Original Article

Hsc70 regulates the nuclear export but not the import of influenza viral RNP: A possible target for the development of anti-influenza virus drugs

Ken Watanabe[1], Naoki Takizawa[1], Saiko Noda[1], Fujiko Tsukahara[2], Yoshiro Maru[2], Nobuyuki Kobayashi[1, 3,*]

[1] *Laboratory of Molecular Biology of Infectious Agents, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan;*
[2] *Department of Pharmacology, Tokyo Women's Medical University, School of Medicine, Tokyo, Japan;*
[3] *Central Research Center, AVSS Corporation, Nagasaki, Japan.*

ABSTRACT: In our previous report, we demonstrated that the matrix 1 (M1) protein of influenza virus directly binds to heat shock cognate protein 70 (Hsc70). The down-regulation of Hsc70 resulted in the reduction of influenza virus production, thus suggesting that Hsc70 plays a crucial role for viral replication. However, the detailed role of Hsc70 in viral replication remains to be elucidated. Hsc70 has been suggested to play a significant role in both the nuclear import and export processes. In this report, using leptomycin B (LMB), a CRM1-mediated nuclear export inhibitor, we demonstrated that Hsc70 forms a complex with vRNP through M1 in infected cells and in the virion, thus playing a significant role in the export of vRNP from the nucleus but not in the import of vRNP into the nucleus. The regulation of Hsc70 may therefore lead to the development of new anti-influenza virus drugs without raising mutant viruses.

Keywords: Influenza virus, Heat shock protein, Hsc70, Nuclear export signal, Leptomycin B

Introduction

The type A influenza virus genome has eight segmented single-stranded RNAs in negative polarity. Its genome in the virion exists as a vRNP complex with RNA-dependent RNA polymerase (RdRp) and nucleocapsid protein (NP). The vRNP is surrounded by matrix protein 1 (M1), which is also associated with the lipid bilayer. After the adsorption of the virion to the host cell, the incoming vRNPs are imported into the nucleus where early transcription of the influenza virus occurs. The early gene products, three subunits of RdRp (PB1, PB2, and PA), and NP are synthesized immediately after infection and then are subsequently transported to the nucleus where they form new vRNPs. These newly formed vRNPs are used as a template for late transcription. The gene products, such as hemagglutinin (HA), neuraminidase (NA), M1, M2, and NS2, are synthesized at the late stage of infection. These proteins are essential for the formation of mature virus particles in the cytoplasm and plasma membrane (for a review, see *Ref. 1*).

M1 has been suggested to play an important role in the nuclear export of vRNPs (*2*,*3*). Using leptomycinB (LMB), an inhibitor of CRM1-dependent nuclear export, we previously found the export of vRNP to be efficiently inhibited (*4*), although M1, NP and RdRp do not have a nuclear export signal (NES). Recently, we identified heat shock cognate protein 70 (Hsc70) as a host factor which bind to M1 protein in infected cells and our results suggest that Hsc70 directly interacts with M1 (*5*). However, its detailed role still remains unclear.

Heat shock proteins (Hsps) are induced by various types of stimulation, such as heat treatments, chemicals, UV, and viral infection, as a cellular defense mechanism. For example, prostaglandins induce the expression of Hsps (Hsp70, Hsc70) and inhibit the replication of a variety of RNA viruses, including paramyxoviruses, rhabdoviruses, rotaviruses, retroviruses and influenza virus (for a review, see *Ref. 6*). On the other hand, some viruses utilize specific Hsps for their propagation. Hsp70, an inducible form of the Hsp70 family protein, is reported to be involved in the polymerase activity of Canine distemper virus and in the growth of measles virus by associating

**Correspondence to:* Dr. Nobuyuki Kobayashi, Laboratory of Molecular Biology of Infectious Agents, Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan;
e-mail: nobnob@nagasaki-u.ac.jp

with N protein (*7*). Hsc70, a constitutive form of the Hsp70 family protein, is involved in the cell entry of rotavirus (*8*).

Hsc70 is a molecular chaperone which consists of the ATPase domain (residues 1-384 in human), the protein binding domain (residues 385-543), and the variable domain (residues 544-646). Hsc70 mediates not only protein folding but also protein translocation across nuclear membrane. We found that Hsc70 has leucine-rich NES (^{394}LDVTPLSL^{401}) (*9*) and it is also involved in the nuclear export of the import receptors, importin and transportin (*10*). Hsc70 has been reported to have NLS(^{246}KRKHKKDISENKRAVRR^{262}) (*11*). Antibodies against Hsc70 inhibit the nuclear import of karyophilic proteins, suggesting the essential role of Hsc70 in nuclear import (*12*). In the case of viral lifecycle, Hsc70 is required for the nuclear import of Adenovirus DNA (*13*), and papillomavirus capsid (*14*), however, the involvement of Hsc70 in the influenza viral nuclear traffic process is unknown.

