Identification of heat-shock protein 90 beta in Japanese encephalitis virus-induced secretion proteins

Chun-Yu Hung,¹ Meng-Chieh Tsai,¹ Yi-Ping Wu¹ and Robert Y. L. Wang¹,²

¹Department of Biomedical Sciences, Chang Gung University, Tao-Yuan 33302, Taiwan, ROC
²Research Center for Emerging Viral Infections, Chang Gung University, Tao-Yuan 33302, Taiwan, ROC

Five host cellular proteins were identified in the secretion medium from Japanese encephalitis virus (JEV)-infected baby hamster kidney-21 (BHK-21) cells, including three molecular chaperones: Hsp70, GRP78 and Hsp90. Hsp90 isoforms were characterized further. Hsp90α was observed to be retained inside the nuclei, whereas Hsp90β associated with virus particles during assembly and was released into the secretion medium upon JEV infection. The association of Hsp90β and viral E protein was demonstrated by using sucrose-density fractionation and Western blot analysis. Moreover, JEV viral RNA replication was not affected by treatment with geldanamycin, an Hsp90 inhibitor, but impaired virus infectivity that was determined by a plaque-forming assay. Our results show that Hsp90β, not Hsp90α, is present in the JEV-induced secretion medium and is required for JEV infectivity in BHK-21 cells.

INTRODUCTION

Heat-shock protein 90 (Hsp90) is one of the most abundant proteins in eukaryotic cells (Welch & Feramisco, 1982), comprising about 1–2% of total cellular protein under non-stress conditions. The most extensively characterized functions of Hsp90 are contributing to various cellular processes including signal transduction (Pratt & Toft, 2003; Richter & Buchner, 2001), protein folding (Picard, 2002), protein degradation and morphological evolution (Lanneau et al., 2010). Higher eukaryotes express two Hsp90 isoforms, Hsp90α (inducible form/major form) and Hsp90β (constitutive form/minor form) (Taherian et al., 2008). The Hsp90α and Hsp90β homologues in human and other mammalian species show approximately 86% identity to each other (Gupta, 1995). Although there is high amino acid similarity between Hsp90α and Hsp90β, the specificity between Hsp90α and Hsp90β is not only restricted to the biochemical level but also extends to the functional role(s) of Hsp90 with regard to the cell differentiation as well as cell development. For instance, the specific Hsp90-binding agent, geldanamycin, was used to examine the requirement for Hsp90 during zebrafish development, revealing a regulatory role for Hsp90α in muscle cell differentiation (Lele et al., 1999), and reduction of Hsp90α caused instability of cdc2 (cyclin-dependent kinase) under high temperature stress (Nakai & Ishikawa, 2001). It has been reported that overexpression of the Hsp90α inhibited cellular differentiation of embryonal carcinoma cells to trophoectoderm (Sreedhar et al., 2004; Yamada et al., 2000), while Hsp90β played a major role in trophoblast differentiation (Voss et al., 2000). In addition, the high expression level of Hsp90β is observed throughout the germ cell lineage from early stages of development to adult oocytes and spermatocytes, whereas Hsp90β-deficient homozygous mice with normal expression of Hsp90α failed to differentiate to form placental labyrinth (Voss et al., 2000). Further studies confirmed these observations in different models and even from different stages of development, suggesting that Hsp90β is required for early embryonic development (Hilscher et al., 1974). Altogether, there are multiple differences between Hsp90 isoforms with regard to cell differentiation as well as embryonic development in various organisms (Dugyala et al., 2002; Sidera et al., 2004; Vanmuylder et al., 2002).

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus and causes human epidemic encephalitis mostly found in Asian countries, leading to a 5–40% fatality rate (Unni et al., 2011; Van Den Hurk et al., 2006). The JEV genome is a positive-sense, ssRNA of approximately 11 kb in length, encoding a large polyprotein of about 3400 aa. There are many studies that have shown that host factors are involved in the JEV life cycle and numerous reports have shown that host factors participate in RNA virus replication. In our previous study, we first reported that three chaperones, Hsp90, Hsp70 and GRP78 were detected in the secretion medium from JEV-infected BHK-21 cells. GRP78 was further demonstrated as playing roles in viral maturation and in subsequent cellular infections (Wu et al., 2011).

