Title: Identification of Hsp90 as a stimulatory host factor involved in influenza virus RNA synthesis

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Running Title: Hsp90 stimulates influenza virus RNA synthesis
Abstract

Efficient transcription and replication of the influenza virus genome are dependent upon host-derived factors. Using an in vitro RNA synthesis system, we have purified and identified Hsp90 as one of the host factors that stimulate viral RNA polymerase activity. Hsp90 interacted with the PB2 subunit of the viral RNA polymerase through the amino terminal chaperone domain and the middle region containing a highly acidic domain. The acidic middle region was also responsible for its stimulatory activity. We found that a portion of Hsp90 is re-localized to the cell nucleus after viral infection. A PB2 fragment containing a Hsp90 binding domain inhibited viral gene expression in a dominant-negative manner. These results suggest that Hsp90 is a host factor for the influenza virus RNA polymerase.
Introduction

Influenza A virus belongs to the *Orthomyxoviridae* family and its genome consists of eight segmented, single-stranded RNA of negative polarity (1). The transcription promoter and the replication signal of the viral genome exist at the 3' and 5' termini of each of the eight segments. Components associated with ribonucleoprotein complexes (vRNP) purified from virions are the minimum factors required for primary transcription. The genome RNA forms vRNP with the viral RNA polymerases consisting of three subunits, PB2, PB1, and PA (2), and nucleocapsid protein (NP). Transcription of the influenza virus genome is initiated with host-derived oligo RNA containing a cap structure. PB2 contains cap recognition domains at its carboxyl terminal region. The capped RNA bound to PB2 is cleaved by the PB1 subunit 10-15 bases downstream from the 5' end (2, 3, 4) and the capped RNA fragment serves as a primer for viral mRNA synthesis catalyzed by PB1 (5). Elongation of the RNA chain proceeds until the polymerase reaches a polyadenylation signal consisting of 5-7 uracil (U) residues located near the 5' terminal region of the vRNA (6). The viral RNA polymerase polyadenylates the nascent RNA chain possibly by a slippage mechanism at the U-stretch (7). Replication of the vRNA is thought to take place by a primer-independent, two-step reaction, namely, the complementary RNAs (cRNA) are first synthesized from vRNA templates, and then the progeny vRNAs are amplified from cRNA templates. Genetic analyses suggest that PA participates in the replication process (8). However, vRNP complexes isolated from virions are incapable of catalyzing replication reactions.

A variety of host proteins have been identified as factors involved in the regulation of the RNA synthesis of viral genomes of *Paramyxoviridae*, the genome of which contains non-segmented and single-stranded RNA of negative polarity. Tubulin, an acidic cytoplasmic
structural protein, is one of the host factors for RNA synthesis of the measles virus, VSV, and Sendai virus genomes (9, 10). RNA synthesis of these viral genomes is catalyzed by viral RNA polymerases consisting of L and P subunits. Tubulin interacts with L protein, a catalytic subunit of the viral RNA polymerase, and is present in isolated transcription initiation complexes (11). Since replication and regulated transcription of the influenza virus genome do not occur only by influenza viral components associated with virions, it has long been thought that some factor(s) present in infected cells is required to carry out these processes. In fact, several host cellular proteins were identified as factors that interact with influenza virus NP and nonstructural protein 1 (12).

We have identified host factors that are involved in the regulation of influenza virus RNA synthesis; these factors were derived from uninfected HeLa cell nuclear extracts by biochemical complementation using an \textit{in vitro} RNA synthesis assay with vRNP complexes isolated from virions (13, 14). In this system, we have used a 53 base-long exogenously added model viral genome (53-merVwt) that contains 15 nucleotides of the 3'-terminal sequence and 22 nucleotides of the 5'-terminal sequence of A/Puerto Rico/8/34 segment 8 vRNA. The stimulatory activity in the extract was examined and fractionated by measuring RNA synthesized from this model viral genome template. With this system, we identified host factors designated as RAF (RNA polymerase activating factor) -1 and RAF-2 that stimulate viral RNA synthesis (14). Subsequently, we revealed that RAF-2 consists of two polypeptides designated as RAF-2p48 and RAF-2p36; moreover, we found that RAF-2p48 is identical with UAP56/BAT1, a factor that is generally thought to be related to RNA splicing (15). RAF-2p48 interacts with NP and is involved in NP-RNA complex formation, thereby stimulating viral RNA synthesis by viral RNA polymerase.
Here, we purified RAF-1 and identified it as Hsp90, a known heat shock protein that functions as a molecular chaperone, together with some conjugating factors (16). Recently, it was reported that Hsp90 functions as a capacitor of phenotypic variation in both insects as well as plants (17, 18). In this report, we demonstrated that Hsp90 interacts with the PB2 subunit through its amino terminal chaperone domain and its middle region containing a highly acidic domain. The RAF-1 stimulatory activity of Hsp90 depended on this acidic middle region. Furthermore, we found that a portion of Hsp90 is re-localized to the nucleus after viral infection. In this context, we have addressed the function of heat shock proteins in terms of host factors involved in the replication of viruses.
Experimental procedures