This study examined the mechanism of Hsc70 in the nuclear localization of influenza viral RNP. We showed that Hsc70-M1-vRNP complex is detected in both infected cells and in virions. NP accumulates in the nucleus in transfected cells without nuclear accumulation of Hsc70. The nuclear export of Hsc70 is inhibited by LMB treatment in infected cells. These results suggest that Hsc70 is thus required for the nuclear export of influenza viral RNP components, but not for the nuclear import thereof.

Materials and Methods

Virus, cells, antibodies, chemicals and plasmids

Influenza virus A/WSN/33 (H1N1) was propagated in 10-day-old embryonic eggs and the allantoic fluid (~1 × 10^7 PFU/mL) was used for the infection procedures. Purified virions were obtained as described previously (*5*). For the preparation of vRNP, purified virions (8 mg total protein) were treated with 1 mL of disruption buffer consisting of 100 mM Tirs-HCl (pH 8.0), 100 mM NaCl, 5 mM $MgCl_2$, 1% lysolecitin (Sigma Aldrich), 1% Triton X-100, 1 mM DTT and 5% glycerol for 30 min at 30°C. The solution was directly subjected to centrifugation on a 30-60% linear gradient of glycerol on an 80% glycerol cushion in 50 mM Tris-HCl (pH 7.9), 100 mM NaCl, and 1 mM DTT in a Beckman SW40Ti rotor at 36,000 rpm for 4 h at 4°C. Madin-Darby canine kidney (MDCK) cells and HeLa cells were maintained in MEM supplemented with 10% FBS. The preparation of anti-M1 and anti-NP rabbit polyclonal Ab were previously described (*15*). Anti-NP mouse mAb was kindly supplied by Dr. Fumitaka Momose (Kitazato University) (*16*). Anti-Hsc70 (SPA-815) rat mAb was purchased from Stressgen (Victoria BC, Canada). Anti-B23 rabbit antiserum was kindly supplied by Dr. Mitsuru Okuwaki (Tsukuba University). LMB was purchased from LC Laboratories, Inc (Woburn, MA).

Immunoprecipitation

HeLa cells grown in 90-mm dish (~1 × 10^7 cells) were infected with influenza virus at a MOI of 1 for 12 h. The cells were washed twice with 3 mL of PBS and then were lysed with 400 μL of the ice-cold lysis buffer (50 mM Tris (pH7.5), 150 mM NaCl, 0.5% Triton X-100, 2 mM EDTA, 1 mM DTT, and 1 mM PMSF) for 30 min, followed by centrifugation at 15,000 g for 10 min. In a total volume of 200 μL, the reaction mixture containing 20 μL of the cell lysate, 10 μL of Ab-bound proteinA sepharose beads, and the lysis buffer containing 0.1% BSA was incubated at 4°C for overnight. After successive washings with a wash buffer (the lysis buffer containing 300 mM NaCl), Ab-bound proteins were eluted by Laemili SDS sample buffer and loaded onto SDS-PAGE and then they were analyzed by Western blotting.

Permeabilization of MDCK cells and in vitro export assays

In vitro export assays were performed as previously described (*4*) with some modifications. Briefly, MDCK cells were washed with an ice-cold transport buffer (20 mM Hepes-KOH (pH 7.4), 100 mM potassium acetate, 5 mM sodium acetate, and 1 mM EGTA). The cells were treated with the ice-cold transport buffer containing 100 μg/mL digitonin (Wako) for 20 min at 0°C. After successive washings at 0°C, the export assays were performed in the transport buffer by incubating the cells at 30°C for indicated time in each figure legends. The supernatant was recovered as an "exported fraction", and then it was analyzed by Western blotting. Digitonin-permeabilized cells before performing export assays were also lysed with Laemili SDS sample buffer and loaded onto SDS-PAGE to estimate the total protein remaining in nucleus ("Permeabilized cells" in each figure).

Indirect immunofluorescence

HeLa cells were grown on glass coverslips and incubated for 24 h to a density of ~50% confluence. The cells were then transfected with pCAGGS-M1 or pCAGGS-NP (Figure 3A) using lipofectamine 2000 (Invitrogen) or were infected with influenza virus and then were further incubated at 37°C for 9 h. The following procedure was performed at room temperature: The cells were fixed with 4% paraformaldehyde in PBS for 10 min and treated with a wash buffer (0.1% NP-40 in PBS) for 20 min. The cells

were immersed for 1 h in a blocking solution (1% skim milk in PBS) and then were incubated for 1 h with anti-Hsc70 rat mAb, anti-NP mouse mAb, or anti-M1 rabbit antiserum in the blocking solution. After washing with the wash buffer, the cells were incubated for 1 h with the secondary antibodies, Alexa488-conjugated anti-rat Ig, Alexa546-conjugated anti-mouse Ig or Alexa546-conjugated anti-rabbit Ig. The cells were washed with the wash buffer, and then were observed by microscopy (Axiophot, Carl Zeiss).