In this study, the Hsp90 isoforms Hsp90α and Hsp90β were characterized separately by using antibodies specific
for each isoform. Both isoforms were present in the cell lysates; however, only Hsp90β was detected in the secretion medium from JEV-infected cells, indicating that only Hsp90β had been released upon JEV infection. Furthermore, the functional role of Hsp90β was demonstrated to be a required host factor for the mature JE viral infectivity in BHK-21 cells.

RESULTS

Hsp90β, not Hsp90α, was present in the JEV-induced secretion medium

We have reported that there were at least five proteins: EF-2, Hsp90, GRP78, Hsp70 and cysteine ligase identified in the secretion medium from JEV-infected BHK-21 cells (Wu et al., 2011). GRP78 was demonstrated as playing roles in viral maturation and in subsequent cellular infections. Hsp90 is another host chaperone that is recruited by the RNA virus for promoting correct folding of viral proteins, preventing the induction of unfolded protein response (UPR) as well as facilitating virus-induced intracellular membranes for the replication purpose (Davenport et al., 2008). To validate and further characterize the secreted Hsp90 isoforms, both intracellular lysates and secretion medium samples from mock-infected and JEV-infected cells were analysed by Western blot using isoform-specific antibodies. As shown in Fig. 1, Hsp90 (this antibody was able to recognize α- and β-forms) was present in the cell lysates as well as the secretion medium from JEV-infected cells. In contrast, Hsp90α was present only in the cell lysates, suggesting that Hsp90β had been released into the secretion medium upon JEV infection. To further characterize the specific Hsp90 isoforms, which are present in the secretion medium, both Hsp90α and Hsp90β were detected separately using antibodies specific for each isoform. As expected, only Hsp90β, but not Hsp90α, was detected in the secretion medium from JEV-infected cells. Since both of the anti-Hsp90α and anti-Hsp90β antibodies did not cross-react, the Hsp90α/β-specific antibodies allowed us to conclude that only Hsp90β is present in the secretion medium from JEV-infected cells.

Hsp90β co-migrates with JEV particles

We tested whether Hsp90β was associated with JEV particles. The secretion medium was collected from JEV-infected cells 3 days post-infection and subjected to 20–60% continuous sucrose-density-gradient centrifugation. A total volume of 1 ml per fraction was collected and subjected to Western blot analysis for the detection of Hsp90β and viral E protein. Co-migration of Hsp90β with viral E protein was observed in fractions 3 and 4 (Fig. 2a).

Subcellular localization of Hsp90 isoforms in JEV-infected cells

The interaction between Hsp90 and viral proteins has been reported as playing a role in the virus life cycle. For instance, hepatitis C virus (HCV)–NS3 formed a complex with Hsp90, which is critical for HCV replication (Ujino et al., 2009). Also, Okamoto et al. (2006) reported that immunoprecipitation analyses revealed that FKBP8 formed a complex with Hsp90 and NS5A, suggesting that a complex consisting of NS5A–FKBP8–Hsp90 or NS3–Hsp90 plays an important role in HCV RNA replication. To explore if the Hsp90 isoform co-localizes with viral structural proteins within the cell, we performed immunofluorescence staining on Hsp90 and the viral E protein in mock- and JEV-infected cells, using antibodies specific for viral E protein, the secretion medium was treated with high salt prior to sucrose-density fractionation. The viral E protein was observed in fraction 7, while the Hsp90β was detected in fractions 2–5 (Fig. 2b). These results indicate that the association of Hsp90β and viral E protein occurs during viral particle release instead of during the centrifugation process.
amounts of Hsp90 function. To further characterize that only Hsp90
JEV E protein by Western blotting. (b) Disassociation of Hsp90
antibodies shown in (a).

