Active Participation of Cellular Chaperone Hsp90 in Regulating the Function of Rotavirus Nonstructural Protein 3 (NSP3)∗

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Heat shock protein 90 (Hsp90) has been reported to positively regulate rotavirus replication by modulating virus induced PI3K/Akt and NFκB activation. Here, we report the active association of Hsp90 in the folding and stabilization of rotavirus nonstructural protein 3 (NSP3). In pCD-NSP3-transfected cells, treatment with Hsp90 inhibitor (17- β-N,N-dimethyllethylendiamine-geldanamycin (17DMAG)) resulted in the proteasomal degradation of NSP3. Sequence analysis and deletion mutations revealed that the region spanning amino acids 225–258 within the C-terminal eIF4G-binding domain of NSP3 is a putative Hsp90 binding region. Co-immunoprecipitation and mammalian two-hybrid experiments revealed direct interaction of the C-terminal 12-kDa domain of Hsp90 (C90) with residues 225–258 of NSP3. NSP3-Hsp90 interaction is important for the formation of functionally active mature NSP3, because full-length NSP3 in the presence of the Hsp90 inhibitor or NSP3 lacking the amino acid 225–258 region did not show NSP3 dimers following coupled transcription-translation followed by chase. Disruption of residues 225–258 within NSP3 also resulted in poor RNA binding and eIF4G binding activity. In addition, inhibition of Hsp90 by 17DMAG resulted in reduced nuclear translocation of poly(A)-binding protein and translation of viral proteins. These results highlight the crucial role of Hsp90 chaperone in the regulation of assembly and functionality of a viral protein during the virus replication and propagation in host cells.

Molecular chaperones are believed to be present as large macromolecular complexes with varied functions such as the prevention of protein aggregation and the folding and assembly of newly synthesized proteins. Two candidate members of the chaperone family are heat shock proteins Hsp70 and Hsp90. Hsp70 preferentially binds to short extended peptides possessing alternative hydrophobic residues (1), a finding consistent with the view that chaperones interact with exposed hydrophobic regions of unfolded proteins that are otherwise buried in native proteins. The relative lack of more stringent recognition requirements apparently contributes to the promiscuous nature of substrate binding, as seen with all classes of chaperones. Hsp90 also plays a key role in the conformational maturation of many cellular proteins. Hsp90 displays an ATP-dependent folding capacity and has been shown to bind during the later stages of protein folding to facilitate the activation-competent state, with the help of its various co-chaperones and ATP (2–7).

There is overwhelming evidence that suggests the direct or indirect dependence of both DNA and RNA viruses on one or more cellular chaperones such as Hsp40 (8, 9), Hsp70 (10), and Hsp90 (11–19) for their growth and replication. The mechanism behind the cooperation of chaperone proteins and the viruses varies among the different viruses (10, 11, 13, 15).

Rotavirus (RV),6 a member of the Reoviridae family, is a double-stranded RNA virus associated with severe gastroenteritis in children worldwide (20, 21). This virus, with its 11-segmented dsRNA genome, encodes six structural proteins (VP1–VP4, VP6, and VP7), which form the virion, and six nonstructural proteins (NSP1–NSP6), which have a role in improving virus replication and infection in host cells (22–30). Hsc70 (70-kDa heat shock cognate protein) has been reported to involve in multistep entry of RV into intestinal epithelial cells (31, 32), whereas Hsp70 negatively affects RV infection by ubiquitin-mediated proteasomal degradation of viral proteins (33). Our group has previously shown that the inhibition of virus replication by Hsp90 inhibitors is correlated with the inhibition of...
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virus-induced PI3K/Akt activation (19). However, whether Hsp90 interacts directly with any rotavirus-encoded protein could not be determined.

In the present study, we have analyzed the direct interaction between Hsp90 and NSP3 protein. NSP3 of group A rotavirus is a 34–36-kDa protein (21) with two structurally and biochemically distinct domains. The N-terminal portion (NSP3-N; aa 1–149) forms an asymmetric homodimer creating a single, highly basic RNA-binding tunnel lined by residues from both monomers (34). The C-terminal domain (NSP3-C) (aa 206–313) forms a symmetric homodimer, composed of three pairs of α-helices (helix 1, aa 208–251; helix 2, aa 255–261; and helix 3, aa 273–304) (35), that interacts with eukaryotic translation initiation factor eIF4GI, evicts poly(A)-binding protein (PABP) from eIF4GI into the nucleus, and shuts off host cell translation but allows efficient expression of viral proteins (36–38).

In this report we show that the C-terminal 12-kDa domain (C90) of Hsp90 binds to the 225–258-aa region of NSP3 monomers. This interaction leads to the formation of functionally active mature NSP3 dimers. In the presence of the Hsp90 inhibitor, dimerization of NSP3 and translocation of PABP to the nucleus was inhibited. Deletion or point mutations within the aa 225–258 region failed to form biologically active NSP3 protein. The results suggest the crucial role of Hsp90 in regulating the assembly and functionality of a virus-encoded protein during the virus replication cycle in host cells.

EXPERIMENTAL PROCEDURES

Reagents—17-N,N-Dimethylethlenediamine-geldanamycin (17DMAG) was purchased from Invivogen (San Diego, CA). Other fine chemicals and buffers were purchased from Sigma-Aldrich.

Cell Culture and Virus Infection—The monkey kidney cell line (MA104) was cultured in minimal essential medium (MEM). Human embryonic kidney epithelial cell line (293T) was cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with U. S.-certified 10% bovine serum and a 1% antibiotic-antimycotic solution (Invitrogen). For virus infection, MA104 cells were infected with the SA11 strain at an m.o.i. of 3. The time of virus removal was taken as 0 h post-infection (hpi). At different time points cells were either fixed for immunofluorescence experiments or freeze-thawed for cell lysis. Extracted and purified virus (39) preparations were titrated by plaque assay.