**In vitro Viral RNA Synthesis**

In this report, all viral resources were derived from influenza A/Puerto Rico/8/34 virus. vRNP was purified from virions as previously described (19) and was used as the enzyme source in the *in vitro* RNA synthesis assays. The 53 base-long model vRNA designated as 53-merVwt (5'-AGUAGAAAACAAGGGUGUUUUUCAUAUUAUUACUACCACCUUUUGCU-3') was synthesized by transcription with MEGAscript T7 Kits (Ambion) and synthetic DNA templates, as previously described (13). *In vitro* RNA synthesis was carried out at 30°C for 60 minutes in 25 µl of a reaction mixture containing 50 mM HEPES-NaOH (pH 7.9); 3 mM MgCl₂; 50 mM KCl; 1.5 mM dithiothreitol (DTT); 500 µM each of ATP, GTP, and CTP; 25 µM UTP; 5 µCi of [α-³²P] UTP (400 Ci/mmol); 10 U of RNase inhibitor; 25 µg/ml of actinomycin D; 250 µM ApG; 5 ng of a 53-merVwt; and vRNP (10 ng NP equivalents) in the presence or absence of the host factor fractions. A limited elongation assay was carried out in the same reaction mixture, with the exception that UTP and [α-³²P] UTP were omitted, and 500 µM each of ATP and CTP were included, as well as 25 µM GTP, and 5 µCi of [α-³²P] GTP (400 Ci/mmol). Run-off RNA synthesis was carried out with a 35-mer adenine (A) residue-less template (35-merV-a; 5'-GUUCUUCUUUCUUUCUUUCUGCCUGCUUUUGCU-3'), which contained the 12 base-long conserved promoter sequence for the viral RNA polymerase at the 3’ terminal region and the subsequent 23 base-long sequence lacking the A-residues. The other materials for the run-off RNA synthesis were the same as those used for the standard or limited elongation assays.
The nucleotide sequences of the plasmids used in this study were confirmed by DNA sequencing. The plasmid containing full-length Hsp90α and β cDNAs were the kind gift of Dr. I. Yahara (the Tokyo Metropolitan Institute of Medical Science, Japan). The Hsp90α cDNA portion was amplified using LA-Taq polymerase (TaKaRa), with the plasmid as the template and specific primers, 5'-CCTGAGGAAACCCAGACCCA-3' and 5'-GCGTCTACTTCTTCCATGCGTGAT-3', corresponding to the Hsp90α amino-terminal and carboxyl-terminal regions, respectively. For the preparation of the carboxyl-terminal hexa-histidine-tagged Hsp90α expression vector, the amplified cDNA fragment was phosphorylated with polynucleotide kinase (TOYOBO) and ligated into pET-14b (Novagen) that had been digested with Nco I and filled with Klenow fragment (TaKaRa). For the preparation of the carboxyl-terminal GST-tagged Hsp90α expression vector, the Hsp90α cDNA fragment was ligated into a pET-GST vector, a pET-14b derivative containing GST cDNA in place of the DNA sequence for the hexa-histidine tag. Hsp90α deletion mutant and Hsp90β expression vectors were constructed via the same method as that used for constructing a wild-type Hsp90α expression vector, with a primer combination corresponding to the amino-terminal and carboxyl-terminal regions of each mutant. The GST-tagged human Hsp70 (heat shock 70 KDa protein 1A, GenBank accession number 5579469) expression vector, pGEX6P-Hsp70, was constructed with a pGEX-6P-1 vector and an Hsp70 cDNA fragment. The Hsp70 cDNA was amplified from a HeLa cell cDNA library using specific primers, 5'-GCGGATCCCATATGGCCAAAGCCGCGGCGA-3' and 5'-CCGGATCCTAATCCACCTCCTCAATGGTA-3', corresponding to the Hsp70 amino-terminal
and carboxyl-terminal regions, respectively. The amplified fragments and the pGEX-6P-1 vector were digested with *Bam*H I and were ligated.

The plasmids thus constructed were used for the transformation of the *E. coli* BL21 strain for the preparation of recombinant proteins. Expression and purification of histidine- or GST-tagged proteins were carried out according to the manufactures’ instructions (Novagen and Amersham Biosciences, respectively). Recombinant proteins were further purified by Mono Q column chromatography as native RAF-1, and dialyzed against Buffer H (50 mM HEPES-NaOH (pH 7.9), 10% Glycerol, 1 mM DTT) containing 50 mM KCl. Recombinant Hsp70 protein was prepared from purified GST-Hsp70 by digestion with PreScission protease (Amersham Biosciences) and dialyzed as described above. Concentrations of these recombinant proteins were determined by electrophoretic separation on 10% SDS-polyacrylamide gels and Coomassie Brilliant Blue staining.

The labeled PB2 was prepared by *in vitro* translation of PB2 mRNA with rabbit reticulocyte lysates (TNT Quick Coupled Transcription/Translation Systems, Promega), and [³⁵S]-methionine (1,000 Ci/mmol at 10 mCi/ml), according to the manufacturer’s instructions. PB2 mRNA was synthesized in a transcription and translation reaction mixture that contained T7 RNA polymerase, and plasmid pET3a-PB2 containing PB2 cDNA, which was prepared from the A/Puerto Rico/8/34 PB2 gene. The PB2 fragments, N515 and N383, were prepared with mRNAs synthesized from pET3a-PB2 digested with *Ava* I and *Pvu* II, respectively.

**GST Pull-Down Assay**

Ten picomole of GST or GST-tagged recombinant protein was fixed on 10 µl (bed volume) of glutathione sepharose beads (Amersham Biosciences). The binding reaction was carried out at 37°C for 60 minutes in a final volume of 100 µl, which contained 50 mM HEPES-
NaOH (pH 7.9), 3 mM MgCl₂, 50 mM KCl, 1.5 mM DTT, and the affinity beads in the presence or absence of 5 µl of vRNP (approximately 250 ng of the NP equivalent). After adsorption, the beads were washed three times with a NETN buffer containing 20 mM Tris-HCl (pH7.9), 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40. Proteins bound to the affinity beads were eluted by boiling them in an SDS-PAGE loading buffer and then they were subjected to 7.5% SDS-PAGE. In order to identify each viral RNA polymerase subunit, rabbit anti-PB1, anti-PB2, and anti-PA antisera (gifts from Dr. T. Toyoda) were used for the immunoblotting analyses.

To calculate the amount of [³⁵S]-labeled PB2 bound to the affinity beads, the proteins were separated by 10% SDS-PAGE. The gels were subjected to autoradiography, and the [³⁵S]-labeled PB2 was quantified using an image analyzer, BAS2000 (Fuji Film).
Results

Identification of RAF-1 as Hsp90α and -β

Using an in vitro influenza virus RNA synthesis system with an exogenously added 53 base-long model virus genome (53-merVwt), we found stimulatory host factors for the viral RNA polymerase, designated as RAF (RNA polymerase activating factor) -1 and RAF-2, in nuclear extracts prepared from uninfected HeLa cells (14). Previously, we demonstrated by biochemical fractionation that RAF-2 consists of 48 kDa and 36 kDa polypeptides; we found that the former peptide is identical with BAT1/UAP56, a putative splicing factor (15). Here, we purified RAF-1 as a host factor that stimulated viral RNA synthesis; purification proceeded to apparent homogeneity through sequential column chromatographies (14) (Fig. 1A). The native molecular mass of RAF-1 was estimated as 350 kDa on gel filtration chromatography (14) (data not shown) and the major polypeptide in the active fraction showed a molecular mass of 90 kDa, as estimated by SDS-PAGE (Fig. 1B). This observation suggests that the purified RAF-1 exists as an oligomer, presumably a tetramer, of the 90 kDa polypeptide.