Hsp90α/β and then viral E protein, respectively. Hsp90α
mainly localized in the cytoplasm of mock-infected cells
(Fig. 3a(i)), whereas only partial co-localization of Hsp90α
with the viral E protein in the cytoplasm was observed in
the JEV-infected cells [Fig. 3a(v) and (vi)]. However, higher
amounts of Hsp90α were observed inside the nuclei in JEV-
infected cells [Fig. 3b(ii)], compared with that of mock-
infected cells [Fig. 3b(i)], where it did not co-localize with
the viral E protein. It has been reported that only Hsp90α
enters the nucleus upon heat-stress treatment in 3T3 cells
(Langer et al., 2003), indicating that Hsp90α served as a
protective protein and/or interacted with other heat-shock
proteins in response to stress conditions. In contrast to the
nuclear localization of Hsp90α in JEV-infected cells, Hsp90β
remained in the cytosolic localization and co-localized with
the E protein in JEV-infected cells [Fig. 3c(i)]. Therefore,
Hsp90α was retained inside the nuclei, whereas Hsp90β
might be associated with the E protein and released into the
secretion medium upon JEV infection.

Hsp90β binds to the E protein in JEV-infected cells
It is thought that molecular chaperones, such as Hsp70 and
Hsp90, bind directly to substrate polypeptide and assist its
function. To further characterize that only Hsp90β is
associated with the E protein, we therefore performed a co-
immunoprecipitation experiment from mock-infected and
JEV-infected BHK-21 cells. Hsp90β was precipitated by
the anti-E antibody from JEV-infected cells (Fig. 4). In
contrast, Hsp90α was not present in the precipitates from
JEV-infected cells (Fig. 4), indicating that only Hsp90β is
associated with the E protein.

Suppression of JEV infectivity by GA, an inhibitor
of Hsp90
It has been reported that Hsp90 inhibitors act as either
preferential clients for protein degradation or as Hsp70
inducers (McClellan & Frydman, 2001). The benzoquinone
ansamycin antibiotic, GA, directly binds to the ATP/ADP-
binding pocket of Hsp90 (Grenert et al., 1997). GA has been
explored in the development of antiviral strategy. For
instance, Geller et al. (2007) reported that the pharmacological
inhibition of Hsp90 by GA impairs the replication of
poliovirus, rhinovirus and coxsackie virus in cells, and in vivo administration of GA significantly decreased the virus
load in poliovirus-infected mice without the emergence of
drug-resistant escape mutants (Geller et al., 2007). To
evaluate the potential antiviral function of the Hsp90
inhibitor GA, BHK-21 cells were treated with different
concentrations of GA prior to JEV infection. As shown in
Fig. 5(a), the expression level of both Hsp90α and Hsp90β
was not decreased upon GA treatment, indicating that the
effect of GA on Hsp90 isoforms is by means of the inhibitor
binding, leading to a block in the Hsp90 activity instead of
the suppression of translation activity. As a control, the NS1
expression level was comparable under different concentra-
tions of GA-treated culture conditions (Fig. 5a), indicating
that viral RNA replication was not affected by GA treatment.
Next, the collected secretion medium was separated from
cells and cellular debris by centrifugation and subjected to
infection with other BHK-21 cells for the determination of
viral infectivity using a plaque-forming assay. An approx-
imate 2 × 10^4-fold reduction in plaque formation was
observed in GA-treated cells in comparison with cells treated
with DMSO only (Fig. 5b, c), suggesting that Hsp90β is
required for JEV infectivity in BHK-21 cells.

Silencing Hsp90β expression reduces JEV
infectivity, but has no effect on viral RNA
replication
To further characterize and distinguish the Hsp90 isoform-
specific function with regard to the JE life cycle, the effect
of reduced Hsp90β expression on viral replication and
virus infectivity was investigated. The siRNA targeting of
Hsp90β significantly reduced the protein level of Hsp90β
and that of JEV infection (Fig. 6a), indicating that the
endogenous expression of Hsp90β was not affected upon
JEV infection. The comparable expression level of two viral
proteins, the E and NS1 proteins, when the Hsp90β protein
level in JEV-infected BHK-21 cells was downregulated (Fig.
6a), indicates that JE viral replication was not affected by
the knocking down of Hsp90β. In contrast, a reduction in
plaque formation was observed in Hsp90β-siRNA cells
compared with that of scramble siRNA-treated cells (Fig.
6b, c), suggesting that Hsp90β is required for JEV infectivity in BHK-21 cells.
DISCUSSION

