**FINAL REPORT**

**ชื่อโครงการ (ภาษาไทย) การค้นหาและการแยกลักษณะของตัวตอบรับของเชื้อไวรัส**

**ไข้เลือดออกโดยใช้เทคนิค affinity chromatography**

# Identification and characterization of dengue virus receptors using affinity chromatography

**PROFESSOR DUNCAN R. SMITH PHD.**

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**Professor Duncan R. Smith PhD.**

**Institute of Molecular Biology and Genetics**

**Mahidol University**

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**Investigator:**

Professor Duncan R. Smith

Institute of Molecular Biology and Genetics

Mahidol University

**Email address**: duncan_r_smith@hotmail.com; frdrs@mahidol.ac.th;
duncan_at_work@yahoo.co.uk

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# บทคัดย่อ

การติดเชื้อไวรัสเด็งกี่เป็นการติดเชื้อไวรัสที่มีแมลงเป็นพาหะนำโรคที่พบมากที่สุดโรคหนึ่ง มีการประมาณการว่ามีประชากรกว่าสามพันล้านคนที่อาศัยอยู่ในพื้นที่ที่มีความเสี่ยงต่อการติดเชื้อไวรัสเด็งกี่ และในแต่ละปีมีผู้ติดเชื้อไวรัสเด็งกี่มากกว่าหนึ่งร้อยล้านคน แม้ว่าจะมีความพยายามในการศึกษาเกี่ยวกับไวรัสเด็งกี่อย่างกว้างขวาง แต่ความรู้เกี่ยวกับปฏิสัมพันธ์ของไวรัสเด็งกี่และเซลล์เจ้าบ้านยังคงไม่ชัดเจน หนึ่งในปฏิสัมพันธ์ของไวรัสและเซลล์เจ้าบ้านที่มีความสำคัญต่อพยาธิวิทยาการเกิดโรคได้แก่ ปฏิสัมพันธ์เบื้องต้นของไวรัสและเซลล์เจ้าบ้านที่บริเวณโปรตีนตัวตอบรับ ซึ่งปฏิสัมพันธ์เบื้องต้นนี้จะ เป็นตัวกำหนดการเกิดปฏิสัมพันธ์ในขั้นตอนต่อไป โดยเฉพาะอย่างยิ่งในการกำหนดตำแหน่งเฉพาะของไวรัสภายในเซลล์ ซึ่งโดยทั่วไปแล้วรูปแบบการเข้าสู่เซลล์และปฏิสัมพันธ์ของไวรัสกับองค์ประกอบภายในเซลล์เจ้าบ้านประกอบไปด้วยรูปแบบที่แตกต่างกันหลากหลายแบบ

ผู้วิจัยได้มีการพิสูจน์โปรตีนตัวตอบรับสองชนิดของไวรัสเด็งกี่ที่ใช้ในการเข้าสู่เซลล์ตับอย่างจำเพาะต่อซีโรไทป์ของไวรัส [Jindadamrongwech et al., 2004; Thepparit and Smith, 2004] เพื่อให้เกิดความเข้าใจมากขึ้นเกี่ยวกับปฏิสัมพันธ์ของไวรัสและเซลล์ตับ ผู้วิจัยได้ใช้เทคนิคแอฟฟินิตี้คอลัมน์โครมาโตกราฟฟี่เพื่อศึกษาความจำเพาะของซีโรไทป์ต่อปฏิสัมพันธ์ของไวรัสและแสดงความจำเพาะเจาะจงของปฏิสัมพันธ์ พบว่าคอลัมน์ที่ใช้ไวรัสเด็งกี่ซีโรไทปป์สองสามารถจับกับโปรตีนตัวตอบรับที่ได้รับการพิสูจน์ก่อนหน้านี้ คือ GRP78 แต่ในเด็งกี่ไวรัสซีโรไทป์หนึ่งไม่มีการจับกันของโปรตีนตัวตอบรับ 37/67kDa high affinity laminin receptor กับไวรัสเด็งกี่ซีโรไทป์หนึ่งในคอลัมน์ [Upanan et al.,2008] ในงานวิจัยที่เกี่ยวข้องผู้วิจัยได้แสดงให้เห็นว่าเซลล์ตับของมนุษย์เป็นเซลล์เป้าหมายชนิดหนึ่งของไวรัสเด็งกี่ [Suksanpaisan et al., 2007] อีกทั้งยังแสดงให้เห็นว่า heat shock protein ไม่มีบทบาทในการเข้าสู่เซลล์ตับของไวรัสเด็งกี่ [Cabrera-Hernandez et al.,2007]

ในการศึกษาเกี่ยวกับเหตุการณ์ที่เกิดขึ้นหลังการจับของไวรัสกับโปรตีนตัวตอบรับ ผู้วิจัยได้ทำการศึกษาครอบคลุมถึงกระบวนการการเข้าสู่เซลล์ของไวรัสเด็งกี่โดยใช้สารยับยั้งทางชีวเคมี, การแสดงออกของการกลายพันธุ์ของยีนส์และ เทคโนโลยี siRNA ซึ่งผู้วิจัยได้แสดงให้เห็นว่าไวรัสเด็งกี่สามารถเข้าสู่เซลล์ตับได้หลายวิธีโดยวิธีหลักได้แก่ clathrin mediated endocytosis [Suksanpaisan et al., 2009] และในการศึกษาปฏิสัมพันธ์ระหว่างไวรัสเด็งกี่กับออโตฟาจีในเซลล์แสดงให้เห็นการเชื่อมโยงของการเข้าสู่เซลล์ของไวรัสและเพิ่มจำนวนของไวรัสภายในเซลล์ [Khakpoor et al., 2009; Panyasrivanit et al.,2009a] จากผลการวิจัยที่ได้กล่าวมานี้ผู้วิจัยได้เสนอว่า การเข้าสู่เซลล์ของไวรัสเด็งกี่และเพิ่มจำนวนไวรัสภายในเซลล์เป็นกระบวนการที่ต่อเนื่องที่เป็นผลจากการเกิดปฏิสัมพันธ์ของไวรัสกับเยื่อหุ้มจากกระบวนการ endocytosis และ autophagy [Panyasrivanit et al.,2009b]

**คำสำคัญ: ออโตฟาจี, แคทริน, เดงกี่, เอนโดไซโตซิส, แมคโครพิโนไซโตซิส, ตัวตอบรับ**

## ABSTRACT

It has been estimated that some 3 billion people live in areas at the risk of infection with the dengue virus, and that up to 100 million infections occur each year, making dengue the most common arthropod-borne viral disease. Despite significant effort worldwide, much of how the dengue virus interacts with a host cell remains unclear. One of the most critical interactions that defines pathogenicity is the initial interaction of the virus with the host cell at a receptor or receptors. The subsequent interactions of the virus with the host cell will in a large part be determined by this initial interaction, and in particular by the specific cellular compartment into which the virus is deposited. As such, the nature of the receptor interaction, mode of entry and interaction with the host cell machinery constitute a continuous spectrum of interactions.

We have previously identified two dengue virus receptor proteins utilized in a serotype specific manner by the dengue virus to gain entry into liver cells [Jindadamrongwech et al., 2004; Thepparit and Smith, 2004]. To further understand this interaction we utilized affinity column chromatography to probe both the serotype specificity of the dengue virus interaction, as well as to determine the specificity of the reaction. A dengue virus serotype 2 affinity column was able to bind the dengue serotype 2 receptor previously identified (GRP78), but not the dengue serotype 1 receptor, the 37/67kDa high affinity laminin receptor protein [Upanan et al., 2008]. In associated work, we were able to provide substantive evidence that human hepatocytes are a legitimate dengue virus target [Suksanpaisan et al., 2007], as well as ruling out a role for heat shock proteins in the internalization of dengue virus into liver cells [Cabrera-Hernandez et al., 2007].

To investigate events immediately subsequent to receptor binding, we undertook a comprehensive analysis of the mechanism of dengue virus entry. Using a combination of biochemical inhibitors, dominant negative mutant expression and siRNA technology we were able to demonstrate entry of the dengue virus into liver cells by multiple pathways. The majority of dengue virus entry was determined to be via clathrin mediated endocytosis [Suksanpaisan et al., 2009]. Studies we initiated on the interaction between the dengue virus and the cellular autophagy pathway linked dengue virus entry and subsequent translation and replication [Khakpoor et al., 2009; Panyasrivanit et al., 2009a]. As a result of this, we have recently proposed that dengue virus entry and translation replication are a continuous process resulting from an interaction with membranes of an endosomal-autophagosomal lineage [Panyasrivanit et al., 2009b].



**Key words: autophagy, clathrin, dengue, endocytosis, macropinocytosis, receptor**

# Executive Summary

## Introduction

Approximately 2.5 billion people live in areas at risk of infection with the dengue viruses, and up to 100 million infections are believed to occur annually [Guzman and Kouri, 2002]. While the majority of these infections are believed to be asymptomatic, infection may result in a febrile disease termed dengue fever (DF) or it may result in hemorrhagic manifestations which are classified as either dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) dependent upon severity [Halstead, 1989]. The causative agent of DF, DHF and DSS are the dengue viruses. These viruses are classified in the family Flaviviridae, genus Flavivirus, and species Dengue virus. There are four antigenically distinct viruses, termed dengue serotypes 1, 2, 3 and 4. The dengue viruses are enveloped positive-sense single-stranded RNA viruses of approximately 11 kb and encode three structural proteins (core, pre-membrane and envelope) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) in one open reading frame [Chang, 1997]. The dengue viruses are trasnsmitted primarily by Aedes aegypti mosquitoes, although other Aedes species such as Aedes albopictus may also serve as transmission vectors.

In humans, the impact of the dengue viruses on liver function is prominent as shown by high rates of hepatomegaly [Mohan et al., 2000], liver enzyme abnormality [Kuo et al., 1992; Mohan et al., 2000; Nguyen et al., 1997; Wahid et al., 2000], occasional fulminant hepatic failure [Subramanian et al., 2005] and histological changes including hepatocellular necrosis [Bhamarapravati, 1989].

In previous studies, we have established that the pattern of binding of the dengue virus to cell surface proteins on liver cells is different when different dengue serotypes are used [Jindadamrongwech and Smith, 2004], and in extension of this have identified

two serotype specific dengue virus receptor proteins [Jindadamrongwech et al., 2004; Thepparit and Smith, 2004]. Both of these proteins were identified through initial virus overlay protein binding assays (VOPBA), and as such there is some question as to whether these proteins represent bone fide receptor proteins given that the initial protein separation step requires denaturation of cell surface proteins. To address this, in this project affinity column chromatography was utilized to bind, under physiological conditions, liver cell surface expressed proteins. Bound and subsequently eluted proteins were analyzed by western blotting. We were able to demonstrate that dengue serotype 2 specifically bound GRP78, our previously identified dengue serotype 2 receptor [Jindadamrongwech et al., 2004], but that the 37/67kDa high affinity laminin receptor, which we have identified as a dengue serotype 1 receptor was not bound by dengue 2 [Upanan et al., 2008]. This result critically supports our previous work that dengue virus receptor usage in liver cells is serotype specific, as well as providing further evidence for a physiologically relevant interaction between dengue E protein and GRP78. While this and our previous studies have utilized liver cell lines, the issue of whether liver cells represent a bone fide cellular target of the dengue virus is of significant importance. This issue was conclusively addressed in our study showing that primary human hepatocytes can be productively infected with dengue serotype 2 [Suksanpaisan et al., 2007].

To attempt to provide further supporting evidence for our work showing that the 37/67kDa high affinity laminin receptor protein is a dengue serotype 2 receptor, we attempted to down regulate this gene in liver cells. Unfortunately siRNA mediated ablation of expression of the 37/67kDa high affinity laminin receptor resulted in the induction of apoptosis, a result we subsequently published [Susantad and Smith, 2008]. In parallel studies, we ruled out a role for hsp70 and hsp90 as dengue receptor proteins on liver cells [Cabrera-Hernandez et al., 2007].

In other work, the method of entry of the dengue virus, post receptor was investigated using a combination of specific biochemical inhibitors of various entry mechanisms (endocytosis, macropinocytosis and cavaeola mediated entry), over expression of dominant negative mutants of Eps15 (for functional knock out of clathrin mediated endocytosis) as well as siRNA mediated ablation of gene expression to knock down clathrin heavy chain expression. Combined, these results showed that the dengue virus can enter into liver cells by multiple pathways. To determine where internalization of the virus deposits the virus, and how this links to the host cell machinery, we initiated studies on the subcellular compartmentalization of the dengue virus. Using parallels with poliovirus, we investigated the process of autophagy, and in particular how this linked with virus entry. We identified a key vesicle, amphisomes, which represent convergence of the endocytosis and autophagy pathways as a site for dengue replication and translation [Khakpoor et al., 2009; Panyasrivanit et al., 2009a; Panyasrivanit et al., 2009b]. Overall, our work is progressing towards linking virus receptor binding, internalization, replication and translation and exit from the cell in a single, unified model [Panyasrivanit et al., 2009b].

**Results and Discussion**

To investigate dengue virus receptor binding under physiological conditions, whole, live dengue virus serotype 2 was used as a ligand after attachment to a sepharose matrix to trap dengue virus binding proteins present in cell membrane extracts of human hepatoma cells. Initial experiments showed a significant amount of non specific binding, and therefore a 2 column strategy was applied, with a pre-absorption column of bovine serum albumin as the ligand being employed. We were able to demonstrate specific binding of GRP78, but not the 37/67kDa high affinity receptor to dengue 2 both confirming our earlier work with regards to serotype specificity of the initial host cell

virus interaction, as well as more directly demonstrating a direct, and physiologically relevant interaction between dengue-2 E protein and GRP78 [Upanan et al., 2008]. At the same time we were able to rule out a role for hsp70 and hsp90 as receptors mediating dengue entry into liver cells [Cabrera-Hernandez et al., 2007] and using purified human primary hepatocytes, we were able to confirm that liver cells are a valid target for dengue virus infections [Suksanpaisan et al., 2007].

After receptor binding, the dengue virus is internalized into the cell by one of a number of possible different mechanisms, including clathrin mediated endocytosis, macropinocytosis or caveolae mediated endocytosis. To investigate the process by which the dengue virus enters into cells, we first used a full range of biochemical inhibitors specific to different pathways to analyze the entry of all four dengue serotypes. These inhibitors provided evidence suggesting that multiple pathways are possibly used by dengue to enter into cells, and moreover that different serotypes use these pathways in different proportions. More detailed genetic experiments with dengue virus serotype 2 and either dominant negative constructs of Eps15 (which inhibit clathrin mediated endocytosis) or siRNA constructs designed to silence clathrin heavy chain expression showed that DEN-2 primarily entered into cells by clathrin mediated endocytosis. However, a significant proportion of virus entry (some 20%) was found to occur when clathrin mediated entry was abolished, and moreover that entry was only finally knocked out when both clathrin mediated and macropinocytosis were inactivated, confirming our experiments which suggest that dengue virus used multiple pathways to enter into cells [Suksanpaisan et al., 2009].

