49) and peroxidase-conjugated goat antibodies against mouse immunoglobulins (DAKO) as primary and secondary antibodies, respectively. ECL Western blotting detection reagents (Amersham Biosciences) were used in accordance with the instructions from the manufacturer.

Immunofluorescence Studies—An immunofluorescence study was performed as described by Ferrigno and Silver (50) with minor modifications. Yeast cells expressing core were grown to mid-logarithmic phase and then fixed in formaldehyde at a final concentration of 4% at room temperature for 1 h. The fixed cells were harvested by centrifugation and washed with phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4) and solution P (PBS containing 1 M sorbitol). The cells were then resuspended in solution P and incubated with 0.3 mg/ml zymolyase 100T (Seikagaku Co.) in the presence of 25 mM dithiothreitol at room temperature for 30 min. The cells were placed on poly-L-lysine-coated slides, treated with a mixture of methanol and acetone, and subjected to indirect immunofluorescence analysis. After treatment with blocking buffer (PBS containing 3% bovine serum albumin (BSA)) at room temperature for 15 min, the cells were incubated with 100 μg/ml core-specific monoclonal antibody 515S, which can react to core in both the cytoplasm and the nucleus (27), at room temperature for 1 h. The cells were then washed several times with PBS and allowed to react with fluorescein-labeled affinity-purified antibodies raised in goat against mouse IgG (KPL Inc.), which had been diluted 100-fold, at room temperature for 1 h. The cells were then stained with 5 μg/ml propidium iodide (Sigma) at room temperature for 15 min after treatment for 30 min with 1 mg/ml RNase A. Finally, cells were washed several times, mounted in 90% glycerol, and observed with a confocal laser scanning microscope (MRC1024, Bio-Rad).

Assay of β-Galactosidase Activity—The assay of β-galactosidase activity was performed as described previously (51). Cells in a culture of yeast (1–1.5 ml) at mid-logarithmic phase were harvested by centrifugation and resuspended in 50 μl of Tris-Triton buffer (0.1 M Tris-HCl, pH 7.5, and 0.05% Triton X-100). Cells were then frozen, thawed, and incubated with 250 μl of buffer Z (10 mM KCl, 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, and 1 mM $MgSO_4$) that contained 0.8 mg/ml *o*-nitrophenyl-β-D-galactopyranoside at 30 °C for 2 min. The reaction was stopped by the addition of 125 μl of 1 M Na_2CO_3. The absorbance of *o*-nitrophenol at A_{420} was measured, and after adjustments for cell density (A_{600}) and reaction time (min), the value was taken as an index of β-galactosidase activity. Activities were normalized by subtraction of the background β-galactosidase activity. The assay was repeated in at least three separate replicate experiments.

Assay of Nuclear Import in Vivo—The assay of nuclear import *in vivo* was performed using 2xGFP-fused Yap1p-(2–59) driven by an inducible *met3* promoter (52). Yeast cells were grown at 30 °C to mid-logarithmic phase with the activity of the *met3* promoter suppressed by the presence of methionine. Then the cells were harvested by centrifugation, washed once with methionine-depleted medium, and incubated at 30 °C for 30 min in methionine-depleted medium to induce the expression of 2xGFP-Yap1p-(2–59). Fluorescence caused by GFP was examined with the confocal laser scanning microscope.

We performed a kinetics-based assay of nuclear import *in vivo* essentially as described by Roberts and Goldfarb (53). Yeast cells were

TABLE I
The yeast strains used in this study

Strain	Genotype	Source (ref.)
PSY580	*MAT***a** *ura3–52 leu2Δ1 trp1Δ63*	Winston *et al.* (64)
PSY688	*MATα ade2 ura3 leu2 trp1 his3 srp1–31*	Loeb *et al.* (65)
PSY962	*MATα gsp1::HIS3 gsp2::HIS3 ura3–52 leu2Δ1 trp1Δ63 gsp1–1* (on p-*CEN TRP1*)	Wong *et al.* (54)
PSY967	*MATα ura3–52 leu2Δ1 his3Δ200 kap123Δ::HIS3*	Seedorf and Silver (66)
PSY1103	*MAT***a** *ura3–52 leu2Δ1 trp1Δ63 rel1–4*	Ferrigno *et al.* (67)
PSY1199	*MATα ade2Δ::hisG ade8Δ100::KAN^R ura3Δ leu2Δ1 his3Δ200 nmd5Δ::HIS3*	Ferrigno *et al.* (67)
PSY1200	*MAT***a** *ura3–52 leu2Δ1 trp1Δ63 his3Δ200 lys2 sxm1Δ::HIS3*	Seedorf and Silver (66)
PSY1201	*MAT***a** *ura3–52 leu2Δ1 trp1Δ63 pse1–1*	Seedorf and Silver (66)
mtr10::HIS3	*MATα ade2 ura3 leu2 trp1 his3 mtr10Δ::HIS3*	Senger *et al.* (68)
kap104–16	*kap104–16* cells are the derivatives of DF5 cells as described in source	Aitchison *et al.* (69)
DY	*MATα can1–100 ade2 leu2 trp1 his3 ura3::(3xSV40AP-1-lacZ)*	Kuge and Jones (57)
DWYU	*MATα can1–100 ade2 leu2 trp1 his3 ura3::(3xSV40AP-1-lacZ) yap1::URA3*	Kuge and Jones (57)
C3	*MATα can1–100 ade2 leu2 trp1 his3 ura3::(3xSV40AP-1-lacZ) yap1::(cup1cp-GFP-YAP1)*	Kuge *et al.* (45)