Plasmid Construction, Transfection, and Protein Purification—Full-length NSP1, NSP2, NSP3, NSP5, and VP6 from rotavirus SA11-H96 were amplified from extracted RNA by RT-PCR with the respective primers (Table 1) and cloned into the pCDNA6 (Invitrogen) mammalian expression vector under the control of the CMV promoter (The GenBank accession numbers for the cloned cDNAs are JF791801–JF791803 and JF791805–JF791806, respectively). All primers used in the study for the respective constructs are given in Table 1. NSP3 point mutants were generated by site-directed mutagenesis (Stratagene) of the full-length pCD-NSP3 plasmid using the respective primers. Deletion mutant Δ225–258 of NSP3 (ΔNSP3) was constructed following deletion of a specific region within pCD-NSP3. Using pcDNA-NSP3 constructs, full-length NSP3, ΔRB (N-terminal deletion of the RNA-binding domain), ΔeIF4GB (C-terminal deletion of eIF4G-binding domain), Δ350–380 (internal deletion of the coiled-coil region known to be involved in dimerization), and Δ225–258 (internal deletion of the putative Hsp90 binding region) were cloned in-frame with the N-terminal FLAG epitope of the pFLAG-CMV expression vector (Sigma-Aldrich). PCDS-A11-RRV-225–258-NSP3, PCDS-A11-Ku-225–258-NSP3, and PCDS-A11-OSU-225–258-NSP3 chimeric expression plasmids were prepared through insertion mutagenesis by replacing cDNA encoding the SA11 225–258 region with equivalent portion of NSP3 of the RRV, Ku, and OSU strains. Three gel-purified PCR fragments that included the internal chimeric cDNA encoding the NSP3-(225–258) region of each of the three virus strains along with two adjacent SA11 fragments were mixed in equimolar amounts to perform an overlap extension PCR; this produced recombinant chimeric NSP3 products that were cloned in-frame into the pCDNA-6 vector. ProLabel-NSP3 fusion constructs (p-proN-NSP3 and p-NSP3-proC) were produced by cloning the PCR-amplified full-length NSP3 in-frame with the ProLabel tag of ProLabel-N and ProLabel-C vectors. All of the clones were confirmed by sequencing. The vectors were transfected into 293T cells using the PrimeFect transfection reagent (Lonza) according to the manufacturer’s instructions. Hsp90-tagged full-length NSP3 and Δ225–258 deletion and point mutants were purified under native conditions using the QIAexpressionist protein purification kit (Qiagen) following the manufacturer’s instructions. Briefly, 293T cells (4 x 107) expressing recombinant protein were lysed in lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, and 0.7% Tween 20, pH 8.0) through sonication on ice followed by centrifugation at 4 °C. Cleared cell lysate was mixed into nickel-nitrilotriacetic acid magnetic agrose beads (Qiagen) on an end-over-end shaker for 4 h at 4 °C, and recombinant protein was separated using a magnet. After the separated protein-bead mixture was washed four times with washing buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole, and 0.7% Tween 20, pH 8.0), protein elution buffer (50 mM NaH2PO4, 300 mM NaCl, 250 mM imidazole, and 0.7% Tween 20, pH 8.0) through magnetic separation. A second round of purification was performed from the eluate after adjusting the imidazole concentration to 20 mM using binding (lysis) buffer without imidazole. Proteins were finally made imidazole-free by step dialysis.

Synthesis of RNA Probe and Gel Retardation Assay—The templates used to produce 46-base 3’ NSP3 and 3’ NSP5 probes were generated from purified rotavirus RNAs using the respective primers with a T7 promoter attached to the forward primers (Table 1). The PCR products were treated with Klenow enzyme to remove overhang A residues and purified by electrophoresis on a 1.5% agarose gel. RNA probes were synthesized by runoff transcription with a TranscriptAid T7 high yield transcription kit (Fermentas Life Sciences, Opelstrasse, Germany) using 50 mM biotin-16-UTP (Ambion, Foster City, CA) and purified following the manufacturer’s instruction.

For the gel retardation assay, purified proteins (100 nM) were incubated with biotinylated NSP5 RNA probe (5 nM) in 25 μl of RNA binding buffer (10 mM HEPES, pH 7.9, 40 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), and 20% glycerol) for 20 min
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TABLE 1

| Name                | Sequence                                                                 | Vector          |
|---------------------|--------------------------------------------------------------------------|-----------------|
| SA11-Vp6-pc-F       | 5′-TAA GCC TCA CTC TGC TAT CTA TAC TGA TCA GAC CAC-3′                    | pCDNA6B         |
| SA11-Vp6-c-F        | 5′-TAA GCC TCA CTC TGC TAT CTA TAC TGA TCA GAC CAC-3′                    | pCDNA6B         |
| SA11-Nsp1-pc-F      | 5′-AAG CGC TCG TGC TAT CTA TAC TGA TCA AAG TAC CAC-3′                    | pCDNA6B         |
| SA11-Nsp1-c-F       | 5′-AAG CGC TCG TGC TAT CTA TAC TGA TCA AAG TAC CAC-3′                    | pCDNA6B         |
| SA11-Nsp1-pc-R      | 5′-CTG GAT CTC AGA TTC CAC-3′                                            | pCDNA6B         |
| SA11-Nsp1-c-R       | 5′-CTG GAT CTC AGA TTC CAC-3′                                            | pCDNA6B         |
| SA11-Nsp5-pc-F      | 5′-AAG CGT GTC TGC TAT CTA TAC TGA TCA AAG TAC CAC-3′                    | pCDNA6B         |
| SA11-Nsp5-c-F       | 5′-AAG CGT GTC TGC TAT CTA TAC TGA TCA AAG TAC CAC-3′                    | pCDNA6B         |
| FLAG2a-N53-F        | 5′-TAA GCG GCG ACG TCA TGC TAT CTA TAC TGA TCA GAC CAC-3′                 | pCDNA6B         |
| FLAG2a-N53-R        | 5′-TAA GCG GCG ACG TCA TGC TAT CTA TAC TGA TCA GAC CAC-3′                 | pCDNA6B         |
| FLAG2a-DΔRB-N53-F   | 5′-TAA GCG GCG ACG TCA TGC TAT CTA TAC TGA TCA GAC CAC-3′                 | pCDNA6B         |
| FLAG2a-DΔFB-N53-R   | 5′-TAA GCG GCG ACG TCA TGC TAT CTA TAC TGA TCA GAC CAC-3′                 | pCDNA6B         |

at room temperature and resolved by electrophoresis on non-denaturing 8% polyacrylamide gel in 0.5× TAE buffer (27 mm Tris, pH 7.9, 13.2 mM sodium acetate, pH 7.9, 4 mM EDTA, and 10% glycerol). Samples were electrophoresed at 4°C at 15 mA with recirculation of the 0.5× TAE running buffer. The RNA-protein complex in the gel was then transferred to a nylon membrane, cross-linked, and developed with Pierce® high sensitivity streptavidin-HRP (Thermo Fisher Scientific, Rockford, IL).

Mammalian Two-hybrid Assay—The mammalian two-hybrid test (CheckMateTM mammalian two-hybrid system, Promega, Madison, WI) was used to analyze protein-protein interactions. All procedures described below were carried out according to the manufacturer's instruction. The C90 coding sequence of Hsp90α (aa 629–731), amplified through PCR, was cloned in-frame into the pACT vector with the yeast Gal4 DNA-binding domain (pBIND-C90α). Similarly, the coding sequence of aa 225–258 of NSP3 was PCR-amplified and fused in-frame into the pACT vector containing the yeast VP16 activation domain (pACT-NSP3225–258). Deletion constructs of Hs90 lacking the C90 region (pBIND-ΔC90α) and NSP3 without the aa 225–258 region (pACT-ΔNSP3) were also prepared in the pBIND and pACT vectors, respectively. Similarly, C90α and NSP3225–258 were cloned reciprocally in pACT and pBIND vectors. pBIND-C90α and pACT-NSP3225–258 were cotransfected along with the pG5Luc vector into 293T cells with PrimeFectTM DNA transfection reagent (Lonza). At 48 h post-transfection, the cells were lysed, and the firefly luciferase and Renilla luciferase activities were quantitated using the Dual-Luciferase® reporter assay system (Promega) against negative control where empty vectors (pBIND, pACT, and pG5Luc) were transfected. Transfection with the pBIND getId and pACT-MyoD vectors that encode the GAL4Id and VP16-MyoD fusion proteins, respectively, was done as a positive control.