After the virus has internalized, there is considerable doubt as to the fate of the released nucleocapsid. Using parallels from other viruses such as poliovirus which utilizes the cellular autophagy mechanism to facilitate its replication, we sought to

investigate the relationship between the dengue virus and autophagy. While the process of autophagy is primarily considered as the host cell reclamation system for old or unwanted cellular constituents, several viruses are known to hijack this system. Using a number of biochemical modulators of autophagy and numerous cellular markers, we were able to show colocalization of constituents of the dengue virus replication-translation machinery with the autophagy pathway. Of particular significance, we were able to identify amphisomes, a vesicle that links endocytosis and autophagy as a key player in the replication strategy of dengue [Panyasrivanit et al., 2009a]. We were moreover able to show that the interaction between dengue and autophagy is modulated, at least in part by the specific serotype of the dengue virus, and while amphisomes are critical in dengue serotype 2 replication, autophagolysosomes, a post lysosomal fusion vacuole are important in dengue 3 replication [Khakpoor et al., 2009].

Combining our results on virus entry and autophagy, we have recently proposed a novel mechanism of the dengue life cycle in which the life cycle can be defined in terms of a continual association with membranes of an endosomal-autophagosomal lineage [Khakpoor et al., 2009; Panyasrivanit et al., 2009a; Panyasrivanit et al., 2009b]. This model is particularly attractive, as it obviates the need for a free floating nucleocapsid stage in dengue replication. It is hoped that this model will, over time be expended to link exocytosis (release of the virus particle) in a model that explains the entire life cycle of the dengue virus in a single unified model.

## Details of research

### Receptor binding studies

While the involvement of the liver in cases of dengue fever, and particularly in the severe forms of the disease has been well documented, the precise mechanism of involvement remains a subject of considerable controversy, particularly in determining

whether the involvement is a primary condition, resulting from direct infection of liver cells, or a secondary involvement, perhaps resulting from an immune reaction to the infection. As our work primarily investigates the dengue virus:liver cell interaction, and our work sought to investigate the binding of dengue virus to liver cell expressed receptors, the question is of considerable significance. We therefore initially sought to address the question of whether liver cells, and specifically hepatocytes, are infectable by the dengue virus. Commercially purchased human primary hepatocytes (sourced from donor liver transplant cut downs) were experimentally infected with dengue virus serotype 2, and de novo virus production assessed by plaque assay technique. Infection of hepatocytes was confirmed by immunoflourescence with antibodies directed against dengue virus E protein and dengue virus NS1 protein. The results confirmed that primary human hepatocytes are infectable by the dengue virus. It was more over established that infection resulted in morphological changes characteristic of apoptosis and that a significant cytokine response, including IL-8 was induced by dengue virus infection. These results established that liver cells in humans are a viable target of the dengue virus, and as such probably contribute to the pathogenesis of the disease.

To investigate the nature of dengue virus receptors expressed on the surface of lvier cells, further experiments were undertaken with standard transformed liver cells. Membrane proteins from liver cells were passed down a column with DEN-2 bound as a ligand. Potential binding proteins (including recetor protein) would bind specifically to the dengue virus, and could be later eluted by increasing concentrations of NaCl used as a wash. To construct a column with dengue serotype 2 as the ligand for use in receptor isolation studies, live, infectious DEN-2 was covalently linked to activated sepharose 4B using CNBr crosslinking. The efficiency of binding was assessed by plaque assay on samples taken both pre and post linking. Membrane proteins extracted from hepG2 cells

were then passed down the column, and, after washing, eluted with increasing concentrations of NaCl. Eluted fractions were concentrated and run on SDS-PAGE gels and proteins subsequently transferred to nitrocellulose membranes. Membranes were used in western blotting analysis with antibodies against GRP78 and the 37/67kDa high affinity laminin receptor protein. A band eluting from high salt fractions was seen in western analysis with an anti GRP78 antibody, but no band was seen when the western was undertaken with an antibody directed against the 37/67kDa high affinity laminin receptor. Subsequent western blotting with an antibody against hsp70 showed both significant numbers of cross reacting bands, as well as a significant number of bands eluting in higher salt concentration, suggesting that non-specific protein-protein interactions could be sufficient to cause retention on the column.

We therefore established a two column system, with a "pre-"column being constructed with bovine serum albumin as the ligand. Membrane proteins from liver cells were applied to the column and the flow through (proteins not binding to an unrelated ligand through protein-protein interactions) were applied to a new sepharose-dengue 2 column. Again, after binding, proteins were eluted with increasing concentrations of NaCl and analyzed by western blotting using antibodies against GRP78 and hsp70. We were able to show specific retention of GRP78 by dengue-2, and a dramatically reduced retention of hsp70. These results support our earlier work suggesting that dengue virus receptor usage in liver cells in serotype specific, and that there is a physiologically relevant interaction between GRP78 and dengue 2.

As we had observed some retention of hsp70 on the dengue-2 column, the role of hsp70 and hsp90, which have been implicated as dengue receptors in monocytes [Reyes-del Valle et al., 2005], was further investigated. Using a combination of antibody mediated inhibition of infection, and lipopolysaccharide (LPS) pretreatment the entry of

all four dengue serotypes into liver cells was investigated. Lipopolysaccharide was used as an inhibitory molecule as hsp70 and hsp90 have previously been shown to be the CD14-independent cell surface receptor in human monocytes and macrophages [Triantafilou et al., 2001; Triantafilou and Triantafilou, 2002]. Results clearly demonstrated no inhibition of infection with either pretreatment against antibodies directed against hsp70 or hsp90, or by pretreatment with lipopolysaccharide [Cabrera-Hernandez et al., 2007]. A control experiment, using an antibody directed against GRP78 as an inhibitory molecule for dengue serotype 2 infection again produced a significant deficit in virus entry, confirming our earlier results [Jindadamrongwech et al., 2004]. We also showed that treatment to induce hsp70 and hsp90 (a heat shock) did not increase dengue virus entry [Cabrera-Hernandez et al., 2007]. Collectively these results supported our contention that GRP78 is a receptor molecule for dengue virus serotype 2.

To further expand our results showing that the 37/67kda high affinity laminin receptor protein is a dengue virus serotype 1 receptor in liver cells, we attempted to use siRNA to inhibit expression of this protein. If our model was correct, inhibition of the 37/67kda high affinity laminin receptor would reduce entry of dengue 1 into liver cells, but leave the entry of other serotypes unaffected. Extensive optimization was required to silence expression of the 37/67kda high affinity laminin receptor, and, surprisingly, when this was achieved, the cells underwent apoptosis, suggesting this protein is a critical one for cell viability. While this results was disappointing from the aspect of our dengue work, it was sufficiently important in understanding cancer cell biology to warrant publication [Susantad and Smith, 2008].

**Post receptor binding studies**

To complement our studies on dengue virus receptor binding, we initiated studies to investigate the immediate post receptor events. Viruses can enter into cells through a number of different routes, including clathrin mediated endocytosis, macropinocytosis and caveolae mediated endocytosis. A number of biochemical inhibitors of different pathways have been characterized, and we determined the effect of several of these on the entry mechanism of all four serotypes of the dengue virus [Suksanpaisan et al., 2009]. Initially screened were the effects of cytochalasin D, amiloride, LY294002 and wortmanin to inhibit macropinocytosis, nystatin to inhibit caveolae mediated entry and chlorpromazine to inhibit clathrin mediated endocytosis. For each biochemical agent, cytotoxicity was determined using Annexin V staining and flow cytometery, and the highest non-toxic concentration was used.

Results suggested that a significant, but variable reduction of entry was seen for all four serotypes when infection occurred in the presence of an inhibitor of clathrin mediated entry. Interestingly however, inhibitors of macropinocytosis also reduced virus entry to a variable amount depending both upon the specific inhibitor used, as well as the specific serotype of dengue virus.

Given that biochemical inhibitors can cause broad ranging cellular effects, virus entry was investigated using more specific, genetic methodologies. Initially cells were transfected with dominant negative constructs of Eps15, which are able to inhibit clathrin mediated endocytosis, while not affecting other pathways. Dengue virus entry of all four serotypes was significantly reduced in the presence of Eps15 mutants, but, critically, while transferring (a clathrin mediated endocytosis control) was completely excluded, a significant (up to 20%) amount of dengue virus entry was still observed. This suggests that entry of the dengue virus into liver cells can occur by multiple pathways

[Suksanpaisan et al., 2009].

To confirm that dengue virus entry can occur in the absence of clathrin mediated endocytosis, use was made of siRNA technology to down regulate the expression of clathrin heavy chain, and integral part of the clathrin vesicle. After optimization of silencing, clathrin heavy chain silenced cells were exposed to the dengue virus. Consistent with the results seen for Eps15 mutants, while entry of the dengue virus was largely excluded, a significant level of entry was still observed. Finally, a combination of siRNA treatment to knock out clathrin mediated endocytosis and wortmanin treatment to inhibit macropinocytosis was used which almost completely inhibited dengue virus entry. Collectively, these results support a model where by dengue virus entry occurs by multiple pathways (clathrin mediated endocytosis and macropinocytosis) where clathrin mediated entry is the dominant pathway (some 80% of entry) while macropinocytosis is responsible for some 20% of virus entry in culture [Suksanpaisan et al., 2009].

Further experiments were aimed at exploring how virus entry, and subsequent replication and translation are linked. The process of autophagy, the cellular mechanism used to recycle the constituents of old or unwanted proteins and organelles has been suggested to be involved in the replication of several viruses, including poliovirus. We therefore sought to determine whether autophagy was induced in response to dengue virus infection, and subsequently whether there was an interaction between the autophagy pathway and constituents of the dengue virus replication-translation complex. By biochemically manipulating of the process of autophagy, we were able to show both that autophagy is induced by dengue virus infection, and moreover that disruption of autophagy resulted in a significant reduction of dengue virus yield, suggesting that the process of autophagy was intimately linked with the dengue virus replication strategy [Panyasrivanit et al., 2009a]. A combination of further biochemical manipulation and

confocal microscopy was able to show that constituents of the dengue virus replication complex (NS1 protein and double stranded [replicative form] RNA) colocalized with markers of autophagy such as LC3 [Panyasrivanit et al., 2009a] . We were further able to show that the particular autophagic vesicles of primary significance in DEN-2 replication were amphisomes [Panyasrivanit et al., 2009a]. Amphisomes represent a vesicle formed between the autophagic pathway and the endocytic pathway, and as such were able to provide a link between the processes of virus entry and replication/translation [Panyasrivanit et al., 2009a; Panyasrivanit et al., 2009b], and we have recently proposed a model in which the dengue virus life cycle can be seen in terms of a continual and ongoing interaction with membranes of an endosomal-autophagosomal lineage. This model has significant advantages over the previous models of dengue virus replication which had no direct link between the processes of virus entry and replication (on endoplasmic reticulum membranes). It is perhaps not entirely un-coincidental that autophagic membranes are known to display several ER markers.

Further studies with DEN-3 have shown that DEN-3 similarly induces autophagy, but that there are some serotype specific differences. Critical to these are that the primary vesicle for replication-translation are autophagolysosomes, the post lysosomal fusion vesicle of autophagy [Khakpoor et al., 2009].

## Conclusions

The work described in this report has produced several significant advances in our understanding of how the dengue virus interacts with human liver cells. We have confirmed that hepatocytes are a valid a target, as well as the serotype specific nature of the interaction between dengue virus and its cognate receptor proteins. More critically, our studies on the mechanism of dengue virus entry, and subsequent translation and

replication have produced an entirely new model of the dengue virus life cycle. We have proposed for the first time that dengue virus entry and replication and translation are linked, and that the dengue virus life cycle can be described in terms of a continual and ongoing association with membranes of an endosomal-autophagosomal lineage.

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## OUTPUT: ABSTRACTS PRESENTED AT SCIENTIFIC MEETINGS

**Invited speaker**

Duncan R. Smith. The dengue virus host cell interaction. Plenary speaker at the 2nd Health and Medical Sciences Congress. 18th-20th June, 2008. University Sains Malaysia, Penang, Malaysia.

**Oral Presentations**

1. Duncan Smith, Atefeh Khakpoor and Mingkwan Panyasrivanit. The dengue virus translation-replication complex is located in close association with autophagic membranes. Second International Conference on Dengue and Dengue Haemorrhagic Fever. October 15-17, 2008. Phuket, Thailand,

2. Maneerat Ekkapongpisit, Lukkana Suksanpaisana, Mingkwan Panyasrivanicha, Giuseppina Nicotra, Ciro Isidoro and Duncan R. Smith. Subversion of the cellular autophagy machinery by the dengue virus and location of the dengue virus replication complex on autophagic membranes. RGJ-PhD Congress IX. April 3 -5, 2008. Pattaya, Thailand.

**Poster presentations**

1. Duncan R. Smith, Chutima Thepparit and Atefeh Khakpoor. Dengue virus genotype specific induction of apoptosis in transformed liver cells. Third Asian Regional Dengue Research Network Meeting. 22nd – 24th August, 2007. Taipei, Taiwan.

2. Atefeh Khakpoor, Chutima Thepparit, Duncan R. Smith. Mechanism of Dengue virus induced apoptosis in liver cells. 8th National Graduate Research Conference. September 7th -8th, 2007. Mahidol University, Thailand.

3. Smith, D.R., Suksanpaisan, L., Cabrera-Hernandez, A., Ekkapongpisit, M., Wannatung, T., Kanokporn Triwittayakorn, K. , Susantad, T. Response of primary and transformed liver cells to dengue virus infection. TRF Junior-Senior Meeting. 11th -13th October, 2007. Pattaya, Thailand. .

4. Maneerat Ekkapongpisit, Giuseppina Nicotra, Lukkana Suksanpaisan, Ciro Isidoro and Duncan R. Smith. Autophagy-lysosomal protein degradation pathway plays a role in dengue virus infection in HepG2 cells. Early steps of the Virus life cycle: Molecular and cellular insights. 4th-5th October 2007. Paris, France.

5. Maneerat Ekkapongpisit, Ciro Isidoro, Duncan R. Smith. The cellular responses of hepatocyte to dengue virus Infection: Autophagy vs Dengue virus interaction. RGJ-PhD Congress VIII. April 4 -6, 2007. Pattaya, Thailand.

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10. Mingkwan Panyasrivanit, Atefeh Khakpooh, Nitwara Wikan, Lukkana Suksanpisan and Duncan R. Smith. Localization of dengue virus translation replication complex at autophagic vacuole. Second International Conference on Dengue and Dengue Haemorrhagic Fever. October 15-17, 2008. Phuket, Thailand.

11. Mingkwan Panyasrivanit, Atefeh Khakpoor, Nitwara Wikan, and Duncan R. Smith.The Interaction between Autophagy and the Dengue virus. RGJ-PhD Congress X. April 3 -5, 2009. Pattaya, Thailand.

## Students.

The following students completed their studies during the course of this grant:

**Doctor of Philosophy (Ph.D.)**

Maneerat Ekkapongpisit. Thesis: The cellular responses of hepatocytes to dengue virus infection: Autophagy vs dengue virus interaction. (Completed, April, 2008)

Atefeh Khakpoor. Thesis: Mechanism of dengue virus induced apoptosis in liver cells. (Completed, April 2009).

