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Specific Incorporation of Heat Shock Protein 70 Family Members into Primate Lentiviral Virions

Cagan Gurer,[1] Andrea Cimarelli,[2] and Jeremy Luban[1]*

Departments of Microbiology and Medicine, Columbia University College of Physicians and Surgeons, New York, New York 10032,[1] and Ecole Normale Supérieure de Lyon, 69364 Lyon, France[2]

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To determine if any heat shock proteins are incorporated into human immunodeficiency virus type 1 (HIV-1) virions in a manner similar to that of the peptidyl-prolyl isomerase cyclophilin A, we probed purified virions with antibodies against heat shock proteins Hsp27, Hsp40, Hsp60, Hsp70, Hsc70, and Hsp90. Of these proteins, Hsp60, Hsp70, and Hsc70 associated with virions purified based on either particle density or size and were shown to be incorporated within the virion membrane, where they were protected from digestion by exogenous protease. Virion incorporation of Hsp70 was also observed with HIV-2 and with simian immunodeficiency viruses SIV_{MAC} and SIV_{AGM}, but it appears to be specific for primate lentiviruses, since Hsp70 was not detected in association with Moloney murine leukemia virus virions. Of the HIV-1 genes, *gag* was found to be sufficient for Hsp70 incorporation, though Hsp70 was roughly equimolar with *pol*-encoded proteins in virions.

The Gag proteins of human immunodeficiency virus type 1 (HIV-1) play numerous roles in the viral life cycle from assembly to early postentry steps (10). By an unknown mechanism which most likely utilizes host factors, the Gag polyprotein is transported to the plasma membrane, where it directs the formation and release of enveloped virions from infected cells. Concurrent with the release of nascent virions, the viral protease cleaves the Gag polyprotein into matrix (MA), capsid (CA), nucleocapsid (NC), and p6 proteins. Upon entry into a new cell, the viral ribonucleoprotein complex is released into the cytoplasm, where *gag*-encoded proteins participate in reverse transcription, transport of the viral preintegration complex into the nucleus, and possibly establishment of the provirus.

Since the roles played by HIV-1 Gag proteins are numerous and complex, it has been hypothesized that host factors might be required for *gag*-encoded functions. Many Gag-interacting factors have been identified, including actin (20, 25, 29, 37), ubiquitin (24), calmodulin (28), the motor protein KIF-4 (33), the nuclear transporter karyopherin-alpha (11), the human nuclear shuttling protein VAN (14), translation elongation factor 1-alpha (7), translation initiation factor 2 (38), and the HO3 histidyl-tRNA synthetase (18). Via interaction with the PTAP motif in the p6 domain of Gag, the ubiquitin-conjugating enzyme homologue Tsg101 is targeted to the site of virion budding, where it is required for fission of the nascent virion membrane from the host cell membrane (12, 23, 34). Cyclophilin A (CyPA) is incorporated into HIV-1 virions via interaction with the CA domain of the Gag polyprotein and is required for wild-type viral replication kinetics (5). CyPA is a member of a large family of proteins which catalyze the isomerization of peptidyl-prolyl bonds (13) and protect cells from heat shock (32).

Several stress proteins have been shown to play roles in the life cycle of a variety of RNA and DNA viruses (30), and there is growing evidence that some of these proteins may also be important for HIV-1 replication. Heat shock protein 60 (Hsp60) copurifies with HIV-1 and simian immunodeficiency virus (SIV) virions, though it was not demonstrated that this cellular protein is a bona fide virion component (2). Hsp70 and Hsp27 expression is selectively increased following infection with HIV-1 (35), and it has been suggested that Hsp70 plays a role in the nuclear import of HIV-1 preintegration complexes (1). In addition, Mason-Pfizer monkey virus assembly in tissue culture and HIV-1 assembly in vivo and in vitro are ATP-dependent processes (16, 19, 36), consistent with a requirement for the ATPase activity of a heat shock protein.