Characterization of secreted virus-associated host cell proteins has been performed in several viruses, such as dengue virus (DENV) (Higa et al., 2008), HCV (Parent et al., 2009), respiratory syncytial virus (RSV) (Radhakrishnan et al., 2010) and JEV (Wu et al., 2011). Among them, cytoskeleton or cytoskeleton-associated proteins have been the most abundant in the analysis of the identification of RSV particle-associated host proteins (Radhakrishnan et al., 2010). Most of these proteins were actin-related, indicating that these host cytoskeleton proteins are relevant to virus particle formation. In DENV, a total of 24 secretion proteins was identified from DENV-infected HepG2, a hepatic cell lineage, including the signal peptide-containing proteins, insulin-like growth factor-binding protein 1, cyclophilin B and serum albumin, revealing that it might be a result of classical secretion upon virus infection (Higa et al., 2008). Apart from the virus-induced host cytoskeleton proteins secretion or host classical secretion in response to virus infection, three molecular chaperones, Hsp90, Hsc70 and Hsp70, were detected in different virus-induced cell culture medium, and a role for chaperones in the replication cycle of several viruses has been described elsewhere (Mayer, 2005). In our group, we first reported that three chaperones, Hsp90, Hsp70 and GRP78, were detected in the secretion medium from JEV-infected BHK-21 cells (Wu et al., 2011). Hsp90 has been detected in RSV preparations (Radhakrishnan et al., 2010), but whether one or both isoforms were present in that preparation due to the high
(85%) amino acid sequence identity between Hsp90α and Hsp90β is unknown. The peptides present in Hsp90α/β isoforms that were identified in our proteomic LC/MS analysis reflects the apparent degeneracy of the Hsp90 peptides between both Hsp90α and Hsp90β (Chen et al., 2005). However, in this study, we determined that only Hsp90β is presented in the JEV-induced secretion medium using an Hsp90β-specific antibody.

Intracellular localization of Hsp90 has been demonstrated in many studies (Langer et al., 2003; Schlatter et al., 2002). It has been reported that under normal condition, Hsp90α is primarily localized in the cytoplasm; after heat stress, an increased amount of Hsp90α can be detected inside the nucleus (Langer et al., 2003). For most of the protein, including chaperones, a so-called nuclear localization signal (NLS) is mandatory in order to move inside the nucleus (Sreedhar et al., 2004). An analysis of the amino acid sequence of Hsp90α reveals two putative NLS (275-KKKKKK-280 and 356-RKKK-359), whereas only one putative NLS (346-NKKKK-350) is present in Hsp90β. Only Hsp90α transfers to the nucleus upon JEV infection, which is consistent with the results described above. Yet, no transfer of Hsp90β to the nucleus upon virus infection (data not shown) was observed.

Although the current understanding of Hsp90 as a molecular chaperone explains its role in many cellular functions both in normal and pathophysiological conditions, the detailed characterization of Hsp90α and Hsp90β function alone has failed to specialize the isoform-specific function of these heat-shock proteins. Hsp90α emerges as a fast-response and cellular protective isoform, while Hsp90β seems to be associated with long-term cellular adaptation and facilitated cellular evolution (Sreedhar et al., 2004). In particular, one of the specific functions of Hsp90β is the cytoskeleton stabilization, thus we can speculate about its possible role during virus assembly and virus morphogenesis based on our understanding of its isoform-specific function. Actin plays a role in the morphogenesis of several viruses (Döhner & Sodeik, 2005), and recent studies have identified an association between actin and both inclusion bodies and virus filaments in virus-infected cells (Kallewaard et al., 2005; Ulloa et al., 1998). In the JEV-induced proteomics analysis, actin was also detected in the secretion medium (data not shown), supporting earlier studies showing that
Fig. 6. Knockdown of Hsp90β by siRNA reduces JEV infectivity. (a) BHK-21 cells were transfected with a non-silencing (scramble) siRNA or with siRNA against Hsp90β. Two days post-transfection, protein expression was visualized by Western blots using an anti-Hsp90 antibody. The siHsp90β-transfected cells were then infected with JEV at an m.o.i. of 10 and cell lysates were collected at 48 h for the determination of viral E, NS1 and host Hsp90β proteins expression level. (b) Plaque formation by JEV particles collected from JEV-infected scramble siRNA-treated cells or cells treated with siRNA of Hsp90β. (c) Quantitative measurement of viral progeny produced from JEV-infected scramble siRNA-treated cells or cells treated with siRNA of Hsp90β as shown in (b). The virus titre was defined as p.f.u. ml⁻¹. Results are derived from three independent experiments.