**Master of Science (M.Sc.)**

Tharinee Susantad. Thesis: SiRNA mediated silencing of proteins involved in dengue virus internalization into liver cells. (Completed, October 2007)

Nitwara Wikan. Investigations into the dengue virus-host cell interaction in insect and human cell lines. (Completed March 2009).

# Appendix

# presentations

**The dengue virus- host cell interaction**

Duncan R. Smith

Institute of Molecular Biology and Genetics

Mahidol University, Thailand

Viruses are known to interact with the host cell machinery in a number of ways, and it is becoming increasingly clear that one critical mediator of the virus-host cell interaction is the interaction with the cellular autophagy machinery. Autophagy is a lysosomal degradation pathway involved in the cellular turnover of macromolecules and organelles that is conserved amongst eukaryotes. Viruses generally interact with the autophagic process in one of three ways: defense, avoidance or subversion. In a defense interaction autophagy is triggered to reduce or clear the invading pathogen, while in avoidance interactions the virus down regulates autophagy through the expression of a specific viral gene product. Subversion is perhaps the most interesting interaction, and in this process the virus is able to use the autophagic process as an integral part of the viral replication strategy. The dengue virus represents a significant public health hazard in many tropical and subtropical countries and infection with the dengue virus can result in a wide range of clinical manifestations. We have established that autophagy is induced by the dengue virus and that down regulation of autophagy substantially reduces dengue virus yield, indicating that the dengue virus subverts the autophagy process. Moreover, using a number of specific autophagic and endosomal markers we have been able to co-localize the dengue virus transcription and replication complexes with autophagic membranes. The entry of the dengue virus by receptor mediated endocytosis and trafficking to late endosomes is well characterized. We have been able to co-localize endosomal and autophagic markers in dengue infected cells and this allows us to propose a novel model describing the dengue virus replication strategy in terms of a continuing association with membranes of the endosomal – autophagic pathway. This new model has broad implications not only for the dengue virus but also for other RNA viruses.

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Basic Virology

# THE DENGUE VIRUS TRANSLATION-REPLICATION COMPLEX IS LOCATED IN CLOSE ASSOCIATION WITH AUTOPHAGIC MEMBRANES.

Duncan Smith[1] , Atefeh Khakpoor[1] , Mingkwan Panyasrivanit[1]

[1] *Institute of Molecular Biology and Genetics, Mahidol University, Thailand*

**BACKGROUND:** Dengue virus is a positive single stranded RNA virus, belonging to the flaviviridae family. Transmitted by mosquitoes, dengue virus infections can cause a broad spectrum of disease presentation in humans ranging from a mild febrile disease to severe life threatening manifestations. While the details of dengue virus replication in human cells remains obscure, some RNA viruses, such as corona virus and poliovirus have been shown subvert the cellular autophagy machinery as part of their replication strategy and to use autophagic membranes as sites for viral replication.

**OBJECTIVES:** This study sought to determine whether autophagy was induced in response to dengue virus infection, and whether parts of the dengue replication and translation complex co-localized with autophagic markers.

**METHODS:** Autophagy induction was determined by colocalization of LAMP1 and LC3 and analysis by confocal microscopy. Autophagy was modulated by biochemical treatment using rapamycin as an autophagy inducer and 3-methyladenine (3-MA) and L-asparagine (L-Asn) as autophagy inhibitors. Location of the dengue virus replication complex was assessed by confocal microscopy using antibodies directed against double stranded RNA and dengue virus NS1 protein in conjunction with autophagic markers.

**RESULTS:** Results demonstrated significant co-localization between LC3 and LAMP1 by 15 hours post infection. Further co-localization was observed between LC3 and double-stranded RNA, and between LC3 and NS1. Down regulation of autophagy resulted in a decrease in virus production and NS1 levels. Co-localization of ribosomal markers and LC3 was observed in response to dengue virus infection but not starvation-induced induction of autophagy.

**CONCLUSIONS:** These results support a model in which the dengue virus utilizes autophagic membranes as a site for the dengue virus replication- translation complex.

# Subversion of the cellular autophagy machinery by the dengue virus and location of the dengue virus replication complex on autophagic membrane

**Maneerat Ekkapongpisit,[a] Lukkana Suksanpaisan[a], Mingkwan Panyasrivanich[a], Giuseppina Nicotra [b], Ciro Isidoro,[b] Duncan R. Smith[a]**

[a]*Institute of Molecular Biology and Genetics, Mahidol University, Nakornpathom 73170, Thailand*
[b]*Department of Medical Science, "Amedeo Avogadro" University, Novara 28000, Italy*

## Introduction and Objective

Autophagy, a lysosomal protein degradation pathway, has been considered as host defense mechanism. The role of autophagy upon virus infection had been recently reported. This study aims to study the role of autophagy in dengue virus infection on liver cell lines.

## Method

To determine whether autophagy is induced in response to Dengue virus infection in HepG2 cells, and whether it modulates the production of the Dengue viruses, firstly characterization of Autophagy in HepG2 induced by nutrient starvation and drug treatment were studies. Then, Dengue virus induced Autophagy and it role in dengue virus infection were characterized using immuno co-localization, Western blot analysis. And the Biological effect of autophagy modulation on Dengue virus production was investigated.

## Results

Characterization of Autophagy in HepG2 cells showed efficient regulated Autophagy by nutrient starvation and drug treatment. Dengue virus infection rapidly induced Autophagy and was independent to virus viability as shown by UV-inactivated virus infection. Co-localization of the Dengue specific viral protein NS1 and dsRNA with the autophagosome marker, LC3 was observed after 15, 24 and 72 hours post infection indicating the role of autophagy in RNA replication complexes. Reduction of NS1 protein expression in the presence of autophagy inhibitors 3-methyladenine (3-MA) and L-asparagine (L-Asn) was observed as shown by Western blot analysis and immunofluorescence. Co-localization of ribosomal protein (L28) with dsRNA and E protein was shown. In addition, immunocolocalization between Cathepsin D, a lysosome/autolysosome marker, and dengue viral proteins showed no co-localization between CD and NS1 but it was strongly co-localized with dengue E proteins. The virus production studies showed that while Rapamycin (Rap), the inhibitor of mTOR that up-regulates autophagy, did not modify the production of virus, both 3-MA and L-Asn reduced Dengue virus yield about 4-5 fold.

## Conclusion

These studies support the subversion of autophagy by dengue virus infection in HepG2 and provide the re-interpretation of dengue virus life cycle from viral RNA replication, translation and employ the lineage of virus trafficking via endosomal-autophagic pathway.

**Key words:** Dengue virus, Autophagy, replication complex, HepG2

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# Dengue virus genotype specific induction of apoptosis in transformed liver cells

Duncan R. Smith, Chutima Thepparit and Atefeh Khakpoor

Abstract

The induction of apoptosis in response to dengue virus infection of transformed liver cells has been well documented. Despite this however, all studies to date have used dengue virus serotype 2 strains. We have examined the apoptotic response of transformed liver cells (HepG2) to dengue virus serotype 2 (strain 16681) and dengue virus serotype 3 (strain 16562). The apoptotic response of the cells was determined by analysis of cell morphology, chromatin condensation, DNA ladder production, Annexin V/propidium iodide staining, activation of caspases 3, 7, 8 and 9 by either western blotting or ELISA assay and investigation of mitochondrial transmembrane potential using $DiOC_6(3)$. All of the results showed that while there was a significant induction of apoptosis with dengue virus serotype 2 (strain 16681), there was little or no induction of apoptosis with dengue virus serotype 3 (strain 16562). These results support a genotype specific induction of apoptosis in transformed liver cells.

To investigate the role of ER stress in the response to dengue virus infection, HepG2 cells were infected with either dengue serotype 2 (strain 16681) or 3 (strain 16562) and samples examined by western blotting for the up regulation of GRP78 and the activation of caspase 12, while the activation of the unfolded protein response (UPR) IRE1 stress response pathway was investigated using RT-PCR to detect the IRE1 mediated stress specific splicing event that results in the excision of a 26 nucleotide intron from XPB-1. Neither dengue serotype 2 nor dengue serotype 3 were seen to induce either GRP78, nor activate caspase 12. However, dengue virus serotype 2 (strain 16681) was shown to activate the unfolded protein response with the IRE1 mediated splicing of XPB-1, while infection with dengue virus serotype 3 did not result in the activation of this pathway.

These results support a genotype specific induction of apoptosis in dengue virus infected liver cells. Interestingly, the trigger for the induction of apoptosis appears to be through ER stress pathways, but the execution of apoptosis appears to be through the mitochondria-mediated apoptosis pathway. We are currently examining genes involved in the link between ER mediated stress and the mitochondrial apoptotic pathway.

Apoptosis, caspase, dengue, ER, mitochondria, stress

# Mechanism of Dengue virus induced apoptosis in liver cells

Atefeh Khakpoor, Chuttima Thepparit and Duncan R. Smith
Molecular Pathology Laboratory, Institute of Molecular Biology and Genetics, Mahidol University

**Abstract:**
Infection of human liver cells with dengue virus, the causative agent of dengue fever, dengue hemorrhagic fever, and Dengue shock syndrome, has been shown to result in the induction of apoptosis in the host cells. Few studies have investigated the mechanism of induction of apoptosis, and none have examined the influence of different dengue serotypes on this process. This study has focused on determining the mechanism of dengue virus induction, as well as the influence of dengue serotype by infecting liver cells with either dengue virus serotype 2 or 3. HepG2 cells were infected with dengue virus serotype 2 or 3 at MOI of 10 and cells examined for a number of apoptotic markers between 2 and 5 days post infection. DNA laddering, chromatin condensation and a high percentage of late apoptotic cells as determined by AnnexinV/propidium iodide staining and flow cytometry were all observed in response to dengue virus 2 serotype, but not dengue virus serotype 3 infection. Similarly, activation of caspases 8, 9, 3 and 7 was observed upon infection with Dengue virus serotype 2, but not with serotype3. Since activation of the caspase cascade is a hallmark of the ER stress response, this response pathway was also studied. Unfolded protein accumulation in ER triggers ER stress response by activation of IRE1 kinase, which leads to splicing of XBP-1 transcript by cutting out a 26 nt intron. Total RNA was extracted from dengue infected cells as well as HepG2 cells which were treated with Tunicamycin (an ER stress inducer). RT-PCR for XBP-1 gene interestingly showed the splicing form of transcript in dengue virus serotype 2 infected cells similar to what was observed in tunicamycin treated cells after 48 hours postinfection but not in Dengue virus serotype 3. Suggesting that the activation of apoptosis by dengue serotype 2, but not serotype 3 maybe the result of ER stress induction and that this induction may well be determined by the specific viral genotype.

Key words: Dengue virus, apoptosis, ER stress response, IRE1 pathway, Caspase cascade, XBP-1 gene.

# Response of primary and transformed liver cells to dengue virus infection

Smith, D.R.[1*], Suksanpaisan, L.[1], Cabrera-Hernandez, A.[1], Ekkapongpisit, M.[1], Wannatong, T.[1], Kanokporn Triwittayakorn, K.[2], Susantad, T.[1]

[1]*Molecular Pathology Laboratory, Institute of Molecular Biology and Genetics, Mahidol University, Thailand*

*2Genomics Laboratory, Institute of Molecular Biology and Genetics, Mahidol University, Thailand*

**Abstract**

While significant evidence including hepatomegaly, liver enzyme abnormality and occasional fulminant hepatic failure suggest a pathological role for the liver in severe cases of dengue infection, it is still unclear as to whether this role is as an active participant in the infection process, or as a consequence of bystander effects. To address this issue we initially sought to determine whether isolated primary human hepatocytes (the major cellular component of the liver) are able to be infected by the dengue virus. Infection of primary human hepatocytes was established by a combination of observing *de novo* production of viral progeny by standard plaque assay as well as by RT-PCR and immunocytochemistry. The cellular consequences of infection of primary human hepatocytes included the up-regulation of the cytokines TRAIL, MIP-1α, IFN-β, MIP-1β, IL-8 and RANTES. Significant secretion of immunoreactive IL-8 was also observed. To further understand the effects of dengue virus infection on transformed hepatocytes the technique of cDNA-AFLP was employed to isolate and characterize transcript derived fragments from genes transcriptionally regulated upon dengue virus infection. From 73 primer combinations over 5000 transcription derived fragments (TDF's) were observed, of which approximately 10% were differentially regulated in response to infection. Sixty-five TDF's were subsequently cloned and sequenced and 27 unique gene transcripts identified. Semi-quantitative RT-PCR was used to validate the expression of 12 of these genes and 10 transcripts (CK2, KIAA509, HSP70, AK3L, NIPA, PHIP, RiboS4, JEM-1, MALT1 and HSI12044) were confirmed to be differentially regulated, with 4 transcripts (HSP70, NIPA, RiboS4 and JEM-1) showing a greater than 2 fold regulation. These results suggest that the expression of a large number of genes is altered in response to dengue virus infection of liver cells, and that further exploration of the response of both transformed and primary hepatocytes will provide valuable information in understanding the pathobiology the dengue virus-liver cell interaction.

**Keywords:** dengue, flavivirus, hepatocyte, primary cell, cytokine, transcription,

# Autophagy-lysosomal protein degradation pathway plays a role in dengue virus infection in HepG2 cells

Maneerat Ekkapongpisit[1], Giuseppina Nicotra[2,] Lukkana Suksanpaisan[1], Ciro Isidoro[2] and Duncan R. Smith[1]

1 Molecular Pathology Laboratory, Institution of Molecular Biology and Genetics, Mahidol University, Nakornpathom, Thailand 73170.
2 Molecular Pathology Laboratory, Department of Science and Medicine, "Amedeo Avogadro" Institutute, Novara, Italy 28000

Autophagy is a membrane trafficking process that leads to the lysosomal degradation of cytoplasmic structures and whose hallmark is the emergence of double-membrane autophagic vacuoles. The autophagic vacuole originates from a crescent shaped double layered membrane structure which engulfs a portion of the cytosol and subsequently forms an enclosed autophagic vacuole. These enclosed vesicles then fuse with lysosomes to form the autophagolysosome where the cargo inside the vacuole is eventually degraded. It has been shown that in cases of viral infection, the process of autophagy is activated. While this process is an important defense against some viruses such as the Sindbis virus, other viruses such as poliovirus subvert the autophagy process and use the double membrane structures as part of the viral replication complex. In this case autophagy is required for efficient replication and maturation of the virus. So far, whether and how autophagy are involved in dengue virus infection and replication have not been investigated. To determine whether autophagy is induced in response to dengue virus infection, and it modulates the production of the dengue viruses, dengue virus serotype 2 was used to infect the human liver cell line HepG2 in the presence or absence of pharmacological inhibitors and activators of the autophagy pathway. The virus production was assessed by standard plaque assay on Vero cells. Autophagy was studied by analyzing the expression and localization of autophagy proteins. Detection of LC3 and Beclin-1 aggregates in infected cells indicated the induction of autophagy by dengue virus infection, a finding confirmed by electron microscopy. Both autophagy inhibitors 3-methyadenine and asparagine reduced dengue virus yield of about 4-5 fold. Our data suggest that Autophagy is involved in dengue virus infection in HepG2 cells.