Next, we analyzed the amino acid sequences of five peptides that were derived from the 90 kDa RAF-1 polypeptide by cleavage with a lysyl endopeptidase. The determined sequences were exactly identical with portions of human Hsp90α and Hsp90β (Table I), indicating that RAF-1 is a mixture of Hsp90α and Hsp90β. Hsp90 is a typical molecular chaperone highly conserved among species, and mainly exists in the cytoplasm as a homodimer or as an oligomer consisting of αα or ββ homodimers (20, 21, 22). To perform a functional analysis of RAF-1/Hsp90 as a stimulatory host factor for influenza virus RNA synthesis, we prepared recombinant human Hsp90α and Hsp90β, each containing a histidine tag at the carboxyl
terminus. We then examined their stimulatory activity as regards the *in vitro* viral RNA synthesis. The recombinant Hsp90α (Fig. 1C, lanes 6-10) demonstrated approximately the same level of stimulatory activity as that of Hsp90β (data not shown), and recombinant Hsp90α demonstrated slightly higher stimulatory activity than did purified RAF-1/Hsp90 (lanes 2-5). We assume that a portion of purified RAF-1/Hsp90 might have been inactivated during the process of column chromatography. These results confirmed that Hsp90α and Hsp90β are the active components in RAF-1. Hsp90α and -β have quite similar domain structure organization, and the similarity of amino acid sequences between Hsp90α and -β is approximately 93%.

A limited elongation assay was carried out, in which UTP was omitted from the reaction mixture of the *in vitro* RNA synthesis system, and only initiation and subsequent elongation reactions occurred, producing short oligoribonucleotides. The results of this assay suggested that the RAF fraction recovered in fractions eluted from the Mono Q column with 0.3 M KCl (Fig. 1A) stimulated the initiation of viral RNA synthesis (14). In the limited elongation assay with the 53-merVwt, the viral RNA polymerase proceeded up to the first A residue on the template and generated 13-mer RNA (Fig. 2A, upper panel) (14), since the sequence of the 53-merVwt contained the 5' and 3' terminal sequences of segment 8 (13). To further confirm the effects of RAF-1/Hsp90 on RNA synthesis in the UTP-limited system and thereby to gain information about the function of RAF-1/Hsp90, we designed a novel 35-mer RNA template (35-merV-a) (Fig. 2A, lower panel). This RNA template consisted of 12 bases of the viral minimum promoter and an A-free 23 base-long tail, such that a 35-mer run-off RNA product was to be synthesized both in the presence and absence of UTP. Run-off RNA synthesis assays with the 35-merV-a were then carried out (Fig. 2B). As expected, the 35-mer RNA product was detected in the standard reaction mixture containing 4 NTPs (lane 1), suggesting that this model viral
RNA was functional. RAF-1/Hsp90 stimulated synthesis of the 35-mer RNA product (lane 2). In the limited elongation condition lacking UTP, the 35-mer RNA was synthesized, along with short RNAs of 12-19 bases derived from endogenous viral RNA segments (lane 3). This finding suggests that the RNA synthesis from exogenously added model RNA templates was catalyzed by not only an RNA polymerase that proceeded toward and fell off of the end of the endogenous RNA template, but also by an RNA polymerase that paused and fell off at the first A residue. RAF-1/Hsp90 also stimulated the limited elongation reaction and synthesis of the 35-mer RNA product (lane 4). These results suggest that RAF-1/Hsp90 exerted its activity at the steps prior to the early elongation stage (see Discussion).

Interaction of Hsp90 with PB2, a Viral RNA Polymerase Subunit

Since RAF-1/Hsp90 was identified as a stimulatory factor in the in vitro viral RNA synthesis system with vRNP as viral factors, a target(s) of Hsp90 must have been the viral RNA polymerase, NP, and/or the viral genome. When partially purified RAF-1/Hsp90 was mixed with vRNP and incubated under the RNA synthesis condition without ApG dinucleotide primer and NTPs, a portion of Hsp90 co-sedimented with vRNP in a glycerol density gradient centrifugation assay (Fig. 3A, arrowheads in lanes 6 and 7). To determine the viral factor that interacts with Hsp90, we carried out GST pull-down assays with purified vRNP and a recombinant Hsp90α that contains the glutathione S-transferase at its carboxyl terminus (Hsp90α-GST). After binding and washing steps (see Discussion), one of the viral RNA polymerase subunits, PB2, was observed bound to Hsp90α-GST (Fig. 3B). A trace level of PB1 subunit was detected, while the PA subunit remained for the most part unbound. The PB2-Hsp90 interaction was also detected in the presence of ribonuclease A (data not shown),
suggesting that these proteins do not interact via RNA. Hsp90β also bound to PB2 as Hsp90α, indicating that PB2 would interact with a homologous region between Hsp90α and β (Fig. 3C).

Although RAF-1/Hsp90 was purified by monitoring the stimulatory activity for the viral RNA polymerase, other heat shock proteins may show similar activity. Thus, we tried to examine the effect of Hsp70 (heat shock 70 KDa protein 1A), one of the major Hsp70 proteins, in our system. In the GST pull-down assays, Hsp70 was unable to bind to PB2 as strongly as did Hsp90 (Fig. 4A). Slight GST-Hsp70 and PB2 interaction was detected only when a low-salt buffer (containing 50 mM NaCl) was used for washing (lane 6). In contrast, this slight interaction was completely abolished when a high-salt buffer (containing 1.0 M NaCl) was used for washing, a condition under which Hsp90 was bound to PB2 (lane 4). The other subunits were not bound to Hsp70 under either the low- or the high-salt buffer conditions. We then carried out in vitro RNA synthesis with recombinant Hsp70 (Fig. 4B; lanes 6-9), which could not stimulate viral RNA synthesis as effectively as could Hsp90α (lanes 2-5). The low level of stimulatory activity in the case of Hsp70 could be the result of its general chaperone activity. Thus, the RAF stimulatory activity of RAF-1/Hsp90, possibly through PB2, could not be replaced by Hsp70.