To find out whether any heat shock proteins other than CyPA are incorporated into HIV-1 virions, we transfected 293T cells with the proviral DNA-containing plasmid pNL4-3 and harvested and purified virions from the culture supernatant by sedimentation through 25% sucrose cushions as previously described (4). Proteins associated with the purified virions were then probed in Western blots with antibodies specific for Hsp27, Hsp40, Hsp60, Hsc70, Hsp70, and Hsp90 (all from Santa Cruz Biotechnology, Santa Cruz, Calif.) as well as for CA (National Institutes of Health AIDS Reagent Program no. 740) and gp41 (NEN Life Science Inc., Boston, Mass.). Using the subtilisin protease protection assay (26), we demonstrated that, of these proteins, Hsp60, Hsc70, and Hsp70 were incorporated within the virion membrane (Fig. 1). Since the Western blot signal was strongest with the anti-Hsp70 antibody, we concentrated on this protein for our subsequent experiments.

To provide further evidence that Hsp70 is a bona fide virion protein, we examined its localization with respect to a virion marker (CA) on a 20 to 60% linear sucrose density gradient as

* Corresponding author. Mailing address: Departments of Microbiology and Medicine, Columbia University College of Physicians and Surgeons, 701 W. 168th St., New York, NY 10032. Phone: (212) 305-8706. Fax: (212) 305-0333. E-mail: JL45@columbia.edu.

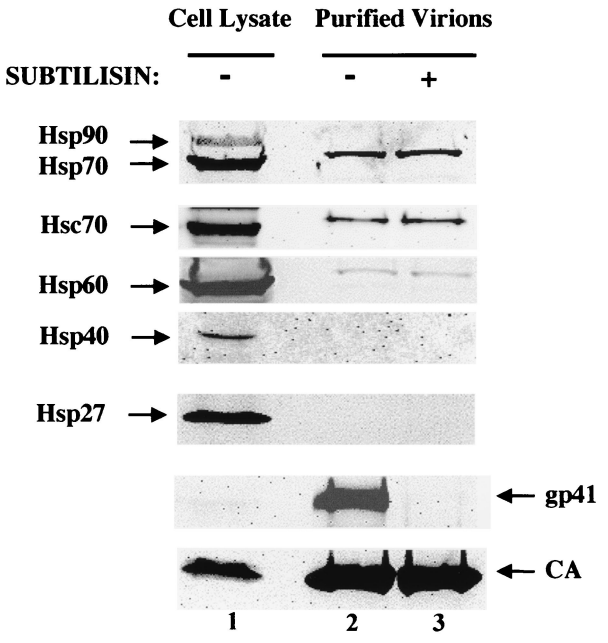

FIG. 1. Presence of heat shock proteins in HIV-1 virions. Virions produced by 293T cells transfected with HIV-1_{NL4-3} proviral DNA were purified by centrifugation through a 25% sucrose cushion. The purified virions were either mock treated (lane 2) or treated with 0.2 mg of subtilisin/ml (lane 3). Subsequently, the transfected cell lysates (lane 1) and virions (lanes 2 and 3) were analyzed by Western blotting with antibodies against gp41, HIV-1 CA, and the indicated heat shock proteins.