Results

Detection of Hsc70-M1-vRNP complex

Recently, we identified Hsc70 (Figure 1A) to be a M1 binding protein using MALDI-TOF MS (*5*). An X-ray structure analysis showed helix 6 (amino acid residues 91-105) of M1 to be involved in the binding with viral RNP (*17*). The interaction between Hsc70, M1 and vRNP was confirmed using co-immunoprecipitation assay (Figure 2A). The infected cells were lysed with the lysis buffer and subjected to immunoprecipitation using anti-Hsc70, NP, or M1 antibody. These results showed that Hsc70 co-precipitates with M1 and NP. Next, the purified virion was lysed by the addition of Triton X-100 and lysolecitin, and then it was separated by glycerol density gradient centrifugation (Figure 2B). The vRNP fractions were pooled, and then were subjected to a Western blotting analysis (Figure 2C). Hsc70 was detected both in the virion and in the vRNP fraction. A far-western blotting analysis confirmed that Hsc70 directly interacts with M1 *in vitro* (Figure 2D). These results suggest that Hsc70-M1-vRNP complex exist in infected cells and in the virion.

LMB inhibits the nuclear export of vRNPs and Hsc70 and suppresses virus production

We hypothesized that Hsc70 shuttles between nucleus and cytoplasm, thus utilizing its NES, NLS, and NLRS (see Figure 1A), and the export of Hsc70 is also mediated by CRM1-dependent pathway in both uninfected cells and influenza virus infected cells. To monitor the nuclear export processes directly, we established a novel *in vitro* nuclear export assay using digitonin-permeabilized cells (*4*) (Figure 3). LMB-treated uninfected cells (Figure 3A) or infected cells (Figure 3B) were permeabilized with digitonin at 0°C and washed successively with the ice-cold transport buffer to remove soluble cytosol components such as viral proteins and nuclear import factors, then subjected to the nuclear export assay. We confirmed that digitonin-permeabilization (100 μg/mL) specifically permeabilize the plasma membrane, whereas the nuclear envelope was kept intact by monitoring GFP-Hsc70 fluorescence (data not shown). The export of Hsc70 from the nucleus occured in a temperature-dependent manner, and it was inhibited by LMB treatment (Figure 3B, lanes 5-7 and 9-11), whereas

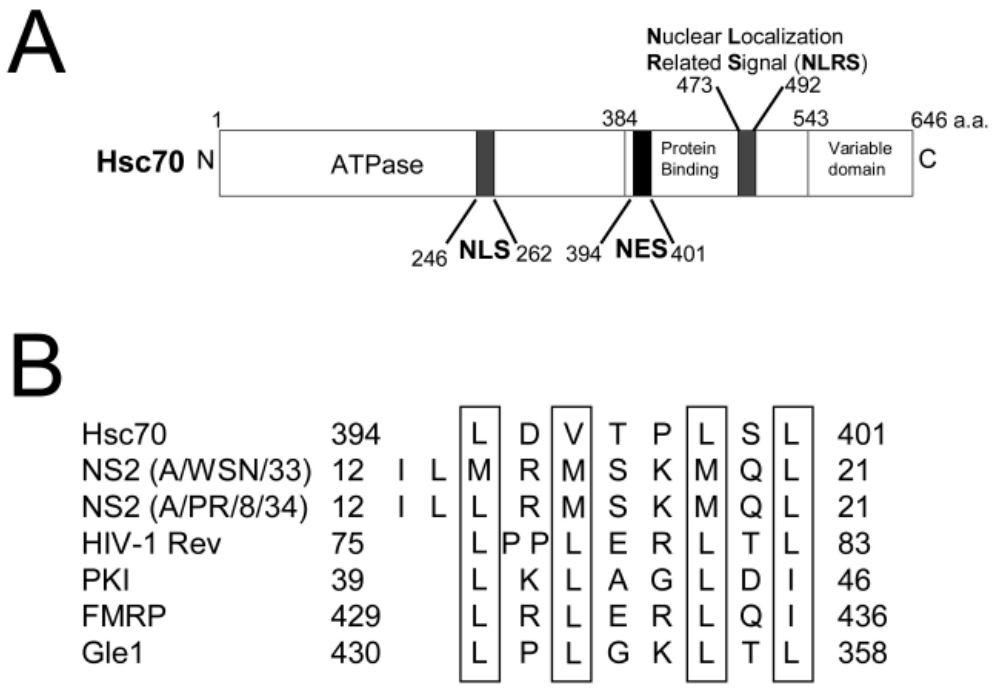

Figure 1. (A) Diagram of functional domain of human Hsc70. Hsc70 contains NLS, NES and Nuclear localization related signal (NLRS) (*9*). (B) Comparison of the NES of Hsc70, influenza NS2, HIV-1 Rev and representative host proteins. The consensus sequence for leucine- rich NESs is shown. The consensus sequence contains hydrophobic residues, including leucine, isoleucine methionine and valine. PKI; protein kinase inhibitor, FMRP; fragile X metal retardation protein.