**Analysis of the Domains Involved in Hsp90α-PB2 Interaction**

Next, we tried to determine the domains involved in the interaction between Hsp90α and PB2. Based on the reports (20, 21, 22, 23, 24) concerning the domain organization of Hsp90, we divided Hsp90α into three regions, a hydrophobic amino terminal region (N), a highly acidic middle region (M), and a carboxyl terminal homo-dimerization region (C), as shown in Fig. 5A. GST pull-down assays were carried out with three Hsp90α deletion mutants and wild-type
Hsp90α containing GST at their carboxyl termini in binding buffers containing 50 mM (low-salt) or 1.0 M (high-salt) KCl. After the binding reaction was carried out at 37°C for 60 minutes, the affinity beads were washed twice with NETN buffer containing 100 mM (low salt) or 1.0 M NaCl (high salt). Hsp90α mutants and wild-type Hsp90α interacted with the PB2 subunit (Fig. 5B), but not with the PB1 and PA subunits (data not shown). Wild-type Hsp90α was able to interact with PB2, irrespective of low- or high-salt binding and low- or high-salt washing conditions (lane 9), although the high-salt wash slightly decreased the PB2-Hsp90α interaction. All of the Hsp90α mutants were capable of interacting with PB2 under low-salt washing conditions (1st and 3rd panels from the top), but with less efficiency than that observed in the case of wild-type Hsp90. Under the high-salt washing condition, the Hsp90α carboxyl terminal mutant (C) released PB2 (2nd and 4th panels, lane 7). Thus, the N and M regions were capable of binding to PB2 more strongly than was the C region. These results indicate that each Hsp90 region was involved with a different affinity in the Hsp90α-PB2 interaction.

The stimulatory activities of these mutants were examined in an in vitro RNA synthesis system using equal moles of Hsp90α deletion mutants (Fig. 5C). Although these three mutants were able to interact with PB2 under the low-salt condition, which was also used in the RNA synthesis system, their stimulatory activities were significantly different. The N and C regions of Hsp90α showed a quite low level of stimulatory activity (lanes 3 and 5), and their activities were much lower than that of either purified RAF-I/Hsp90 (lane 1) or full-length recombinant Hsp90α (lane 9). In contrast, the specific activity of the M region was clearly higher than that of the others (lane 4). Mutant proteins containing the M region in connection with other regions, such as NM (lane 8) and MC (lane 6) regions, showed higher activity than did N or C alone. Densitometric scanning analysis revealed that NM possessed slightly higher
activity than M. On the other hand, the NC mutant lacking the M region was as inefficient at stimulation as was N or C mutant alone. Taken together with results of GST pull-down assays (Fig. 5B), it is suggested that Hsp90 interacts with PB2 through N, M, or NM; moreover, the Hsp90 middle region, which contains a highly acidic region, is responsible for the RAF-1 stimulatory activity of Hsp90α.

Next, we tried to roughly estimate a Hsp90 binding site in PB2. GST pull-down assays were carried out with PB2 proteins and either an Hsp90α NM region or wild-type Hsp90α tagged with GST at their carboxyl termini (Fig. 6). The labeled wild-type PB2 and PB2 fragments, N515 and N383, (Fig. 6A) were produced with rabbit reticulocyte lysates, [35S]-methionine, and PB2 mRNAs encoding each PB2 protein, because recombinant wild-type and mutant PB2 proteins could not be synthesized efficiently in E. coli cells. The binding efficiencies of wild-type PB2 and N515 fragments to wild-type Hsp90α and the NM region were approximately equal. The N383 fragment showed less binding activity toward the NM region. Therefore, it is possible that the NM region, which is fully active as regards RAF-1 activity (Fig. 5C), interacts with PB2 through the region between its amino-terminus and the amino acid position 515. N383 was able to bind to wild-type Hsp90α more efficiently than to the NM region. Although we do not know the exact reason for this at present, we assume that wild-type Hsp90 binds to the N383 fragment as a chaperone as well as RAF-1. The structure of the PB2 fragment would be much less stable than that of the wild-type protein, and thus would be targeted by the Hsp90 chaperone. Since dimerization and/or oligomerization of Hsp90 is required for its chaperone activity (20, 22), Hsp90α NM lacking the carboxyl terminal region that is involved in dimerization and/or oligomerization was unable to behave as a molecular chaperone.
There appears to be a contradiction regarding the observation that the majority of Hsp90 molecules is localized in the cytoplasm of uninfected cells (Fig. 7B) (24), while viral RNA synthesis takes place in the nuclei of infected cells (Fig. 7C). We hypothesized that the intracellular localization of Hsp90 could be changed during viral infection. This was indeed the case (Fig. 7D). In infected cells, Hsp90 was localized in both the nucleus and the cytoplasm, although the total amounts of Hsp90 protein determined by Western blot analysis with rabbit anti Hsp90 antiserum remained unchanged (data not shown). At present, the exact mechanism of translocation of Hsp90 from the cytoplasm to the nucleus upon infection has not been determined (see Discussion).

Next, we tried to examine whether inhibition of the Hsp90-PB2 interaction has an effect on viral gene expression or not. It has been shown with GST pull-down assays that the PB2 region spanning the area between amino acid positions 383 and 515 was involved, in part, in the interaction with Hsp90α (Fig. 6B). Based on this observation and the functional domain map of PB2 (Fig. 6A), we prepared a PB2 deletion mutant spanning amino acid positions 258 and 401 with an additional myc-tag sequence at its amino terminus (myc-258-401). This deletion mutant contained a putative Hsp90α binding domain, but not contained the functional domains already reported, such as the cap structure recognition sequence, PB1 binding domain, and nuclear localizing signals (NLS) (25). It is expected that in infected cells, myc-258-401 disturbs the interaction between PB2 and Hsp90 in a dominant negative-manner but does not inhibit the assembly of viral RNA polymerase subunits, nor does it inhibit the cap structure recognition activity of wild-type PB2. Furthermore, myc-258-401 may inhibit the nuclear localization of
Hsp90 if the nuclear localization of Hsp90 depends on PB2-Hsp90 interaction, because myc-258-401 does not contain the NLS sequence.

