Heat Shock Protein 40 Is Necessary for Human Immunodeficiency Virus-1 Nef-mediated Enhancement of Viral Gene Expression and Replication*

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The human immunodeficiency virus-1 (HIV-1) Nef protein, originally identified as a negative factor, has now emerged as one of the most important viral proteins necessary for viral pathogenesis and disease progression. Nef has been also implicated in viral infectivity and replication, however, the molecular mechanism of Nef-induced viral gene expression and replication is not clearly understood. Although involvement of heat shock proteins in viral pathogenesis has been reported earlier, a clear understanding of their role remains to be elucidated. Here we report for the first time that Nef not only interacts with heat shock protein 40 (Hsp40) but it also induces the expression of Hsp40 in HIV-1-infected cells. The interaction between Nef and Hsp40 is important for increased Hsp40 translocation into the nucleus of infected cells, which seems to facilitate viral gene expression by becoming part of the cyclin-dependent kinase 9-associated transcription complex regulating long terminal repeat-mediated gene expression. The finding is consistent with the failure of the *nef*-deleted virus to induce Hsp40, resulting in reduced virus production. Our data further shows that, whereas, Hsp40 overexpression induces viral gene expression, silencing of Hsp40 reduces the gene expression in a Nef-dependent manner. Thus our results clearly indicate that Hsp40 is crucial for Nef-mediated enhancement of viral gene expression and replication.

Viruses are known to modulate cellular proteins for successful replication within the host cells. The sequence of events in the establishment of a productive infection by human immunodeficiency virus type 1 (HIV-1)[3] not only involves interaction between a number of viral and cellular factors but is also accompanied by complex and dynamic changes in the patterns of cellular gene expression. Nef, a 27–30-kDa myristoylated phosphoprotein, encoded by HIV-1 has been shown to play a crucial role in viral pathogenesis by modulating cellular gene expression and signaling pathways (1, 2). *nef*-deleted viruses fail to replicate efficiently *in vivo*, and do not develop symptoms of acquired immunodeficiency syndrome (AIDS) (3, 4). Nef is also thought to contribute to viral pathogenesis by down-regulation of CD4 and major histocompatibility complex class I surface molecules preventing viral

superinfection and by helping the virus to evade host immune system. Nef has also been implicated in the activation of T cells, making the cells permissible to the virus (5, 6). All these functions of Nef are manifested by a number of important events such as activation of upstream signaling molecules, inhibition of apoptosis in the infected cell, activation and up-regulation of transcription factors, alleviation of repressors of transcription, as well as increase of the infectivity of newly produced virions (1, 7). Even though these functions of Nef have been well studied, controversy exists on its role in viral infectivity and replication as both negative (8, 9) and positive (10, 11) effects are available in the literature. Although a number of reports show that Nef increases viral replication by activating T cells, the molecular basis of Nef-induced viral gene expression and replication remains to be clearly elucidated.

Cellular heat shock proteins (Hsp) are chaperone molecules known to participate in protein folding, transport, and assembly. Hsps are induced in cells during stress conditions like heat shock, UV irradiation, and even microbial/viral infections (12, 13). Recent studies have revealed that Hsps are also involved in apoptosis and immune response (14, 15). In mammalian cells, expression of a number of Hsps, including Hsp40, Hsp70, Hsp90, and Hsp100, is enhanced by heat shock and regulated at the transcriptional level (16). Hsp40, a heat-inducible DnaJ homologue, has been found to co-localize with Hsp70 (17, 18) and assists Hsp70 in folding of nascent proteins (19–21). The major function of Hsp40 is to regulate the ATP-dependent polypeptide binding by Hsp70 (19, 22). Among all the heat shock proteins studied to date, Hsp70 has been shown to play an important role in the HIV-1 life cycle. Hsp70 is overexpressed in lymphocytes of HIV-positive individuals and is also found abundantly in HIV virions (23, 24). Hsp70 and Hsp90 have been shown to stabilize the assembly of the active cdk9-cyclin T1 complex responsible for positive transcription elongation factor b (P-TEFb) or Tat-associated kinase complex-mediated Tat transactivation (25). However, the role of Hsp40 in the HIV life cycle remains to be studied.

Nef was previously reported as a negative factor for viral replication in T cell lines (26), but recent reports demonstrated Nef as an enhancer of viral replication (27–31). However, the molecular mechanism of this positive effect needs to be clearly elucidated. It is well known that Nef performs most of its functions by interacting with different proteins (1, 7). Although the functions attributed to Nef remain conserved across different HIV-1 subtypes, most of the functional studies for Nef to date have been performed with subtype B Nef protein. There is scant literature regarding subtype C Nef, the most prevalent subtype in the world. In the reported study, we have attempted to identify novel Nef interacting host cell factors using subtype C Nef as bait in the yeast two-hybrid system. Our results showed that both subtype C and subtype B Nef interacted with a human DnaJ homologue, Hsp40, *in vitro* and *in vivo*. This interaction led to increased viral gene expression and virus production. Furthermore, Hsp40 expression was increased in HIV-1-infected cells in a Nef-dependent manner and this up-regulation was nec-

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[3] The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Hsp, heat shock protein; P-TEFb, positive transcription elongation factor b; HA, hemagglutinin; cdk9, cyclin-dependent kinase 9; GST, glutathione *S*-transferase; LTR, long terminal repeat; GFP, green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; PBMC, peripheral blood mononuclear cells; siRNA, small interfering RNA; RT, reverse transcriptase.

essary for increased viral replication. Finally, Hsp40 seems to form part of the cdk9-associated transcription complex that regulates viral gene expression; an observation that provides a molecular basis for Nef-mediated enhancement of viral gene expression and replication.

EXPERIMENTAL PROCEDURES

Plasmids, Cell Lines, and Antibodies—The *nef* gene from HIV-1 subtype C Indian isolate IN301904 (32) was amplified by PCR using the following primers: subtype C Nef, forward, 5′-GAGGATCCCGAT-GGGGGGCAAG-3′; and reverse, 5′-CGGAATTCGCATGTCAG-CAGTC-3′. This PCR product was first cloned into pGEMT Easy vector (Promega) according to the manufacturer's instructions. The subtype C Nef fragment was taken from pGEMT Easy-NefC and cloned into the BamHI and EcoRI sites of pCDNA3.1 (Invitrogen). The pAS2–1NefC expression vector was constructed by cloning subtype C Nef from pGEMT Easy-NefC into BamHI and SalI sites of pAS2-1 (Clontech). The sequence of cloned *nef* was confirmed by DNA sequencing (ABI 310 Genetic Analyzer, ABI). Expression of Nef from both vectors was confirmed by immunoblotting. The pAS2–1NefC plasmid expresses subtype C Nef and Gal4 DNA binding domain as a fusion protein, which was used as the bait protein in yeast two-hybrid library screening. A cDNA library of human leukocytes in the pACT2 vector was obtained from Clontech. pCDNA-Hsp40, pCDNA3 expressing human Hsp40, was a kind gift of Dr. Margarida D. Amaral (33). NL4-3 Nef cloned in pCDNA (pCDNA-Nef) and NL4-3 Nef tagged with HA (HA-Nef) plasmids were kind gifts of Dr. M. Federico and Dr. W. C. Greene, respectively. The NL4-3 molecular clone (pNL4-3) was obtained from the National Institutes of Health AIDS repository (34). The *nef*-deleted NL4-3 molecular clone (pNL4–3ΔNef) and glutathione *S*-transferase (GST)-Nef plasmids were obtained from Dr. K. Saksela. 293T cells (human embryonic kidney cell line) were obtained from the NCCS Cell Repository, India. CEM-GFP, a CD4$^+$ human T cell line, was obtained from the National Institutes of Health AIDS repository (35). Monoclonal and polyclonal Nef antibodies were obtained from the National Institutes of Health AIDS repository (36, 37). Antibodies against Hsp40, Hsp70, Hsp90, cdk9, and rabbit anti-HA antibody were obtained from Santa Cruz Biotechnology.