transformed with pRS cp-GFP-yap1-(1–373) for expression of GFP fused to Yap1 NLS (45) or with pGAD-GFP for expression of GFP fused with an NLS of SV40 T antigen. These cells were further transformed with pKT10-191, pKT10-173, or pKT10-151. Cells were grown in the presence of 2% glucose at 30 °C to early logarithmic phase, harvested by centrifugation, and washed with distilled H_2O. The cells were resuspended in glucose-depleted medium that contained 10 mM NaN_3 and 10 mM 2-deoxy-D-glucose as metabolic inhibitors, and then they were incubated at 30 °C for 45 min for inhibition of ATP-dependent transport, with resultant nucleocytoplasmic equilibration of NLS-containing proteins by passive diffusion. Cells were harvested by centrifugation and washed with ice-cold distilled H_2O. At time 0, the import assay was initiated by resuspending in the medium with 2% glucose, and incubation at 30 °C. Samples were observed at each time point under the confocal laser scanning microscope. The percentage of cells with distinct nuclear localization of NLS-containing proteins was determined. Scoring of the cells for nuclear or cytoplasmic localization was performed as described by Roberts and Goldfarb (53). At least 30 cells were examined of each time point. The assay was performed with at least three separate sets of each transformant.

Expression and Purification of Recombinant Proteins—Recombinant proteins were prepared as described by Isoyama *et al.* (52). *Escherichia coli* BL21 cells were transformed with pGEX-PSE1 (52) for expression of Kap121p as a GST fusion protein, and grown at 37 °C in the presence of ampicillin. At an absorbance at 600 nm (A_{600}) of 0.5, isopropyl-β-D-thiogalactoside was added to the culture to a final concentration of 1 mM and cells were cultured at 37 °C for another 4 h. The cells were harvested by centrifugation, resuspended in PBS, and disrupted by sonication. Cell lysates were incubated with 1% Triton X-100 at 4 °C for 30 min. GST fusion protein was purified with glutathione-Sepharose beads according to the instructions from the manufacturer (Amersham Biosciences). For isolation of His-tagged core or Yap1p-(1–244), *E. coli* BL21 (DE3) cells that harbored pET-191 or pET-yap1-(1–244) (52) were grown in the presence of kanamycin, and expression of the genes was induced by incubation with 1 mM isopropyl-β-D-thiogalactoside at 20 °C for 20 h. Cells were harvested and sonicated in lysis buffer (50 mM Na_2HPO_4, pH 8.0, 300 mM NaCl, 1 mM 2-mercaptoethanol, and 20 mM imidazole). Each cell lysate was loaded onto a nickel-nitrilotriacetic acid-agarose column according to the instructions from the manufacturer (Qiagen). The column was washed, and His-tagged proteins were eluted with elution buffer (lysis buffer containing 250 mM imidazole). Purified proteins were concentrated by VIVASPIN™ (Vivascience) and further purified by chromatography on Superdex 200 (Amersham Biosciences) in 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, and 2 mM 2-mercaptoethanol using a SMART chromatography system (Amersham Biosciences).

Binding Assays—Binding assays *in vivo* were performed as described by Isoyama *et al.* (52). For the assays, recombinant proteins were expressed and purified from bacterial lysates as described above. Five μg of GST-Kap121p was bound to 20 μl of a 50% slurry of glutathione-Sepharose beads by incubation in binding buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 10% glycerol, and 0.5 mg/ml BSA) for 2 h at 4 °C. The beads with bound GST-Kap121p were washed with washing buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 10% glycerol) and incubated with 5 μg of His-Yap1p-(1–244) and indicated amounts of core in 250 μl of binding buffer for 3 h at 4 °C. After extensive washing, bound proteins were eluted from beads with 10 mM reduced glutathione and analyzed by SDS-PAGE, with subsequent staining with Coomassie Brilliant Blue or immunoblotting. His-Yap1p-(1–244) was detected with rabbit antibody against His tag (MBL) and peroxidase-conjugated antibody raised in goat against rabbit immunoglobulins (DAKO) as primary and secondary antibodies, respectively.

RESULTS

Subcellular Localization of HCV Core Proteins in Yeast Cells—Core proteins p23 (core 191), p21 (core 173), and p19 (core 151) of HCV were expressed in *S. cerevisiae* cells. Extracts from yeast cells that expressed individual core proteins were analyzed by immunoblotting with a core-specific monoclonal antibody. As shown in Fig. 1*A*, core proteins were detected in lysates of cells that harbored core expression plasmids (*lanes 2–4*), but not a vector plasmid (*lane 1*). Approximately 40% of core 191 expressed in yeast cells was processed to a more rapidly migrating form that corresponded to core p21 (Fig. 1*A*, *lane 2*), suggesting that processing of core 191 by a signal peptidase occurs in yeast. Because the mobility of the processed form was slightly greater than that of core 173 (Fig. 1*A*, compare *lanes 2* and *3*), it is possible that the processing site is amino acid position 179/180 or position 182/183 (9) rather than position 173/174 (8, 26).