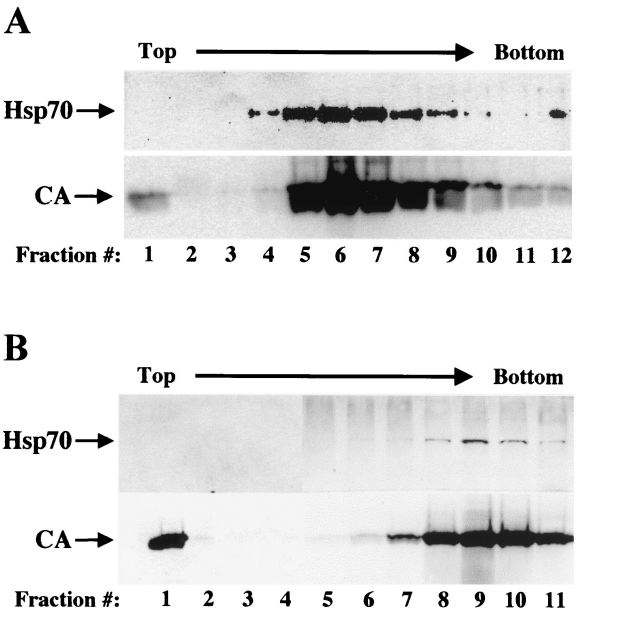

FIG. 2. Hsp70 copurifies with HIV-1 virions after purification based on either density or particle size. Virions in culture supernatant from HIV-1_{NL4-3} proviral DNA-transfected 293T cells were resuspended after sedimentation through a 25% sucrose cushion and then layered onto either of two gradients as follows: (A) 20 to 60% linear sucrose gradient accelerated to equilibrium (16 h at 100,000 × *g*) to monitor particle density, or (B) 6 to 18% linear iodixanol gradient accelerated for a shorter duration (250,000 × *g* for 1.5 h) to monitor velocity of particle sedimentation. Fractions were collected from the top of each gradient (as indicated by numbers across the bottom of each pair of panels) and analyzed by immunoblotting with anti-Hsp70 and anti-CA antibodies (as indicated).

previously described (9). Gradient fractions were collected and probed by immunoblotting, and it was found that Hsp70 cosedimented with CA (Fig. 2A). Nonetheless, cell membrane-microvesicles are of comparable density to virions and may contaminate HIV-1 particles purified on linear sucrose density gradients (3). To determine whether the Hsp70 signal detected in the density gradient fractions reflected contamination with microvesicles, we performed a velocity sedimentation gradient with 6 to 18% iodixanol (OptiPrep; Invitrogen), which separates HIV-1 virions from the majority of contaminating microvesicles (9). Once again, both Hsp70 and CA cosedimented in the same fractions, providing further evidence for the specific incorporation of Hsp70 into the virions (Fig. 2B).

We next examined two other common HIV-1 laboratory strains (HIV-1_{LAI} and HIV-1_{HXB2}), as well as a more distantly related, primary HIV-1 isolate (HIV-1_{ELI}), and showed that virions encoded by these viruses also incorporate Hsp70-family members (Fig. 3A and data not shown). Hsp70 incorporation into HIV-1 was not specific to the virions produced by 293T cells, since virions produced by transfected HeLa cells or Jurkat T cells harboring a spreading infection gave an equally strong signal for Hsp70 (data not shown). Thus, virions produced by T cells also contain Hsp70. We also checked several proviral clones from different subgroups of primate lentiviruses and found that HIV-2_{ROD}, SIV_{MAC239}, SIV_{AGM}Vervet, and SIV_{AGM}Grivet incorporated Hsp70 with roughly the same efficiency as HIV-1 (Fig. 3B and data not shown).

To determine whether Hsp70 packaging into virions is specific to HIV-1 and related primate lentiviruses, we examined Moloney murine leukemia virus (MLV) virions purified from the supernatant of chronically infected Rat-2 cells. Immunoblot analysis was performed using anti-Hsp70 antibody (catalogue no. sc-1060, human and rat cross-reactive; Santa Cruz) along with an antibody that recognizes MLV CA (79S-804; National Cancer Institute). Unlike the results of our experiments with primate lentiviruses, we were unable to detect Hsp70 in association with MLV virions (Fig. 3C), despite the fact that our MLV virion preparation was three to four times more concentrated than our HIV-1 virion preparation (compare lanes 1 and 2 in Fig. 3D).

Expression of the HIV-1 Gag polyprotein is sufficient for the assembly and release of virus-like particles (VLPs) from the plasma membrane. To check whether VLPs formed by HIV-1 Gag incorporate Hsp70 in the absence of other viral proteins, we PCR amplified and cloned a previously described *gag* cDNA (that was modified to be Rev independent) (31) into mammalian expression vector pEF /*myc*/*cyto* (Invitrogen) such that it was in-frame with a *myc* tag at the carboxyl terminus. Since MLV does not incorporate Hsp70, we also cloned MLV *gag* into the same expression vector as a negative control. 293T cells were transfected with these two constructs, and VLPs were purified from the supernatant through a 25% sucrose