Yeast Two-hybrid Assay—A human leukocyte cDNA library in pACT2 vector (Clontech) was screened for Nef interacting proteins by co-transformation with the pAS2–1NefC bait plasmid into yeast strain AH109 (Clontech). Positive clones were selected based on growth in medium lacking adenine, histidine, tryptophan, and leucine and also by expression of β-galactosidase, which gives a blue color on plates containing X-gal. Co-transformants in AH109 yeast strains were screened three times for growth on these selection plates and also for β-galactosidase activity on plates to exclude false positive clones. Liquid β-galactosidase assay was finally performed to confirm the interaction, using the yeast β-galactosidase assay kit from Pierce, as per the manufacturer's protocol. The interacting protein in the positive clone was identified by rescuing the gene fragment, using PCR amplification with pACT2-specific primers followed by DNA sequencing.

Transient Transfection and Luciferase Assay—HEK-293T cells were transfected with LTR-reporter vector (pLTR-Luc) along with other expression vectors using calcium phosphate precipitation and harvested 36 h post-transfection for luciferase assay. After 4 h of transfection, the transfected cells were either treated or mock treated with HSP inhibitor KNK437 (Calbiochem) at 100 μM final concentration for 32 h. The cells were then lysed in cell lysis reagent (Promega), and luciferase assays were performed using Luclite substrate (PerkinElmer Life Sciences). Normalization of transfection efficiency was done using the

enhanced green fluorescent protein reporter (pEGFP-N1) co-transfection and quantitation as described earlier (38). The culture supernatants were also collected at the time of luciferase assay from HIV-1 NL4-3 and the *nef*-deleted NL4-3 molecular clone-transfected cells to determine virus production.

HIV-1 Infection and Virus Quantitation—5 × 10^6 CEM-GFP cells were infected with HIV-1 NL4-3 virus at a multiplicity of infection of 0.1 in the presence of Polybrene (1 μg/ml) as described earlier (39). Peripheral blood was collected from normal seronegative donors and PBMCs were isolated by Ficoll-Hypaque (Amersham Biosciences) gradient centrifugation. Cells were activated with 5 μg/ml phytohemagglutinin (Sigma) for 36–48 h. 5 × 10^6 activated PBMCs were infected with 0.5 multiplicity of infection of NL4-3 virus for 4 h in the presence of Polybrene (1 μg/ml) with intermittent mixing as described earlier (39). After washing, the cells were plated in Complete medium supplemented with human interleukin-2 (Roche Applied Bioscience) at 20 units/ml concentrations and incubated at 37 °C in a humidified CO$_2$ incubator. The culture supernatants from infected and molecular clone-transfected cells were used to determine virus production by p24^{gag} antigen capture ELISA (PerkinElmer Life Sciences).

Immunoprecipitation, GST Pull-down, and Immunoblotting—Recombinant [^{35}S]methionine-labeled Nef and Hsp40 proteins were synthesized from pCDNA-NefC and pCDNA-Hsp40 plasmids using the coupled *in vitro* transcription and translation kit as per the manufacturer's instructions (Promega). These proteins were incubated together in binding buffer (20 mM HEPES, pH 7.4, 1 mM dithiothreitol, 2 mM MgCl$_2$, 100 μg/ml bovine serum albumin) and were then immunoprecipitated with polyclonal antibody against Nef. The antigen-antibody complex was pulled down by an equal mixture of protein A- and protein G-agarose beads and resolved on 12% SDS-PAGE. The gel was then prepared for fluorography as described elsewhere (40) and exposed to x-ray film (Kodak).

Escherichia coli BL21(DE3) cells expressing either GST or GST-Nef were induced with isopropyl β-D-thiogalactoside followed by purification of proteins using glutathione-Sepharose beads (Amersham Biosciences). Transfected 293T cells overexpressing Hsp40 were lysed in radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl, pH 7.2, 0.15 M NaCl, 1% Triton X-100, 0.15% SDS, and 1% sodium deoxycholate) with a protease inhibitor mixture (Roche Applied Bioscience). The clarified lysates were incubated with either GST or GST-Nef protein immoblized on glutathione-Sepharose beads at 4 °C and subjected to five washes with RIPA buffer. The complexes were resuspended in Laemmli's sample buffer, boiled, and resolved on 12% SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride membrane and the membrane was probed with polyclonal Hsp40 antibody.

HEK-293T cells overexpressing HA-Nef and Hsp40 and CEM-GFP-infected cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 0.12 M NaCl, 0.5% Nonidet P-40, 0.5 mM NaF, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) on ice for 45 min. Clarified lysates were incubated with Hsp40 or Nef polyclonal antibody, and the antigen-antibody complex was pulled down by an equal mixture of protein A- and protein G-agarose beads followed by resolution on 12% non-reducing SDS-PAGE. The proteins were transferred onto polyvinylidene difluoride membrane and the membrane was probed with either HA antibody or Hsp40 antibody. The blots were developed by using the ECL Plus system (Amersham Biosciences). Furthermore, equal amounts of protein were taken from the CEM-GFP-infected cell lysates or lysates from 293T cells transfected with viral molecular clones and were run on SDS-PAGE, followed by immunoblotting for Hsp40 and other proteins.

FIGURE 1. **Amino acid homology between subtype C Nef and NL4-3 Nef.** The amino acid alignment of subtype C Nef used in the present work (Indian isolate 301904) and subtype B NL4-3 Nef shows about 76% identity.
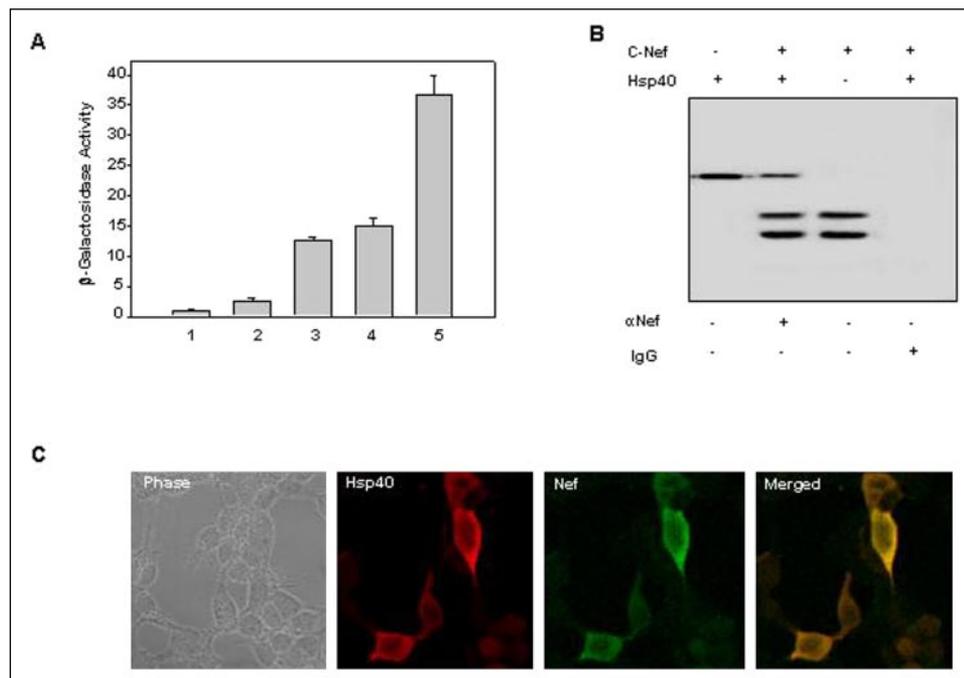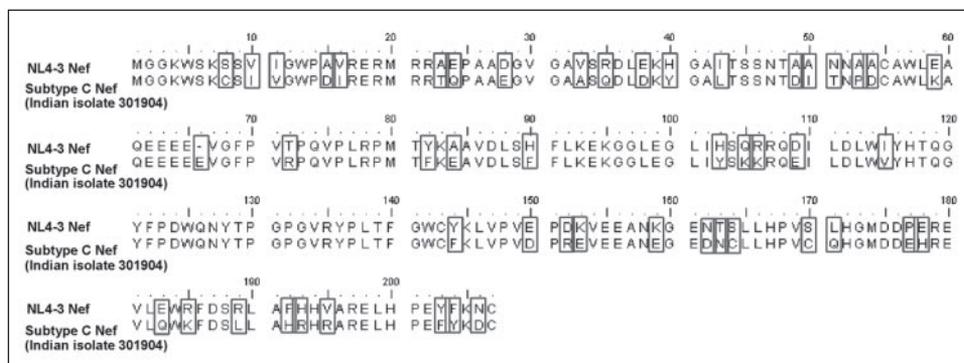
FIGURE 2. **HIV-1 subtype C Nef physically interacts with Hsp40 protein.** *A*, Nef interacts with Hsp40 in the yeast two-hybrid assay. A human leukocyte cDNA library was screened using subtype C Nef as bait in the yeast two-hybrid system. One of the clones co-expressing Hsp40 and Nef was found positive after repeated screening. Liquid β-galactosidase activity assay was performed using lysates from yeast co-transformants as described under "Materials and Methods." *Lane 1*, pAS2.1 subtype C Nef and pACT2; *lane 2*, pTD1 and pLAM5; *lane 3*, pTD1 and pVA3; *lane 4*, the identified Hsp40 clone; *lane 5*, pCL1, the full-length GAL4 plasmid transformant. *B*, Nef interacts with Hsp40 *in vitro*. Recombinant [^{35}S]methionine-labeled Nef and Hsp40 proteins were synthesized using the *in vitro* transcription and translation kit as described in text. The two proteins were incubated together followed by immunoprecipitation with Nef antibody. *C*, co-localization of Hsp40 and subtype C Nef in co-transfected 293T cells. 293T cells were grown on coverslips and transfected with pCDNA-NefC and pCDNA-Hsp40. After 36 h, transfected cells were stained with monoclonal Nef antibody and polyclonal Hsp40 antibody as described in the text. The indirect immunofluorescence was visualized by confocal microscopy.