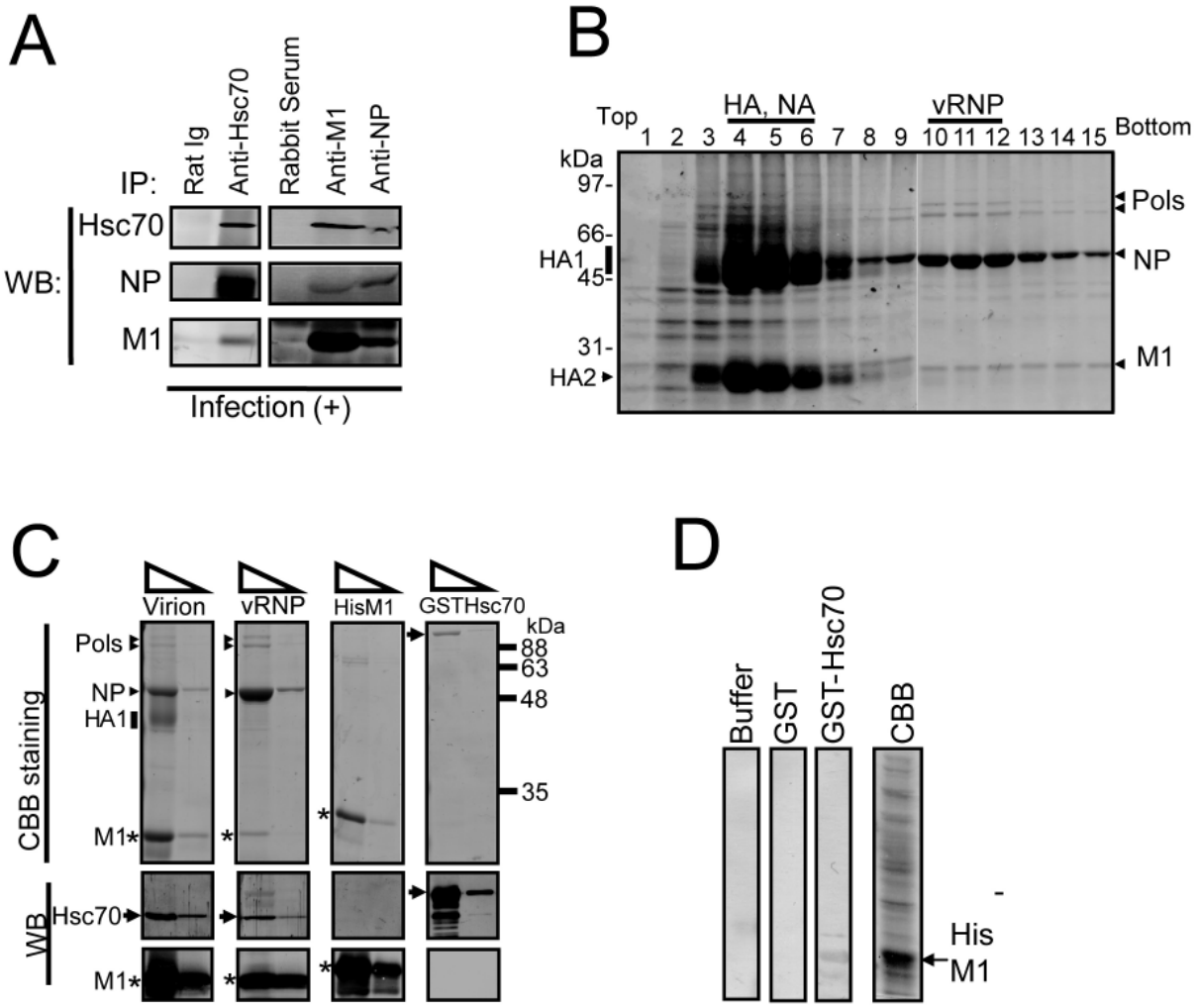

Figure 2. Detection of Hsc70-M1-vRNP interaction. (A) Immunoprecipitation. HeLa cells were infected with the influenza virus A/WSN/33 (MOI = 1) for 12 h. The total cell lysates were immunoprecipitated with anti-M1 antiserum, anti-NP antiserum, or anti-Hsc70 mAb and then were visualized with anti-M1 antiserum, anti-NP mAb, or anti-Hsc70 mAb after 10% SDS-PAGE as indicated. (B) Separation of vRNP-associated factors by glycerol density gradient centrifugation. Virions were purified on a sucrose step gradient (indicated as virion in Figure 2C) and were disrupted by the addition of Triton X-100 and lysolecitin as described in materials and methods. Fractions (1-15) were collected, then separated by a 10% SDS-PAGE, followed by visualization by CBB staining. The vRNP fractions (Fraction numbers 10-12) were pooled, mixed, and subjected to Western blotting using anti-Hsc70 antibody (see Figure 2C). Separately, marker proteins were also subjected to the same glycerol density gradient. Trypsin inhibitor (21 kD), BSA (66 kD) and myosin (200 kD) sediments in fraction number 2, 3 and 4-5, respectively. (C) Detection of virion- and vRNP-associated Hsc70. Purified virion (5 μg and 500 ng), purified vRNP, purified recombinant His-M1 (1 μg and 100 ng), or GST-Hsc70 (300 ng and 30 ng) were loaded onto 10% SDS-PAGE. The gels were either stained with CBB (upper panel) or subjected to Western blotting using anti-Hsc70 (middle panel) or anti-M1 antibody (lower panel). The asterisks and arrows indicate M1 protein and Hsc70, respectively. The arrowheads indicate NP and Polymerases (Pols; PA, PB1 and PB2). (D) Far-Western Blotting. *E. coli*-expressed crude lysate of His-M1 was separated