We next examined the subcellular localization of core in yeast cells by immunofluorescence microscopy using a core-specific monoclonal antibody. No fluorescence was detected with the core-specific antibody in cells that harbored the vector plasmid (data not shown). The cytoplasmic and nuclear localization of core was evident in cells that expressed core 191 or core 173 (Fig. 1*B*, *upper* and *middle panels*). The cytoplasmic localization of core was observed as a reticular or punctate pattern that is typical of endoplasmic reticulum-associated proteins around the nucleus. The nuclear localization in cells expressed core 173 was more efficient than that in cells expressed core 191. As expected, core 151 was concentrated in the nucleus (Fig. 1*B*, *lower panels*). Taken together, our observations demonstrate that the processing and subcellular localization of core in yeast cells are similar to those in mammalian cells reported by a number of groups (24–27).

Nuclear Accumulation of HCV Core Protein Requires Ran—The core protein has NLS-like sequences within its amino-terminal region. Moreover amino-terminal region of core, when fused to other proteins, results in concentration of these proteins in the nucleus (25). In most cases, nuclear transport involves active transport and requires the activity of small GTPase Ran (36–38). Therefore, we next examined whether the nuclear import of core also requires Ran using a yeast mutant with a temperature-sensitive *GSP1* gene (*gsp1–1*) for the yeast orthologue of Ran. In *gsp1–1* cells, NLS-containing proteins are not transported into the nucleus at a restrictive temperature (54). We expressed the nuclear localized form, core 151, in *gsp1–1* cells, and cultured the cells at the permis-

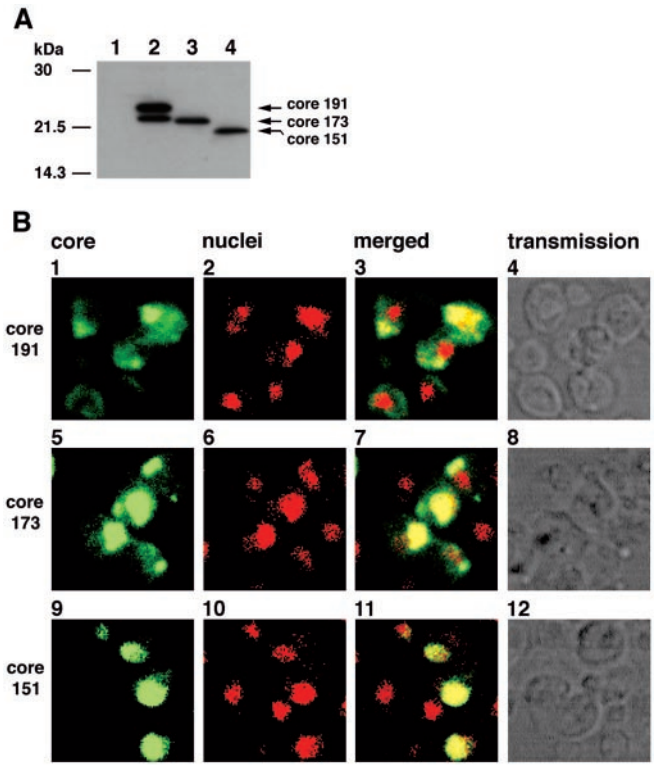

FIG. 1. **Expression of HCV core protein in yeast cells.** *A*, processing of core in yeast cells. Yeast extracts were prepared from cells (DY) that had been transformed with the following plasmids: *lane 1*, pKT10 (vector plasmid); *lane 2*, pKT10-191; *lane 3*, pKT10-173; *lane 4*, pKT10-151. Extracts were fractionated by SDS-PAGE (15% polyacrylamide) and subjected to immunoblotting with core-specific monoclonal antibody. Bands of core 191, 173, and 151 are indicated by *arrows*. Positions of molecular mass markers are shown on the *left*. *B*, subcellular localization of core in yeast cells. Yeast cells (DY) were grown to mid-logarithmic phase and fixed for indirect immunofluorescence studies as described in the text. Immunofluorescence patterns are shown for cells transformed with pKT10-191 (*upper panels*), pKT10-173 (*middle panels*), and pKT10-151 (*lower panels*). Core was detected with the core-specific monoclonal antibody and fluorescein isothiocyanate-conjugated antibody against mouse IgG as primary and secondary antibodies, respectively (*panels 1*, *5*, and *9*). The images of cells stained with propidium iodide are shown in *panels 2*, *6*, and *10*. *Panels 3*, *7*, and *11* show merged images from *panels 1* and *2*, *panels 5* and *6*, and *panels 9* and *10*, respectively. Transmitted images are shown in *panels 4*, *8*, and *12*.