# THE CELLULAR RESPONSES OF HEPATOCYTE TO DENGUE VIRUS INFECTION: AUTOPHAGY VS DENGUE VIRUS INTERACTION

**Maneerat Ekkapongpisit,[a] Ciro Isidoro,[b] Duncan R. Smith[a]**
[a]*Institute of Molecular Biology and Genetics, Mahidol University, Nakornpathom 73170, Thailand*
[b]*Department of Medical Science, "Amedeo Avogadro" University, Novara 28000, Italy*

## Introduction and Objective

Autophagy, a lysosomal protein degradation process is a host defense mechanism that has been shown to be activated upon virus infection. This study aims to investigate the role of autophagy in dengue virus infected liver cells.

## Method

To study whether autophagy plays a role in dengue virus infection, liver cells were infected with the dengue virus and cells examined with MDC fluorescence dye labeling to observe autophagic vacuoles. In addition, dengue virus production and cell viability was examined in the presence of biochemical inhibitors of a number of different stages of the autophagy process and samples assayed by standard plaque assay, trypan blue exclusion and flow cytometry.

## Results

The results showed that the virus production of all 4 dengue serotypes from HepG2 was altered upon biochemical modulation of the autophagy pathway, while no alteration was observed for DEN-2 production from Hep3B cells. MDC-labeled vacuoles were detected in DEN-2 and DEN-3 infected HepG2 cells. Cell viability studies showed that autophagy inhibition reduced cell viability in DEN-2 infected HepG2 but not Hep3B cells as compared with DEN-3 infection. However, no effect of autophagy enhancement on cell viability was observed in dengue virus infected HepG2 and Hep3B cells.

## Conclusion

The alteration of dengue virus production upon autophagy modulation together with the observed of MDC-labeled vacuoles in infected cells reveals the induction of autophagy upon dengue virus infection. The cell viability studies suggest the involvement of autophagy in dengue virus infection induced cell death. These imply that both apoptotic and autophagic cell death are triggered as a cellular response to dengue virus infection of liver cell lines, although this is dependent upon both cell and serotype specific factors. The exact role of autophagy in modulating dengue virus infection needs to be further investigated.

**Key words:** Dengue virus, Autophagy, Apoptotic cell death, HepG2, Hep3B

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# การศึกษาการเข้าสู่เซลล์ตับของไวรัสไข้เลือดออกซีโรไทป์2

# CHARACTERIZATION OF DENGUE VIRUS SEROTYPE 2 ENTRY INTO LIVER CELL

ฐารินี สุสันทัด,[1] ลักขณา สุขสันต์ไพศาล[1] และ ดันแคน อาร์ สมิทธ์[1]

Tharinee Susantad,[1] Lukkana Suksanpaisan[1] and Duncan R. Smith[1]
[1] Molecular Pathology Laboratory, Institute of Molecular Biology and Genetics, Mahidol University, Nakorn Pathom, 73170. Email: jomjam_aus@hotmail.com

**บทคัดย่อ:** ไวรัสไข้เลือดออกเป็นสาเหตุสำคัญของโรคไข้เลือดออก เชื่อว่าการติดเชื้อของไวรัสนี้ในเซลล์ของสัตว์เลี้ยงลูกด้วยนมสามารถเกิดโดยผ่านหนึ่งในสามกระบวนการดังต่อไปนี้ คือ Clathrin coated pit, Caveolae และ Macropinocytosis ในงานวิจัยนี้มีการใช้สารยับยั้งทางชีวเคมีเพื่อยับยั้งกระบวนการดังกล่าว พบว่าไวรัสไข้เลือดออกซีโรไทป์2 สามารถเข้าสู่เซลล์ตับได้หลายวิธี การส่ง mutant ของยีน Eps15 ซึ่งเป็นยีนที่มีบทบาทในกระบวนการ Clathrin coated pit เข้าไปในเซลล์ พบว่าส่งผลให้ไวรัสเข้าสู่เซลล์ได้ลดลงถึง 80% เพื่อเป็นการยืนยันผลการทดลองดังกล่าวจึงมีการใช้เทคนิค RNAi เพื่อไปลดการแสดงออกของยีนของ Clathrin heavy chain ก่อนที่จะมีการติดเชื้อด้วยไวรัสไข้เลือดออก ผลการศึกษายืนยันว่า ไวรัสประมาณ 20% ยังสามารถเข้าสู่เซลล์ตับได้ เมื่อใช้เทคนิค RNAi ซึ่งยับยั้งกระบวนการ Clathrin coated pit ร่วมกับการใช้สารยับยั้งต่อกระบวนการ Macropinocytosis พบว่าสามารถยับยั้งการติดเชื้อของไวรัสได้เกือบสมบูรณ์ จากผลการทดลองทั้งหมดสามารถสรุปได้ว่าไวรัสไข้เลือดออกซีโรไทป์ 2 เข้าสู่เซลล์ตับโดย 2 กระบวนการโดยผ่านกระบวนการหลักคือ Clathrin coated pit 80% และอีก 20% โดยกระบวนการ Macropinocytosis

**Abstract:** Dengue viruses, the cause of dengue fever (DF), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS), are believed to enter into mammalian cells through one of three endocytic pathways which are clathrin coated pit mediated endocytosis, caveolae and macropinocytosis. Using a variety of biochemical inhibitors shows DEN-2 entry via multiple pathways. Transfection of dominant negative mutant of Eps15 was investigated to determine the role of clathrin coated pit mediated endocytosis. Virus entry was significantly reduced, approximately 20% entry was observed. To confirm this result RNAi was used to silence the expression of clathrin heavy chain prior to infection. Again some 20% virus entry was still observed. A combination of RNAi and biochemical treatment was used to inhibit both clathrin coated pit mediated endocytosis and macropinocytosis served to almost completely exclude DEN-2 entry to liver cells. From the results, we concluded that the virus enter into liver cells via two independent pathways which are clathrin coated pit mediated endocytosis as the predominant pathway and macropinocytosis contributing some 20% of virus entry.

**Keywords:** Dengue virus, Endocytosis, RNAi, Liver cells.

# Identification and Characterization of the human hepatocyte receptor for dengue virus infection

Duncan R. Smith*, Chutima Thepparit, Lukkana Suksanpaisan, Waranyoo Phoolcharoen, Pornpilas Sakoonwatanyoo, Sumalee Jindadamrongwech, Pimjai Chingsuwanrote, Arturo Cabrera-Hernandez, Verawan Boonsanay and Supranee Upanan

Institute of Molecular Biology and Genetics, Mahidol University, Salaya Campus 25/25 Phuttamontol Sai 4, Nakorn Pathom, Thailand 73170

**Abstract**

This work seeks to understand how the dengue virus enters into target cells, and our interest focuses primarily on human liver cells, a known target of dengue virus infections. Additionally, we also seek to understand the mechanism by which the dengue virus enters into insect cells, particularly those of mosquito species. We have recently identified a second liver expressed dengue virus receptor protein, in addition to our earlier work identifying the molecule GRP-78. This protein, the 37-kDa/67-kDa high affinity laminin receptor has been clearly identified as a dengue virus serotype 1 receptor (1). The work suggests that different dengue virus serotypes use different receptors to enter into cells. This is supported by our observations that different dengue serotypes bind to different proteins in virus overlay assays (2). Additionally, we have shown that entry of the virus into mammalian cells is cell cycle modulated (3), although the relationship between the cell cycle and the expression of dengue virus receptor proteins requires further investigation. Entry of the dengue into insect cells is somewhat less well characterized, although we have identified a laminin binding protein as playing a role in dengue virus serotype 3 and 4 entry (4). Additional receptor elements in both human and insect cells are currently being investigated.

**Keywords— dengue, Flavivirus, hepatocyte, insect, receptor**

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*Corresponding author. Tel (662) 800-3624 ; Fax (662) 441-9906; email: frdrs@mahidol.ac.th

**Identification and characterization of dengue virus receptors**

Duncan R. Smith, Chutima Thepparit, Lukkana Suksanpaisan, Pornpilas Sakoonwatanyoo, Waranyoo Phoolcharoen, Arturo Cabrera-Hernandez and Verawan Boonsanay

Molecular Pathology Laboratory, Institute of Molecular Biology and Genetics, Mahidol University, Salaya Campus, Nakorn Pathom.

**Abstract**: Infection with the Dengue viruses is a significant public health hazard in Thailand and world-wide. The mechanism by which the dengue virus enters into either insect (mosquito) or human cells is as yet poorly characterized and our work has focus on identifying the receptor molecules expressed on the surface of permissive cells of both an insect and a human origin. To date we have identified two proteins expressed on the surface of human hepatocyte cells, GRP78 and the 37/67kDa high affinity laminin receptor as dengue virus serotype 2 and 1 receptors respectively. In other work we have undertaken the preliminary identification of molecules expressed on the surface of insect cells, and implicated laminin binding proteins as potential dengue serotype 2, 3 and 4 and Japanese encephalitis virus binding proteins.

**Introduction:** Dengue viruses are a mosquito-borne viruses that causes a critical globally endemic disease; dengue fever and in its more severe forms dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). The dengue virus belonging to the genus flavivirus and comprises of four antigenically distinct serotypes; DEN-1, DEN-2, DEN-3, and DEN-4. Despite the liver being reported as a target organ of dengue virus infection, especially in severe and fatal cases, there is little information on the interaction between the dengue virus and liver cells, especially with regards to the nature of moieties facilitating the entry of the virus.

**Methodology:** Cell membrane proteins were separated through either native gels or SDS-polyacrylamide gels and after transfer to solid matrix were incubated with purified DEN or JEV. Subsequently membranes were incubated with a specific anti-dengue virus (HB-114) or anti-flavivirus (HB-112) monoclonal antibody. The viral binding band was visualized by incubation with a secondary rabbit anti-mouse IgG-HRP and the band subsequently extracted from the gel and analyzed by peptide mass spectroscopy.. Cells were subsequently incubated with either antibodies directed against candidate receptor proteins or directly with the receptor ligand and then infected with the dengue virus. The infected cells were then incubated for 1.5 virus replication cycles at that time point growth medium was taken and assayed for level of infectious virus by standard plaque assay. For immunofluorescence, the cells grown on glass slide were treated with 20 μg of antibody or reagent as indicated prior to infection with dengue virus at MOI of 10. The slides were viewed under the fluorescence microscope.

**Results, Discussion and Conclusion:** Using the technique of Virus overlay protein binding assay (VOPBA), two HepG2 cell membrane proteins with sizes approximately 37 and 78 kDa were found to be able to bind to dengue virus serotype 1 and 2, respectively. These two proteins were later identified as the 37/67 kDa high affinity laminin receptor and GRP-78 by mass spectrometry. Infection inhibition studies showed the inhibitory effect of blocking GRP-78 on DEN-2 entry to HepG2 cells. Similarly, blocking the 37/67 kDa high affinity laminin receptor produced a significant inhibition of DEN-1 entry. Recently, laminin binding proteins have also been implicated in the binding of Japanese encephalitis virus as well as DEN-3 and 4 onto mosquito cells. These results suggest that common mechanisms of virus internalization may occur in human and insect cells.

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**Keywords**: Dengue, flavivirus, receptor, liver, insect

# MECHANISM OF ER STRESS INDUCTION BY DENGUE INFECTION IN HepG2 CELLS

Atefeh Khakpoor, Duncan Richard Smith

*Molecular Pathology Laboratory, Institute of Molecular Biology and Genetics, Mahidol University, Salaya Campus, Nakhon Pathom, Thailand*

**BACKGROUND:** Dengue viruses, comprised of four closely antigenically related serotypes (DEN- 1, 2, 3, and 4), belong to the family flaviviridae are the causative agent for dengue fever and the more severe forms of the disease dengue hemorrhagic fever and dengue shock syndrome, which are a significant public health threat in tropical and subtropical regions. ER stress is thought to be activated upon viral infection and to lead to either adaptation of cells to the stress or cell death if the stress is prolonged. Several studies have shown a relationship between ER stress and autophagy induction, termed ER-phagy.

**OBJECTIVES:** This study aimed to look at ER stress induction after dengue virus infection (DEN-2 and DEN-3) in HepG2 cells and its relation to autophagy induction.

**METHODS:** The activation of ER stress and its relation to autophagy was investigated by a combination of RT-PCR, western blotting and confocal microscopy to examine genes involved in the ER stress signaling pathways in dengue infected cells in the presence and absence of the autophagy inhibitor L-asparagine. Control cells treated with the ER stress inducer tunicamycin were also investigated.

**RESULTS:** The results showed ER stress activation upon DEN-2 but not in DEN-3 infection, as demonstrated by the stress specific splicing of XBP-1 and phosphorylation of eIF2α. Results of confocal microscopy showed significant co-localization of dengue proteins and ER resident proteins and between ER resident proteins and autophagic vacuole markers. Significantly, down regulation of autophagy abolished the stress specific splicing of XBP-1 in DEN-2 infected cells.

**CONCLUSIONS:** It can be concluded that the serotype specific ER stress induction upon dengue infection results as a by-product of interactions occurring between dengue proteins and ER resident proteins located on autophagic vacuoles.

# LOCALIZATION OF DENGUE VIRUS TRANSLATION REPLICATION COMPLEX AT AUTOPHAGIC VACUOLE

Mingkwan Panyasrivanit, Lukkana Suksanpisan, Maneerat Ekkapongpisit, Duncan Richard Smith

*Molecular Pathology Laboratory, Institute of Molecular Biology and Genetics, Mahidol University, Salaya campus, Nakhon pathom, Thailand.*

**BACKGROUND:** Dengue viruses are causative agents of dengue fever, dengue hemorrhagic fever, and dengue shock syndrome. About 100 million humans are infected with dengue viruses each year. The dengue translation and replication mechanism in cells is not clearly characterized. In recent years, many RNA viruses such as coronavirus, poliovirus and hepatitis C virus have been proposed to subvert the autophagy pathway for their replication. Autophagy is the conserved lysosomal degradation pathway which cells used to degraded their own cytoplasmic material.

**OBJECTIVES:** This study aims to investigate the relationship between dengue virus and autophagy and the localization of dengue virus translation replication complex (DTRC) and autophagic vacuoles (AV) in hepatocyte (HepG2) and monocyte (U937) cell lines.

**METHODS:** The relationship between dengue virus and autophagy was investigated by using L-Asparagine (L-Asn) to inhibit autophagy. Briefly, HepG2 or U937 were infected with dengue virus serotype 2 (DEN-2) in presence or absence of L-Asn. The medium were collected every 24 hours for 3 days. Dengue virus production was investigated by standard plaque assay.

To investigate location of DTRC and AV, immunofluorescent assay was performed using antibodies against LC3 (autophagic marker), L28 ribosomal protein, dengue protein (NS1), and double-stranded RNA (dsRNA). Briefly, HepG2 or U937 were infected or mock-infected with dengue virus serotype 2 (DEN-2). At 15 hours (HepG2) or 3 days (U937) post infection, cells were fixed and permeabilized followed by stained with the pairs of antibodies as indicated on each figure. Fluorescent signals were observed using a confocal microscope.