Immunofluorescence Microscopy—HEK-293T cells grown on coverslips were transfected with both pCDNA-NefC and pCDNA-Hsp40 by calcium phosphate precipitation. Cells were harvested 36 h post-transfection and stained with polyclonal Hsp40 antibody and monoclonal Nef antibody after fixing with 2% paraformaldehyde. The secondary antibodies used for Hsp40 and Nef were indocarbocyanin (Cy3)-conjugated to anti-goat IgG (Chemicon) and fluorescein isothiocyanate-conjugated to anti-mouse IgG (Sigma), respectively. After washing, cells were mounted in antifade on the slide, and the samples were analyzed with a confocal microscope (Zeiss LSM 510, Germany).

Reverse Transcription-PCR—RNA was prepared from 2×10^6 HIV-1 NL4-3-infected and uninfected PBMCs using TRIzol Reagent (Invitrogen). The cDNA was made using Moloney murine leukemia virus reverse transcriptase (Invitrogen) followed by amplification by PCR for *HSP40* and human β-ACTIN with *Taq* polymerase (Invitrogen) using standard conditions and gene-specific oligonucleotide primers as described: human *β-ACTIN*: forward, 5′-TGACGGGGGTCACCCAC-ACTGTGCCCATCTA-3′, and reverse, 5′-CTAGAAGCATTTGCG-GTGGACGATGGAGGG-3′; human *HSP40*: forward, 5′-CAGGATC-CATGGTGGATTACTATGAAG-3′, and reverse, 5′-GTGGAAGA-GAATGAAGTGAGG-3′.

Quantification of HSP40 Expression by Real-time PCR—*HSP40* expression level was analyzed by quantitative real-time PCR of cDNA in

a 25-μl reaction mixture containing SYBR Green IQ supermix (Bio-Rad) and 10 pmol concentration of each of the human *β-ACTIN* and *HSP40* primer pairs listed above, using the Icycler IQ real-time thermal cycler (Bio-Rad). The amplification was performed using one cycle of 95 °C for 2 min and 40 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min followed by melt curve analysis. The changes in the threshold cycle (C_T) values were calculated by the equation $\Delta C_T = C_{T,\text{target}} - C_{T,\text{input}}$. The -fold difference was calculated as follows: -fold difference = $2^{-\Delta(\Delta C_T)}$.

Preparation of Nuclear and Cytoplasmic Extracts—10^7 infected or uninfected CEM-GFP cells were pelleted down and washed with ice-cold phosphate-buffered saline. Cells were resuspended in 400 μl of ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM *p*-aminobenzoic acid, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 μg/ml pepstatin, 50 μg/ml aprotinin) and kept on ice for 15 min to allow the cells to swell. After that 25 μl of 10% Nonidet P-40 was added to the cells and vortexed for 10 s. Then samples were centrifuged at 4 °C for 30 s to remove the cytoplasmic fraction. The nuclear fraction was prepared by resuspending the remaining pellet in 200 μl of ice-cold buffer C (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM *p*-aminobenzoic acid, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 μg/ml pepstatin, 50 μg/ml aprotinin) and kept on ice

for 20 min. Equal amounts of nuclear and cytoplasmic extracts from both infected and uninfected CEM-GFP cells were used for immunoblotting of Hsp40, Nef, RNA pol II, and actin as described above.

HSP40 Gene Silencing Using SiRNA—293T cells were transfected with 100 nM control siRNA duplex made against the non-target gene or SMARTpool siRNA duplexes made against *HSP40* (Dharmacon) using Lipofectamine 2000 reagent according to the manufacturer's instruction (Invitrogen). After 24 h of siRNA transfection pNL4-3 or pNL4-3ΔNef vectors were transfected using Lipofectamine 2000 reagent according to manufacturer's instructions (Invitrogen). After 72 h of siRNA transfection, supernatant was collected, and virus production was determined by p24^{gag} antigen capture ELISA (PerkinElmer Life Sciences). RNA was also isolated from these 293T cells and expression of *HSP40* and p24^{gag} was analyzed by RT-PCR using gene-specific primers.

RESULTS

HIV-1 Nef protein interacts with a large number of cellular proteins, most of which perform critical functions in signaling pathways. Although Nef-mediated signaling has been well studied, however, the molecular mechanism of Nef-mediated transcriptional enhancement remains to be clearly elucidated. Furthermore, the majority of studies to date have been performed with Nef protein from subtype B, so we initiated studies with subtype C Nef, which is not only the most prevalent subtype in Africa and Asia but currently accounts for almost 50% of infections worldwide. Although the majority of the functional domains of Nef are well conserved across the subtype, considerable sequence differences are observed. The subtype C Nef from Indian isolate IN301904 used in the present study has about 76% identity at the amino acid level with Nef from the well studied subtype B isolate NL4-3, whereas both proteins show almost 84% similarity in amino acid sequence (Fig. 1). We initiated the present study with screening for subtype C Nef interacting cellular proteins.

Identification of a Novel HIV-1 Subtype C Nef-interacting Protein—To identify novel proteins interacting with subtype C Nef, we screened a human leukocyte cDNA library using full-length subtype C Nef as bait in a yeast two-hybrid system. Repeated screening on X-gal containing dropout plates led to the identification of a specific positive clone. The gene expressed in this clone was amplified by PCR using the pACT2-specific primers. Sequence analysis and data base comparisons revealed that this clone expressed a gene encoding for a human *DnaJ* homologue that is *HSP40*. To further confirm this interaction, liquid β-galactosidase assay was performed as described under "Materials and Methods." The yeast clone expressing Hsp40 and subtype C Nef clearly showed β-galactosidase activity in liquid β-galactosidase assay, confirming the protein-protein interaction in the yeast system (Fig. 2A).