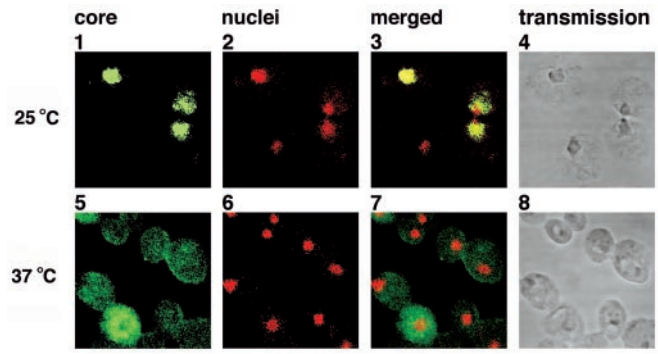

FIG. 2. **Nuclear import of HCV core protein requires Ran/Gsp1p.** Mutant yeast with a temperature-sensitive *GSP1* allele, *gsp1–1*, cells (PSY962) were transformed with pKT10-151. The cells were grown to mid-logarithmic phase at the permissive temperature of 25 °C (*upper panels*) and then incubated at the restrictive temperature of 37 °C for 1 h (*lower panels*). Cells were fixed and subjected to indirect immunofluorescence analysis. Core 151 was detected with the core-specific monoclonal antibody and fluorescein isothiocyanate-conjugated antibodies against mouse IgG as primary and secondary antibodies, respectively (*panels 1* and *5*). The images of cells stained with propidium iodide are shown in *panels 2* and *6*. *Panels 3* and *7* show merged images from *panels 1* and *2* and from *panels 5* and *6*, respectively. Transmitted images are shown in *panels 4* and *8*.

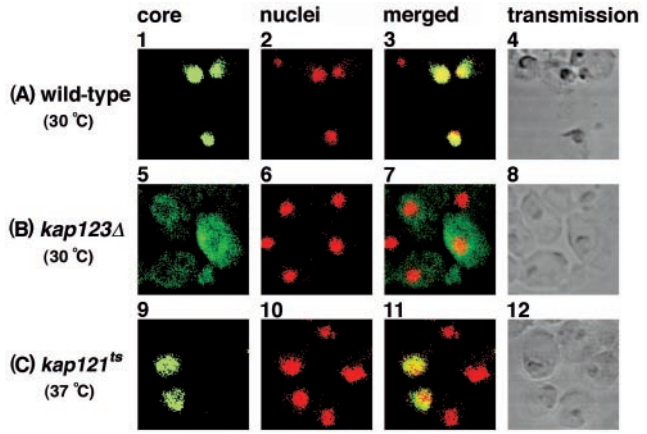

FIG. 3. **Nuclear transport of HCV core protein is mediated by Kap123p in yeast cells.** Wild-type cells (PSY580) (*A*), *kap123*Δ cells with a disrupted gene for *KAP123* (PSY967) (*B*), and *kap121*^{ts} cells with a temperature-sensitive *KAP121* allele, *pse1–1* (PSY1201) (*C*) were transformed with pKT10-151. Cells were grown to mid-logarithmic phase and then incubated at 30 °C (*A* and *B*) or at the restrictive temperature of 37 °C (*C*) for 1 h. The cells were fixed and subjected to indirect immunofluorescence studies. Core 151 was detected with the core-specific monoclonal antibody and fluorescein isothiocyanate-conjugated antibodies against mouse IgG as primary and secondary antibodies, respectively (*panels 1*, *5*, and *9*). The images of cells stained with propidium iodide are shown in *panels 2*, *6*, and *10*. *Panels 3*, *7*, and *11* show merged images from *panels 1* and *2*, *panels 5* and *6*, and *panels 9* and *10*, respectively. Transmitted images are shown in *panels 4*, *8*, and *12*.

sive temperature of 25 °C. As observed in the wild-type cells (see below, Fig. 3*A*), core 151 accumulated in the nucleus at 25 °C (Fig. 2, *upper panels*). By contrast, nuclear accumulation of core 151 was inhibited at the restrictive temperature of 37 °C (Fig. 2, *lower panels*). These results indicate that the nuclear import of core is dependent on Ran.

Nuclear Import of HCV Core Protein Is Mediated by Kap123p in Yeast Cells—Our results suggested that core is imported into the nucleus by transport receptor(s) that require Ran for their transport activities. To identify the import receptor(s) responsible for the nuclear transport of core in yeast cells, we examined the localization of core 151 in various strains of yeast cells with mutations in individual transport receptors, including the members of the importin β family, as well as importin α. In wild-type cells, core 151 accumulated in the nucleus (Fig. 3*A*). We found that *KAP123*-disrupted mutant (*kap123*Δ) cells failed to transport core 151 into the nucleus (Fig. 3*B*), an indication that Kap123p mediates the nuclear import of core. Yeast cells with a mutation in the yeast orthologue of importin α (*srp1–31*) or of importin β (*rsl1–4*) were not defective with respect to the nuclear accumulation of core 151 (data not shown), suggesting that the nuclear import of core is independent of the classical NLS-import pathway that involves the importin α-importin β complex. In addition, cells with mutations in genes for other members of the importin β family, namely *sxm1*Δ, *nmd5*Δ, *mtr10D*, and *kap104–16,* were also not defective in the nuclear accumulation of core 151 (data not shown).