by 10% SDS-PAGE, transferred onto the PVDF membrane, then followed by denaturation with 6 M guanidine HCl in TBST for 10 min. After stepwise renaturing, the membrane was incubated with 1 μg/mL of each purified GST-Hsc70 or GST in TBST at 4°C for 4 h. Specific signals were detected by a Western blotting procedure using anti-Hsc70 mAb.

Nucleophosmin/B23, which is known to localize in the nucleolus, did not come out of the nucleus. Next, the infected MDCK cells (7 hpi) were also subjected to the *in vitro* export assays and the exported fraction was detected by Western blotting (Figure 3B). Viral RNP, M1, and Hsc70 were exported from nucleus at 30°C and the export processes were inhibited when the cells were pretreated with LMB (lanes 5-7 and 9-11). These results suggest that Hsc70, M1, and vRNP are exported from the nucleus to the cytoplasm *via* the CRM1-dependent pathway. To determine whether LMB affects the influenza virus production, MDCK cells were infected with the influenza virus A/WSN/33 at a MOI of 0.1 in the presence (1 or 10 ng/mL) or absence of LMB (Figure 3C). At 12 or 24 h post infection, the supernatants of the infected cells were recovered and subjected to the plaque assay. The virus titer in the supernatant was markedly reduced when cells were treated with LMB in dose dependent manner. In uninfected cells, 10 ng/mL of LMB treatment did not affect cell viability significantly (data not shown). In infected cells, at 12 h post infection, the cell viability was > 90% in the presence or absence of LMB. At 24 h post infection, the cell viability was 32.6% in the absence of LMB whereas that of LMB treated cells (1 ng/mL or 10 ng/mL) was 40.8% or 61.8%, thus suggesting that cell death by the