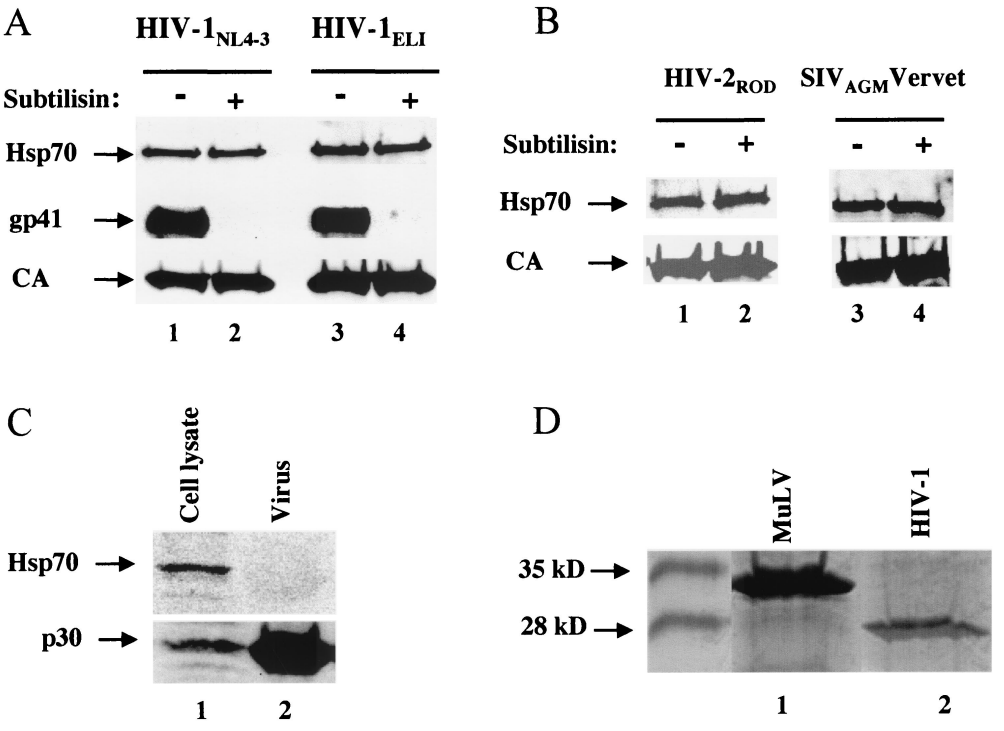

FIG. 3. Hsp70 is incorporated into virions produced by three different subgroups of primate lentiviruses. Virions were purified from the supernatant of 293T cells transfected with the following indicated proviral DNAs: (A) HIV-1_{NL4-3} or HIV-1_{ELI} or (B) HIV-2_{ROD} or SIV_{AGM}Vervet. After subtilisin treatment, Western blot analysis was performed using antibodies against Hsp70 or CA (A and B) or HIV-1 gp41 (A). (C) MLV virions were harvested from the supernatant of chronically infected Rat-2 cells. Immunoblot analysis was performed on the infected cell lysate and purified virions with antibodies against Hsp70 and MLV p30 CA. (D) The same amounts of HIV-1_{NL4-3} and MLV virion samples as used in the gels shown in panels A and C, respectively, were processed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and proteins were visualized with Coomassie blue to directly compare the relative amounts of the two viral CAs. The arrows point at the molecular mass standards.

cushion. The cell lysates and purified VLPs were analyzed by Western blotting with antibodies against the *myc* tag (Santa Cruz) and Hsp70. We found that HIV-1 Gag is sufficient for the incorporation of Hsp70 (Fig. 4C, lane 1). Even though MLV Gag is well expressed and forms VLPs just as efficiently as HIV-1 Gag, it does not incorporate Hsp70 (Fig. 4C, lane 2), consistent with the fact that infectious MLV virions do not incorporate Hsp70 (Fig. 3C).