It has been suggested that Kap123p (Yrb4p) and the closely related transport receptor Kap121p (known as Pse1p) (19% homology, 48% similarity) have overlapping functions in nuclear import. For example, ribosomal protein L25 is mainly transported into the nucleus by Kap123p, but Kap121p can substitute functionally for Kap123p (38, 55). To examine the possibility that Kap121p might also play a role in the nuclear import of core, we examined the localization of core 151 in

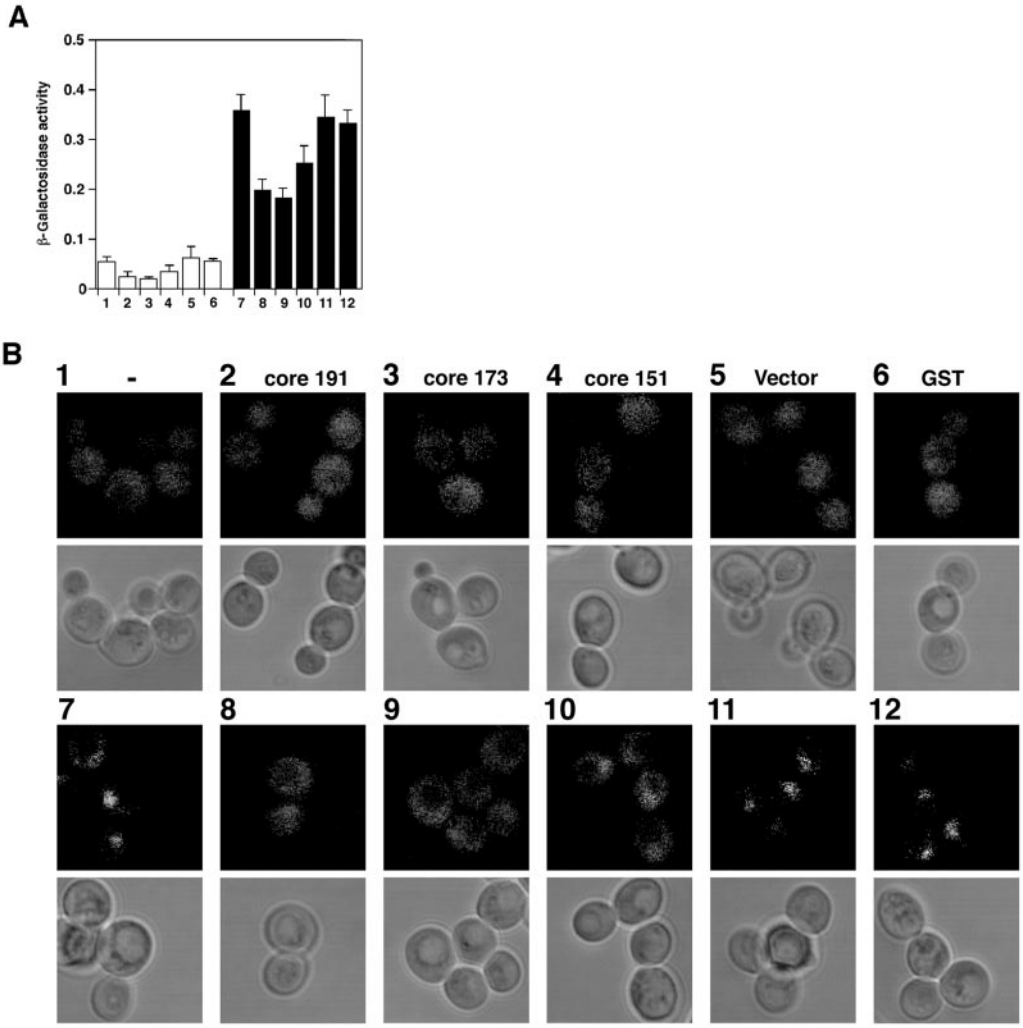

FIG. 4. **HCV core protein inhibits nuclear transport of Yap1p.** *A*, core protein suppresses the transcriptional activity of Yap1p. Yeast cells (C3) carrying a *lacZ* reporter gene specific for Yap1p and a gene for GFP-Yap1p were transformed with the following plasmids: *columns 1* and *7*, none; *columns 2* and *8*, pKT10-191; *columns 3* and *9*, pKT10-173; *columns 4* and *10*, pKT10-151; *columns 5* and *11*, pKT10 (vector plasmid); *columns 6* and *12*, pKT10-GST. Cells were grown to mid-logarithmic phase and then incubated in the absence (*columns 1–6*) or presence (*columns 7–12*) of 1.5 mM diamide at 30 °C for 1 h. Finally, cells were subjected to assays of β-galactosidase activity as described in the text. β-Galactosidase activity in DWYU cells was taken as the background value. Measured activities of β-galactosidase were normalized by subtraction of the background value. Each *column* and *bar* represent the average of the results of at least three separate experiments and the standard deviation, respectively. *B*, core inhibits nuclear translocation of Yap1p upon exposure of cells to oxidative stress. C3 cells were transformed with the following plasmids: *panels 1*and *7*, none; *panels 2* and *8*, pKT10-191, *panels 3* and *9*, pKT10-173; *panels 4* and *10*, pKT10-151; *panels 5* and *11*, pKT10 (vector plasmid); *panels 6* and *12*, pKT10-GST. The cells were grown to mid-logarithmic phase and then incubated in the absence (*panels 1–6*) or presence (*panels 7–12*) of 1.5 mM diamide at 30 °C for 1 h. GFP-Yap1p was monitored by fluorescence microscopy. Fluorescent images (*upper panels*) and transmitted images (*lower panels*) are shown. *C*, core inhibits nuclear import by Yap1p NLS. DY cells were transformed with the following plasmids: *panels 1*, pKT10 (vector plasmid); *panels 2*, pKT10-191; *panels 3*, pKT10-173; *panels 4*, pKT10-151. 