Nuclear localization of a fraction of Hsp90 was also detected (Fig. 7J and N) in cells co-transfected with pCAGGS-PB2-Myc, encoding a PB2-containing carboxyl-terminal myc-tag (Fig. 7F), and pCAGGS-FLAG-Hsp90α, encoding an Hsp90α-containing amino-terminal FLAG-tag (Fig. 7J). On the other hand, nuclear localization of FLAG-Hsp90α was less often observed in cells co-expressing myc-258-401 (Fig. 7I and M). Myc-258-401 was present around the outside periphery of the nucleus and/or in spots in the cytoplasm (Fig. 7E and G) and it co-localized with FLAG-Hsp90α at the outside periphery of the nucleus (Fig. 7M). Cytoplasmic vacuole-like spaces where both FLAG-Hsp90 and myc-258-401 are not present were observed in a few percent of the cells expressing the myc-258-401 (panel M, cell on the right).

HeLa cells that had been transfected with myc-258-401 or NP-myc expression vector (Fig. 7G, K, and O and Fig. 7H, L, and P, respectively) were infected at 12 hours post-transfection with influenza virus A/Puerto Rico/8/34 at a m.o.i. of 3. At 9 hours post-infection, cells were fixed and subjected to indirect immunofluorescence assays. Myc-258-401 was localized around the outside periphery of the nucleus (Fig. 7G), as shown in panel E. In myc-258-401-negative cells, the cell membrane was reactive to anti-influenza virus A/Puerto Rico/8/34 HA antiserum (Fig. 7K). In sharp contrast, myc-258-401-positive cells were markedly reduced in their expression of HA (Fig. 7K, arrowheads). Expression of NP-myc (Fig. 7H) did not have such an effect (Fig. 7L and P, arrowheads). This was also the case in cells expressing GFP (data not shown). These observations suggest that the inhibitory effect of myc-258-401 may be interpreted as related to the competitive binding of myc-258-401 to Hsp90,
resulting in a decrease or loss of the viral RNA synthesis stimulatory activity of Hsp90. Alternatively, the decrease in the Hsp90-PB2 interaction may cause destabilization of nascent PB2. Basic subunits of the viral RNA polymerase would tend to aggregate and to be inactivated without an appropriate chaperone-like molecule (12).
Discussion

In this manuscript, we demonstrated the possibility that influenza virus requires the host cellular stress protein, Hsp90, as a stimulatory host factor for its RNA synthesis. Hsp90 is a typical molecular chaperone highly conserved among higher eukaryotes, and mutations in this protein cause serious disorganization of cellular metabolism (26). The ubiquity of this protein would be advantageous to viral infections among animals, such as the influenza virus. In this report, we demonstrated that Hsp70 could not function as a stimulatory host factor, in spite of its functional similarity as a molecular chaperone (Fig. 4B). Hsp70 is highly conserved among species and also functions as a molecular chaperone, as does Hsp90. Therefore, it is speculated that the protein chaperone activity of Hsp90 may not be responsible for the RAF-1 stimulatory activity. In fact, we found that the stimulatory activity of Hsp90 resides in its middle region but not in its amino terminal chaperone domain (Fig. 5C), although both regions would be required for stable interaction with PB2 (Fig. 5B). Furthermore, Hsp70 is known as a general molecular chaperone, whereas interactors with Hsp90 are more specific to that protein (27). Thus, the interaction between Hsp90 and PB2 might be specific (Figs. 3 and 4), even if Hsp90 functions as a chaperone for PB2.

In this report, we used a newly developed 35-mer model viral RNA, 35-merV-a, to examine the mechanism of stimulation of viral RNA synthesis. The efficiency of RNA synthesis with the 35-merV-a seemed to be greater than that with the 53-merVwt template (Fig. 2B). This observation could be due to the assumption that the recycling of the RNA polymerase on the run-off template was more effective than that on the 53-merVwt template. In the limited elongation assay condition lacking UTP, RAF-1/Hsp90 was capable of stimulating RNA synthesis not only from 35-merV-a, but also from endogenous vRNA (Fig. 2B), suggesting that
RAF-1/Hsp90 plays a role during the steps leading to the early elongation stage. RAF-1/Hsp90 may facilitate dissociation of the RNA polymerase on vRNP; RNA polymerase is located at the promoter region but does not yet initiate RNA synthesis, or RNA polymerase pauses on the template during elongation. However, this hypothesis could not be the case, since the RAF fraction stimulated RNA synthesis from the 53 base-long RNA template by vRNA-free purified RNA polymerases (14). Instead, it is thought that RAF-1/Hsp90 facilitates the association of RNA-free RNA polymerases to template RNA and/or stabilizes the RNA polymerase during its translocation between templates. This assumption is supported by our preliminary result that pre-incubation of vRNP resulted in inactivation of the RNA polymerase activity, whereas this inactivation was suppressed to some extent in the presence of RAF-1/Hsp90 (data not shown).