METHODS

Cell culture and viruses. The culturing of BHK-21 and JEV stock preparations has been described elsewhere (Wu et al., 2011). The viral stocks were generated via γ-ray treatment of the Taiwan JEV NT109 strain, called RP-9 (provided by Dr Ching-Len Liao, National Defense University, Taiwan).

Western blotting. The protein sample collection and separation have been described elsewhere (Wu et al., 2011). The primary antibodies used were the following: a mouse anti-JEV NS1/E (1:3000 dilution) (Wu et al., 2011) (YaoHong Biotechnology), or rabbit anti-β-actin antiserum (1:10 000 dilution; Sigma) or rabbit anti-HSP90 antibodies (catalogue# ADI-SPA-836), Hsp90α (catalogue# ADI-SPS-771) and Hsp90β (catalogue# ADI-SPA-844). All of the anti-Hsp90 isoform antibodies were purchased from Enzo Life Sciences International.

Viral plaque assay. BHK-21 cells were seeded in six-well plates at 4 × 10⁴ cells per well, followed by incubation overnight in RPMI 1640 medium containing 2% FBS to a form a monolayer. The serial 10-fold dilutions of the supernatant of JEV-infected medium were prepared in serum-free RPMI medium before infection. After 1 day, the monolayer of BHK-21 cells was incubated with serum-free RPMI 1640 medium for 1 h and then 0.5 ml of 10-fold dilutions and 0.5 ml of serum-free RPMI 1640 medium was added to monolayer of BHK-21 cells for 1.5 h. The six-well tissue culture plates were incubated at room temperature for 30 min to allow the 0.3% agarose overlay to solidify, followed by incubation at 37 °C for 3 days. Finally, cells were washed with 2 ml 10% formaldehyde and kept at room temperature (22–25 °C) for 30 min and then the overlay of 0.3% agarose was removed. The monolayer of cells was stained with crystal violet stain solution for 2 min and washed with ddH₂O. The p.f.u. ml⁻¹ was calculated with the virus titre formula, where virus titre equals the number of plaque × (1 ml/0.5 ml) × dilution factor.

Sucrose-density gradient analysis. The secretion medium of infected BHK-21 cells (2 days post-infection at an m.o.i. of 10) was centrifuged and concentrated prior to sucrose-density gradient analysis. The secretion and concentrated medium were layered onto a 20–60% sucrose-linear gradient in HEPES buffer (20 mM HEPES, 0.5 mM EDTA and 50 mM KCl) and centrifuged at 31 100 g for 17 h at 4 °C. A total of 10 fractions (1 ml per fraction) were harvested from the top of the sucrose gradients.

Immunofluorescence and antisera. For immunofluorescent staining, cells were cultured on glass coverslips, rinsed with PBS twice, fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and then permeabilized with 0.1% (v/v) Triton X-100 in PBS for 30 min and incubated in 2% blocking buffer (Roche) for 1 h. The cells were then incubated sequentially with primary antibodies: mouse anti-E protein (Yao-Hong Biotechnology), rabbit anti-HSP90 (Enzo Life Sciences International) and secondary antibodies: conjugated with Rodamine and FITC. After immunostaining, coverslips were mounted onto slides in gelvatol medium containing DAPI (Vector Laboratories), 500 ng ml⁻¹ in PBS. Images were acquired using a Zeiss confocal microscope (LSM 510) and processed with Adobe Photoshop software.

ACKNOWLEDGEMENTS

The authors thank Drs Yu-sun Chang, Jau-song Yu, and faculties of the Proteomics Core facility in the Molecular Medicine Center, Chang Gung University for the proteomics analyses of secretion medium from JEV-infected cells. This work was supported by grants from the National Science Council in Taiwan (NSC-99-2321-B-002-025-) and from the Chang Gung Memorial Hospital Research Funding (CMRPD180092) to R.W.
REFERENCES