2xGFP-Yap1p-(2–59) was expressed from the inducible *met3* promoter as described in the text and monitored by fluorescence microscopy. Fluorescent images (*upper panels*) and transmitted images (*lower panels*) are shown. *D*, core inhibits nuclear import that involves the NLS of Yap1p, but not by the NLS of SV40. DY cells were transformed with pRS cp-GFP-yap1-(1–373) (*left graph*) or pGAD-GFP (*right graph*). Cells were then transformed with no plasmid (*squares*), pKT10-191 (*diamonds*), pKT10-173 (*circles*), and pKT10-151 (*triangles*) and subjected to a kinetics-based assay of nuclear import *in vivo*, as described in the text. Each *point* and *bar* represent the average of results of at least three separate experiments and the standard deviation, respectively.

temperature-sensitive *KAP121* mutant (*pse1–1*) cells. However, as shown in Fig. 3*C*, Kap121p appeared not to be involved in the nuclear accumulation of core 151. These results demonstrate that Kap123p is a receptor for the nuclear import of core in yeast cells.

HCV Core Protein Inhibits Nuclear Import of Yap1p—We used the yeast AP-1-like transcription factor Yap1p as an indicator molecule to examine whether core might affect nucleocytoplasmic transport. Yap1p is a shuttling protein; it is imported into the nucleus by Kap121p (52), a homologue of Kap123p, and is exported from the nucleus by the export receptor Crm1p (51, 56). When yeast cells are exposed to diamide, the latter step is inhibited, with resultant nuclear accumulation of Yap1p (51, 56). Thus, Yap1p seems to be an appropriate indicator of both nuclear import and export.

Expression of core 191, of core 173, and of core 151 did not affect the level of Yap1p detected by immunoblotting, as described by Kuge *et al.* (45) (data not shown). Next, we examined the transcriptional activity of Yap1p in cells with a *lacZ* reporter gene specific for Yap1p (57). We found that β-galactosidase activity was reduced upon expression of core 191, core 173, and core 151 (Fig. 4*A*, *columns 1–4*), whereas a vector plasmid and GST had no effect on the activity (Fig. 4*A*, *columns 5* and *6*). The effects of core 191 and core 173 were more

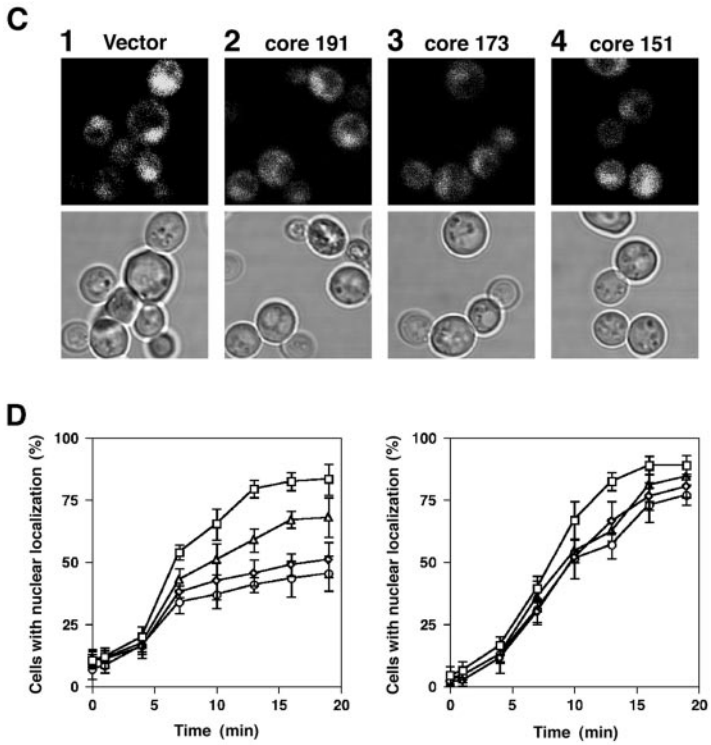

FIG. 4—*continued*

significant than that of core 151. Because the activity of the reporter gene depended on the amount of Yap1p in the nucleus (45), these results suggested two possibilities: either import of Yap1p was inhibited or export of Yap1p was accelerated. To distinguish between these possibilities, we specifically inhibited the nuclear export of Yap1p by exposing cells to diamide (51, 52). The diamide-induced level of β-galactosidase activity was specifically reduced by core 191, by core 173 and by core 151 (Fig. 4*A*, *columns 7–12*), whereas core did not affect the Yap1p-independent basal level of β-galactosidase activity in *YAP1*-disrupted cells (data not shown). These data suggested that nuclear import step of Yap1p was affected by core.