**RESULTS:** The results from standard plaque assay showed the reduction of DEN-2 production in both HepG2 and U937 cells when the infections were done in the presence of the autophagy inhibitor, L-Asn. Immunofluorescent assay results showed co-localization of LC3 with NS1 and J2, and L28 with LC3 and dsRNA in both HepG2 and U937 cells infected with DEN-2.

**CONCLUSIONS:** These suggest that dengue viruses subvert autophagy for their replications and the DTRC is located on autophagic vacuoles.

# The Interaction between Autophagy and the Dengue virus

**Mingkwan Panyasrivanit, Atefeh Khakpoor, Nitwara Wikan, and Duncan R. Smith**
*Molecular Pathology Laboratory, Institute of Molecular Biology and Genetics, Mahidol University, Nakorn Pathom 73170, Thailand.*

## Introduction and Objective

Autophagy, a lysosomal degradation pathway, has been reported to play a role in viral infection. Induction of autophagy was shown in dengue virus infection. This work seeks to characterize the role of autophagy in dengue virus replication and the difference of dengue virus serotype 2 and 3 (DEN-2 and -3) replication in relation to autophagy.

## Methods

To characterize the role of autophagy in DEN-2 and -3 replication, the induction of autophagy in DEN-2 and -3 infected HepG2 cells was investigated by immunoblot assay of the autophagic marker (LC3). The effect of autophagy modulators on DEN-2 and -3 production was determined by standard plaque assay. Double and triple staining-immunofluorescence was used to investigate the location of the DEN-2 and -3 translation replication complex (TRC) and its association with autophagic membranes.

## Results

The induction of autophagy was observed in both DEN-2 and DEN-3 infections. Virus production studies showed that both DEN-2 and -3 production decreased and increased in the presence of autophagy inhibitor and inducer respectively whereas the increase of DEN-2 and decrease of DEN-3 production was observed when autophagosome-lysosome fusion was inhibited. Suggesting that autophagy plays a role in both DEN-2 and -3 replication, but the lysosomal fusion step has deleterious effects to DEN-2, whereas DEN-3 requires the post-lysosomal fusion step for its replication. Colocalization of dengue nonstructural protein 1 or double stranded RNA (dsRNA) with LC3 and L28 ribosomal protein were observed, supporting the association of DEN-2 and -3 TRC and autophagic membranes. To specify the types of autophagic membrane associating with DEN-2 and -3 TRC, colocalization of triple staining for LC3, dsRNA and endosomal marker were observed in both DEN-2 and -3, suggested the TRC associate in pre lysosomal fusion membranes. However colocalization of lysosomal marker and dsRNA was observed in DEN-3, but not DEN-2 infection. This result support the data from virus production study that show the importance of the post lysosomal fusion step in DEN-3 replication.

## Conclusion

These studies support a role for autophagy in dengue virus replication in respect of the site of DEN-2 and -3 translation replication complexes on autophagic membranes. Moreover the difference in relationship between autophagy and replication mechanism among the different serotypes of dengue virus was shown. These data provide the important information in understanding the dengue virus life cycle.

**Keywords:** autophagy, dengue virus, replication, autophagic membrane

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# Publications

Journal of Medical Virology 79:386–392 (2007)

# Dengue Virus Entry Into Liver (HepG2) Cells Is Independent of hsp90 and hsp70

**Arturo Cabrera-Hernandez, Chutima Thepparit, Lukkana Suksanpaisan, and Duncan R. Smith***

*Molecular Pathology Laboratory, Institute of Molecular Biology and Genetics, Mahidol University, Nakorn Pathom, Thailand*

Recently, several stress-related proteins including GRP78, hsp70, and hsp90 have been implicated as dengue virus receptors in various cell types, with hsp90/70 being implicated as a receptor complex in monocytes and macrophages, while GRP78 has been implicated as a liver cell expressed dengue virus receptor. To assess whether the hsp90/70 complex plays a role in the internalization of the dengue viruses into liver cells, we undertook infection inhibition studies with lipopolysaccharide and antibodies directed against both hsp70 and hsp90, individually and in combination. No inhibition of any dengue serotype was seen in the presence of lipopolysaccharide or antibodies directed against either hsp70 or hsp90 either singly or in combination. A moderate inhibition of dengue virus serotype 2 entry into liver cells was observed in the presence of antibodies directed against GRP78. These results confirm a proposed role for GRP78 as a dengue virus serotype 2 receptor protein and suggest that the recently identified hsp90/70 complex does not play a role in dengue virus internalization into liver cells. ***J. Med. Virol. 79:386–392, 2007.*** 

**KEY WORDS:** flavivirus; GRP78; heat shock; liver; receptor

## INTRODUCTION

Approximately 100 million people are believed to be infected with the dengue viruses each year [Guzman and Kouri, 2002] making it the most prevalent arthropod borne viral disease. While the majority of these infections are believed to be asymptomatic, infection may result in a febrile disease termed dengue fever (DF) or it may result in hemorrhagic manifestations which are classified as either dengue hemorrhagic fever or dengue shock syndrome dependent upon severity [Halstead, 1989]. The dengue viruses are four antigenically distinct viruses that belong to the family *Flaviviridae*, genus *Flavivirus*, and species *Dengue virus* and are termed dengue virus serotypes 1, 2, 3, and 4. The dengue viruses are enveloped positive-sense single-stranded RNA viruses of approximately 11 kb that encode three structural proteins (core, premembrane, and envelope) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) in one open reading frame [Chang, 1997].

In severe cases of dengue, the impact of the dengue virus on liver function is prominent as shown by hepatomegaly [Mohan et al., 2000] and elevated levels of serum alanine aminotransferase and alkaline phosphatase [Nguyen et al., 1997; Wahid et al., 2000]. Although the nature of the cellular targets in the liver is somewhat unclear, several studies based upon autopsy specimens have suggested the involvement of both hepatocytes and Kupffer cells [Couvelard et al., 1999; Rosen et al., 1999; Huerre et al., 2001]. Two serotype-specific dengue virus receptors, GRP78 and the 37/67 kDa high affinity laminin receptor, have been identified in transformed liver cells [Jindadamrongwech et al., 2004; Thepparit and Smith, 2004] and evidence suggests that, in liver cells at least, the serotype may play a role in receptor usage [Jindadamrongwech and Smith, 2004; Jindadamrongwech et al., 2004; Thepparit and Smith, 2004].

The first liver cell receptor identified, GRP78 or BiP, is a stress protein related to the heat shock family of proteins [Lindquist and Craig, 1988; Little et al., 1994] and recently other members of this family, heat shock protein 70 (hsp70) and heat shock protein 90 (hsp90) have been identified as parts of a receptor complex mediating the internalization of the dengue virus into monocytes and macrophages [Reyes-Del Valle et al., 2005]. Interestingly, hsp70 and hsp90 have been previously identified as the CD14-independent cell

*Correspondence to: Duncan R. Smith, Molecular Pathology Laboratory, Institute of Molecular Biology and Genetics, Mahidol University, Salaya Campus, 25/25 Phuttamonthol Sai 4, Salaya, Nakorn Pathom, Thailand 73170.
E-mail: duncan_r_smith@hotmail.com

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surface receptor for lipopolysaccharide (LPS) in human monocytes and macrophages [Triantafilou et al., 2001; Triantafilou and Triantafilou, 2002] and the identification of hsp70 and hsp90 as dengue virus receptors in these cells provides an elegant explanation of the LPS-mediated inhibition of dengue virus entry to monocytes and macrophages observed previously [Chen et al., 1999].

Given that GRP78, hsp70, and hsp90 are all related homologous stress proteins [Lindquist and Craig, 1988; Little et al., 1994], it has been suggested that the identification of GRP78 as a liver cell receptor may have resulted in error from a cross reaction with hsp70 or even hsp90 [Reyes-Del Valle et al., 2005]. To address this issue, we sought to assess the role of hsp70 and 90 in entry of the dengue virus into liver cells.

## MATERIALS AND METHODS

### Cells and Viruses

The African green monkey cell line, Vero, was cultivated in M-199 medium supplemented with 5% heat-inactivated fetal calf serum (FCS, PAA, Pasching, Austria) and 100 units of penicillin–streptomycin (PAA) per ml at 37°C with 5% $CO_2$. The human hepatoma cell line HepG2 was cultivated in Dulbecco's modified eagle's medium (DMEM, HyClone, Logan, UT) supplemented with 10% FCS (PAA) and cells incubated under 10% $CO_2$. Dengue virus serotypes 1 (strain 16007), 2 (strain 16681), 3 (strain 16562), and 4 (strain 1036) were propagated in Vero cells. Virus was either partially purified by centrifugation and stored frozen at −80°C, or purified through sucrose step gradients as described previously [Sithisarn et al., 2003; Suksanpaisan and Smith, 2003].

### Viral Assays

Levels of extracellular viruses were determined by standard plaque assay as described previously [Sithisarn et al., 2003].

### Infection Inhibition Assays

HepG2 cells were grown on 6-well plates until the cells number reached $1 \times 10^6$, following which the cells were incubated with either *E. Coli* LPS purified from *E. coli* 055:B5 (Product code L2880; Sigma Chemical Co., St. Louis, MO) and reconstituted for cell culture experiments at the indicated concentration for 24 hr or with 2.5, 5, or 10 μg/ml of a rabbit anti-human hsp90 polyclonal antibody (H114, Santa Cruz Biotechnology, Santa Cruz, CA), a rabbit anti-human hsp70 polyclonal antibody (K20R, Santa Cruz Biotechnology) or a goat polyclonal antibody directed against human GRP78 (N20, Santa Cruz Biotechnology) or with a 10 μg/ml of a pool of antibodies directed against both the amino-terminal (N-17, Santa Cruz Biotechnology) and carboxyl-terminal (H-114) of hsp90 as well as hsp70 (K20R) for 1 hr prior to infection with dengue virus serotype 2 at MOI of 1 or 5 as indicated for 90 min at 37°C. After viral adsorption, the extracellular viruses were inactivated by washing with PBS, followed by acid glycine treatment [Hung et al., 1999]. The infected cells were washed again with PBS and fresh growth medium was added. The infected cells were then incubated for a further one and a half virus propagation cycles [Thepparit et al., 2004] at which point growth medium was taken and assayed for level of infectious virus by standard plaque assay. Each sample was titered in duplicate, and each experiment undertaken three times independently.

### Cytokine RT-PCR

HepG2 cells were either untreated with LPS or treated with 1 μg/ml LPS (Sigma) for 24 or 48 hr under standard conditions at 37°C following which the cells were harvested and total RNA extracted with Trizol reagent (Molecular research center, Inc., Cincinnati, OH) according to the manufacturer's instructions. First strand cDNA was synthesized from total RNA using Improm II™ reverse transcriptase (Promega, Madison, WI) with Oligo (dT) primer. For the cytokine genes, first strand cDNA was amplified for 35 cycles with specific primers for IFN-β [Li et al., 2005], IL1-β [Bosch et al., 2002], IL-8 [Bosch et al., 2002], IL-6 [Abdallah et al., 2005] or 25 cycles for actin (primers: 5′-GAA GAT GAC CCA GAT CAT GT-3′ and 5′-ATC TCT TGC TCG AAG TCC AG-3′) using Taq DNA polymerase (Promega, WI). Cycling conditions were denaturation: 94°C for 15 sec, annealing at 55°C for 20 sec, and extension at 72°C for 30 sec. All PCR products were separated on 1.8% agarose gels and visualized by ethidium bromide staining and quantitated by the ImageJ software [Abramoff et al., 2004].

### Statistical Analysis

All data were analyzed using the GraphPad Prism program (GraphPad Software, Inc., San Diago, CA).

## RESULTS

We sought initially to determine whether dengue virus infection of liver (HepG2) cells was inhibited by the presence of lipopolysaccharides (LPS). Lipopolysaccharides are characteristic components of Gram-negative bacteria made up of a hydrophobic lipid, a hydrophobic core polysaccharide chain and a hydrophilic O-antigenic polysaccharide side chain. LPS is extremely heterogenous and tends to form aggregates of varying sizes depending upon the specific chemical environment. The action of LPS on HepG2 cells is poorly characterized, so to verify the interaction of LPS with the HepG2 cells and to demonstrate biological activity of the particular LPS preparation to be used in future experiments, monolayers of HepG2 cells were either not treated or treated with LPS at a concentration of 1 μg/ml for 24 or 48 hr under standard conditions at 37°C and the levels of gene expression of IFN-β, IL-1β, IL-6, and IL-8 determined semi-quantitatively by RT-PCR in comparison to levels

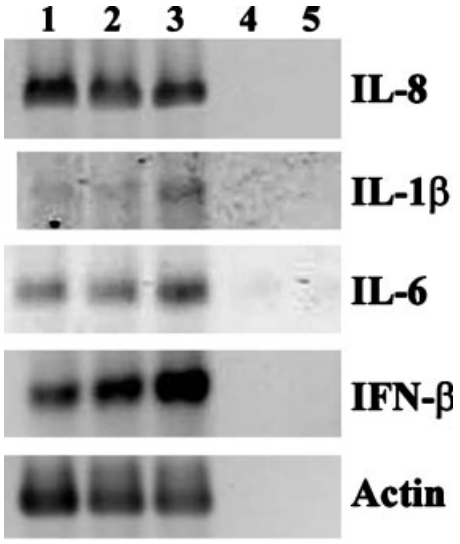


Fig. 1. Induction of cytokine gene expression in response to LPS treatment. HepG2 cells were either not treated (**lane 1**) or treated with 1 μg/ml LPS for 24 (**Lane 2**) or 48 (**lane 3**) hr after which the levels of mRNA for IL-8, IL-1β, IL-6, and IFN-β were determined semi-quantitatively by RT-PCR in comparison with β-actin. Experiment was undertaken in parallel with a RT control (no RNA, **lane 4**) and a PCR control (no cDNA, **lane 5**).

of β-actin. Results (Fig. 1 and Table I) show that LPS was able to induce changes in expression of these cytokine genes in HepG2 cells in agreement with the observations of other authors [Thornton et al., 1990; Gutierrez-Ruiz et al., 1999; Nanbo et al., 1999] and demonstrating a specific action between LPS and HepG2 cells in our system.

To determine whether LPS was able to block the entry of the dengue virus monolayers of approximately $1 \times 10^6$ HepG2 cells grown as described previously [Jindadamrongwech et al., 2004; Thepparit and Smith, 2004; Thepparit et al., 2004] were pre-incubated with varying amounts of *E. Coli* LPS (1, 5, and 10 μg/ml) for 24 hr at 37°C prior to the addition of dengue virus serotype 2 at an MOI of 1 for 90 min at 37°C followed by an acid glycine (pH 3) wash to inactivate un-internalized viruses [Hung et al., 1999] as described previously [Thepparit and Smith, 2004; Thepparit et al., 2004]. After one and a half virus replication cycles [Thepparit et al., 2004], the growth media was assayed by standard plaque assay as described previously [Sithisarn et al., 2003]. Experiment was undertaken in triplicate, with duplicate assay of plaque titer. Results (Fig. 2) show no inhibition of dengue virus production, and indeed are consistent with an increase in dengue virus production in the presence of LPS, although no statistically significant variation from control samples was noted. Under these conditions, Chen et al. [1999] reported a 2 log inhibition of dengue virus production from monocytes/macrophages. However, other investigators have also noted either no effect or a slight enhancement of dengue virus infection following pretreatment of a range of cells with LPS [McBride and Bielefeldt-Ohmann, 2000].