ProLabel Protein Quantitation—Expression of NSP3 ProLabel fusion proteins was quantitated using a ProLabelTM detection kit (Clontech) according to the manufacturer’s instruction. The assay is based on an enzyme system derived from β-galactosidase enzyme fragment complementation. Briefly, 48 h post-transfection, homogeneous cell lysates or renatured proteins (with the ProLabel tag encoding α fragment of β-galactosidase enzyme) in lysis buffer were mixed with EA

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In Vitro Coupled Transcription-Translation and Chase—Plasmids encoding the full length and mutants of NSP3 under the T7 promoter (pCDNA constructs) were subjected to in vitro coupled transcription-translation using TNT® Quick Coupled Transcription/Translation System (Promega) according to the manufacturer’s specifications. Briefly, 2 μg of circular plasmid was added to the TNT® Quick Master Mix and incubated in the presence of Transcend™ biotin-lysyl-tRNA (Promega) in a 50-μl reaction volume for 50–90 min at 30 °C. The labeled product was then analyzed by SDS-PAGE followed by Western blotting with Pierce® High Sensitivity streptavidin-HRP (Thermo Fisher Scientific).

To follow the fate of the synthesized protein, reaction mixtures were centrifuged at 159,000 × g for 58 min at 4 °C to pellet down the ribosomes. The supernatants, which had no translation activity, were then incubated with cold lysine at 30 °C for increasing amounts of time (5–30 min) and subsequently analyzed by SDS-PAGE. To analyze the role of Hsp90, 17DMAG (5 μM) was added to the post-ribosomal supernatant before chase. In a control reaction, an equal volume of water was used.

SDS-PAGE and Western Immunoblotting—Whole-cell lysates, nuclear or cytoplasmic extracts, and in vitro translated or immunoprecipitated products were prepared. SDS-PAGE followed by immunoblotting was performed according to standard protocols (40). Samples were incubated in protein sample buffer (final concentration: 50 mM Tris, pH 6.8, 1% SDS, 10% glycerol, 1% β-mercaptoethanol, and 0.01% bromphenol blue) for 30 min at either 4 °C or, alternatively, boiled for 5 min before SDS-PAGE at room temperature. Under denaturing conditions NSP3 dissociated and migrated as monomers. Under non-dissociating conditions (4 °C), both NSP3 dimers and monomers were observed. For immunoblotting, mouse monoclonal antibody to Hsp90 (BD Biosciences), PABP (Santa Cruz Biotechnology, Inc.), PCNA (Cell Signaling, Inc., Danvers, MA), anti-eIF4G (Cell Signaling), anti-rotavirus VP6 antibody (3C10) (HyTest, Ltd., Turku, Finland), and rabbit monoclonal antibody to GAPDH (Cell Signaling) were used at the concentrations recommended by the manufacturer. Polyclonal antibodies against rotavirus nonstructural proteins (NSP1, NSP2, NSP3, and NSP5) raised in rabbit were described previously (31). The monoclonal primary antibodies were detected by using HRP-conjugated secondary antibodies (Thermo Fisher Scientific) and chemiluminescent substrate (Millipore, Billerica, MA). In vitro translated products or chased samples containing biotinylated proteins were detected by immunoblotting using Pierce® High Sensitivity streptavidin-HRP (Thermo Fisher Scientific) followed by chemiluminescence. Where necessary, blots were reprobed with β-actin, GAPDH, or PCNA to confirm equal protein loading. The immunoblots shown are representative of three independent experiments. Blots were scanned and quantitated by using the GelDoc XR system and Quantity One software version 4.6.3 (Bio-Rad).

Immunoprecipitation—For overexpression, transfected cells were lysed and clarified. The in vitro coupled transcription-translation reaction (50 μl) was stopped by the addition of a similar volume of TEM buffer (20 mM Tris, pH 7.4, 5 mM EDTA, 10 mM ammonium molybdate, and 50 mM NaCl) and clarified. Clarified samples were then incubated overnight at 4 °C with protein A-Sepharose (GE Healthcare), which were preincubated with the appropriate antibodies (anti-FLAG M5 antibody (Sigma-Aldrich), anti-NSP3, anti-Hsp90, or anti-GAPDH) for 1 h at 4 °C. The beads were washed five times either with 1× lysis buffer or TEM buffer containing 0.1% Triton X-100. The immunoprecipitates were analyzed by SDS-PAGE followed by Western immunoblotting.

For detection of NSP3 intermediates, immunoprecipitates were first released under low pH conditions (0.1 M glycine, pH 3, for 10 min at room temperature) followed by neutralization with a one-tenth volume of 1 M Tris, pH 9.5, before nondenaturing SDS-PAGE.

Nuclear and Cytoplasmic Protein Extraction—Nuclear and cytoplasmic lysates were prepared using a ProteoJET cytoplasmic and nuclear protein extraction kit (Fermentas Life Sciences) for the PABP translational study.

Immunofluorescence Microscopy—MA104 cells seeded in four-well chamber slides (BD Biosciences) were either mock infected or infected with rotavirus SA11 in the presence or absence of 17DMAG. Cells were fixed with paraformaldehyde (4% (w/v) in PBS) for 10 min at room temperature and permeabilized with 0.1% Triton X-100 for 20 min at 4 °C. The samples were then incubated in blocking solution (PBS supplemented with 5% (v/v) horse serum and goat serum) for 1 h at room temperature. The cells were washed with cold PBS and incubated with monoclonal PABP and polyclonal RV-NSP3 antibodies at room temperature for 2 h followed by Rhodamine Red X-conjugated anti-mouse and fluorescein isothiocyanate-conjugated anti-rabbit antibodies (The Jackson Laboratory, West Grove, PA) for 1 h. After being washed five times with PBS, the slides were mounted with Vectashield-DAPI (Vector Laboratories, Burlingame, CA) and examined under a fluorescence microscope (Carl Zeiss, Gottingen, Germany). Excitation and emission detection for each fluor was performed sequentially to avoid cross-talk. The specificity of the NSP3 antibody was tested by immunoblotting cell extracts expressing VP6 and NSP3, respectively (supplemental Fig. 1).

Bioinformatics—The NSP3 protein sequence of SA11-H96 and other simian (RRV), porcine (OSU), human (Ku and Wa), and rotavirus strains was examined using the SMART program (41), Win Gen/Win Pep (42), and ELM server (43) to identify the sequence from aa 225 to 258 as being partially tetratricopeptide repeat (TPR)-like. The sequence (aa 225–258) was used to search for related sequences in the GenBank™ database and the Protein Data Bank to predict the possible binding partner of cellular proteins by using the BLAST program (Gapped BLAST and PSI-BLAST) (44). SA11, OSU, Ku, RRV, Wa, A64, RF, human group C, and chicken group D rotavirus NSP3 protein sequences were aligned by using the ClustalX program to check the similarity among the predicted sequence regions. The GenBank™ accession numbers for the NSP3 sequences are as follows: simian SA11 H[96], DQ838610; simian RRV,
Polyribosome Fractionation and RNA Analysis—Cytoplasmic extract preparation and polyribosome analysis was performed and interpreted as described previously (45–48). Briefly, MA104 cells were infected with SA11 (m.o.i. = 3). At the indicated time (hpi), cells were incubated at 4 °C for 15 min in low salt buffer A (10 mm Tris-HCl, pH 8.0, 140 mm NaCl, 1.5 mM MgCl₂, 0.5% NP-40, 20 mm dithiothreitol, 150 µg/ml cyclohexamide, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 500 units/ml RNasin). The swollen cells were then homogenized with 15 strokes of a Dounce homogenizer, and the nuclei were removed by spinning at 1000 g for 10 min. The supernatant was supplemented with 665 µg/ml heparin and centrifuged at 12,000 g for 5 min to remove mitochondria. Postmitochondrial extracts (200 µl) were then layered on the top of a 4-ml sucrose gradients (0.5–1.5M) prepared in buffer A. The gradients were centrifuged for 58 min at 159,000 g in a Sorvall TH660 rotor. Nine fractions (450 µl) were collected, and absorbance was monitored at 254 nm; each of the fractions was then treated with proteinase K (80 µg) in 1% SDS for 15 min at 37 °C. RNA was extracted from individual fractions by phenol-chloroform extraction. The RNA was analyzed by electrophoresis on a 1.2% formaldehyde agarose gel and subjected to Northern blot hybridization analysis, where hybridizations were performed overnight at 42 °C in the presence of 50% formamide. The biotinylated RNA probe was identified by streptavidin-HRP during development. The experiment was repeated three times independently, and representative data are shown.

