Virology 443 (2013) 236–247

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Heat shock protein-90-beta facilitates enterovirus 71 viral particles assembly

Robert Y.L. Wang [a,b,*], Rei-Lin Kuo [a,c], Wei-Chieh Ma [b], Hsing-I Huang [a,c], Jau-Song Yu [d,e], Sih-Min Yen [b], Chi-Ruei Huang [a,b], Shin-Ru Shih [a,c]

[a] Research Center for Emerging Viral Infections, College of Medicine, Chang Gung University, Tao-Yuan 333, Taiwan
[b] Department of Biomedical Sciences, College of Medicine, Chang Gung University, Tao-Yuan 333 Taiwan
[c] Department of Medical Biotechnology and Laboratory Science & Graduate Program of Biomedical Sciences, College of Medicine, Chang Gung University, Tao-Yuan 333, Taiwan
[d] Department of Cell and Molecular Biology, College of Medicine, Chang Gung University, Tao-Yuan 333, Taiwan
[e] Molecular Medicine Research Center, Chang Gung University, Tao-Yuan 333, Taiwan

ARTICLE INFO

Article history:
Received 12 February 2013
Returned to author for revisions
1 March 2013
Accepted 2 May 2013
Available online 24 May 2013

Keywords:
Heat shock protein 90
Hsp90β
Enterovirus 71
Viral particles assembly
Geldanamycin

ABSTRACT

Molecular chaperones are reported to be crucial for virus propagation, but are not yet addressed in Human Enterovirus 71 (EV71). Here we describe the specific association of heat shock protein-90-beta (Hsp90β), but not alpha form (Hsp90α), with EV71 viral particles by the co-purification with virions using sucrose density gradient ultracentrifugation, and by the colocalization with viral particles, as assessed by immunogold electron microscopy. The reduction of the Hsp90β protein using RNA interference decreased the correct assembly of viral particles, without affecting EV71 replication levels. Tracking ectopically expressed Hsp90β protein associated with EV71 virions revealed that Hsp90β protein was transmitted to new host cells through its direct association with infectious viral particles. Our findings suggest a new antiviral strategy in which extracellular Hsp90β protein is targeted to decrease the infectivity of EV71 and other enteroviruses, without affecting the broader functions of this constitutively expressed molecular chaperone.

Introduction

There is growing evidence that infectious virions contain some intracellular host proteins. The acquisition of host proteins allows viruses to evade the host immune response, and may also function in mechanisms of virus entry or the release of new virions from cells. Throughout the virus life cycle, it is believed that viral, host, and environmental factors contribute to the pathogenesis and the progression of virus-induced diseases.

Human enterovirus 71 (EV71), one of two *Enterovirus* serotypes that are most often associated with large outbreaks of hand-foot-and-mouth disease (HFMD), causes a variety of neurologic diseases, such as aseptic meningitis, encephalitis, and poliomyelitis-like paralysis. Many reports have indicated that EV71 has caused epidemics of severe neurologic disease in Asia, Europe, and the USA (Abubakar et al., 1998; Alexander et al., 1994; da Silva et al., 1996; Gilbert et al., 1988; Ishimaru et al., 1980; Komatsu et al., 1999). During 1997 and 1998, EV71 was considered the primary agent that was associated with fatal cases of brain-stem encephalitis during large HFMD outbreaks in Malaysia (Cardosa et al., 2003; Chan et al., 2000) and in Taiwan (Ho et al., 1999; Lin et al., 2003). EV71 belongs to the member of the genus *Enterovirus* in the family *Picornaviridae*, and is classified as Human Enterovirus species A. EV71 contains a single-stranded and positive-sense RNA genome that is approximately 7400 bases long. It is genetically related to coxsackieviruses, showing the greatest similarity to another major etiological agent of HFMD in Asia, Coxsackievirus A16 (CV-A16).