The acidic middle domain of Hsp90 is involved in RAF-1 activity. We have proposed the term “acidic molecular chaperone” to describe proteins that contain highly acidic regions and function as chaperones for basic proteins, thanks to their acidic properties, which allow them to imitate the nature of a nucleic acid (12). Transcription and replication from basic protein-nucleic acid complexes, such as ribonucleoprotein and chromatin templates, require the dissociation and re-association of nucleic acid-binding proteins from and to nucleic acids. The acidic molecular chaperone prevents aggregation and inactivation of basic proteins, and facilitates association and dissociation of basic proteins to/from nucleic acids. A variety of host factors involved in viral genome functions could be categorized as acidic molecular chaperones. Acidic cytoskeletal proteins such as tubulin (9, 10) and actin (28, 29) are stimulatory host factors for viral RNA synthesis of vesicular stomatitis virus, Sendai virus, and measles virus (tubulin) and for respiratory syncytial virus, human parainfluenza III viruses (actin) (30). Interestingly, the P subunit of the paramyxoviridae family RNA polymerase contains the conserved functional acidic domain at its amino-terminal region (31, 32, 33, 34). The amino-terminal region of VSV P
protein, referred to as domain I, has been shown to facilitate viral transcription in conjunction with carboxyl-terminal regions designated as domains II + III \textit{in trans} (35). The acidic region of VSV P protein is functionally exchangeable with a full-length of \(\beta\)-tubulin (36). The chimeric P protein in which domain I is replaced with \(\beta\)-tubulin is capable of functioning as the viral RNA polymerase subunit (36). In the case of influenza virus, RAF-2p48/UAP56/BAT1 containing an acidic region has been found to function as a chaperone for NP (15). This has also been demonstrated in the case of DNA virus. Template activating factors-I, -II, and -III contain functional highly acidic regions and stimulate adenovirus transcription and replication from viral DNA complexed with basic core proteins or histones (37, 38, 39). Along these lines, we hypothesized that Hsp90 may function as the acidic molecular chaperone for PB2, a basic polymerase subunit.

An alternative aspect of the Hsp90 function in influenza virus RNA synthesis is that Hsp90 may be involved in the modulation of the activity and structure of viral RNA polymerase. It is reported that Hsp90 is involved in hepadnavirus reverse-transcription (40). Hepatitis B virus genome replication includes a reverse-transcription step of the pre-genomic RNA that is catalyzed by viral reverse transcriptase-Hsp90 complexes (41). The influenza virus RNA polymerase complex, comprised of PB2, PB1, and PA, is stably formed by strong binding between PB2 and PB1 and between PB1 and PA (25). However, it has been reported using cells expressing these subunits that a combination of PB2 and PB1 can catalyze transcription, whereas the combination of PB1 and PA supports replication (42, 43). Dissociation of the complex into each subunit has not been observed under physiological conditions. It remains of interest that the PB1 and PA subunits were found to be easily released from the polymerase complex that was bound to Hsp90 in GST pull-down assays (Fig. 3\textit{B} and Fig. 4\textit{A}). It seems likely that binding of
Hsp90 to PB2 in the polymerase complex loosens PB2-PB1 interactions. It would be worthwhile to examine whether or not Hsp90 is one of the triggers involved in a possible conversion of the RNA polymerase complexes as regards their structure and function. Immunoprecipitation assays have revealed the interaction between Hsp90 and the RNA polymerase \textit{in vivo} (unpublished observation). However, no Hsp90 molecule was detected in 1 µg NP-equivalent purified vRNP or in virions when tested by Western blot analysis with anti-Hsp90 polyclonal antiserum and an enhanced chemiluminescence system (data not shown). Therefore, we suggest here that there may be a mechanism(s) for the dissociation of PB2-Hsp90 interactions when nascent vRNPs are packaged in progeny virions and/or there may be a mechanism for the elimination of the Hsp90-vRNP complex from the packaging process.

It was unexpected that Hsp90/RAF-1 could be purified from uninfected HeLa cell nuclear extracts, since a major fraction of Hsp90 is localized in the cytoplasm, and only a low level of Hsp90 is present in nuclei (Fig. 7\textit{B}) (24). We might have purified Hsp90 present in the nuclei, giving this minor fraction of Hsp90, and/or Hsp90 might have contaminated in the nuclear fractions during the course of cell fractionation. In infected cells (Fig. 7\textit{D}), Hsp90 is re-localized to nuclei, where the transcription and replication of influenza virus take place. At present, it remains unclear whether the re-localization of Hsp90 is caused by cellular stress during infection or by co-migration with viral nuclear protein(s). Recently, it was reported that in vaccinia virus-infected cells, Hsp90 was transiently associated with virosomes that were localized in cytoplasm (44). Along these lines, it is possible that the nuclear re-localization of Hsp90 in influenza virus-infected cells takes place as a co-migration with viral RNA polymerase, possibly with the PB2 subunit. The possibility that newly synthesized PB2 is a carrier candidate (Fig 7\textit{N}) is being examined further by our group.
Expression of the PB2 deletion mutant, myc-258-401, exerted a significant effect on the localization of co-expressed FLAG-Hsp90α and on the expression of influenza virus HA protein. In some cells expressing myc-258-401 PB2 fragment, vacuole-like spaces were generated (Fig. 7M), which have also been observed in cells that were cultured in the presence of excess concentrations of geldanamycin, a potent Hsp90 inhibitor (unpublished observation) (45). Thus, in some myc-258-401 positive cells, not only PB2-Hsp90 interactions, but also the essential function(s) of Hsp90, might have been suppressed as a result of the interactions between myc-258-401 and Hsp90. Expression of recombinant proteins such as NP (Fig. 7P) and GFP (data not shown) did not affect viral gene expression. In contrast, the expression of viral proteins such as HA (Fig. 7O) or nascent PB2 (data not shown) was inhibited in cells expressing myc-258-401. We have considered the possibility that myc-258-401 inhibits the interaction between PB2 wild-type and Hsp90 proteins, such that Hsp90 can no longer function as a host factor. We have not yet examined whether or not myc-258-401 inhibits the production of progeny virions, since cells stably expressing myc-258-401 are not currently available. Since Hsp90 is essential for living cells, the development of knock-out models is impossible (26); we are now establishing cell lines in which the level of Hsp90 is inducibly down-regulated. With such cells in hand, we will examine the function of Hsp90 in the virus multiplication cycle.
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Figure Legends