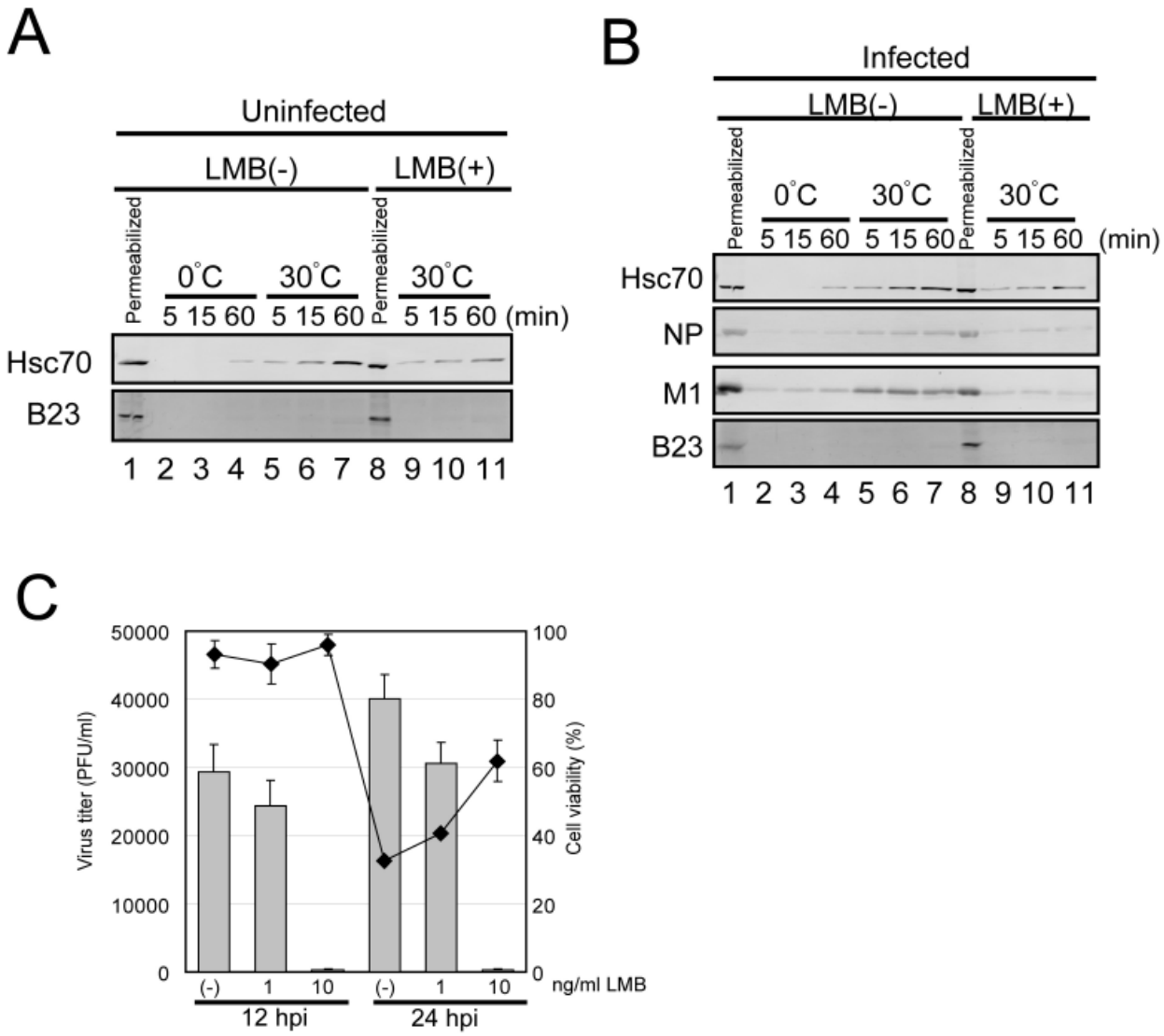

Figure 3. (A) (B) *In vitro* export assays. MDCK cells were uninfected or infected in the absence or presence of 10 ng/mL LMB for 7 h as indicated. The cells were treated with ice-cold 100 μg/mL Digitonin, the permeabilized cells were incubated at 0°C or 30°C. (A) The export of the endogenous Hsc70 was detected by the *in vitro* export assays using uninfected MDCK cells. The permeabilized cells lysed by Laemili SDS sample buffer (indicated as "Permeabilized", lanes 1 and 8) or the exported supernatant fraction was recovered at 5, 15 and 60 min were loaded onto 10% SDS-PAGE, then detected by the Western blotting using anti-Hsc70 (upper panel) and anti-B23 antibodies (lower panel). (B) Export of the endogenous Hsc70 and viral proteins. MDCK cells were infected with A/WSN/33 at a MOI of 1 for 7 h. The cells were treated with digitonin and subjected to the *in vitro* export assays. The permeabilized cells lysed by Laemili SDS sample buffer (indicated as "Permeabilized", lanes 1 and 8) or the exported supernatant fractions from digitonin-permeabilized cells were separated by 10% SDS-PAGE and then visualized by Western blotting using anti-Hsc70 mAb, anti-M1 antiserum and anti-NP antiserum as indicated. (C) The effect of LMB on the propagation of the influenza virus. MDCK cells were infected with A/WSN/33 at a MOI of 0.1 for 60 min. After successive washing, the cells were further incubated without or with 1 ng or 10 ng/mL of LMB. The culture supernatant was recovered at 12 and 24 h after infection, then subjected to plaque assays. The viability of the cells was determined by trypanblue staining. The virus titer (PFU/mL) is shown as solid bar, and the viability (%) was shown as a line. The experiments were performed in triplicate and the mean + SD was shown.

infection was suppressed by LMB treatment.