Subtype C Nef Interacts with Hsp40—Recombinant [^{35}S]methionine-labeled subtype C Nef and Hsp40 protein were prepared from pCDNA-NefC and pCDNA-Hsp40 plasmids, respectively, using *in vitro* transcription and translations as described earlier. These two proteins were then incubated together and immunoprecipitation was carried out with anti-Nef antibody. Fig. 2B clearly showed that subtype C Nef and Hsp40 proteins interacted with each other *in vitro* as both the proteins were present in *lane 2*, where Nef antibody was used for immunoprecipitation in contrast to no detectable band in immunoprecipitation performed with control IgG. Whereas the larger Nef (~27 kDa) is the full-length Nef protein, the smaller Nef (~25 kDa) corresponds to the Nef protein synthesized as a result of translation initiation from an internal methionine start site (30). This result further confirms that Nef and Hsp40 interact with each other *in vitro*.
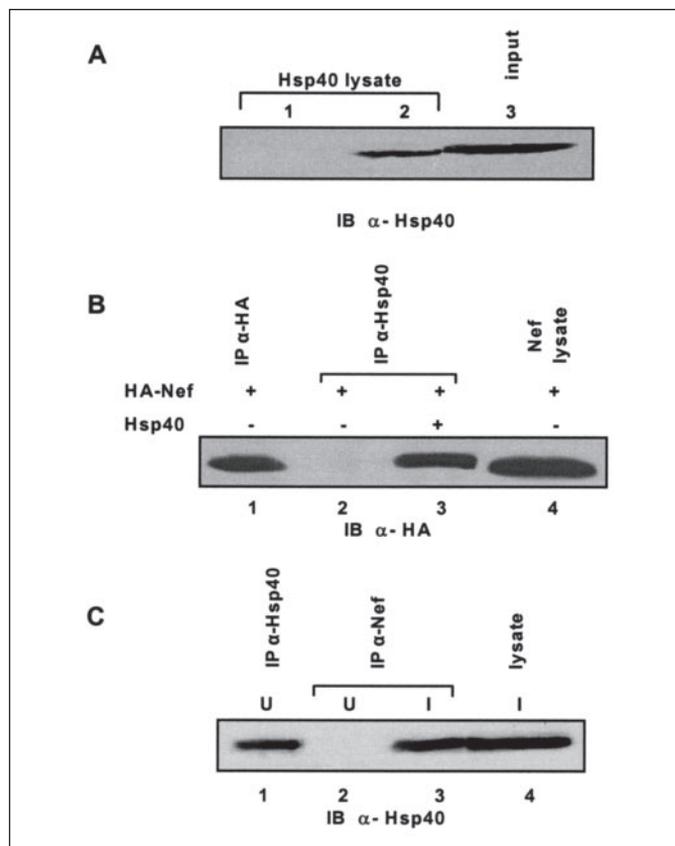
FIGURE 3. **Hsp40 physically interacts with subtype B NL4-3 Nef both *in vitro* and *in vivo*.** *A*, GST-Nef pulls down Hsp40 from Hsp40-transfected 293T cell lysate. GST (*lane 1*) and GST-Nef beads (*lane 2*) were incubated with 293T cells lysate overexpressing Hsp40 followed by SDS-PAGE and immunoblotting (*IB*) with Hsp40 antibody. The Hsp40-transfected 293T cell lysates (*lane 3*) were run in parallel as control. *B*, Hsp40 and Nef co-immunoprecipitates from the transfected cell lysate. 293T cells were transfected with the HA-NL4-3 Nef expression vector alone or with Hsp40 expression vector. After 48 h, the cells were lysed, immunoprecipitated with anti-Hsp40 antibody (*lane 2* and *3*), and followed by immunoblotting with anti-HA antibody as indicated in the figure. *C*, Nef co-immunoprecipitates with Hsp40 in HIV-1-infected CEM-GFP cell lysate. CEM-GFP cells were infected with HIV-1 NL4-3 virus as described in text. The cells were lysed on day 7 post-infection. The lysates of uninfected CEM-GFP cells (*lane 2*) and infected CEM-GFP cells (*lane 3*) were immunoprecipitated with anti-Nef antibody followed by immunoblotting with anti-Hsp40 antibody.

Hsp40 has been reported to be localized predominantly in the cytoplasm before heat shock and to relocalize in the nucleus after heat shock (18, 41, 42), whereas Nef has been reported to be a predominantly cytoplasmic protein but has also been shown to be in the nucleus of HIV-1-infected and transfected cells (36, 43, 44). As these proteins are reported to be present both as cytoplasmic and nuclear proteins during different cellular conditions, it would be interesting to know if these two proteins co-localize. Therefore, we performed immunofluorescence staining for both Hsp40 and Nef in co-transfected 293T cells as detailed under "Materials and Methods." As shown in Fig. 2C, confocal microscopic images of immunostained cells clearly indicated that both proteins were co-localized in the cytoplasm of transfected 293T cells. Co-localization was further confirmed by Z-stack analysis of confocal images (data not shown), which could probably be explained by predominantly cytoplasmic localization of both proteins in the normal cellular environment. We then tested whether this interaction was specific for subtype C Nef or whether this interaction can also be observed with subtype B Nef, for which we have used Nef from a common laboratory derived subtype B isolate, NL4-3.

NL4-3 Nef Also Interacts with Hsp40 Both in Vitro and in Vivo—To test whether Hsp40 interacts with NL4-3 Nef, purified GST and GST-
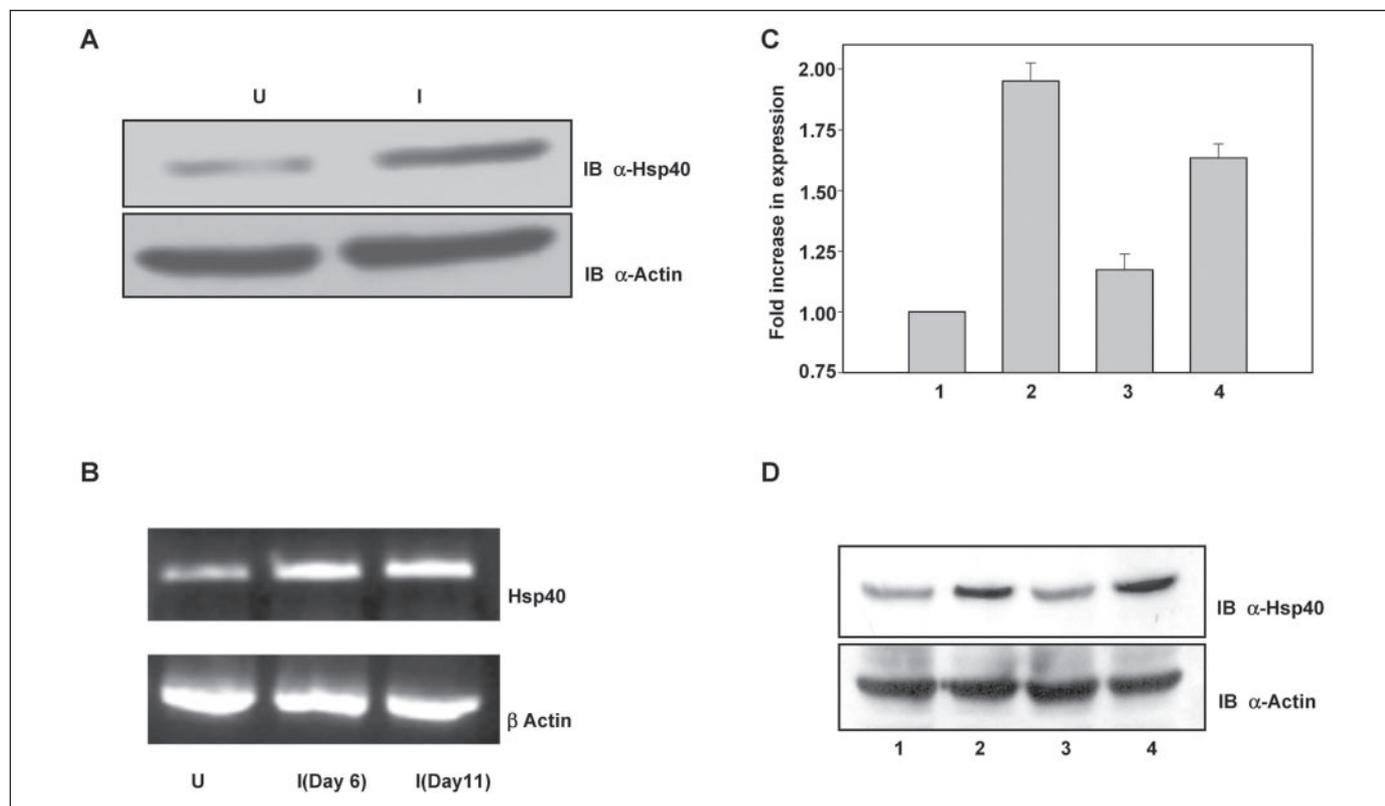
FIGURE 4. **Hsp40 is up-regulated in HIV-1 infection, which is dependent on Nef expression.** *A*, Hsp40 is up-regulated in HIV-1-infected CEM-GFP cells. HIV-1 NL4-3-infected CEM-GFP cells were lysed on day 7 post-infection and equal amounts of protein from uninfected and infected cells were used for immunoblotting of Hsp40. *B*, Hsp40 expression is up-regulated in HIV-1-infected human PBMCs. RNA was isolated from uninfected and NL4-3-infected PBMCs on days 6 and 11 post-infection followed by RT-PCR for *HSP40* using gene-specific primers, as listed under "Materials and Methods." Human *β-ACTIN* was used as an internal control. *C*, Nef is required for up-regulation of Hsp40 expression. 293T cells were transfected with empty vector (*lane 1*), HIV-1 NL4-3 molecular clone (*lane 2*), and *nef*-deleted NL4-3 molecular clone (*lane 3*) or pCDNA-Nef along with the *nef*-deleted NL4-3 (*lane 4*). After 36 h of transfection, RNA was isolated followed by real-time PCR for *HSP40* expression using human *β-ACTIN* as a control. *D*, Hsp40 expression is reduced in the absence of Nef and reverts back in the presence of Nef in NL4-3-transfected cells. 293T cells lysates from transfections mentioned in *C* were prepared after 48 h of transfection and equal amounts of protein were used in immunoblotting (*IB*) for Hsp40. Actin was used as an equal loading control.