FIG. 1. Identification of the host factor that stimulated influenza virus RNA synthesis activity. 
A, A purification scheme of RAF-1. For the details regarding the column chromatography and 
biochemical complementation assay, see elsewhere (14, 15). B, SDS-PAGE analysis of RAF-1 
fractions of each purification step. The loaded amounts were 10-fold those used in the in vitro 
RNA synthesis assay; these amounts were adjusted to the equal level of stimulatory activity 
attained in the in vitro RNA synthesis assay. Lane 1, uninfected HeLa cell nuclear extracts (5 
µl); lane 2, 0.05 M KCl flow through fraction from a phosphocellulose column (7 µl); lane 3, 0.4 
M KCl eluate from a Mono Q column (5 µl); lane 4, 0.3 M (NH₄)₂SO₄ eluate from a phenyl 
superose column (2 µl); lane 5, 350 kDa-fraction of gel filtration chromatography (purified RAF-
1 fraction) (1 µl); lane M, molecular weight markers (BIO-RAD). The gel was stained with 
Coomassie Brilliant Blue. C, The stimulatory activity of recombinant Hsp90α. In vitro viral 
RNA synthesis was carried out in the absence (lane 1) or the presence of purified RAF-1 fraction 
(lanes 2-5), recombinant Hsp90α (lanes 6-10), or BSA (lanes 11-14). The amounts of 90 kDa 
polypeptide in purified RAF-1, recombinant Hsp90, and the BSA per assay were 4 ng (lanes 2, 6 
and 11), 8 ng (lanes 3, 7 and 12), 16 ng (lanes 4, 8 and 13), 32 ng (lanes 5, 9 and 14), and 64 ng 
(lane 10). RNA products from endogenous viral RNA present in vRNP are shown at the top of 
10% polyacrylamide gel in the presence of 6 M urea, and the RNA products from the 53 base-
long model viral genome (53-merVwt) are indicated by arrowhead.

FIG. 2. Limited elongation assay with a 35-mer model viral RNA. A, Illustration of in vitro 
viral RNA synthesis without UTP (limited elongation) from the segment 8 RNA and 53-merVwt
template (upper) and 35-merV-a template (lower), used as templates. In the absence of UTP, RNA synthesis was paused at the first adenine residue on the template, whereas RNA polymerase dropped off from the 5’ end of the 35-merV-a template. For additional details about this system, see the text.  

**B.** RAF-1/Hsp90 stimulated viral RNA synthesis with a 35-merV-a template. RNA synthesis was carried out with 1.7 ng of 35-merV-a in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of UTP and in the presence (lanes 2 and 4; 200 ng of Hsp90) or absence (lanes 1 and 3) of RAF-1/Hsp90. The short RNA derived from the endogenous viral genome and the model RNA template are indicated by arrowheads and numerals (12-mer, segments 1, 3 and 7; 13-mer, segments 5 and 8; 14-mer, segment 6; 18-mer, segment 4; and 19-mer from segment 2 of A/Puerto Rico/8/34 strain). The RNA products that appeared between 19-mer and 35-mer (lanes 2 and 4) may have been immature and/or inaccurately initiated transcripts from the 35-merV-a template.

**FIG. 3.** The interaction between Hsp90α and viral proteins.  

**A.** Co-sedimentation of Hsp90 in RAF-1 fraction with vRNP upon glycerol density gradient centrifugation. Aliquots of the RAF-1/Hsp90 fraction (6 µl of phenyl superose eluate) were incubated in the absence (lower panel) or presence of 1 µg NP equivalent vRNP (upper panel) in 100 µl of a reaction buffer containing 50 mM HEPES-NaOH pH7.9, 50 mM KCl, 6 mM MgCl₂, and 3 mM DTT. Subsequently, the mixtures were loaded onto 1.2 ml of a 30% to 60% glycerol density gradient buffer (50 mM HEPES-NaOH pH7.9, 50 mM KCl, 6 mM MgCl₂, 3 mM DTT and 30% to 60% glycerol) and centrifuged at 4°C in a SW50.1 rotor at 45,000 rpm for 5 hours. Fractionation (200 µl) was carried out from the top of the gradient. One hundred microliters of each fraction were precipitated with 10% trichloroacetic acid and were loaded onto 7.5% SDS-PAGE.  

Proteins
were visualized by silver staining. Viral RNA polymerase subunits (lane 1, 5 µl) and Hsp90 (lane 2, 0.5 µl) are shown as the control for gel mobility. A portion of the 90 kDa-polypeptide that sedimented with the vRNP fractions (lanes 6 and 7) is indicated by arrowheads. B, Hsp90α interacted with the PB2 subunit. GST pull-down assays were carried out with recombinant Hsp90α fused with GST at its carboxyl terminus (Hsp90α-GST; lane 3) or with GST (lane 2). Eluates and input vRNP (lane 1, 5 µl) were loaded onto 7.5% SDS-PAGE and Western blot analysis was carried out using rabbit anti-PB2, -PB1, and -PA polyclonal antisera. C, Hsp90β also interacted with the PB2 subunit. GST pull-down assays were carried out with GST (lane 2), recombinant Hsp90α-GST (lanes 3 and 4), or Hsp90β-GST (lanes 5 and 6) in the presence (lanes 2, 4, and 6) or absence (lanes 3 and 5) of vRNP, as described above. Western blot analysis was carried out with anti-PB2 antiserum. Control vRNP (1.3 µl) is also shown (lane 1).

FIG. 4. Effect of Hsp70 on viral RNA synthesis. A, GST pull-down assays were carried out with GST (lane 2), Hsp90α-GST (lanes 3 and 4), or GST-Hsp70 (lanes 5 and 6) in the presence (lanes 2, 4, and 6) or absence (lanes 3 and 5) of vRNP (5 µl). The affinity beads were washed with NETN buffer containing 50 mM (Low) or 1 M NaCl (High). Western blot analysis was carried out with anti-PB2, -PB1, and PA antisera. Control vRNP (lane 1, 1.3 µl) is also shown. B, In vitro viral RNA synthesis was carried out in the absence (lane 1) or presence of recombinant Hsp90 (lanes 2-5; 6.3, 12.5, 25, and 50 ng, respectively) or Hsp70 (lanes 6-9; 6.3, 12.5, 25 and 50 ng, respectively). An RNA product derived from 53-merVwt is indicated by an arrowhead.
FIG. 5. Domain analysis of Hsp90α. A, Schematic representation of Hsp90α consisting of 732 amino acids (a.a.) and its mutants. The chaperone domain, (approximately 1-210 a.a.), highly acidic domain (approximately 220-270 a.a.), and homo-dimerization domain (approximately 540-732 a.a.) are indicated by closed squares. Three mutants, N (1-213 a.a.), M (214-432 a.a.), and C (433-732 a.a.) are indicated by thick bars. B, The PB2 binding efficiency of Hsp90 mutants. GST pull-down assays were carried out with N (lanes 2 and 3), M (lanes 4 and 5), and C (lanes 6 and 7) mutants, and with wild-type Hsp90-GST (lanes 8 and 9) in the absence (lanes 2, 4, 6, and 8) or presence (lanes 3, 5, 7, and 9) of vRNP (5 µl). Western blot analysis was carried out with anti-PB2 antiserum. Control vRNP (lane 1, 1.3 µl) is also shown. C, The stimulatory activities of Hsp90α mutants. In vitro viral RNA synthesis was carried out in the absence (lane 2) or presence of equivalent moles (0.6 pmol) of the purified RAF-1/Hsp90 (lane 1), recombinant wild-type Hsp90α (lane 9), or mutants (lanes 3-8).