To examine in further detail the effects of core on the transport of Yap1p, we monitored the fluorescence of GFP-fused Yap1p in living yeast cells. As observed previously (45), Yap1p accumulates in the nucleus in response to diamide (Fig. 4*B*, *panels 1* and *7*). However, cells expressing core 191, core 173, or core 151, respectively, were defective in the nuclear accumulation of Yap1p upon exposure of diamide (Fig. 4*B*, *panels 2–4* and *8–10*). A vector plasmid and GST had no effect in the nuclear translocation of Yap1p (Fig. 4*B*, *panels 5*, *6*, *11*, and *12*). These data demonstrate that core inhibits the nuclear translocation of Yap1p, with resultant suppression of Yap1p-dependent transcription.

To eliminate the influence of diamide, we performed a nuclear import assay *in vivo* using a region of Yap1p that include the NLS; Yap1 domains that included the DNA-binding domain and the carboxyl-terminal nuclear export signal region were deleted in this construct. Yap1p NLS-tagged GFP (2xGFP-Yap1p-(2–59)) was induced to localize in the nucleus, and we examined the effect of core on the nuclear import of Yap1p-NLS. As shown in Fig. 4*C*, the nuclear localization of Yap1p-NLS was inhibited by core 191, core 173, or core 151, but not by a vector plasmid.

For a quantitative study of the effects of core on the nuclear import of Yap1p-NLS (GFP-Yap1-(1–373)), we performed a kinetics-based assay of nuclear import *in vivo*. When yeast cells were exposed to azide and deoxyglucose as metabolic inhibitors in the absence of glucose, active transport was blocked, with resultant equilibration of GFP-Yap1p-(1–373) between the nuclear and cytoplasmic compartments by passive diffusion. Cells were washed and incubated in the presence of glucose, and then GFP-Yap1p-(1–373) was imported into the nucleus by active transport (Fig. 4*D*, *left graph*). The NLS of SV40 T-antigen, which is known as a classical basic NLS, is recognized by the importin α/β complex and is transported into the nucleus even in yeast cells (58). We found that, core proteins 191, 173, and 151 each inhibited nuclear import by the Yap1p NLS, but had no significant effect on import by the SV40 NLS (Fig. 4*D*, compare *left graph* and *right graph*). The inhibitory effects of core 191 and core 173 on the nuclear import by Yap1p NLS were stronger than that of core 151 (Fig. 4*D*, *left graph*). These data suggest that core inhibits a specialized nuclear transport pathway.

HCV Core Protein Inhibits the Interaction between Yap1p and Its Nuclear Import Receptor—Recently, we showed that Yap1p binds directly to its nuclear import receptor, namely Kap121p (52). To determine whether core affects the interaction between Yap1p and Kap121p, we expressed core, His-tagged Yap1p-(1–244) and GST-fused Kap121p, in *E. coli*, and performed an competition assay *in vitro*. GST-Kap121p, bound to glutathione-Sepharose beads, was incubated with His-Yap1p-(1–244) and various amounts of core. The beads were washed, and proteins that had bound to the beads were resolved by SDS-PAGE. The amount of Yap1p bound to Kap121p was reduced in the presence of core, and the effect was dose-dependent (Fig. 5). No similar effect was observed with BSA (data not shown). These observations suggest that core suppresses the interaction between Yap1p and Kap121p, with resultant inhibition of the nuclear import of Yap1p.

DISCUSSION

Because nucleocytoplasmic transport systems are strongly conserved in *S. cerevisiae* and mammals at the molecular level, we used yeast cells as a model of mammalian cells. We found that the processing and subcellular localization of core in yeast

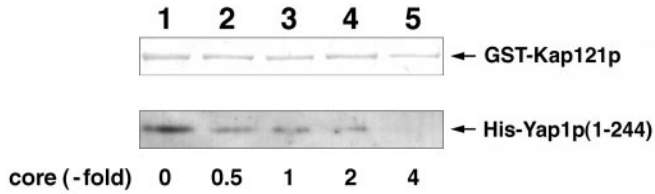

FIG. 5. **HCV core protein inhibits the interaction between Yap1p and its nuclear import receptor.** GST-Kap121p, purified from *E. coli*, was bound to glutathione-Sepharose beads. The beads were then incubated with His-Yap1p-(1–244) and core, also purified from *E. coli*, and washed extensively. The molar excess of core (-fold) relative to His-Yap1p-(1–244) is indicated. Bound proteins were eluted from beads with 10 mM reduced glutathione, and eluted proteins were separated by SDS-PAGE (12% polyacrylamide) with subsequent staining with Coomassie Brilliant Blue (*upper panel*) or immunoblotting with His tag-specific antibodies (*lower panel*). Bands of GST-Kap121p and His-Yap1p-(1–244) are indicated by *arrows*.

cells resemble those in mammalian cells reported by several groups (see Fig. 1; See also Refs. 24–27). Furthermore, our data indicate that the nuclear import of core requires Ran/Gsp1p (Fig. 2), and is mediated by a specific import receptor, Kap123p (Fig. 3). To our knowledge, this study provides the first evidence that the core protein of HCV is transported into the nucleus via a specific transport receptor in a Ran-dependent manner.