Hsc70 does not regulate the nuclear import of vRNP components but regulate the export of vRNP

It has been reported that the dissociation of M1 from vRNP is required for the nuclear import of incoming vRNP in the early stages of infection (*2*) and the nuclear import of newly synthesized vRNP components such as NP is mediated by importin family proteins (for a review, see *Ref. 18*). To rule out the possibility that Hsc70 regulate nuclear import of vRNP components *via* M1, we monitored the localization of Hsc70, NP, or M1 in transiently transfected HeLa cells. Hsc70 and M1 localized in both nucleus and cytoplasm (Figure 4A, panels a and b) where NP accumulated in nucleus (c), suggesting that Hsc70 does not regulate nuclear import processes of viral proteins. Next, we monitored the localization of Hsc70 and NP at the late stages of infection (9 h) when nuclear export of vRNP mainly occurs (Figure 4B). NP and Hsc70 localized dominantly in the nucleus (panels b and e). Clearly, in the presence of LMB, NP was localized exclusively in the nucleus (c). The localization of Hsc70 was more extensively

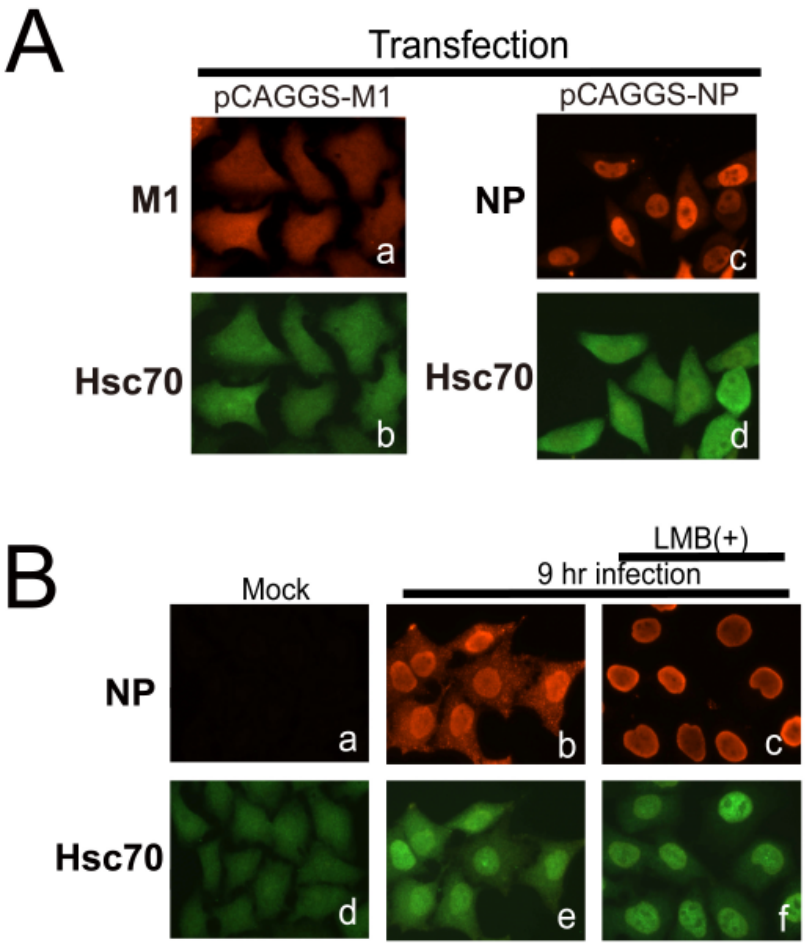

Figure 4. Hsc70 regulates the nuclear export of vRNP. HeLa cells were transiently transfected with NP- or M1-expressing plasmids (A) or were infected with A/WSN/33 (MOI = 5) in the absence or presence of 10 ng/mL LMB (B). The cells were fixed and stained with anti-Hsc70 mAb, anti-NP mAb, and anti-M1 antiserum as indicated. NP (Alexa 546: Red), M1 (Alexa 546: Red) and Hsc70 (Alexa488: Green) were observed under microscopy.