Statistical Analysis—Data are expressed as means ± S.D. of at least three independent experiments (n = 3). In all tests, p < 0.05 was considered statistically significant. Experiments in which p is <0.01 are marked with an asterisk.

RESULTS
Inhibition of Hsp90 Results in Reduced Expression of Transiently Transfected NSP3 and Negatively Affects Nuclear Localization of PABP in Virus-infected Cells—To analyze whether Hsp90 directly affects the expression of any rotaviral protein, plasmids encoding the full-length virus structural gene (VP6) and nonstructural genes (NSP1, NSP2, NSP3, and NSP5) were transfected in 293T cells either untreated or treated with Hsp90 inhibitor (17DMAG). The cells were harvested and lysed after 24 and 36 h of transfection, and expression of viral proteins was analyzed by Western immunoblotting. The concentration of 17DMAG used was determined through a cytotoxicity assay in 293T and MA104 cells (supplemental Fig. 2). Immunoblotting with specific polyclonal antibodies revealed a marked reduction (2–3-fold) in NSP3 expression levels in the presence of 17DMAG compared with untreated controls. However, expression of VP6, NSP1, NSP2, and NSP5 proteins was not affected in the presence of 17DMAG (Fig. 1A), suggesting that

**FIGURE 1. Effect of Hsp90 inhibitor (17DMAG) on rotavirus proteins.** A and B, Western blot analysis showing expression of representative structural and nonstructural proteins in the presence or absence of 17DMAG (5 µM). Cell lysates of transiently transfected (pCD-VP6, NSP1, NSP2, NSP3, and NSP5) 293T cells or SA11-infected (m.o.i. = 3) MA104 cells were separated by SDS-PAGE and immunoblotted using specific antibodies. Equal protein loading was confirmed by reprobing with GAPDH antibody. C, effect of 17DMAG on NSP3 expression in the presence or absence of proteasomal inhibitor. pCD-NSP3-transfected 293T cells were treated with 17DMAG in the presence or absence of MG132 (100 nM), and expression of NSP3 was analyzed by immunoblotting. D, nuclear localization of PABP following rotavirus infection by immunofluorescence microscopy. MA104 cells were mock infected or infected with SA11 (m.o.i. = 3) in the presence or absence of 17DMAG. Cells were fixed and incubated with NSP3- and PABP-specific antibodies followed by fluorescein isothiocyanate-labeled anti-rabbit (NSP3 (green), column 2) and Rhodamine Red X-labeled anti-mouse (PABP (red), column 3) antibodies. Nuclei were stained with DAPI (column 1).

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NSP3 is more sensitive to Hsp90 inhibitor than other rotavirus proteins. To understand whether 17DMAG inhibited transcription of the NSP3 gene, expression of NSP3 mRNA was analyzed by real-time PCR. No significant difference in the level of NSP3 transcript was observed in the transfected cells at 24 and 36 h in the presence of 17DMAG (data not shown), indicating that Hsp90 modulates NSP3 post-transcriptionally. However, when viral protein expression was monitored in MA104 cells infected with SA11 (m.o.i. = 3) in the presence or absence of 17DMAG, a significant reduction (2–3-fold) in NSP3 protein was observed as early as 6 hpi in the presence of 17DMAG compared with controls (Fig. 1B). The level of other viral proteins was not significantly altered at 6 hpi; however, at 12 hpi, a reduction in all viral proteins was observed (Fig. 1B). This could be because of the reduction in viral replication in the presence of Hsp90 inhibitor (supplemental Fig. 3). To determine whether inhibition in the expression of NSP3 protein was due to posttranslational degradation promoted by 17DMAG, cells transfected with NSP3 plasmid were treated with 17DMAG in the presence or absence of proteasome inhibitor MG132 (100 nM). As shown in Fig. 1C, NSP3 protein expression was restored in the presence of proteasomal inhibitor confirming a possible role of Hsp90 in stabilizing NSP3.

Previous studies have shown that expression of NSP3 redirects the subcellular localization of PABP from cytoplasm to nucleus (36–38). To analyze the role of Hsp90 in PABP translocation, MA104 cells infected with SA11 (m.o.i. = 3) in the presence or absence of 17DMAG (5 μM) were fixed 8 h post-infection, and PABP localization was examined by immunofluorescence compared with the control uninfected cells in the presence or absence of drug. Significant localization of PABP to the nucleus was observed in virus-infected cells (Fig. 1D, third panel from top); however, in the presence of 17DMAG, PABP localization to nucleus was greatly reduced (Fig. 1D, fourth panel), indicating that Hsp90 directly or indirectly modulates activity of NSP3. No significant changes in PABP translocation were observed in 17DMAG-treated uninfected cells (Fig. 1D, second panel).

**Association of Hsp90 with NSP3**—Based on the results, we tried to analyze whether Hsp90 interacts physically with NSP3 to modulate its function. Cell lysates of MA104 cells following SA11 infection (8 h) were subjected to immunoprecipitation with Hsp90 antibody followed by immunoblotting with NSP3 antibodies. As shown in Fig. 2A, Hsp90 co-immunoprecipitated with NSP3 in virus-infected cells. Similarly, when lysates from SA11-NSP3 transiently expressing FLAG-NSP3 in 293T cells were subjected to immunoprecipitation with anti-FLAG antibody followed by Western blotting using anti-Hsp90 and anti-FLAG antibodies, Hsp90 co-immunoprecipitated with NSP3 in virus-infected cells. Similarly, when lysates from SA11-NSP3 transiently expressing FLAG-NSP3 in 293T cells were subjected to immunoprecipitation with anti-FLAG antibody followed by Western blotting using anti-Hsp90 and anti-FLAG antibodies, Hsp90 co-immunoprecipitated with NSP3 in virus-infected cells.
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![Diagram of Hsp90-NSP3 interaction]  
**FIGURE 3.** Association of NSP3 of group A rotavirus strains with Hsp90 is independent of sequence variation in group A rotaviruses. A, sequence alignment of the 213–308-aa region or equivalent region of NSP3 of the group A rotavirus strains. Human group C and chicken group D NSP3 sequences are also aligned to show similarities with the group A NSP3 aa 225–258 region. Sequence similarity is encoded by the white-gray-black color gradient. B, the region spanning aa 225–258 of SA11-NSP3 was replaced by the corresponding sequence of simian RRV, human Ku, and porcine OSU strains to produce the rearranged PcD-NSP3 plasmids (pCD-SA11-RRV225–258-NSP3, pCD-SA11-Ku225–258-NSP3, and pCD-SA11-OSU225–258-NSP3). Proteins expressed from these plasmids in 293T cells were immunoprecipitated with NSP3 antibody followed by Western blotting with anti-Hsp90 antibody. Blots were reprobed with NSP3 to confirm expression of the rearranged protein.