Nef were immobilized on glutathione-Sepharose beads and used to pulldown Hsp40 from Hsp40 overexpressing 293T cell lysates. We observed that the Hsp40 protein was specifically pulled down by GST-Nef, whereas GST alone could not bring down Hsp40 (Fig. 3*A*).

To confirm this interaction *in vivo*, we performed co-immunoprecipitation from 293T cells expressing both Hsp40 and HA-Nef. The immunoprecipitation was done with Hsp40 antibody followed by immunoblotting with polyclonal HA antibody. The Nef protein co-immunoprecipitated with Hsp40 (*lane 3*) in cells expressing both proteins (Fig. 3*B*). Similar co-immunoprecipitation experiments with anti-Nef antibody from the lysates of uninfected or HIV-1 NL4-3 virus-infected CEM-GFP cells also immunoprecipitated Hsp40 only from infected CEM-GFP cell lysate but not from uninfected cell lysate (Fig. 3*C*). Thus both Hsp40 and Nef physically interact in HIV-1-infected cells and exist in a complex.

Hsp40 Is Induced in HIV-1-infected Cells—Although modulation of some cellular heat shock proteins is reported in HIV-1 infection, Hsp40 modulation was not reported earlier. Several studies reported increased expression of Hsp70 in HIV-1 infection and showed its presence in the virion (23, 24, 45). Therefore, we assessed the Hsp40 expression level in HIV-1-infected CEM-GFP cells by immunoblotting for Hsp40. The result showed that there was up-regulation of Hsp40 protein in HIV-1-infected CEM-GFP cells (Fig. 4*A*). Because the Hsp40 expression level increased in the infected CEM-GFP cell line, we performed RT-PCR for Hsp40 with RNA prepared from HIV-1 NL4-3 infected (days 6 and 11 post-infection) and uninfected human PBMCs. As shown in Fig. 4*B*,

Hsp40 expression was induced in HIV-1-infected PBMCs. Taken together, these data clearly indicate Hsp40 up-regulation in HIV-1-infected cells.

Nef Is Required for Up-regulation of Hsp40 in HIV-1-infected Cells— Because Nef interacts with Hsp40 in HIV-1-infected cells, we tested whether Nef plays any role in Hsp40 up-regulation in HIV-1-infected cells. We analyzed Hsp40 expression in 293T cells transfected with either the wild type NL4-3 molecular clone or *nef*-deleted NL4-3 or pCDNA-Nef along with the *Nef*-deleted molecular clone. A quantitative RT-PCR using real-time PCR showed about a 2-fold increase in *HSP40* transcription in 293T cells transfected with wild type molecular clone, but not in the cells transfected with *nef*-deleted molecular clone (Fig. 4*C*). Moreover, when Nef was transfected in *trans* along with the *nef*-deleted molecular clone, *HSP40* expression was restored. Furthermore, an exactly similar profile of Hsp40 expression was observed at the protein level by immunoblotting of protein lysates from transfected cells (Fig. 4*D*). These results clearly indicate that HIV-1 up-regulates Hsp40 expression in a Nef-dependent manner.