TABLE I. Quantitation of Cytokine Gene Expression in HepG2 Cells in Response to LPS Treatment

| | Untreated | LPS-treated 24 hr | LPS-treated 48 hr |
|---|---|---|---|
| IL-8 | 525.9729 | 545.5193 | 603.3423 |
| IFN-B | 347.9513 | 1172.867 | 2433.439 |
| IL-6 | 97.83832 | 150.6108 | 257.1417 |
| IL-1B | 52.96511 | 75.89834 | 168.0829 |

Results are expressed as arbitrary absorbance units calculated from the integrated band density using the ImageJ program [Abramoff et al., 2004] after normalization against actin.

Given that no inhibition of dengue infection was noted with LPS pre-treatment despite the biological activity of the LPS, it was possible that there may be differences in numbers of receptor complexes on the surface of the different cell types, and so the inhibition experiment was repeated with dengue virus serotype 2 at an MOI of 5 and over a broader range of LPS concentrations (0.1, 1, 10, 100, and 1,000 μg/ml). Again (Fig. 2), no inhibition was observed even at very high LPS concentrations and results consistent with a slight, but not statistically significant, enhancement of infection in the range 1–10 μg/ml was noted. When the entry of all four dengue serotypes was investigated in the presence of 10 μg/ml LPS, no statistically significant variation from the untreated control samples was seen (Fig. 2).

To examine the involvement of hsp70 and hsp90 in the dengue virus internalization process into liver cells, the presence of these proteins was confirmed first, as well as GRP78 by Western blot analysis. All three proteins (hsp70, hsp90, and GRP78) were present in membranes of HepG2 with a relatively constant abundance (data not shown). Given that hsp70, hsp90, and GRP78 are present in membrane fractions of HepG2 cells, inhibition of infection experiments was carried out subsequently using pre-incubation of HepG2 cells with specific anti-heat shock protein as well as anti-GRP78 antibodies. HepG2 cells (as above) were pre-incubated with increasing concentrations (2.5, 5, or 10 μg/ml) of either a rabbit anti-human hsp90 polyclonal antibody or a rabbit anti-human hsp70 polyclonal antibody or a goat polyclonal antibody directed against human GRP78 for 1 hr at 37°C before infection with dengue virus serotype 2 at an MOI of 5 followed by an acid glycine wash to inactivate uninternalized viruses [Hung et al., 1999]. This range of antibody concentration was chosen to be consistent with our previous work [Jindadamrongwech et al., 2004; Thepparit and Smith, 2004] and is approximately equivalent to 10 to 40 × the dissociation constant (Kd) of antibodies which normally have Kd values in the nanomolar range. Cells were incubated under standard conditions [Jindadamrongwech et al., 2004; Thepparit and Smith, 2004; Thepparit et al., 2004] for one and a half virus replication cycles [Thepparit et al., 2004] before the growth medium was assayed by standard plaque assay. Each experiment was undertaken in triplicate with duplicate assay of samples. The results (Fig. 3) show that there was no inhibition of infection with cells pre-incubated with antibodies directed against hsp70 or hsp90 and no statistically

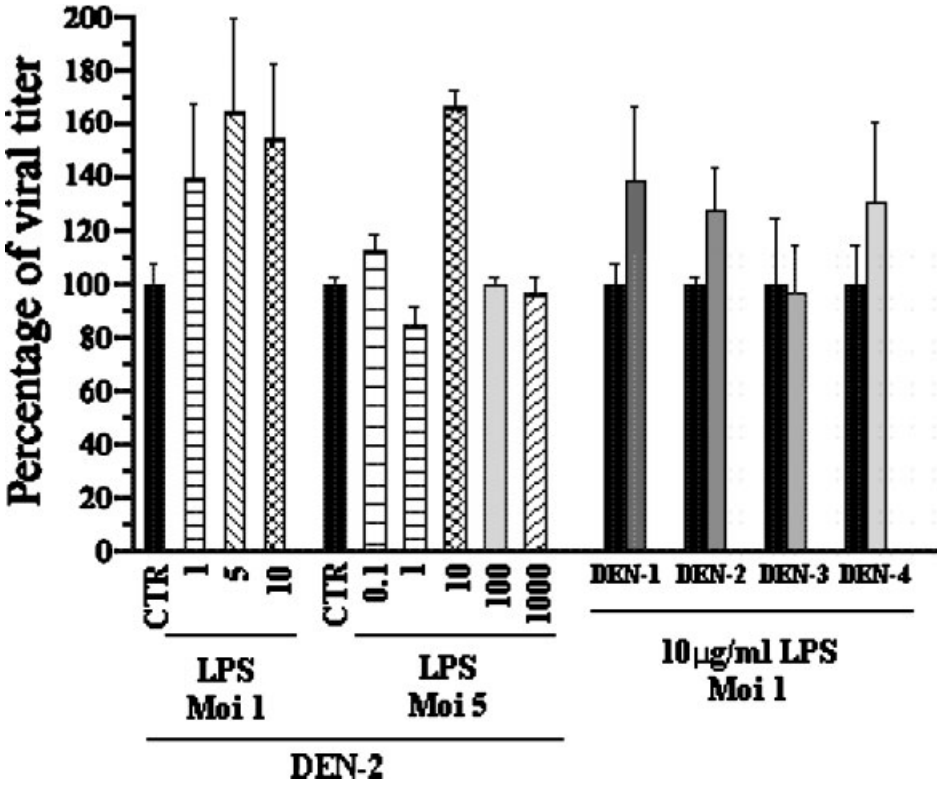


Fig. 2. HepG2 cells were pre-treated with varying concentrations of LPS for 24 hr before infection with dengue serotypes 1 to 4 as indicated. Supernatants were assayed after one and a half replication cycles. Results are expressed as a percentage of virus titer of untreated HepG2 cells (CTR, solid bars) as absolute dengue virus titers from different serotypes vary significantly. Error bars represent SEM of three independent experiments assayed in duplicate.

significant difference from control was seen, while again [Jindadamrongwech et al., 2004] a moderate and statistically significant ($P = 0.0135$, Wilcoxon Signed-rank test) inhibition of infection of dengue virus entry was noted in the presence of antibodies directed against the amino terminus of GRP78. Inhibition of dengue virus infection in the presence of a pool of antibodies directed against both the amino-terminal (N-17) and

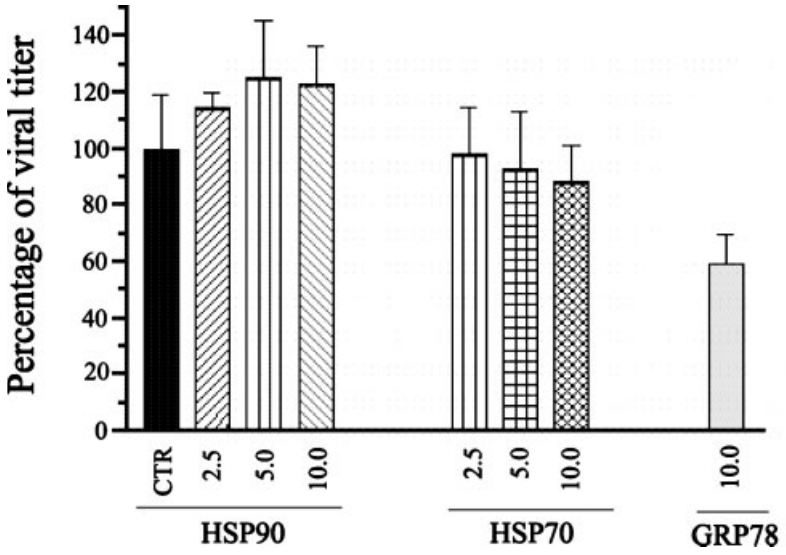


Fig. 3. HepG2 cells were pre-treated with 2.5, 5, or 10 μg/ml of antibodies directed against hsp90, hsp70, or GRP78 for 1 hr before infection with dengue serotypes 2 at an MOI of 5. Supernatants were assayed by standard plaque assay after one and a half replication cycles. Results are expressed as a percentage of virus titer of untreated HepG2 cells (CTR, solid bar) as absolute dengue virus titers from different serotypes vary significantly. Error bars represent SEM of three independent experiments assayed in duplicate.

carboxyl-terminal (H-114) of hsp90 as well as hsp70 (K20R) at a concentration of 10 μg/ml was examined for all four dengue serotypes and in no case was any inhibition seen and no statistically significant variation from control was observed (Fig. 4).

Heat shock proteins in HepG2 cells have been shown to be inducible by a heat shock of 42°C for 30 min [Rada et al., 2005]. Therefore, in order to maximize the expression of hsp70 and 90, HepG2 liver cells were heat shocked at 42°C for 30 min before being infected separately with all four dengue serotypes at an MOI of 1. Cells were incubated under standard conditions before assay of the growth medium. The results (Fig. 4) show no enhancement of infection over control (non-shocked) cells and no statistically significant variation from the control was found.

## DISCUSSION

The ability of the dengue viruses to enter into and productively infect cells is a critical determinant of pathogenicity, and in the last few years, several cell-specific dengue virus receptors have been identified [Tassaneetrithep et al., 2003; Jindadamrongwech et al., 2004; Thepparit and Smith, 2004; Reyes-Del Valle et al., 2005]. The most recent of these, the hsp70/hsp90 complex is believed to mediate the binding and internalization of the dengue viruses to monocytes and macrophages [Reyes-Del Valle et al., 2005]. Monocytes and macrophages have long been identified as the primary cellular target in cases of second infections with a heterologous dengue virus, and infection was shown to occur via the antibody-dependent enhancement (ADE) mechanism [Halstead and O'Rourke, 1977; Halstead et al., 1977] with the entry of the virus occurring through the participation of the FcγR1 and FcγRII receptors recognizing the constant region of cross reacting but non-neutralizing anti-dengue IgG molecules [Littaua et al., 1990; Schlesinger and Chapman, 1999]. However, further work employing adherent human monocytes revealed the existence of receptors sensitive to trypsin, in addition to the trypsin-insensitive Fc receptors [Daughaday et al., 1981]. These trypsin-sensitive receptors were postulated to be high affinity receptors used in primary dengue infections. Subsequently, Chen et al. [1999] detected a CD14-dependent-LPS-inhibition during dengue virus binding to primary human blood monocytes, suggesting the existence of a CD14-coupled molecule acting as a dengue virus receptor.

The identity of the CD-14 coupled molecule was apparently revealed when Reyes-Del Valle et al. [2005] employing a combination of E protein column affinity chromatography and VOPBA assay detected five proteins bands with molecular masses of 45, 60, 75, 84, and 100 kDa and subsequent mass spectroscopy analysis identified the 84 kDa band as hsp90. Although hsp90 is a member of the highly conserved molecular chaperone family who are primarily resident inside the cell, hsp70 and 90 are known to associate at the cell surface membrane where they have been shown to participate in the so-called "CD14-independent LPS receptor

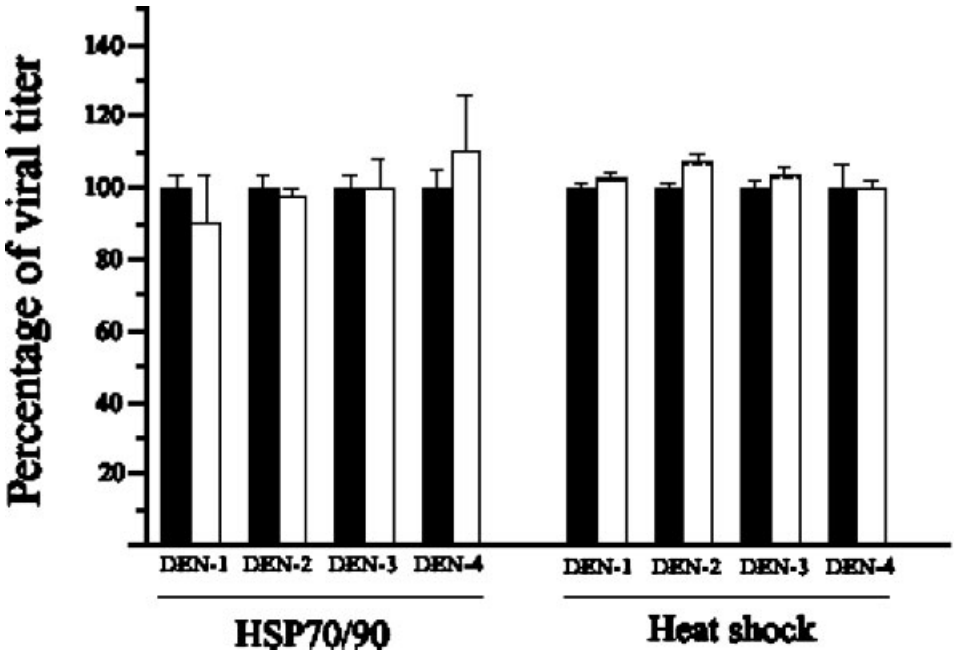


Fig. 4. Hep G2 cells were either pre-treated with 10 μg/ml of a combination of antibodies against hsp70 and hsp90 or heat shocked for 30 min at 42°C before being infected separately with dengue virus serotypes 1 to 4 at an MOI of 5 (antibody pre-treatment) or 1 (heat shock). Supernatants were assayed after one and a half replication cycles. Results are expressed as a percentage of virus titer of untreated (solid bars) HepG2 cells as absolute dengue virus titers from different serotypes vary significantly. Error bars represent SEM of three independent experiments assayed in duplicate.

cluster" [Triantafilou et al., 2001]. This observation, in the light of the LPS-dependent inhibition of dengue virus infection [Chen et al., 1999], permitted Reyes del Valle and colleagues to infer and subsequently demonstrate the co-participation of the lipid raft-associated hsp70 with hsp90 during dengue virus entry to the cell [Reyes-Del Valle et al., 2005].

In a previous study, two serotype-specific dengue virus receptors expressed on the surface of transformed liver cells have been identified [Jindadamrongwech et al., 2004; Thepparit and Smith, 2004]. One of these, GRP78 or BiP, identified as a dengue virus serotype 2 receptor [Jindadamrongwech et al., 2004] is also a member of the same molecular chaperonin family as hsp70 and hsp90, and as such it has been suggested that the identification of GRP78 represents a mis-identification or cross-reaction with hsp70 [Reyes-Del Valle et al., 2005]. The present study, however, does not support a role for the hsp70/90 complex in internalization of the dengue virus into liver cells. First, there is no LPS-mediated inhibition of dengue virus infection of HepG2 cells, which would, in itself tend to argue against the involvement of the hsp70/90 complex. Second, there is no inhibition of infection of HepG2 cells in the presence of antibodies directed against hsp70 and 90 either singly or in combination, and lastly there is no increase in dengue virus infection after treatment known to upregulate heat shock proteins prior to infection. Collectively, the results obtained in the current study provide clear and conclusive evidence that the hsp70/90 complex identified as a dengue virus receptor in monocytes and macrophages [Reyes-Del Valle et al., 2005] does not mediate the internalization of the dengue virus into liver cells.