were identical with the conventional TPR motif (Fig. 2C). To validate the search analysis, FLAG-tagged NSP3 deletion mutants lacking an RNA-binding domain (aa 1–149) (ΔRRB), an eIF4G-binding domain (aa 206–313) (ΔeIF4GB), a central coiled-coil region (aa 150–240) (Δ150–240), and a predicted Hsp90 binding region (aa 225–258) (Δ225–258) were generated (Fig. 2D). Each of the FLAG-NSP3 mutants was transfected into 293T cells, and cell lysates were immunoprecipitated using anti-FLAG antibody followed by immunoblotting with Hsp90 antibody (Fig. 2E). Although FLAG-ΔRRB-NSP3 co-immunoprecipitated with Hsp90, little Hsp90 was observed in the immunoprecipitates of FLAG-Δ150–240-NSP3. Similarly, in FLAG-ΔeIF4GB- and Δ225–258-NSP3-transfected cells, no Hsp90 immunoprecipitates were observed (Fig. 2E). Because FLAG-Δ225–258 had full-length NSP3, except for aa 225–258, this region was predicted as a potential Hsp90 binding site.

**Association of Residues 225–258 of NSP3 with Hsp90 Is Not Entirely Sequence-dependent**—The sequence alignment of NSP3–225–258 aa and its flanking region revealed significant homology (Fig. 3A, shaded region) among the different group A rotavirus strains (including SA11), although some variability was also observed (Fig. 3A). Human group C and chicken group D rotavirus showed a wide range of differences. To confirm whether the corresponding regions of other group A rotavirus strains also function as Hsp90 binding regions, or whether this was SA11 sequence-specific, the SA11-NSP3 225–258-aa region was replaced with the corresponding region of the simian RRV, human Ku, and porcine OSU strains in the pcDNA-SA11-NSP3 construct. Cell lysates from 293T cells expressing the rearranged plasmids (pCD-SA11-RRV225–258–NSP3, pCD-SA11-Ku225–258-NSP3, and pCD-SA11-OSU225–258-NSP3) were immunoprecipitated using NSP3 antibody followed by immunoblotting with anti-Hsp90. Similar to the SA11-NSP3 protein, the rearranged NSP3 proteins also co-immunoprecipitated with Hsp90 (Fig. 3B), indicating an equivalent association of NSP3 with cellular Hsp90 and thus confirming it as a strain-independent phenomenon.

**Direct Interaction of the Hsp90 C-terminal Domain with aa 225–258 Region of NSP3 Is Required for Its Assembly**—To investigate the possible direct interaction of NSP3(225–258) with the C-terminal TPR binding region of Hsp90 (C90) (49), the mammalian two-hybrid system was utilized. The C90 coding sequence of Hsp90 (Hsp90α, aa 629–731) was cloned in-frame with the yeast DNA-binding domain of GAL4 (pBIND-C90α). Similarly the NSP3(225–258) coding sequence was fused in-frame with the herpes simplex virus VP16 activation domain (pACT-NSP3(225–258)). Deletion constructs of Hsp90 without the C90 region (pBIND-ΔC90α) and NSP3 without aa 225–258 (pACT-ΔNSP3) were also prepared. When pBIND-C90α and pACT-NSP3(225–258) were cotransfected in 293T cells with the firefly luciferase reporter plasmid pGLuc +, significant transactivation (>85%) of the luciferase gene was observed compared with the negative control (pBIND + pACT only) (Fig. 4A). The reciprocal constructs pBIND-NSP3(225–258) and pACT-C90α also showed similar transactivation (data not shown). The transactivation was due to C90α-NSP3 (225–258 aa) binding, because deletion mutants of either C90α (pBIND-ΔC90α) or NSP3 (pACT-ΔNSP3) failed to activate the luciferase reporter following cotransfection with the respective wild type partners (pACT-NSP3(225–258) or pBIND-C90α) (Fig. 4A). To rule out the false interaction, unrelated proteins such as nuclear laminin (pBIND-laminin) were cotransfected with pACT-NSP3(225–258) as an internal negative control, which also did not transactivate luciferase (data not shown).

To determine the functional significance of the Hsp90-NSP3 interaction, specific point mutations were introduced in pCD-NSP3 (K237E, S240A, E253K, K237E, and S240A) in addition to the aa 225–258 deletion mutant (pCD-ΔNSP3). Wild type as well as mutant plasmids was subjected to in vitro coupled transcription and translation (IVT) for 90 min in the presence of...
Transcend™ biotin-lysyl-tRNA as a probe. The IVT products (Fig. 4B, upper panel) were immunoprecipitated with anti-Hsp90 monoclonal antibody followed by denaturing SDS-PAGE analysis. Immunoblotting with streptavidin-HRP revealed that other than the wild type full-length NSP3, mutants of NSP3 co-precipitated poorly with anti-Hsp90 antibody, confirming that Hsp90 interaction with NSP3 requires an aa 225–258 intact region (Fig. 4B, lower panel). No co-immunoprecipitation of full-length NSP3 was observed with the internal control GAPDH. Further, when the Hsp90 co-immunoprecipitates were subjected to SDS-PAGE under nondissociating conditions, only the monomeric form of NSP3 was observed with the internal control GAPDH. However, when the NSP3 co-immunoprecipitates were subjected to SDS-PAGE of the pre-co-immunoprecipitation IVT reaction mix. Following immunoprecipitation using Hsp90 antibody, only the monomeric form of NSP3 was observed (lane 2), whereas NSP3 mutants show little or no NSP3 (lanes 3–7).