Hsp40 Enhances HIV-1 LTR-driven Gene Expression in the Presence of Nef—Because previous studies have shown that Hsp70 associates with P-TEFb complex (25) and Nef enhances viral gene expression (30), we examined a possible role of Hsp40 in viral gene expression. Hsp40 co-transfection in 293T cells with Tat and Nef showed a definite increase in HIV-1 LTR-driven viral gene expression as compared with Tat and Nef or Tat alone (Fig. 5*A*). Hsp40 overexpression along with Tat and Nef resulted in at least a 1.5-fold increase in LTR-mediated reporter gene expression than that observed with Tat and Nef and more than a 2.5-
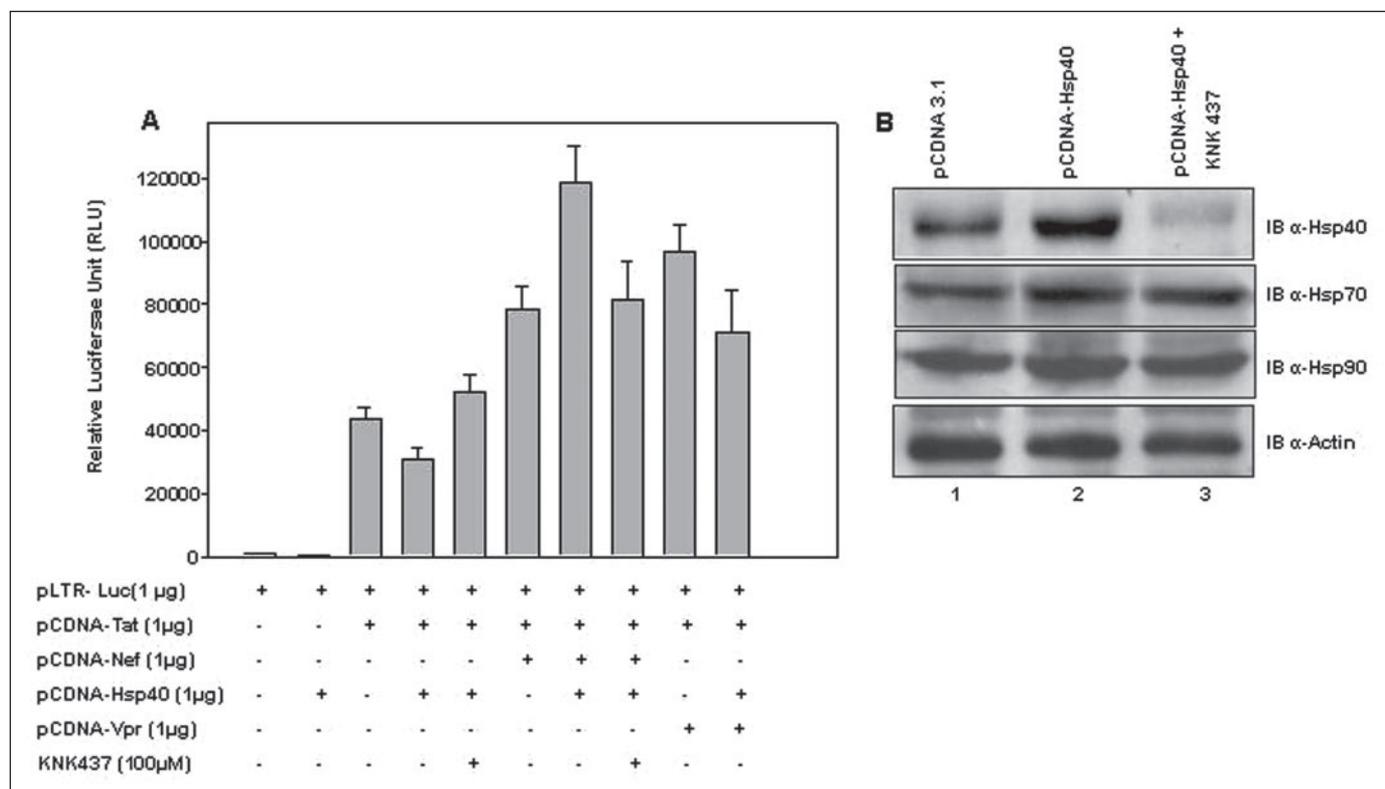
FIGURE 5. **Hsp40 specifically enhances HIV-1 LTR-mediated gene expression in presence of Nef.** *A*, Hsp40 enhances Tat-induced LTR-mediated gene expression specifically in the presence of Nef, which is inhibited by KNK437. 293T cells were transfected with various expression vectors along with the LTR-Luc reporter as indicated in the figure. After 4 h of transfection, cells were either treated or not treated with the Hsp overexpression inhibitor KNK437 (100 μM) for 32 h. Cells were then lysed and luciferase assay was performed as described in the text. *B*, KNK437 specifically inhibits Hsp40 overexpression in Hsp40-transfected cells. 293T cells were transfected with empty vector (*lane 1*) or Hsp40 expression vector treated with either vehicle control (*lane 2*) or KNK437 (*lane 3*) as described above. Cell lysates were used in immunoblotting (*IB*) for Hsp40, Hsp70, and Hsp90. Actin was used as equal loading control.
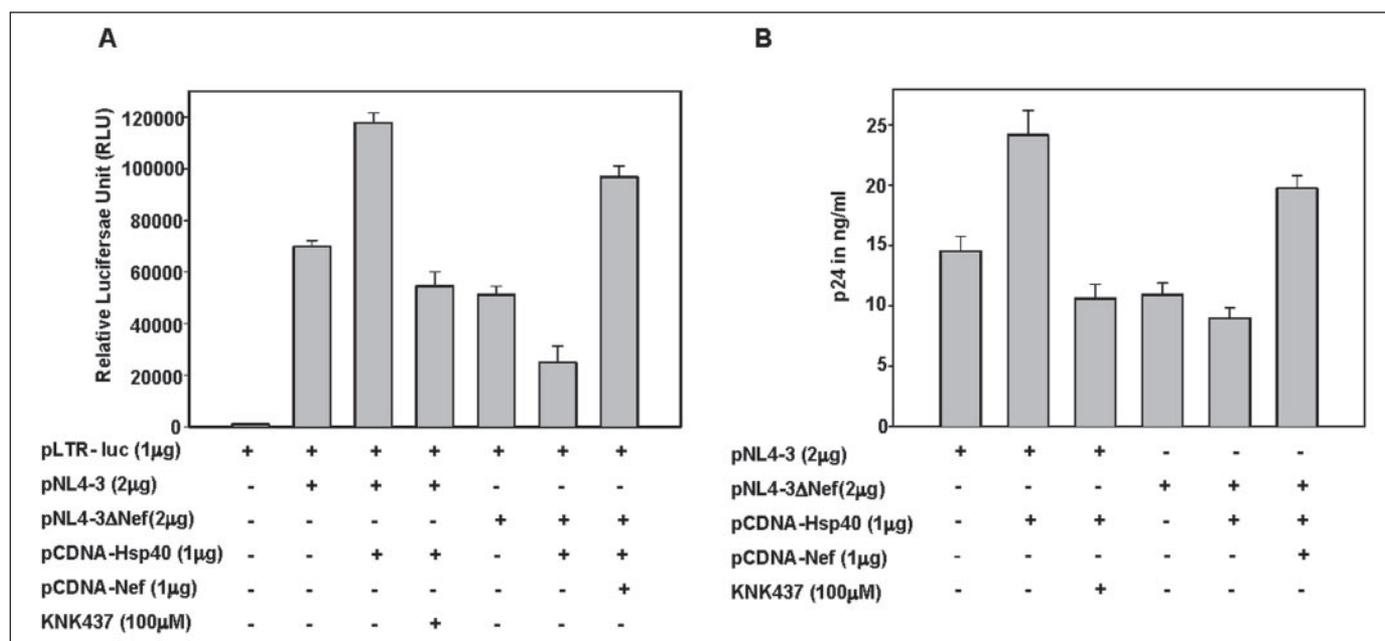
FIGURE 6. **Nef is required for Hsp40-induced HIV-1 LTR-mediated gene expression and virus production.** *A*, Nef is required for Hsp40-induced LTR-mediated gene expression. 293T cells were transfected with different expression vectors along with the LTR-Luc reporter as indicated in the figure. After 4 h of transfection some of the transfected cells were treated with 100 μM KNK437 for 32 h as indicated. The cells were lysed and luciferase assay was performed as described in text. *B*, Nef is required for Hsp40-induced increase in virus production. Virus production was determined in culture supernatants of 293T cells transfected in the experiment in *A*, by using p24 antigen capture ELISA.

fold increase over the Tat-induced gene expression. This increase in LTR-mediated viral gene expression because of Hsp40 is specific to the Nef protein as overexpression of Hsp40 in the presence of either Tat alone or Tat and Vpr together did not increase the reporter gene expression (Fig. 5*A*). Similar experiments with subtype C Tat and Nef along with the subtype C LTR promoter-driven reporter has also shown iden-

tical results (data not shown), indicating that this phenomenon is not restricted to any virus subtype.

The role of Hsp40 in LTR-mediated gene expression was further examined by pharmacological inhibition of Hsp40 overexpression by KNK437, a benzylidene lactam compound, in the transfected 293T cells. KNK437 was shown to inhibit the induction of various Hsps including Hsp40 and Hsp70 after heat shock (46), however, it does not inhibit basal levels of Hsp expression. Therefore, 293T cells were transiently transfected with Hsp40 and were treated with KNK437. As shown in Fig. 5*B*, the induction of Hsp40 by transient transfection was almost completely inhibited by KNK 437, although endogenous expression of Hsp70 and Hsp90 was not affected by this inhibitor, as they were not induced. The HIV-1 LTR-driven gene expression observed during Hsp40 overexpression with Tat and Nef was completely reversed in the presence of KNK437 (Fig. 5*A*). KNK437 also reversed the negative effect of Hsp40 overexpression on Tat-mediated transactivation. Put together, all these results clearly indicate that Hsp40 specifically induces HIV-1 LTR-driven gene expression in the presence of Nef protein.

Hsp40 Is Required for the Nef-induced Increase in Viral Gene Expression and Replication—The molecular basis of Nef-enhanced virus production (6) is not clearly understood. Our observation that Nef interacts with Hsp40 to augment LTR-mediated gene expression led us to investigate whether the enhanced LTR-mediated gene expression leads to increased virus production. We then performed a single cycle replication study in 293T cells by co-transfecting HIV-1 molecular clones, wild type or *nef*-deleted NL4-3 along with Hsp40 and LTR-luciferase reporter plasmid for assessing the LTR-mediated gene expression by luciferase assay. As shown in Fig. 6*A*, Hsp40 induced LTR-mediated gene expression with wild type virus but is unable to induce expression in the case of *nef*-deleted virus. Co-transfection of Nef along with the *nef*-deleted virus and Hsp40 restored the gene expression close to wild type levels. An almost identical profile was obtained when virus production was analyzed by the p24 antigen assay in culture supernatants of these cells (Fig. 6*B*). The wild type HIV-1 molecular clone showed about a 2-fold increase in viral replication with Hsp40 overexpression, whereas there was no increase in virus production in the case of the *Nef*-deleted molecular clone, rather it was decreased (Fig. 6*B*). However, putting *Nef* in *trans* with *Nef* minus molecular clone showed increased virus production, which again confirms that the interaction between Nef and Hsp40 leads to an increase in LTR-mediated gene expression as well as virus production. Furthermore, use of KNK437 in this experiment along with Hsp40 co-transfection leads to reduced gene expression and decreased virus production, clearly indicating the importance of Hsp40 and Nef interaction in the viral life cycle.

To further assess the importance of Hsp40 in virus production, the specific siRNA duplex (Dharmacon) was used in 293T cells for silencing *HSP40* expression. As shown in Fig. 7*A*, expression of the *HSP40* level was significantly decreased at 72 h after SMARTpool siRNA transfection (*lane 4*), whereas no reduction was observed with control siRNA (*lane 1*). As compared with control siRNA-transfected cells, transfection of SMARTpool Hsp40 siRNA along with the pNL4-3 molecular clone in 293T cells showed decreased virus production (Fig. 7*B*). The *nef*-deleted molecular clone-transfected 293T cells did not show any significant change in p24^{gag} antigen capture ELISA (Fig. 7*B*) in Hsp40-silenced cells. An almost similar pattern was also observed at the p24 RNA level (Fig. 7*C*), indicating thereby that Hsp40 down-regulation in the presence of Nef leads to decreased virus production.