Interestingly, while no inhibition of dengue virus entry was noted in the presence of LPS, in agreement with other investigators [McBride and Bielefeldt-Ohmann, 2000], a slight enhancement of infection was observed. Perhaps more interestingly, a slight, but not statistically significant enhancement of dengue virus entry was also observed in the presence of anti-hsp90 antibodies, but not in the presence of anti-hsp70 antibodies or pooled hsp90/70 antibodies. This result, together with the observation that LPS is able to interact with HepG2 cells and produce a biological result would tend to argue that HepG2 cells express the CD14/hsp70/hsp90 complex in a functional form and that while the virus is able to bind to the complex, it does not result in productive dengue virus entry. Blocking this complex with LPS could result in higher dengue virus entry through productive pathways, resulting in the enhancement seen. This observation, together with our observation that the LPS-mediated enhancement of infection is restricted to a narrow range of LPS concentrations requires further study.

This study again noted a modest but definite inhibition of dengue virus serotype 2 entry into HepG2 cells in the presence of antibodies directed against GRP78 in agreement with our earlier study [Jindadamrongwech et al., 2004]. The reproducible inhibition of about 40% of wild type entry again supports our proposal that GRP78 functions as a minor receptor element for dengue virus serotype 2 entry [Jindadamrongwech et al., 2004; Cabrera-Hernandez and Smith, 2005]. However, it is clear that GRP78 acts as at least a minor receptor in dengue virus internalization and that further work is required to identify additional elements mediating the internalization of the dengue virus into liver cells.

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# cDNA-AFLP Analysis of Differential Gene Expression in Human Hepatoma Cells (HepG2) Upon Dengue Virus Infection

**Maneerat Ekkapongpisit,[1] Tirawat Wannatung,[1] Tharinee Susantad,[1] Kanokporn Triwitayakorn,[2] and Duncan R. Smith[1]***

[1]*Molecular Pathology Laboratory, Institute of Molecular Biology and Genetics, Mahidol University, Salaya, Nakorn Pathom, Thailand*
[2]*Genomics Laboratory, Institute of Molecular Biology and Genetics, Mahidol University, Salaya, Nakorn Pathom, Thailand*

In infectious diseases, the disease pathogenesis is the outcome of the interaction between the genome of the host and the genome of the pathogen. Despite the wide distribution of dengue infections in the world, and the large number of annual infections, few studies have investigated how the dengue genome alters the global transcriptional profile of the host cell. To investigate alterations in the liver cell transcriptome in response to dengue virus infection, liver cells (HepG2) were infected with dengue serotype 2 at MOI 5 and at 3 days post-infection RNA extracted and analyzed by cDNA-AFLP in parallel with mock-infected cells. From 73 primer combinations over 5,000 transcription-derived fragments (TDFs) were observed, of which approximately 10% were regulated differentially in response to infection. Sixty-five TDFs were subsequently cloned and sequenced and 27 unique gene transcripts identified. Semi-quantitative reverse transcription (RT)-PCR was used to validate the expression of 12 of these genes and 10 transcripts (CK2, KIAA509, HSP70, AK3L, NIPA, PHIP, RiboS4, JEM-1, MALT1, and HSI12044) were confirmed to be differentially regulated, with four transcripts (HSP70, NIPA, RiboS4, and JEM-1) showing a greater than twofold regulation. These results suggest that the expression of a large number of genes is altered in response to dengue virus infection of liver cells, and that cDNA-AFLP is a useful tool for obtaining information on both characterized and as yet uncharacterized transcripts whose expression is altered during the infection process. ***J. Med. Virol. 79:552–561, 2007.*** © 2007 Wiley-Liss, Inc.

**KEY WORDS:** flavivirus; liver; transcriptome

## INTRODUCTION

The re-emergence of infections with the dengue virus in the 21st century represents a considerable burden to both public health and the economies of developing countries especially those in tropical and sub-tropical regions. Dengue is believed to be distributed in up to 100 endemic countries and it is estimated that there are up to 100 million new infections each year leading to approximately 500,000 severe cases and some 24,000 fatal cases annually [Guzman and Kouri, 2002]. At the present time there is no specific treatment for dengue infection, nor is there a viable vaccine and, as such, an increased understanding of dengue pathogenesis is urgently required to facilitate the development of new treatments for this disease. Infections with the dengue virus maybe asymptomatic, but commonly result in a flu-like illness, termed dengue fever (DF). Progression of the disease to more severe forms can occur and dengue haemorrhagic fever (DHF) is characterized by high fever, haemorrhagic phenomena, liver enlargement, and circulatory failure. If DHF is coupled with shock, typified by a rapid, weak pulse, and by signs of circulatory failure, the infection syndrome is termed dengue shock syndrome (DSS) which is caused by increased vascular permeability that leads to a reduction

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*Correspondence to: Duncan R. Smith, Molecular Pathology Laboratory, Institute of Molecular Biology and Genetics, Mahidol University, Salaya Campus, 25/25 Phuttamonthol Sai 4, Salaya, Nakorn Pathom 73170, Thailand.
E-mail: duncan_r_smith@hotmail.com

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in intravascular fluid volume and then elevated hematocrit, hypotension, and serous effusions [McBride and Bielefeldt-Ohmann, 2000].

DF, DHF, and DSS are all caused by the dengue viruses. These viruses are classified in the family *Flaviviridae,* genus *Flavivirus,* and species *Dengue virus*. There are four antigenically distinct viruses, termed dengue serotype 1, 2, 3, and 4 and the dengue viruses are enveloped positive-sense single-stranded RNA viruses of approximately 11 kb. The genome of the dengue viruses encodes for three structural proteins (core, pre-membrane and envelope) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) in one open reading frame [Chang, 1997].

Several clinical studies have implicated the liver in the pathogenesis of dengue infections [Bhamarapravati, 1989; Nguyen et al., 1997; Mohan et al., 2000; Wahid et al., 2000; Huerre et al., 2001; Pancharoen et al., 2002; Souza et al., 2004; Subramanian et al., 2005]. In particular hepatomagaly is frequently evident in patients with DHF [Bhamarapravati, 1989] and liver dysfunction, as evidenced by increasing serum liver enzymes, is commonly observed in DHF/DSS patients [Kuo et al., 1992; Nguyen et al., 1997; Mohan et al., 2000; Wahid et al., 2000; Pancharoen et al., 2002; Souza et al., 2004; Wichmann et al., 2004; Kularatne et al., 2005]. Examination of liver specimens obtained at autopsy of fatal cases of dengue virus infection have supported the direct involvement of the liver in the pathogenesis of dengue, and the presence of the dengue virus genome has been demonstrated by reverse transcription (RT)-PCR [Rosen et al., 1999; Huerre et al., 2001], as well as the presence of dengue virus antigens in hepatocytes by immunocytochemistry [Couvelard et al., 1999; Huerre et al., 2001]. Moreover the virus itself has been recovered from liver samples from fatal cases of dengue [Rosen et al., 1989].

Transformed liver cell lines are broadly permissive to the dengue viruses [Lin et al., 2000; Thongtan et al., 2004] and infection can result in the upregulation of cytokine and chemokine gene expression and secretion of cytokines from the infected cells [Medin et al., 2005] and high levels of IL-8 secretion from HepG2 cells in response to dengue infection have been documented [Medin and Rothman, 2006], which may be linked to plasma leakage in dengue patients. More broadly, infection of transformed liver cells with the dengue virus results in the induction of cellular death through apoptosis [Marianneau et al., 1997; Thongtan et al., 2004], possibly by a JNK mediated pathway [Hilgard et al., 2004].

The induction of cytokine gene expression in transformed liver cells in response to dengue virus infection is evidence that the normal transcriptional machinery of the cell is perturbed. However, to date few studies have investigated the global nature of the changes in cells in response to dengue virus infection, and no study to date has investigated the broad transcriptional changes in dengue-infected liver cells.

To date only three studies have attempted to determine a picture of the global changes in gene expression in response to dengue virus infection. One of these studies [Liew and Chow, 2004] utilized the technique of differential display RT-PCR (DDRT-PCR) and, as starting material, used RNA from ECV304 (a human endothelial cell line) and identified some 203 differentially expressed genes. The genes identified consisted of those involved in cell cycle and development, components of the cytoskeleton, signal transduction molecules, membrane proteins, components of the translation, and modification pathways, transcriptional regulators, apoptosis regulators as well as a range of genes of miscellaneous or unknown role [Liew and Chow, 2004], suggesting that part of the cellular response to dengue virus infection is a wide spread alteration in the regulation of transcription. More recently, these investigators have undertaken a micro-array analysis of the same cells and identified a total of 111 genes exhibiting at least a 1.5-fold difference in expression level [Liew and Chow, 2006]. Again, genes involved in a wide range of functions including the cell cycle, apoptosis, transcriptional regulation, signal transduction, enzymes, and protein transport were identified. A study by a different group examined HUVEC, and employed a combination of differential display RT-PCR as used by Liew and Chow [2004] as well as a micro-array analysis using Affymetrix oligonucleotide GeneChips [Warke et al., 2003]. In this study, differential display identified only eight differentially expressed genes (inhibitor of apoptosis-1,2′-5′ oligoadenylate synthetase (OAS), a 2′-5′ OAS like gene, galectin-9, myxovirus protein A, regulator of G-protein signaling, endothelial, and smooth muscle cell-derived neutropilin-like protein and phospholipids scramblase I). The Affymetrix GeneChip array analysis of 22,000 human genes identified a further 269 genes that were induced, and 126 that were repressed more than fourfold after dengue virus infection [Warke et al., 2003].

Recently, Bachem et al. [1996] developed a novel cDNA-AFLP technique based on the AFLP technique developed by Vos et al. [1995] to allow gene profiling at the transcriptional level. The technique is a powerful one that enables the identification and isolation of transcripts where there is no prior sequence knowledge. It is a rapid isolation methodology, in that many candidate genes may be generated per reaction run, although the necessity for subsequent cloning and sequence analysis can be relatively labor intensive. This technique has several advantages over classical differential display methodologies, in particular there is no requirement for radioisotopes, and the amplification steps mean that even rare transcripts may be identified, and additionally, relatively small amounts of starting material are required. Given the advantages of this technique, we sought to apply cDNA-AFLP to identify differentially regulated transcripts from dengue virus serotype 2-infected HepG2 cells. As we have described extensively the interaction of dengue serotype 2 strain 16681 with HepG2 cells with respect to its

internalization and production [Thepparit et al., 2004], its ability to induce apoptosis in HepG2 cells [Thongtan et al., 2004] and more recently its ability to infect primary human hepatocytes [Suksanpaisan et al., 2007] this strain was selected as the infecting virus.

## MATERIALS AND METHODS

### HepG2 Cell Culture and Virus Preparation

The human hepatoma cell line (HepG2) was cultivated at 37°C under 10% $CO_2$ in Dubecco's Modified Eagle's Medium (DMEM; Hyclone, Logan, UT) supplemented with 10% heat inactivated fetal bovine serum (FBS; PAA, Pasching, Austria) and 100 U/ml of penicillin–streptomycin (PAA). Dengue virus serotype 2 strain 16681 was propagated from the human hepatoma p53 null cell line Hep3B. The virus was partially purified by centrifugation and stored frozen at −80°C. Titer of virus stock was obtained by standard plaque titration on Vero cells as described previously [Sithisarn et al., 2003].

### Dengue Virus Infection

HepG2 cell were seeded in 56.7 $cm^2$ culture plates and cultured for 24 hr before infection ($\sim 5 \times 10^6$ cells/plate). Cells were washed once with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4$, and 1.4 mM $KH_2PO_4$) and infected with dengue virus at MOI of 5 in BA-1 buffer (1 × M-199 medium, 50 mM Tris-HCl, 1%BSA, 7.5% $NaHCO_3$, and 1% Antibiotic) at 37°C for 2 hr with agitation every 10 min. The cells were then washed once with PBS and new growth medium added. The cells were grown for a further 3 days and then RNA was extracted. Mock infection was performed at the same time with the BA-1 buffer alone. For time course studies, the infections were performed as described above but the cells were collected at 24, 48, 72, and 96 hr post-infection for RNA extraction.

### RNA Extraction and mRNA Purification

Total RNA from mock-infected and infected HepG2 cells were extracted with TRIzol reagent (Gibco BRL, Life Science Technology, Gaithersburg, MA). Equal amounts (500 μg per sample) of total RNA from infected and mock-infected HepG2 were subjected to oligotex direct mRNA kit (QIAGEN, Valencia, CA) purification performed according to manufacturer's protocol.

### cDNA-AFLP Analysis

Equal amount of mRNA (l μg) from mock-infected and infected HepG2 were used as templates for cDNA synthesis using the SuperScript™ Double-Stranded cDNA Synthesis Kit (Invitrogen). For first strand cDNA synthesis, 1 μg each mRNA was pre-heated with 100 ng Primer $T_{25}$ and 0.83 mM dNTPs at 65°C for 5 min and then immediately placed on ice. Each reaction was then incubated with 1× Superscript II reaction buffer supplemented with 0.67 mM DTT at 42°C for 2 min then 200 units Superscript II enzyme was added and samples further incubated at 42°C for 1 hr. After that the reaction was heat-inactivated at 70°C for 15 min. The total reaction was then used to generate the second cDNA strand in a reaction containing 1× T4 polymerase reaction buffer, 0.16 mM dNTP, 3 U RNase H, and 10 units T4 DNA polymerase enzyme. Second strand cDNA synthesis was performed at 16°C for 2 hr, following which the double stranded cDNA was purified, quantitated by spectrophotometry and subjected to double restriction enzyme digestion. For double restriction enzyme digestion the entire purified cDNA product (~1 μg) was digested with 10 units *Mse*I restriction enzyme (New England Biolabs, Beverly, MA) at 37°C for 3 hr followed by digestion with 12 units *Eco*RI restriction enzyme (Promega) for another 3 hr. The double *Eco*RI–*Mse*I digested cDNA products were then purified via phenol/chloroform extraction and ligated with the AFLP adaptor sequences. Each ligation reaction contained 1× rapid ligation buffer, 1 unit T4 Ligase (Promega), 0.5 mM *Eco*RI adaptor and 5 mM *Mse*I adaptor (*Eco*RI adaptor: 5′GACTGCGTACCAATT 3′, *Mse*I-Adaptor: 5′GATGAGTCCTGAGTA 3′ [Vos et al., 1995]) and the ligation reaction was performed at 16°C for 16 hr.

The preamplification step was performed using primers that contain the adaptor sequence plus one extension base with the following PCR amplification conditions: 1 × Taq polymerase buffer (Promega), 24 mM dNTP, 45 mM $MgCl_2$, and 50 ng each primer with Taq DNA polymerase (Promega). Firstly, the preamplification PCR product was pre-heated at 94°C, 45 sec, and amplified by 28 cycles of 94°C, 45 sec; 56°C, 45 sec, and 72°C, 1 min and extension at 72°C for another 5 min. The preamplification products were used as the PCR template for the final amplification step.