Hsp90 Modulates Rotavirus NSP3 Biogenesis

NSP3 Mutants and NSP3 in the Presence of Hsp90 Inhibitors Show Impaired Function—Because the mature dimeric form of NSP3 protein is reported to be functionally active (34, 50), we tried to investigate the association of either full-length NSP3 in the presence of Hsp90 inhibitors (17DMAG or Hsp90 siRNA) or the NSP3 mutants with eIF4G (Fig. 5A). Compared with the NSP3 controls, deletion mutants or full-length NSP3 expressed in the presence of Hsp90 inhibitor (17DMAG) precipitated poorly with eIF4G, indicating that the association of functionally active NSP3 with eIF4G may require NSP3-Hsp90 interac-
Incubation of purified proteins with biotinylated RNA probe (46 bases, NSP5) for 20 min in vitro coupled transcription-translation for 50 min in the presence of Transcend™ biotin-lysyl-tRNA as described under “Experimental Procedures” and subsequently chased with excess (20 mM) unlabeled lysine. At different time points (5–30 min), samples were subjected to sequential immunoprecipitation (IP) with anti-Hsp90 followed by NSP3 antibody. The results (Fig. 6 lane 3, lane 4–7) demonstrate that, Hsp90-NSP3 Complex Is an Intermediate Step toward Formation of Mature NSP3-NSP3 Dimer—To understand how Hsp90 interaction with NSP3 modulates its stability, we tried to assess the interaction of NSP3 during its dimerization. Full-length NSP3 was subjected to in vitro coupled transcription-translation for 50 min in the presence of Transcend™ biotin-lysyl-tRNA (pulse) and subsequently chased with excess unlabeled lysine for an increasing period of time (5–30 min) followed by immunoprecipitation with either Hsp90- or NSP3-specific antibody. The post-immunoprecipitation supernatants were again reciprocally immunoprecipitated with either NSP3 or Hsp90 antibody. The results (Fig. 6A) demonstrate that, with time, NSP3 co-precipitation with anti-Hsp90 decreased, to a gel mobility shift assay (Fig. 5B). Compared with the control reaction lacking NSP3 protein (Fig. 5B, lane 1), significantly higher RNA-protein complex was detected in the reaction mixture that contained full-length NSP3 and the 3’ N5 probe (Fig. 5B, lane 2). ΔNSP3 protein failed to form a complex with RNA (Fig. 5B, lane 3), whereas a variable (low to medium) level of RNA-protein complexes was observed for various NSP3 point mutants (lanes 4–7), indicating that these mutations probably reduced the RNA binding ability of NSP3 either partially or completely.

Furthermore, full-length NSP3, as well as its mutants, was expressed in 293T cells, and the presence of PABP was assessed in the nuclear and cytoplasmic extracts by immunoblotting. Reduced nuclear distribution of PABP was evident in the NSP3 deletion mutant compared with the wild type NSP3-transfected cells (Fig. 5C), which was consistent with observations made in the presence of 17DMAG (Fig. 1D). Point mutants also revealed relatively low levels of PABP in nuclear extracts. The PABP levels in the cytoplasmic extracts were reciprocally comparable with that of the nuclear extracts of both the wild type and mutant NSP3-transfected cells (Fig. 5C). PCNA (nuclear) and β-actin (cytoplasmic) protein expression was measured as an internal loading control (Fig. 5C).
whereas the amount of NSP3 in the supernatant increased. Subsequent SDS-PAGE under nondissociating conditions confirmed presence of only monomeric NSP3 in the immunoprecipitates (data not shown). When these immunoprecipitates were resuspended in fresh translation mixture followed by incubation at 30 °C, a conversion of the monomeric to the mature dimeric form of NSP3 was observed in a time-dependent manner (Fig. 6B), indicating that the observed Hsp90-NSP3 complex represents a true folding intermediate during the formation of mature NSP3 dimers.

Inhibition of Hsp90 Activity or Its Depletion in Vitro Impairs the Formation of Mature Folded Structure of NSP3

The full-length NSP3 (pcD-NSP3) was subjected to in vitro coupled transcription-translation in the presence of Transcend™ biotin-lysyl-tRNA for 50 min. The reaction was ultracentrifuged to remove ribosomes, and the supernatant was incubated further at 30 °C in the presence or absence of 17DMAG (5–30 min). The reaction mixtures were immunoblotted using streptavidin-HRP following nondenaturing SDS-PAGE. B, purified NSP3 ProLabel fusion proteins (ProN-NSP3 and NSP3-ProC) were denatured by guanidine hydrochloride (5 M) followed by renaturation through subsequent dialysis. Renatured proteins were allowed to refold in the presence of either crude rabbit reticulocyte lysate (RRL), Hsp90-immunodepleted RRL, or Hsp90-immunodepleted RRL supplemented with purified Hsp90 protein in vitro. Expression of ProLabel-NSP3 was quantitated by measuring the activity of the ProLabel tag using a ProLabel chemiluminescence detection assay Kit (Clontech). The data are presented as luminescence units/s (RLU/S) (mean ± S.D., n = 3; *, p < 0.05). C, renatured ProLabel-NSP3 proteins in the presence of either crude RRL, Hsp90-immunodepleted RRL, or Hsp90-immunodepleted RRL supplemented with purified Hsp90 were subjected to immunoprecipitation (IP) with NSP3 antibody followed by Western blotting with eIF4G to observe the association of NSP3 with eIF4G as a measure of its function. Blots were reprobed with NSP3 to measure equal expression of NSP3. Pro-N and Pro-C were used as negative controls.

To assess the folding of NSP3 in the presence or absence of Hsp90, NSP3 ProLabel fusion proteins (ProN-NSP3 and NSP3-ProC) expressed in 293T cells were purified with anti-NSP3 antibody-Sepharose beads (supplemental Fig. 7, A–C). The purified protein was then denatured with guanidine hydrochloride (5 M) followed by renaturation with subsequent dialysis. The renatured protein was incubated with crude rabbit reticulocyte lysate or Hsp90-immunodepleted lysate (supplemental Fig. 5A), and ProLabel expression was quantitated. With respect to the only renatured protein, >20-fold higher chemiluminescence was observed in ProN-NSP3 and NSP3-ProC in the presence of crude lysate, whereas 4–4.5-fold less chemilu-
minescence was observed (Fig. 7B) in the presence of Hsp90-
immunodepleted lysate. When purified Hsp90 protein was
added to the Hsp90-immunodepleted lysate, the activity of
ProN-NSP3 and NSP3-ProC was restored (Fig. 7B). The pre-
esence of Hsp90 inhibitor (17DMAG) in the lysate also reduced
the chemiluminescence of the renatured ProLabel-NSP3 fusion
proteins (data not shown), suggesting that spontaneous refold-
ing of NSP3 requires the presence of functionally active Hsp90.
The biological activity of each of the refolded proteins was
tested by measuring its eIF4G binding property (Fig. 7C). NSP3
fusion proteins in the presence of Hsp90-immunodepleted lysate showed poor association with eIF4G, whereas significant
NSP3-eIF4G binding was observed in the presence of crude
erosomal inhibitor (Fig. 1E). The presence of 17DMAG-mediated
reduction of NSP3 expression in the presence of protea-
somal inhibitor (Fig. 1C) provided evidence of a physical inter-
action between these proteins. The presence of a putative TPR-
lke motif (aa 225–258) in SA11-NSP3 (Fig. 2C) further
indicated the probability of a direct interaction between NSP3
and Hsp90. The TPR is a structural motif of 34 amino acid
residues with 3–16 tandem repeats present in a wide range of
proteins that mediates protein-protein interactions and the
assembly of multiprotein complexes (51). Several Hsp90-asso-
ciated cofactors contain multiple copies of the TPR motif (52–
54) that interact with C90 (49).