Finally, we determined the molar ratio of Hsp70 to CA in a purified, subtilisin-treated, HIV-1_{NL4-3} virion preparation. Virion-associated Hsp70 and CA were quantitated by comparing Western blot signal intensities to the intensities obtained by serial dilution of purified Hsp70 (Stressgen, Victoria, Canada) and CA (Intracell Corp.) standards. The concentration and purity of the protein standards were confirmed by using Pierce BCA protein assay reagent (Pierce Chemical, Rockford, Ill.) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, respectively. Using Kodak Image Station 440CF with 1D image analysis software to quantitate the signal intensities, we estimated that the molar ratio of Hsp70 to CA is in the 1:25 to 1:30 range (data not shown). This indicates that the amount of incorporated Hsp70 is similar to the amount of viral *pol* protein that is incorporated into HIV-1 virions.

Hsp70 protein family members, by controlled binding and release, facilitate the folding, oligomeric assembly-disassembly, and intracellular transport of protein complexes (15). Both the stress-inducible protein Hsp70 and its constitutive form, Hsc70, interact with various viral proteins and may be involved in the assembly of adenovirus (21), enterovirus (22), and polyomavirus capsid protein complexes (8). Hence, Hsp70 and Hsc70 could bind to nascent HIV-1 Gag polyprotein chains and hold them in an assembly-competent conformation during transport to the plasma membrane. Alternatively, upon entry into susceptible target cells, virion-associated Hsp70 might participate in early events of infection. For example, Hsp70 might actively uncoat the viral capsid in a manner similar to its role in the uncoating of clathrin cages (6). Hsp60, Hsp70, and Hsp90 have been shown to interact with hepatitis B virus reverse transcriptase and to facilitate the initiation of viral DNA synthesis from hepatitis B virus pregenomic RNA (17, 27). Thus, the Hsp70, Hsc70, and Hsp60 proteins in HIV-1 virions might serve a similar function in the initiation of HIV-1 cDNA synthesis. Finally, the Hsp70 and Hsc70 proteins might target the HIV-1 viral preintegration complex to the nuclear pore complexes, as has been suggested by others (1).

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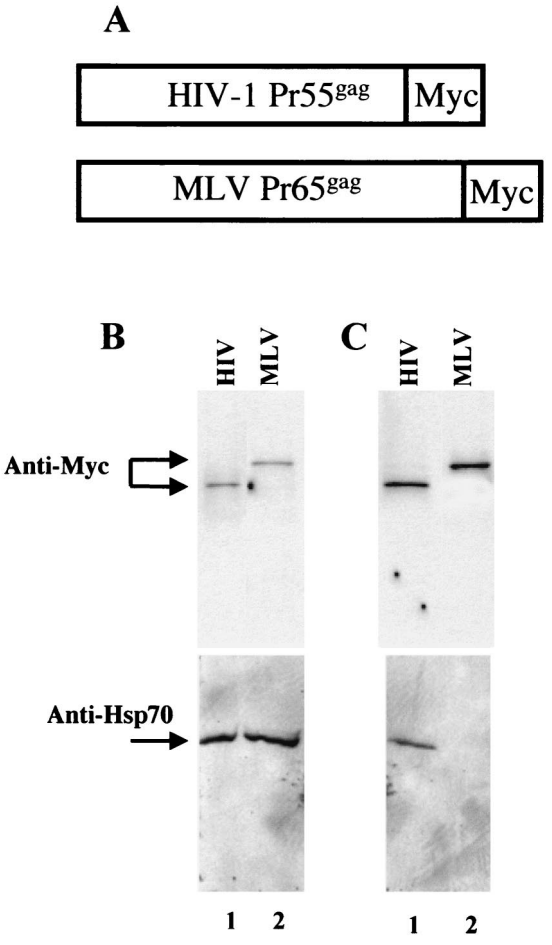

FIG. 4. Gag is sufficient for Hsp70 incorporation into HIV-1 virions. (A) Schematic representation of the HIV-1 and MLV Gag coding constructs. Both constructs were fused to a *myc* tag to permit normalization of the purified VLPs using the same antibody. (B and C) 293T cells were transfected with the Gag-expression constructs shown in panel A. Cell lysates (B) and purified VLPs produced by these cells (C) were analyzed by Western blotting by using anti-myc (top panels) and anti-Hsp70 antibodies (bottom panels).

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