The EV71 viral particle is the structure of the icosahedron (icosahedral), without enveloped membrane (Brown and Pallansch, 1995). The viral genome consists of 11 genes, which encode four structural proteins (VPs) and seven nonstructural proteins ($2A^{pro}$, 2B, 2C, 3A, 3B, $3C^{pro}$ and $3D^{pol}$), flanked by two non-translational regions (NTRs) in which located at the 5′ (5-NTR) and 3′ (3-NTR) termini of the RNA genome (Shih et al., 2011). A clover (cloverleaf) is located at the 5-NTR having a structure with an internal ribosome insertion position (names: internal ribosome entry site; IRES), serving for the binding sites of host factors to regulate the viral RNA replication as well as the viral proteins translation (Bhattacharyya et al., 2008; Chen et al., 2013; Sean et al., 2009). Following by viral internalization and uncoating, the

* Corresponding author at: Department of Biomedical Sciences, College of Medicine, Chang Gung University, Tao-Yuan 333 Taiwan. Fax: +886 32118700.
E-mail addresses: yuwang@mail.cgu.edu.tw, ylwang@ntu.edu.tw (R.Y.L. Wang).

0042-6822/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.virol.2013.05.001

RNA genome induces the production of a viral polyprotein in the host cytoplasm by means of a cap-independent mechanism. The EV71 RNA genome has an open reading area (the open reading frame; ORF) encodes an approximately 200 KDa polyprotein, including the structural proteins (P1) and the non-structural protein (P2 and P3). After the translation, the large polyproteins are then processed by the viral protease 2A and 3C to individual viral proteins (Li et al., 2002). The P1 segment is divided into four capsid proteins (VP1, VP2, VP3 and VP4). The synthesis of the viral genome begins from VPg, at the 5′-NTR, in which a viral protein encoded by the EV71 viral 3B gene acting as a replacement for the eukaryotic 7-methyl cap. The viral 3D protein (a viral RNA-dependent RNA polymerase) then modifies VPg to VPgpU-pU, serving for the initiation of viral RNA synthesis in the 3-NTR region of the EV71 genome and synthesizes the negative-sense RNA strand using the positive-sense RNA as the template (Daijogo and Semler, 2011). Many copies of the positive-sense RNA genome are then formed from the negative RNA strand and are packed into viral capsids, which emerge from the host as progeny viruses to infect new hosts.

Molecular chaperones are necessary for the maintenance of proper protein functions through involvement in such cellular processes as protein folding, activation, transport, and polymerization (Eustace and Jay, 2004; Isaacs et al., 2003; Neckers and Ivy, 2003). Heat shock protein 90 (Hsp90) is one of the abundant molecular chaperones. Hsp90 is thought to primarily function intracellularly, but recently published results indicate that two Hsp90 isoforms, Hsp90α and Hsp90β, were present in the culture medium from HT-1080 fibrosarcoma and MD231 breast carcinoma cells, as well as BHK21 cells infected with the Japanese encephalitis virus (JEV) (Hung et al., 2011; Lietzen et al., 2011). Further studies revealed that extracellular Hsp90α is involved in various pathological processes associated with tumor cell invasion and metastasis, and that virus-induced, extracellular Hsp90β protein is required for JEV infectivity.

In this study, we used a proteomics approach to produce a global profile of the EV71-infected, secretion of host proteins from the human glioblastoma cell line, SF268. We showed that the Hsp90β protein, but not the Hsp90α isoform, was present in the cultured medium of EV71-infected SF268 cells, and that the Hsp90β protein was associated with newly released virions. Inhibition of Hsp90β protein functions decreased virus assembly leading to reduce the EV71 infectivity, indicating a significant role of extracellular Hsp90β in the EV71 life cycle.