To confirm and identify the region of NSP3 that interacts
with Hsp90, different deletion mutants of NSP3 were prepared
based on the reported crystal structure of NSP3 (34, 35). Co-
immunoprecipitation experiments following transfection of
NSP3 constructs confirmed the importance of the aa 225–258
region, as FLAG-NSP3 Δ225−258 failed to co-precipitate with
Hsp90 (Fig. 2F). Interestingly NSP3 deletion within the central
coiled-coil domain Δ250−296 NSP3 also revealed poor associa-
tion with Hsp90 (Fig. 2E). This could be either because of its
partial overlap with residue 225–258 or because of overall
changes in structure of proteins. Furthermore direct evidence
of interaction with the aa 225–258 region and Hsp90 was con-
firmed by mammalian two-hybrid assay, where NSP3(225−258)

**DISCUSSION**

We had previously reported a positive role of cellular chap-
erone protein Hsp90 during rotavirus replication, but whether
the chaperone had any direct or indirect effect on virus was not
determined. The questions addressed were whether Hsp90
interacts directly with one or more rotavirus proteins, affecting
their structure and function, or whether it modulates various
steps indirectly during the maturation of the virus. In the pre-
ence of Hsp90 inhibitor, down-regulation of NSP3 protein
expression was observed in transfected or virus-infected cells
(Fig. 1, A and B). Although Hsp90 does not seem to have any
direct effect on other transfected rotavirus proteins (Fig. 1A), in
virus-infected cells an overall reduction in viral proteins was
observed at later time points (12 hpi), possibly because of
reduced viral titers in the presence of Hsp90 inhibitors (19).

Co-immunoprecipitation of NSP3 or FLAG-NSP3 with
Hsp90 (Fig. 2, A and B) and the lack of 17DMAG-mediated
down-regulation of NSP3 expression in the presence of protea-
somal inhibitor (Fig. 1C) provided evidence of a physical inter-
action between these proteins. The presence of a putative TPR-
expression of NSP3 and NSP5 mRNAs was assessed in
the polysome gradients of MA104 cells infected with SA11 in the

**17DMAG Reduces Translational Efficiency of Rotavirus RNA**—
NSP3 has been shown earlier to be involved in the efficient
translation of rotavirus RNA (36, 37). Thus, we tried to deter-
mine whether viral RNA translation was affected by Hsp90 inhib-
it. The distribution of NSP3 and NSP5 mRNAs was assessed in
the polysome gradients of MA104 cells infected with SA11 in the
presence or absence of 17DMAG. Infected cells were harvested at
6 hpi, and lysates were centrifuged through 0.5–1.5 M linear
sucrose gradients. RNA was extracted from each fraction, and
the distribution of NSP3 and NSP5 RNAs in the gradients was
analyzed by Northern blot hybridization. Fig. 8 shows the poly-
somal sedimentation profiles of the infected cell lysates under
the two conditions and the sedimentation of the NSP3 as well as
NSP5 mRNAs in the gradient. Major amount of both mRNAs
(>70%) were distributed in polysomal fractions (fractions 5–9)
in virus-infected cells in the absence of 17DMAG. However, in
the presence of 17DMAG >50% of the two mRNAs were dis-
tributed in the subpolysomal fractions (fractions 1–4, contain-
ing ribosomal subunits, untranslated messenger ribonucleo-
protein particles (mRNPs), and preinitiation complexes), indi-
cating that the translational efficiency of viral RNA is
reduced in the absence of functionally active Hsp90.
and C-90α -Hsp90 interaction resulted in a significant increase in activation of the luciferase reporter gene, whereas mutants of NSP3 (ΔNSP3) and Hsp90 (ΔC90-α) failed to do so (Fig. 4A). Although sequence alignment of SA11-NSP3 with other group A rotavirus strains revealed some variability within residues 225–258, an equivalent association of the chimeric NSP3 proteins with Hsp90 (Fig. 3B) emphasizes the significant role of the aa 225–258 region. It is possible that the Hsp90 binding property of aa 225–258 is not sequence-specific but depends on the structural conformation of α-helices (helix 1, aa 208–251) in the C-terminal portion of the NSP3 protein. The NSP3 sequence of SA11 was used in this study, but to avoid any strain-specific effect, all of the amino acids point-mutated in NSP3 were based on the conserved residues among different rotavirus strains.

The crystal structure of NSP3 has been confirmed as a dimer (34, 50). NSP3 protein has been shown to have an RNA-binding domain (aa 1–149) as well as an eIF4G-binding domain (aa 206–313) and a central coiled-coil dimerization (aa 150–240) domain (50). All of these domains are important for functionality of NSP3, as binding of a consensus sequence of viral RNA to the NSP3 homodimer not only makes the protein more stable (34) but is also required for translation of viral mRNAs. Although the eIF4G-binding and dimerization domains have been implicated in stimulating the translation of cellular polyadenylated mRNA (55), the simultaneous interaction of NSP3 with eIF4G and the 3′-end of viral RNA has also been shown to result in nuclear translocation of PABP, which shuts off host cell protein synthesis and efficiently translates viral mRNA (36–38). Previous studies have shown the RNA binding property of asymmetric homodimers formed by the N-terminal part of NSP3 (aa 1–149) (49). Surprisingly, despite the intact N-terminal domain, poor RNA binding activity was observed in NSP3-Δ225–258 (ΔNSP3) or the point mutants (Fig. 5A). Because the previous studies were based on the crystal structure of either only the N-terminal or the C-terminal domain (34, 35), it is possible that in full-length NSP3, mutations in the C-terminal region (residues 225–258) result in a changed conformation that prevents dimerization of the N-terminal domains. Further studies on the crystal structure of full-length NSP3 are required for an understanding of the dimerization process. Poor nuclear localization of cytosolic PABP in SA11 rotavirus-infected MA104 cells treated with 17DMAG (Fig. 1C) and little distribution of PABP in the nuclear lysates of 293T cells expressing point mutants within the 225–258 aa region and the Δ225–258 deletion mutant of NSP3 (Δ-NSP3) (Fig. 5B) further confirmed the role of Hsp90 in regulating NSP3 function. In the presence of 17DMAG, reduced translational efficiency of viral mRNA in virus-infected cell was observed, as NSP3 and NSP5 mRNAs were distributed more in subpolysomal fractions than in polysome gradients in 17DMAG-treated cells (Fig. 8).

Co-immunoprecipitation of the in vitro translation products of wild type or mutant NSP3 with Hsp90 antibody revealed the presence of only the monomeric form of WT NSP3 but not with deletion (residue 225–258) mutant (Fig. 4B, lower panel, lanes 2–7). Low levels of monomers co-precipitated in NSP3 point mutants. The IVT reaction mixtures of full-length NSP3 as well as NSP3 point mutants prior to co-immunoprecipitation showed the presence of both NSP3 dimers and monomers (supplemental Fig. 4). This suggests that Hsp90 is associated only with NSP3 monomers and is dissociated as dimerization occurs. This was further confirmed when the levels of NSP3 precipitated with Hsp90 decreased and NSP3 in the supernatant increased in a time-dependent manner following sequential immunoprecipitation of in vitro coupled transcription-translation products after chase (5–30 min) (Fig. 6A); this decrease in NSP3 associated to Hsp90 correlated with the formation of NSP3 dimers (Fig. 6). In addition, the absence of NSP3 dimers in the presence of 17DMAG (Fig 7A), the reduced ProLabel activity of full-length NSP3 ProLabel fusion protein in a low Hsp90 environment, and its restoration after supplementation with purified Hsp90 (Fig. 7B) altogether highlight the significance of Hsp90 during the formation of properly folded, stable NSP3.