Increased Translocation of Hsp40 into the Nucleus and Its Interaction with cdk9 in HIV-1-infected Cells—Although the above mentioned data establishes Nef-Hsp40 interaction and its role in increased gene expres-
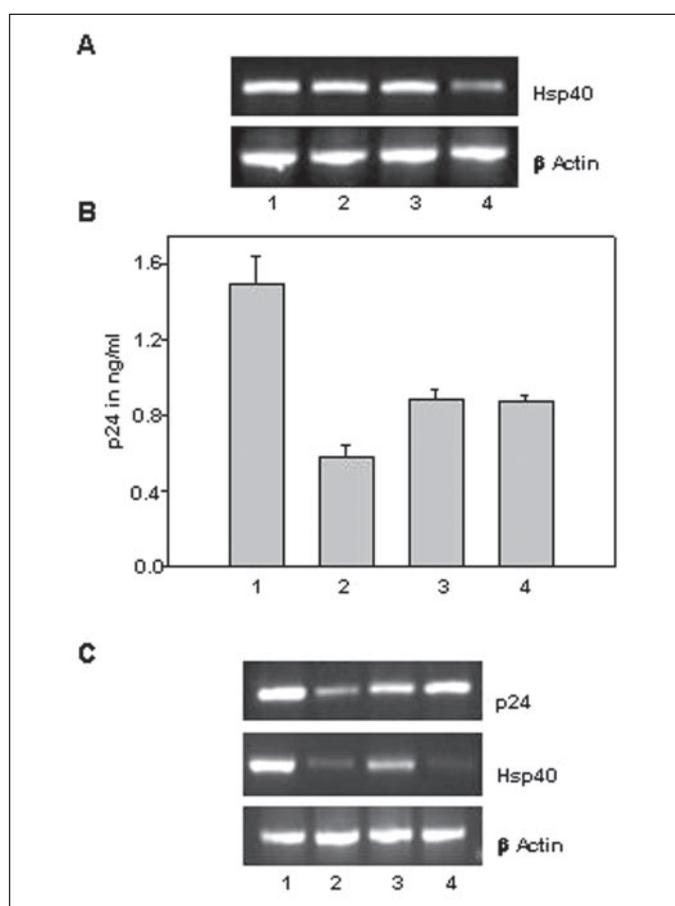
FIGURE 7. **Hsp40 down-regulation inhibits viral replication in the presence of Nef.** *A*, *HSP40* gene silencing by specific siRNA. 293T cells were transfected with 100 nM Hsp40 SMARTpool siRNA or control siRNA duplex. RT-PCR was performed for Hsp40 expression at different time points post-transfection. *Lane 1*, control siRNA (72 h); *lanes 2–4*, Hsp40 siRNA at 36, 48, and 72 h, respectively. *B*, Hsp40 down-regulation by siRNA reduces virus production in the presence of Nef. Control or Hsp40 siRNA-transfected 293T cells as described above were again transfected after 24 h with either the NL4-3 molecular clone (*lanes 1* and *2*) or the *Nef*-deleted NL4-3 molecular clone (*lanes 3* and *4*). Culture supernatants were collected at 72 h of siRNA transfection, and virus production was determined by using p24 antigen capture ELISA. *C*, HIV-1 p24^{gag} expression is down-regulated in *HSP40*-silenced cells in the presence of Nef. RNA was isolated from transfected 293T cells described above (*B*) after 72 h of siRNA transfection, and RT-PCR was performed for HIV-1 p24, *HSP40*, and human *β-ACTIN*.

sion and virus production, the molecular mechanism remains to be elucidated. Because cellular stress such as heat shock mobilizes Hsp40 into the nucleus (18, 41, 42), we tested whether stress in the form of viral infection (13) relocates Hsp40 into the nucleus. Therefore, nuclear and cytoplasmic extracts were prepared from HIV-1-infected and -uninfected CEM-GFP cells and was immunoblotted for Hsp40. The purity of nuclear and cytoplasmic extracts was confirmed by immunoblotting for RNA polymerase II and actin, respectively. Hsp40 was observed in the nucleus and cytoplasm of both uninfected and infected cells; however, more Hsp40 was present in the nucleus of infected cells than uninfected cells (Fig. 8*A*). Because Hsp40 and Nef interact with each other, we looked at the localization of Nef in these cytoplasmic and nucleus extracts. Our results clearly showed that Nef was also present in both the nucleus and cytoplasm of infected cells, which correlated well with previous reports of Nef localization in both the nucleus and cytoplasm (30, 36, 43, 44). It has been reported that Hsp70, which is assisted by Hsp40 for its function, is required for P-TEFb-mediated Tat transactivation of viral gene expression (25). Because there was increased nuclear translocation of Hsp40 in infected cells, we investigated whether Hsp40 could be part of the Tat transcription complex. We performed a co-
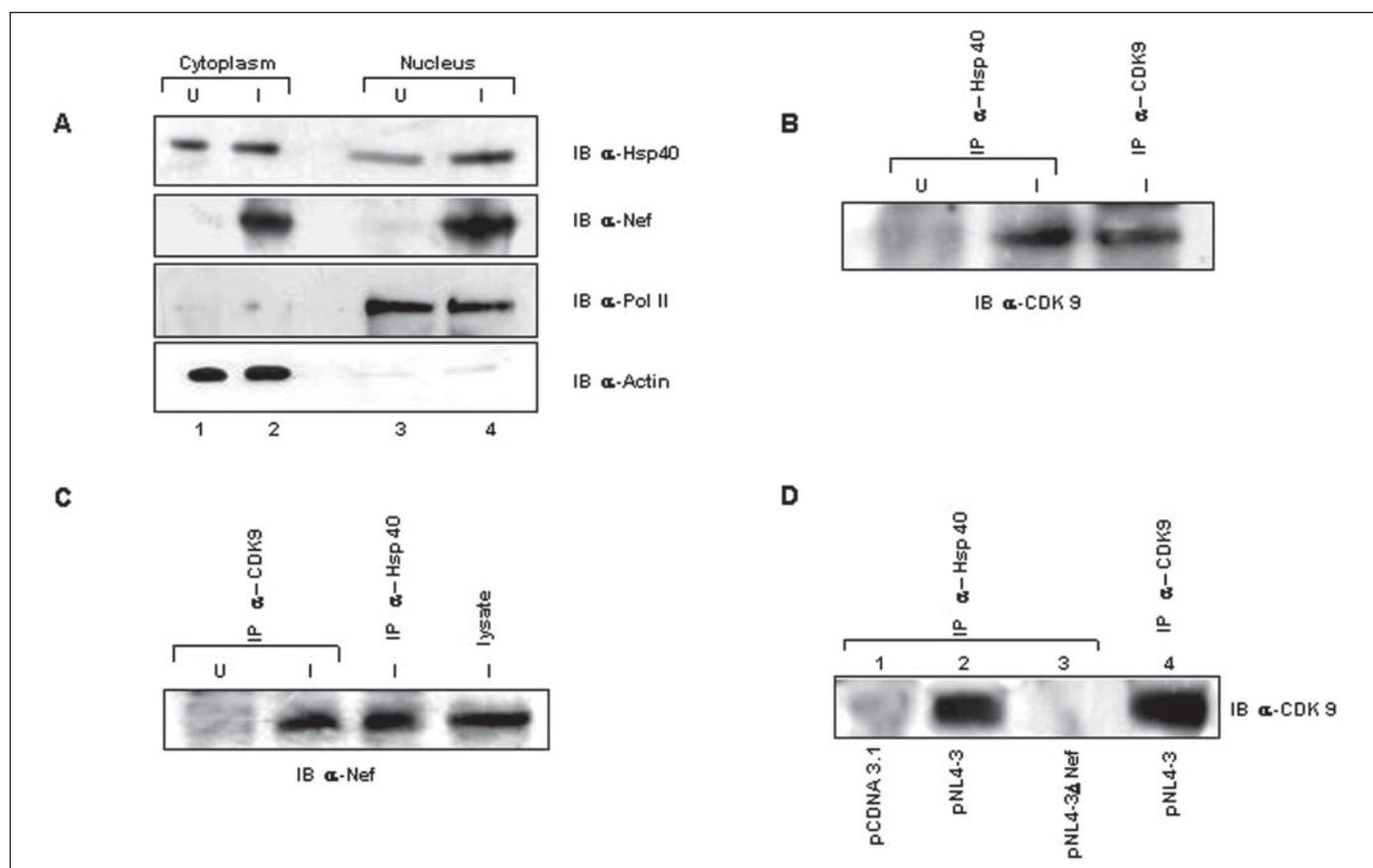
FIGURE 8. **Hsp40 translocates into the nucleus of HIV-1-infected cells and interacts with cdk9 in a Nef-dependent manner.** *A*, Hsp40 increases in the nucleus of HIV-1-infected cells along with Nef. Nucleus and cytoplasm fractions were prepared from uninfected and infected CEM-GFP cells as described under "Materials and Methods." Equal amounts of cytoplasmic and nuclear extracts were used for immunoblotting of Hsp40 and Nef. The same blots were probed with actin and RNA polymerase II antibody as loading controls. *B*, Hsp40 interacts with cdk9 in HIV-1-infected cells. CEM-GFP uninfected and infected lysates were used for immunoprecipitation with Hsp40 antibody followed by immunoblotting (*IB*) with anti-cdk9 antibody. Lysate was also subjected to immunoprecipitation with cdk9 antibody as positive control. *C*, Nef is a part of the Hsp40 and cdk9 complex. CEM-GFP uninfected and -infected lysates were immunoprecipitated with either cdk9 antibody (*lanes 1* and *2*) or with Hsp40 antibody (*lane 3*) followed by immunoblotting with anti-Nef antibody. Lysate from infected cells was used as a positive control (*lane 4*). *D*, Hsp40 interacts with cdk9 in a Nef-dependent manner. Lysates of 293T cells transfected with pCDNA3.1, NL4-3 molecular clone, and *nef*-deleted NL4-3 molecular clone were used for immunoprecipitation with Hsp40 antibody followed by immunoblotting with anti-cdk9 antibody (*lanes 1–3*). Immunoprecipitation with cdk9 antibody was used as a positive control (*lane 4*).