Results

Proteomics profiling of host proteins in the cultured medium from EV71-infected SF268 cells

Previous studies have reported that certain RNA viruses induce the release of host proteins from virus-infected cells (Hung et al., 2011; Lietzen et al., 2011; Wu et al., 2011). To obtain a global profile of host proteins that are released during EV71 infection, we cultured EV71-infected SF268 cells in serum-free media, and probed for the presence of extracellular proteins (Fig. 1A). Following EV71 infection, the cells were initially cultured in DMEM supplemented with 2% fetal bovine serum (FBS) to facilitate viral RNA replication, followed by cultivation of the cells in serum-free medium. To test whether viral replication is affected by the lack of FBS proteins, cell extracts from cells that were cultured in serum-free or serum supplemented cultures were isolated, and analyzed for the presence of EV71 viral nonstructural proteins by western blotting using anti-EV71-3A specific antibody. The expression level of 3A/3AB in cells cultured in the serum-free medium was comparable to that of cells that were provided 2% FBS (Fig. 1B, lane 5 and 6 compared to lane 3 and 4). These results indicate that the EV71 viral RNA replication was not affected by the removal of serum proteins in 24 h. Similarly, the 24 h time point collection of the cultured medium from the EV71-infected cells showed maximal protein accumulation (Fig. 1C) combined with minimal cell lysis (determination by the absence of β-actin in the secretion medium as shown in Fig. 1D) or cell death (determination by the MTT assay as shown in Fig. 1E) resulting from the EV71 infection. SDS-PAGE analysis of proteins in the serum-free cultured medium from EV71-infected cells showed that there were 10 proteins that were up-regulated compared to the cultured medium from mock-infected cells (Fig. 1F). Among them, two of which were consistently expressed in the mock-infected cells (Fig. 1F, bands 5 and 10 compared to bands 4 and 9). Proteins from all 14-gel bands were analyzed by LC-MS/MS. A total of 12 potential protein homologs were identified, and are listed in Table 1. Gel bands 4 and 5, which were present in both EV71- and mock-infected cells were identified as peripherin (Fig. 1F). Gel bands 9 and 10 were identified as plasminogen activator inhibitor. Gel bands 1, 2, and 3 were identified as α-actinin-4, heat shock protein 90β-isoform, and heat shock cognate 71 kD protein, respectively. Peripherin protein is expressed primarily in neurons of the peripheral nervous system (Eriksson et al., 2008), indicating that these data were not the result of artificial loading variations. Moreover, the peripherin protein could be used as an internal control in future studies. The α-actinin-4 is an actin binding protein that plays multiple roles in different cell types. The release of α-actinin-4 during the EV71 infection may be due to the association of virions with cellular cytoskeleton components during the viral release pathway (Kim et al., 2002).

Hsp90β, but not Hsp90α, is identified as the extracellular protein from EV71-infected cells

Many studies have indicated that heat shock proteins were involved in RNA viruses life cycle [reviewed in (Geller et al., 2012; McCready et al., 2010; Xiao et al., 2010)]. The heat shock cognate 71 kDa protein was also reported to be present in the cultured medium from JEV-infected cells (Wu et al., 2011). Hsp90β was also reported to be present in the cultured medium from JEV-infected BHK-21 cells, and was further characterized as promoting JEV virion assembly (Hung et al., 2011). Hsp90 is a cellular chaperone that is known to be involved in the life cycles of RNA viruses. Therefore, to test whether Hsp90 isoforms were present in the intracellular lysates or the cultured medium from EV71-infected cells, the protein samples were analyzed by western blotting using Hsp90 isoform specific antibodies. The Hsp90β protein was detected in the cultured medium from both EV71-infected and heat shocked control cells (Fig. 2A, lane 2 and 3). In contrast, The Hsp90α protein was not detected neither in the cultured medium from EV71-infected cells nor heat shocked control cells (Fig. 2B, lane 2 and 3). Moreover, the expression level of extracellular Hsp90β was greater for EV71-infected cells than that produced by heat shock treatment alone, suggesting an induced secretion of the Hsp90β upon EV71 infection in the SF268 cells (Fig. 2A, lane 2 compared to lane 3). Detection of extracellular Hsp90β in the cultured medium from EV71-infected cells is in a time-dependent manner upon viral infection whereas no significant cell lysis or cell death was observed at these time points as shown above. As seen in Fig. 2C, the extracellular Hsp90β protein was detectable at 18 h and 24 h post-infection (lane 4 and 5), which is consistent with the detection of the EV71 viral structural proteins, VP1 and VP3. Taken collectively; these results indicate that the Hsp90β protein was present in the cultured medium from EV71-infected cells, and that the Hsp90α protein was not.