The symmetric homodimerization of the C-terminal portion of NSP3 (residues 206–313) creates two binding pockets that bind to eIF4G. Both NSP3 C monomers interact with the same fragment of eIF4G (35). Most of the residues involved in eIF4G binding are in the C terminus of α-helix H3 (aa 273–304); however, residues 292–313 seem to be important because C-terminal truncation at aa 292 has been shown to abolish both eIF4G binding and NSP3-mediated stimulation of rotaviral mRNA translation (37). The Hsp90 binding region (residues 225–258) is a part of the eIF4G-binding domain of NSP3 (aa 206–313), and in addition it also partially overlaps the RoXaN binding region (aa 163–237). RoXaN (110 kDa) protein, like other Hsp90-binding proteins, has a TPR region at its N-terminal domain, and it has been shown to form a ternary complex with eIF4G1 and NSP3 proteins (56). Thus one could speculate that mutations within the C-terminal domain (aa 225–258) may alter the conformation of NSP3, resulting in disruption of the overall function of NSP3 including binding with other cellular proteins (eIF4G or RoXaN) irrespective of the presence of Hsp90. However, the reduced association of WT NSP3 with eIF4G in the presence of Hsp90 inhibitors (Fig. 5A), as well as its restoration following the exogenous addition of purified Hsp90 protein (Fig. 7C), suggests the significance of Hsp90 for efficient NSP3 function. It may be either that Hsp90 directly stabilizes the interaction between NSP3 and eIF4G or that the poor interaction in the absence of Hsp90 is due to the improper folding and assembly of NSP3.

Overall, based on our results it can be hypothesized that the binding of Hsp90 to NSP3 monomers probably not only protects NSP3 from proteasomal degradation but also results in a change in conformation that facilitates the dimer formation and the release of Hsp90 from NSP3 (Fig. 9). Even though a few amino acid residues of the predicted Hsp90 binding region of NSP3 are identical
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with respect to the consensus single TPR domain sequence, the post-translational association of Hsp90 with this region probably induces a change that is similar to the folding of other Hsp90-binding proteins. In the case of steroid receptors, evidence from reconstitution experiments suggests that a foldosome (comprising Hsp90, Hsp70, Hsp40, and p23) is first formed, which then associates with (and hence activates) the steroid receptor (57, 58). The fact that Hsp70 and Hsp90 are often found as a complex in the cytosol may render the possible involvement of Hsp70 as a part of the chaperoning function on NSP3. However, we do not yet have any direct evidence as to whether a similar foldosome is involved in NSP3 maturation. The involvement of a foldosome in NSP3 folding and assembly would have significant implications, because it would unify concepts pertaining to the chaperone-associated folding of cytosolic proteins in general. Further analysis is required to explore the detailed structural-functional relationship between NSP3 and the chaperone proteins.

REFERENCES

1. Blond-Elguindi, S., Cwirla, S. E., Dower, W. J., Lipshutz, R. J., Sprang, S. R., Sambrook, J. F., and Gething, M. J. (1993) Cell 75, 717–728
2. Nollen, E. A., and Morimoto, R. I. (2002) J. Biol. Chem. 277, 40041–40050
3. Mayer, M. P. (2005) Annu. Rev. Biochem. 74, 297–326
4. Pearl, L. V., and Workman, P. (2006) Annu. Rev. Biochem. 75, 271–294
5. Groth, C. M., and Morimoto, R. I. (2002) Cell. Mol. Life Sci. 59, 1640–1648
6. Nair, S. C., Toran, E. J., Rimerman, R. A., Hjermstad, S., Smithgall, T. E., Bork, P., Rychlewski, L., Kuster, B., Hunter, W. N., and Lipman, D. J. (1997) J. Virol. 71, 915–923
7. Schultz, J., Mülpetz, F., Bork, P., and Ponting, C. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 5857–5864
8. Henning, L. (1999) BioTechniques 26, 1170–1172
9. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Nucleic Acids Res. 25, 3389–3402
10. Keryer-Bibens, C., Legagneux, V., Namanda-Vanderbeken, A., Cosson, B., Bolte, S., Arold, S. T., and Poncet, D. (2008) J. Virol. 82, 5015–5025
11. Naito, T., Momose, F., Kawaguchi, A., and Nagata, K. (2007) J. Virol. 81, 1339–1349
12. Chase, G., Deng, T., Fodor, E., Leung, B. W., Mayer, D., Schwemmle, M., and Brownlee, G. (2008) Virology 377, 431–439
13. Uijono, S., Yamaguchi, S., Shimotohno, K., and Takaku, H. (2009) J. Biol. Chem. 284, 6841–6846
14. Dutta, D., Bagchi, P., Chatterjee, A., Nayan, M. K., Mukherjee, A., Chattopadhyay, S., Nagashima, S., Kobayashi, N., Komoto, S., Taniuchi, K., and Chavalda-Sarkar, M. (2009) Virology 391, 325–333
15. Rosen, J. W., Bouma, J., Raetzker, R. H., Bülker, H. A., and Einerhand, A. W. (2004) J. Virol. 78, 9712–9730
16. Estes, M. K., and Kapikian, A. Z. (2007) in Fields Virology (Knipe, D. M., Howley, P. M., Griffin, D. E., Lamb, R. A., Martin, M. A., Roizman, B., and Straus, S. E., eds) Vol. 2, 5th Ed., pp. 1917–1974, Lippincott Williams & Wilkins, Philadelphia
17. Autret, A., Martin-Lattil, S., Brissac, L., Mousson, L., Colbère-Garapin, F., and Blondel, B. (2008) J. Virol. 82, 3796–3802
18. Bitko, V., Shulayeva, O., Mazumder, B., Musiyenko, A., Ramaswamy, M., and Mukherjee, A., Chattopadhyay, S., Nagashima, S., Kobayashi, N., Komoto, S., Taniuchi, K., and Chavalda-Sarkar, M. (2009) Virology 391, 325–333
19. Naito, T., Momose, F., Kawaguchi, A., and Nagata, K. (2007) J. Virol. 81, 1339–1349
20. Chase, G., Deng, T., Fodor, E., Leung, B. W., Mayer, D., Schwemmle, M., and Brownlee, G. (2008) Virology 377, 431–439
21. Uijono, S., Yamaguchi, S., Shimotohno, K., and Takaku, H. (2009) J. Biol. Chem. 284, 6841–6846
22. Dutta, D., Bagchi, P., Chatterjee, A., Nayan, M. K., Mukherjee, A., Chattopadhyay, S., Nagashima, S., Kobayashi, N., Komoto, S., Taniuchi, K., and Chavalda-Sarkar, M. (2009) Virology 391, 325–333
23. Rosen, J. W., Bouma, J., Raetzker, R. H., Bülker, H. A., and Einerhand, A. W. (2004) J. Virol. 78, 9712–9730
24. Estes, M. K., and Kapikian, A. Z. (2007) in Fields Virology (Knipe, D. M., Howley, P. M., Griffin, D. E., Lamb, R. A., Martin, M. A., Roizman, B., and Straus, S. E., eds) Vol. 2, 5th Ed., pp. 1917–1974, Lippincott Williams & Wilkins, Philadelphia
25. Autret, A., Martin-Lattil, S., Brissac, L., Mousson, L., Colbère-Garapin, F., and Blondel, B. (2008) J. Virol. 82, 3796–3802
26. Bitko, V., Shulayeva, O., Mazumder, B., Musiyenko, A., Ramaswamy, M., and Mukherjee, A., Chattopadhyay, S., Nagashima, S., Kobayashi, N., Komoto, S., Taniuchi, K., and Chavalda-Sarkar, M. (2009) Virology 391, 325–333