immunoprecipitation experiment for Hsp40 and cdk9, using lysates from HIV-1-infected and -uninfected CEM-GFP cells. The results in Fig. 8*B* showed clearly that Hsp40 can bind to cdk9, the catalytic component of the Tat-associated transcription complex P-TEFb. To further confirm that Nef is part of this transcription complex, we used cdk9 and Hsp40 immunoprecipitates of HIV-1-infected CEM-GFP cells for immunoblotting with Nef antibody. Nef was found to be present in both cdk9 and Hsp40 immunoprecipitates (Fig. 8*C*) clearly indicating its presence in the complex. We then investigated the role of Nef in the interaction between Hsp40 and cdk9 by co-immunoprecipitation of 293T cell lysates transfected with wild type NL4-3 or the *Nef*-deleted NL4-3 molecular clone using Hsp40 antibody followed by immunoblotting for cdk9 (Fig. 8*D*). The data clearly showed that the interaction of Hsp40 and cdk9 was Nef-dependent as Hsp40 was able to bring down cdk9 only in the presence of Nef. These observations indicate that Hsp40 interacts with Nef, translocates into nucleus, and integrates into the viral transcription complex by binding to cdk9 in a Nef-dependent manner, resulting in enhancement of LTR-mediated gene expression and increased virus production.

DISCUSSION

In the present study, we show for the first time that Nef interacts with one member of the heat shock protein family, Hsp40, which has been identified by yeast two-hybrid screening and further confirmed by

immunoprecipitation and co-localization studies. This interaction is not subtype specific as both subtypes B and C Nef bind to Hsp40. Our data corroborates previous studies (47) suggesting that despite significant sequence differences in the Nef from various subtypes, functional properties remain relatively well conserved. Furthermore, our results clearly show that Hsp40 is up-regulated in the HIV-infected cell line as well as in infected PBMCs, and that Nef is necessary for this Hsp40 up-regulation during HIV-1 infection. Although the role of cellular heat shock proteins in HIV infection has been investigated by several groups, little information has been generated about the different Hsp family members except Hsp70. The up-regulation of Hsp70 has been reported in HIV infection along with its presence in the virion (23, 24), however, its role in HIV-1 infection remains to be established. Hsp70 has been shown to interact with the Vpr protein and inhibit Vpr-induced apoptosis (48). Hsp70 has also been implicated in the enhancement of the LTR-mediated gene expression (25). Our single cycle replication studies to identify the biological role of Nef-Hsp40 interaction further shows that Hsp40 enhances viral gene expression and virus production specifically in the presence of Nef, clearly pointing toward the importance of this interaction. Our data clearly indicates that overexpression of Hsp40 enhances viral gene expression, whereas inhibition of Hsp40 expression reduces viral gene expression and virus production in a Nef-dependent manner. Furthermore, immunoblotting analyses of nuclear and cytoplasmic extracts from HIV-1-infected cells show increased transloca-

tion of Hsp40 into the nucleus of infected cells along with the presence of Nef protein. Finally, we also show that Hsp40 interacts with cdk9 in a Nef-dependent manner, one of the important host cell proteins required for viral transcription, which could be the basis for Hsp40-induced Nef-mediated enhancement of viral gene expression.

One of the early functions ascribed to Nef was that it acts as a transcriptional repressor through the HIV-1 LTR, which might contribute to viral latency (9, 26). At least two groups have failed to corroborate this results (49, 50), whereas other groups later reported transcriptional enhancing activity for Nef (10, 51). Nef has also been shown to activate T cells, which increases expression of transcription factors required for viral transcription (31). Recent reports show that Nef directly acts as a positive modulator of HIV-1 LTR through different mechanisms (52, 53) and also micro-RNA for *nef* may down-regulate HIV-1 transcription (54). In our previous study (30), we have shown that Nef enhances viral replication by interacting with its transactivator protein, Tat, and thereby modulates viral gene expression. Both Nef and Hsp40 proteins have been shown to be individually translocated in the nucleus by different groups during different conditions (18, 30, 36, 41–44). In our previous report (30) we have also shown that Tat and Nef co-localize more predominantly in the nucleus and enhance viral replication. Based on the present data, it could be suggested that Tat may be involved in bringing Nef and Hsp40 proteins into the nucleus, as the ability of intracellular trafficking by Tat is well documented and a specific region of Tat is actually used in intracellular delivery of proteins (55–57). Alternatively the role of Hsp40 in chaperoning Nef inside the nucleus may not be ruled out.

The present study provides further insight into the molecular mechanism of the Nef-induced enhancement of viral gene expression and replication. In summary, we conclude that Nef not only interacts with Hsp40 but also up-regulates the expression of Hsp40 in HIV infection. Nef and Hsp40 interaction leads to enhancement of LTR-mediated gene expression and virus production. All our data clearly shows that Hsp40 enhances viral gene expression and replication in a Nef-dependent manner. It is worth mentioning here that similar phenomena have been observed in avian adenovirus CELO, where the viral protein Gam1 activates Hsp40 and thereby induces viral replication (58). Finally, interaction of Hsp40 with cdk9, the catalytic component of P-TEFb, suggests that the Nef-Hsp40 complex could be part of the viral transcription assembly. All together our findings lead us to hypothesize a model in infected cells, where Nef is responsible for Hsp40 up-regulation followed by Nef-Hsp40 interaction. This interaction then may lead to increased translocation of Hsp40 into the nucleus of infected cells along with Nef and Tat where Hsp40 binds to cdk9 and enhances viral replication by modulating the activity of P-TEFb. Finally, the present work not only provides a molecular basis of Nef-mediated enhancement of viral gene expression and replication but also identifies a novel viral strategy to use cellular heat shock response proteins to its own advantage.

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Heat Shock Protein 40 Is Necessary for Human Immunodeficiency Virus-1 Nef-mediated Enhancement of Viral Gene Expression and Replication

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