In addition, core inhibited the Kap121p-dependent nuclear import of Yap1p (Fig. 4). This inhibitory effect of core seems to involve a specialized import system, because importin α/β-dependent import of proteins with a classical NLS was not affected under the same conditions (Fig. 4*D*). Our data suggested that core inhibits the interaction between Yap1p and its import receptor, Kap121p (Fig. 5), with resultant inhibition of the nuclear import of Yap1p (Fig. 4). It is unlikely that core interacts directly to Yap1p domains that included DNA-binding domain, because core could also affect nuclear localization of a limited region of the NLS of Yap1p, amino acid positions 2–59 (Fig. 4*C*).

Two recent reports indicate that expression of core increases cellular oxidation state (59, 60). It is interesting to demonstrate if this is the case in yeast cells. Even though Yap1p is a transcription factor that is induced by oxidative stress, we could observe neither an accumulation of Yap1p in the nucleus nor an induced level of Yap1p-dependent transcription, when core was expressed in yeast cells (Fig. 4). This could be because core inhibits nuclear import step of Yap1p (this study), whereas oxidative stress inhibits nuclear export of Yap1p, leading accumulation of Yap1p in the nucleus and transcriptional activation of Yap1p (51, 52). Thus, another indicator will be required to examine effect of core on oxidative state in yeast cells.

Differences among inhibitory effects of core on Yap1p import are somewhat correlated to the cytoplasmic localization of each core protein; core 191 and core 173, which showed stronger inhibition, were both localized in the cytoplasm and nucleus, whereas core 151, which was less effective, was localized solely in the nucleus (Fig. 1*B*). The region that represents the difference between core 191 and core 151 might be required for the maximal inhibitory effect. It should be noted that the carboxyl-terminal region of core, amino acid positions 176–188, has leucine-rich nuclear export signal-like sequence. Core may shuttle between the nucleus and the cytoplasm by cellular nuclear import and export systems.

Localization of core seems to be controversial. Recent studies indicate that core predominantly localized in cytoplasm not only in cultured cells (28, 29), but also in cells harboring HCV replicons (29), cells from liver biopsy samples of HCV-infected patients (30), as well as a HCV-infected chimpanzee (28). Thus, the relevance of steady-state nuclear localization of core to natural HCV infections remains in doubt. On the other hand, Kohara and co-workers (27) have shown that processed form of core (p21) is identified in the nucleus only by using a specific monoclonal antibody, and that this processed form of core is also identified in the viral particles isolated from HCV-infected patients. These results suggest that specific antibody and/or condition are required to detect core in the nucleus. Nevertheless, our findings suggest that core affects cellular metabolism in the cytoplasm by disturbing specific nuclear transport, because specific binding of cargoes to its importins are carried out in the cytoplasm.

In addition to the sequence homology between Kap123p and Kap121p, these receptors might have overlapping functions in nuclear import because Kap121p can substitute functionally for Kap123p in the nuclear transport of ribosomal protein L25 (38, 55). Unlike ribosomal protein L25, core was not imported into the nucleus by Kap121p (Fig. 3*C*), but core did inhibit Kap121p-dependent nuclear transport (Fig. 4). Karyopherin β3 (also known as importin 5) is the mammalian orthologue of Kap121p and mediates the nuclear import of ribosomal proteins (61). It is likely that core might have an effect on karyopherin β3-dependent nuclear transport in mammalian cells. We are currently investigating this possibility. Furthermore, human importin 4 has been recently identified as a Kap123p orthologue (62).

Recently, Jin *et al.* (63) reported that LZIP, a transcription factor with basic leucine zipper domain that binds cyclic AMP-responsive element, can function as a cofactor of core for transformation of NIH 3T3 cells. They indicated that core can sequester LZIP in the cytoplasm, with resultant morphological changes in NIH3T3 cells. The mechanism of sequestration of LZIP by core in the cytoplasm might be the result of inhibition of the nuclear transport of LZIP by core.

In view of the conservation of nucleocytoplasmic transport between yeast and mammalian cells, it is likely that core plays a role in the nuclear transport of cellular proteins, such as transcription factors and intracellular signaling molecules, in mammalian cells. Nuclear transport is critical for gene expression and cellular responses to intracellular signals. Inhibition of nuclear import results in loss of the regulation of these events in cells, leading to destruction of the cellular metabolism that maintains homeostasis. Core might contribute to the pathogenesis of HCV by disturbing nuclear transport.

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The Core Protein of Hepatitis C Virus Is Imported into the Nucleus by Transport Receptor Kap123p but Inhibits Kap121p-dependent Nuclear Import of Yeast AP1-like Transcription Factor in Yeast Cells

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