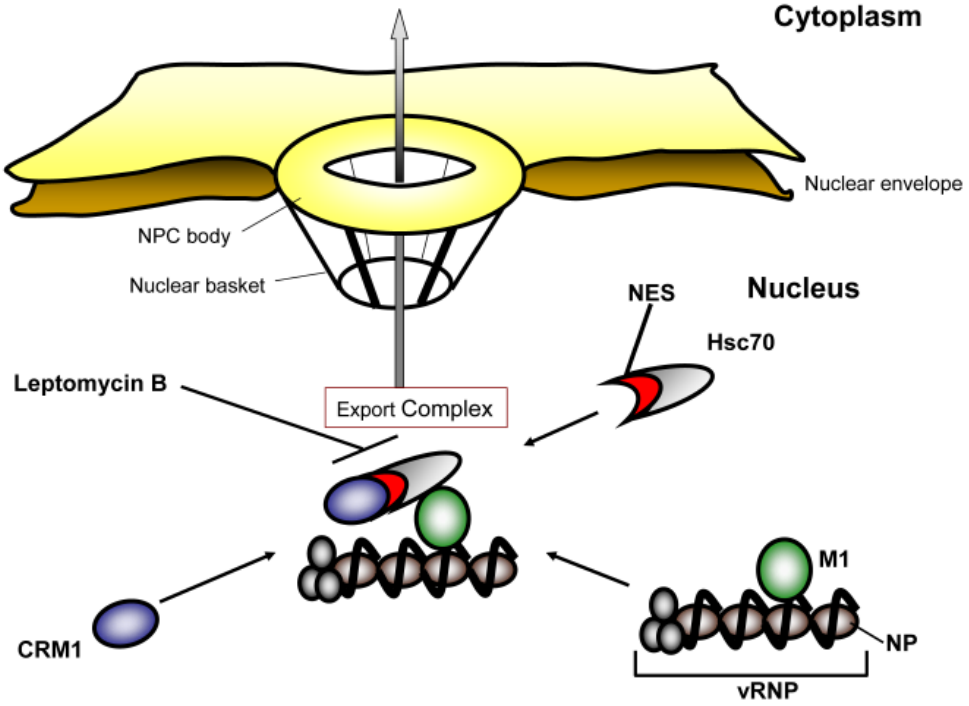

Figure 5. A model for Hsc70-related nuclear export of the influenza virus RNP complexes. Hsc70 interacts with CRM1 *via* its NES, and M1 interacts with Hsc70. CRM1-Hsc70-M1-vRNP complex is formed and exported from the nucleus and concomitantly CRM1 is released from Hsc70. A part of Hsc70 may incorporate into the virions.

accumulated in the nucleus in the presence of LMB in infected cells (f). These results suggest that Hsc70 regulates vRNP export from the nucleus in the late stages of infection, while having no such effect in the early stages of infection.

Discussion

This study demonstrated that Hsc70 regulates the nuclear export of vRNP. The nuclear export of Hsc70 and vRNP was LMB sensitive. In infected cells, M1 and NP co-precipitated with Hsc70, and the Hsc70-M1-vRNP complex exported from the nucleus *via* the CRM1-dependent pathway.

Because the transcription and replication of influenza virus occurs in nucleus, nuclear import and export of vRNP is one of the most important steps for the virus life cycle. It has been reported that nuclear export of vRNP is mediated by M1 and viral NS2 protein (*2,19*), and is inhibited by LMB (*20,21*). Although M1 does not have NES, it plays a key role as a mediator to bind both vRNP and NS2. NS2 have leucine rich NES which binds to a nuclear export factor, CRM1 (Figure 1B). Thus, vRNP is thought to be exported by the vRNP-M1-NS2-CRM1complex (for a review, see *Ref. 18*). However, we previously found that the effect of LMB on the nuclear export of the NS2 was a partial effect (*4*), suggesting the existence of other host factors which is essential for the nuclear export of vRNP. We explored M1 binding protein using MALDI-TOF MS and identified Hsc70 (*5*). Hsc70 was complexed with M1 and NP in infected cells and in the virion (Figure 2). Hsc70 has both NES and NLS, and is known to shuttle between the nucleus and cytoplasm. To monitor the export processes, an *in vitro* export assay system was developed (Figure 3A and 3B) and showed that export of Hsc70 and vRNP was CRM1 dependent. The inhibition of the export of vRNP by LMB (Figure 3B) resulted in the reduction of virus production (Figure 3C) in MDCK cells. MDCK cell is usually used for viral infection experiments, because it support multiple cycle of the virus infection and efficient virus production. However, the cytopathic effect (CPE) occurs very fast in the MDCK cells. Therefore, for the detection of viral protein, HeLa cell was used for immunoprecipitation (Figure 2A) and indirect immunofluorescence (Figure 4). The nuclear import of NP was not related with Hsc70 (Figure 4A), whereas the nuclear export of NP and Hsc70 was inhibited by LMB. Taking these observations into account, we therefore hypothesize that vRNP is exported as Hsc70-M1-vRNP *via* the CRM1-dependent pathway (Figure 5). Further studies are essential to elucidate the involvement of NS2 in the export complex.

The anti-influenza drugs available today are directed against essential viral protein functions. Amantadine blocks the ion-channel activity of the viral M2 protein, which is required for virus entry steps. Zanamivir and oseltamivir, block the receptor-destorying activity of viral NA protein, prevent viral budding form the cell surface. These drugs are approved and commercially available in many countries. However, the influenza virus easily change its genome sequence and many drug-resistant mutated viruses has been reported (for a review, see *Ref. 22*). Specific cellular proteins essential for virus propagation such as Hsc70 may therefore be a good target for the development of drugs which can suppress viral diseases (*23*).

Acknowledgements

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