FIG. 6. Binding of Hsp90α to PB2 fragments. A, Schematic representation of the PB2 subunit (759 a.a.) and its fragments. The PB1 binding domain (51-259 a.a.) and cap binding sequence (544-556 a.a.) are indicated by closed squares (25). The nuclear localizing signals (NLS; 449-495 and 736-739 a.a.) are indicated by horizontal striped squares (25). PB2 fragments (258-401, 258-401 a.a.; N515, 1-515 a.a.; and N383, 1-383 a.a.) are indicated by thick bars. B, The binding efficiency of PB2 fragments to Hsp90α. GST pull-down assay was carried out with the GST-tagged Hsp90α MM region and wild-type Hsp90α in the presence of in vitro-translated PB2 fragments (3 µl). The amount of [35S]-methionine-labeled PB2 mutants was measured using an image analyzer, BAS2000 (Fuji Film). The binding efficiency is shown as ratios of the amounts of PB2 bound to GST-tagged proteins to those of input PB2.
FIG. 7. Localization of Hsp90 and effects of a PB2 fragment containing a Hsp90 binding site on the expression of viral proteins. Indirect immunofluorescence analysis was carried out with MDCK cells (A-D) or HeLa cells (E-P). Cells were mock-infected (8 panels on the left) or infected (8 panels on the right) with influenza virus A/Puerto Rico/8/34 at a m.o.i. of 3. At 9 hours post-infection, the cells were fixed with acetone:methanol=1:1 (A-D) or 3% paraformaldehyde solution (E-P). Samples were incubated with rabbit polyclonal antiserum reactive to the viral RNA polymerases and NP (anti-pol/NP; A and C) or Hsp90 (anti-Hsp90; B and D). This anti-mouse Hsp90 antiserum was able to cross-react with both human and canine Hsp90α and Hsp90β (46, data not shown). In panels E to P, HeLa cells had been transfected at 12 hours before infection with myc-258-401 (E, I, M, G, K and O), PB2-myc (F, J and N), NP-myc (H, L and P), and FLAG-Hsp90α (E, F, I, J, M and N) expression vectors that were constructed from a mammalian expression vector, pCAGGS (47), such that they contained the indicated epitope-tag sequences. The cells expressing myc-258-401 mutants or NP-myc are indicated by arrowheads. Double staining was carried out with a combination of rabbit anti-myc polyclonal antiserum (E and F) and mouse anti-FLAG epitope monoclonal antibody (I and J), or a combination of mouse anti-myc epitope monoclonal antibody (G and H) and rabbit anti-influenza A/Puerto Rico/8/34 HA polyclonal antiserum (K and L). These fluorescence images are shown as merged images (M-P). The fields of view differ among panels A-D, but those of panels E, I, and M, and panels F, J, and N, and those of panels G, K, and O, and panels H, L, and P correspond to each other. Magnifications are indicated by the white bars in panel A (10 µm, for A-D) and in panel E (10 µm, for E-P).
TABLE I

Amino acid sequences of the oligopeptides derived from the 90 kDa-polypeptide in a purified RAF-1 fraction.

| No. | Identified oligopeptide sequences | Amino acid position* (a.a.) |
|-----|----------------------------------|-----------------------------|
| 1.  | SLVSVTK                          | β: 532-538                  |
| 2.  | YIDQEELNK                        | α: 284-292, β: 276-284      |
| 3.  | ESEDKPEIEDVGSDEEEEK              | α: 251-269                  |
| 4.  | LGLGILEDPTADDTSAAV               | α: 694-712                  |
| 5.  | TKIWWTRNPDDITQEYPYGEFYK          | β: 285-306                  |

*α and β indicate Hsp90α and Hsp90β, respectively.
A

5' AGCAAAAAGCAGGGG
3' UCGUUUCGUCCACUUCAAA

Segment #8, 53-merVwt

5' AGCAAAAAGCAGGCCAGAAGAAGAAAGAAAGAAGAAGAAGAAC
3' UCGUUUCGUCCGGUCUUCUUUCUUUCUUUCUUUCUUG

35-merV-a

B

+ + + - - UTP
- + - + + RAF-1/Hsp90

1 2 3 4

(base)
Momose, F. et al. Figure 4

A

|     | Input | GST | Hsp90α-GST | GST-Hsp70 | vRNP |
|-----|-------|-----|-------------|-----------|------|
| 1   | +     |     | -           | -         | -    |
| 2   |       | +   | +           | +         | +    |
| 3   | -     | +   | -           | -         | -    |
| 4   |       |     | +           | +         | +    |
| 5   | -     | +   | -           | -         | -    |
| 6   |       |     | +           | +         | +    |

- **anti-PB2**
  - Low
  - High
  - Low
  - Low

- **anti-PB1**
  - Low

- **anti-PA**
  - Low

B

- **Recombinant Hsp90α**
  - (-)
  - 1
  - 2
  - 3
  - 4
  - 5

- **Recombinant Hsp70**
  - 6
  - 7
  - 8
  - 9

- 53-mer
Momose, F. et al. Figure 7

mock

anti-pol/NP anti-Hsp90

A B

infected

anti-pol/NP anti-Hsp90

C D

myc-258-401 PB2-myc

E F

anti-myc

G H

myc-258-401 NP-myc

anti-myc

I J

anti-FLAG

K L

anti-HA

M N

merge

O P

merge

anti-HA

merge
Identification of Hsp90 as a stimulatory host factor involved in influenza virus RNA synthesis
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