The final amplification was performed by using various dilutions of preamplified products to fine tune the appropriate amount of preamplified product template for each AFLP selective primer pair [Vos et al., 1995]. The selective primers contained three selective nucleotides extending from the adaptor sequence: *Eco*RI primer: 5′GACTGCGTACCAATT$\boldsymbol{N^1NN}$3′ and *Mse*I primer: 5′GATGAGTCCTGAGTA$\boldsymbol{N^1NN}$3′, where $\boldsymbol{N^1NN}$ represent selective nucleotides which can be A, T, C, or G. $\boldsymbol{N^1}$ is the selective base of the preamplification primer in preamplified reaction [Vos et al., 1995].

The final amplification reaction contained 1× Taq polymerase buffer (Promega), 20 mM dNTP, 30 mM MgCl, and 25 ng each primer with Taq DNA polymerase (Promega). The AFLP PCR profile was as follows: 13 cycles, 94°C for 10 sec; 65°C (−0.7°C/cycle) for 30 sec; 72°C for 1 min and 25 cycles, 94°C for 10 sec; 56°C for 30 sec; 72°C for 1 min (−0.01 sec/cycle). These final amplification products were then subjected to electrophoresis through 5% polyacrylamide gels supplemented with 45% (g/ml) urea with mock-infected and infected samples run in parallel. After electrophoresis, the gels were fixed in 10% acetic acid for at least 10 min and then washed in distilled water for 5 min three times and stained in 1 mg/ml silver nitrate solution for another

30 min before developing the signal with ice-cold developing buffer which contained 30 g of sodium carbonate. The cDNA-AFLP gels were air-dried overnight at room temperature and then scanned and scored for the presence of differentially expressed TDFs. For reproducibility, each cDNA-AFLP analysis was undertaken independently in duplicate.

### Isolation, Reamplification, and Cloning of TDFs

Reproducibly expressed transcription-derived fragments (TDFs) (present on both gels) were selected and excised from the one of the duplicate cDNA-AFLP gels. The excised TDFs bands were eluted in distilled water at 37°C overnight and the eluent used in a reamplification step. The reamplification of the TDFs was performed as for the preamplification stage and products electrophoresed through 1.8% agarose gels. The reamplified products were eluted from the agarose gel using the GENECLEAN kit and subsequently cloned into pGEM-T easy vector using the pGEM-T easy vector system I (Promega) according to manufacturer's protocol. Isolated plasmids were sequenced commercially (Macrogen, Seoul, Korea). The TDF sequences were analyzed by comparison with database sequences using the BLAST homology search against mRNA and protein databases (http://www.ncbi.nlm.nih.gov/BLAST/).

### Semi-Quantitative RT-PCR

To confirm the cDNA-AFLP analysis and to expand the expression profiles, gene sequence of the identified TDFs were retrieved from databases and used for primer design. All the specific primers were designed using Primer3 program. For reverse transcriptase (RT) reactions, equal amounts of total RNA from each time point were subjected separately to reverse transcription using 10 pmol reverse primer of each specific gene (see Table I), 10 mM dNTP, 1× Imprompt II buffer and 1 U Imprompt II reverse transcriptase enzyme. Samples minus Reverse transcriptase were pre-heated at 70°C for 3 min, and following addition of the reverse transcriptase samples were incubated at 42°C for 1 hr and heat inactivated at 70°C for another 15 min. The first strand cDNAs from each reaction were used in subsequent PCR amplification reactions with conditions optimized for each particular primer pair. For semi-quantitative analysis, the exponential phase of the amplification reaction was selected for expression profile quantitation. Products were analysed on 1.8% agarose gels and quantitation of the expression level was performed using the band intensities of the PCR products using the Image J program. The intensities were normalized against actin expression, and then calculated as a percentage of relative intensity calculated from ratio of the infected and uninfected band intensity of each time point.

## RESULTS

### cDNA-AFLP Profiling

To obtain a cDNA-AFLP profile of dengue virus serotype 2-infected HepG2 cells, total purified mRNA of infected and mock-infected HepG2 cells at day 3 post-infection were used to prepare double stranded cDNA which was then digested with restriction enzymes *Mse*I and *Eco*RI and subsequently ligated to adapter primer sequences. A subset of these fragments was then amplified by polymerase chain reaction using primers complimentary to the adapter sequences, plus one additional selective base (preamplification step). A second and final round of PCR (the amplification step) was subsequently undertaken using sequences complimentary to the adapter sequences, plus an additional three selective bases. The final amplification products were then separated on 5% denaturing polyacrylamide gels by electrophoresis and visualized by silver staining as shown in Figure 1. Each cDNA-AFLP analysis was undertaken with each primer pair independently in duplicate. All bands, which represent TDFs, between 100 and 500 bases in size, were scored if present in both duplicate reactions. A total of 5,110 bands were scored resulting from 73 different primer combinations. Of these bands a total of 522 bands were scored as differentially regulated, with 296 TDFs deriving from upregulated transcripts (present in infected cells, absent in uninfected cells) while 226 TDFs were derived from downregulated transcripts (absent in infected cells, present in uninfected cells) as shown in Table II.

TABLE I. Primer Sequences Used in Semi-Quantitative RT-PCR

| Gene | Primer sequence (5′ to 3′) | | Product size |
|---|---|---|---|
| | Forward | Reverse | |
| *PPIF* | ACCTCCCCAAGGCTTAGTGT | GAGCACCGAGAGACCTTGTC | 204 |
| *ORF37* | GCCTTTTGGACCAGGTTGTA | TTTCTGGAATGGCTGGATTC | 178 |
| *CK2* | ACGAGTCACATGTGGTGGAA | GGTTCGTGACACAGGGTCTT | 248 |
| *MALT1* | GCCTCAGTTGCCTAGACCTG | GCCAAGACTGCCTTTGACTC | 217 |
| *KIAA0509* | CCCACCTCTGAATCAGCAAT | AGCCCTTGTTTATGCCACAC | 245 |
| *HSP70* | TCTGGACTGAATGTGCTTCG | ATCCCCATTTGTGGATTTCA | 177 |
| *AK3L* | TCTCATTCAGCGTGAGGATG | GCTTTCTGGCTTCTTTGTGG | 198 |
| *NIPA* | TCCTCCCAATCTTTGTCCAG | TGCTTGCAACATTCAAGAGG | 168 |
| *PHIP* | CGCACAAATAAAAGCTGCAA | CAGTTGGAACAAGTCGCTCA | 175 |
| *RiboS4* | CGCTACCCAGATCCTGTCAT | AAATGTTGGAAAGCCTCGTG | 235 |
| *HSI12044* | TCCTGTCTTGACTGCTGACG | CCCCTCCATGAGGTTAGTGA | 162 |
| *JEM-1* | CCATCAACAGTTGAATTCGCA | TGCAGAATTCAACTGTTGATGG | 100 |

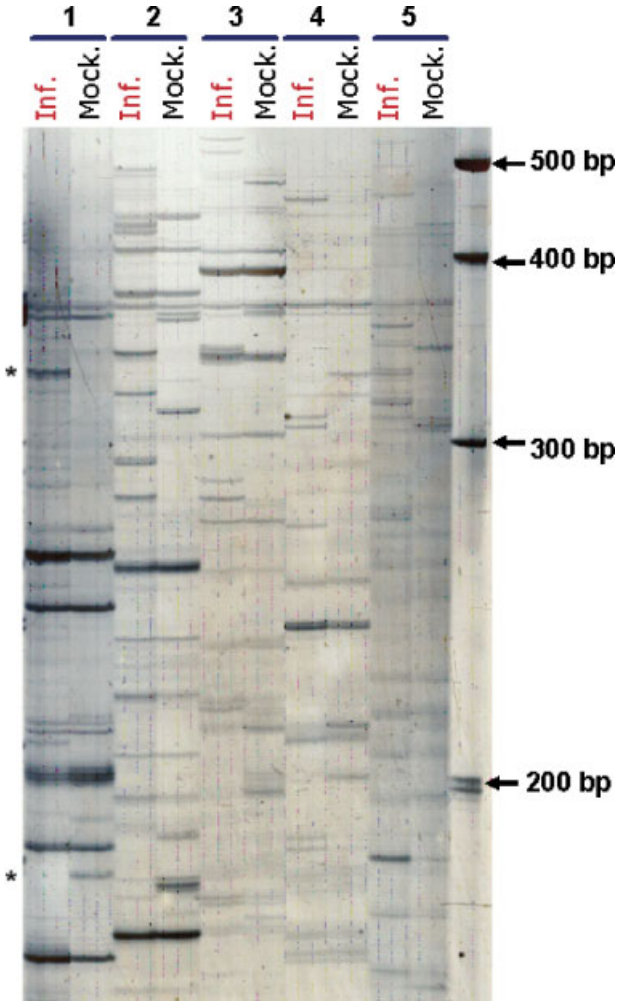


Fig. 1. cDNA-AFLP gel from five different primer combinations (*Eco*RI primer: E_AAC plus *Mse*I primer; 1: M_CCA, 2: M_CAg, 3: M_CAC, 4: M_CAT and 5: M_CAA). One microgram RNA was used for each cDNA-AFLP reaction. Subsequent to adaptor ligation, equal amounts of sample were used in all the following steps and 8 μl each of the final amplification products were electrophoresed through on 5% polyacrylamide gels. Bands were visualized by silver staining. Examples of differentially expressed fragments are marked (*).

## Isolation and Identification of the Differentially Expressed TDFs

After the cDNA-AFLP expression profiles were scored, some 400 differentially expressed TDFs were cut out and the DNA eluted by soaking the gel slice in distilled water. The DNA in the eluent was subsequently reamplified using the original preamplification primer pairs. Of the 400 TDFs originally isolated, approximately 300 were successfully reamplified. The amplified fragments were purified by agarose gel electrophoresis and cloned into pGEM-T easy vector. Of the approximately 300 cloning attempts, only 90 clones were successfully obtained. However plasmid screening indicated that 25 of these clones had only very small inserts and were excluded from further analysis. The inserts for the 65 clones were sequenced in both directions and the derived sequences searched against the BLAST database (http://www.ncbi.nlm.nih.gov/BLAST) to identify the specific nature of the clones. From the search results, 28 of the 65 TDFs matched with know sequences, with one sequence being represented by two independent clones (Table III). At day 3 post-infection, 12 of the 27 TDFs were upregulated while the remaining 15 were downregulated (Table III).

TABLE II. Summary of cDNA-AFLP Analysis of Dengue Virus Serotype 2-Infected HepG2 Cells

| Expression profile after dengue virus infection | Number |
|---|---|
| cDNA fragments displayed (100–500 bp) | 5,110 |
| Differentially expressed fragments | 522 |
| Upregulated transcription-derived fragments | 296 |
| Downregulated transcription-derived fragments | 226 |
| Cloned fragments | ~90 |
| Sequenced fragments | 65 |
| Clones with unique database matches | 27 |

## Expression Profiles of Differentially Expressed Genes Using RT-PCR

To verify the cDNA-AFLP results, 12 of the 27 differentially expressed transcripts (PPIF, ORF37, CK2, MALT1, KIAA0509, HSP70, AK3L, NIPA, PHIP, RiboS4, HSI12044, and JEM-1) were selected for further analysis by semi-quantitative RT-PCR. HepG2 cells were infected with dengue serotype 2 at MOI 5 and total RNA extracted at days 1 and 3 post-infection in parallel with mock-infected cells. Equal amount of total RNA from both time points were used for semi-quantitative RT-PCR using the specific primers given in Table I. PCR conditions for each selected transcript were optimized individually to give exponential phase amplification, as shown in the example of RiboS4 (Fig. 2) and the expression calculated as a percentage increase over mock after normalization with actin. Results of semi-quantitative RT-PCR for the 12 selected TDFs showed that 2 transcripts (PPIF and ORF37) showed no alteration of expression in response to infection, while 8 transcripts (CK2, KIAA509, HSP70, AK3L, NIPA, PHIP, RiboS4, and JEM-1) were upregulated in response to infection and 2 transcripts (MALT1 and HSI12044) were downregulated in response to infection (Fig. 3).

Finally, a more detailed profile was established for four of the identified genes (*CK2*, *NIPA*, *HSP70*, and *MALT1*) and the expression profile over 4 days post-infection was established in parallel with non-infected cells. As this experiment was not normalized with respect to the prior experiment, differences in the absolute values of percent relative expression were observed between the two experiments, however results (Fig. 4) showed that CK2, NIPA, and HSP70 were upregulated at every time point post-dengue virus infection. The expression of HSP70 was seen to be highly upregulated by day 3 post-infection, and the expression of CK2 and NIPA gradually increased in response to dengue virus infection. In contrast MALT1

TABLE III. Description of Identified Transcripts From cDNA-AFLP Analysis

| Accession no. | Gene description | Expression |
|---|---|---|
| | Transcriptional regulators | |
| BC026220 | TATA binding interacting protein (TIP120A) | ↓ |
| NM_003666 | Basic leucine zipper nuclear factor 1 (JEM-1 or BLZF1) | ↑ |
| NM_016282 | Similar to nuclear zinc finger protein Np95 (UHRF1) | ↑ |
| | Apoptosis | |
| AL031259 | Programmed cell death 2 gene (*PDCD2*) | ↑ |
| BC050036 | Casein kinase (CK2) | ↑ |
| | Signal transduction | |
| NM_173844 | Mucosa associated lymphoid tissue lymphoma translocation gene 1 (*MALT1*) | ↑ |
| NM_005766 | RhoGEF (ARHGEF) | ↓ |
| AF037360 | Growth hormone/chorionic somatomammotropin gene cluster | ↓ |
| BC024034 | HSP70 9B (mortalin-2) | ↑ |
| NM_017934.4 | Pleckstrin homology domain interacting protein (PHIP) | ↓ |
| | Protein synthesis | |
| BC010286 | Ribosomal protein S4, Y-linked 1 | ↓ |
| | Cellular metabolism | |
| AF100785 | GTP:GMP phophotransferase (*AK3L* gene) | ↑ |
| HS44A20 | A novel protein similar to C1 - tetrahydrofolate synthases | ↓ |
| | Miscellaneous and unknown functions | |
| AK026351 | Human cDNA clone no. HSI12044 | ↓ |
| AF264036 | ADG-90 mRNA | ↓ |
| AC007640 | Chromosome 11 clone no. RP11-89A1 | ↓ |
| AC104370 | Homo sapiens chromosome 8, clone RP11-367L7 | ↓ |
| NM_020448 | NIPA like protein | ↑ |
| AL603910 | Clone RP11-505P4 | ↓ |
| AC_083884.6 | BAC clone RP11-813J7 from chromosome 7 | ↓ |
| AL_391056.25 | Clone RP11-492E3 on chromosome 9 | ↓ |
| AB00 7978.1 | Chromosome 1 specific transcript KIAA0509 | ↓ |
| AL391665.28 | Clone RP11-519K18 on chromosome 10 | ↑ |
| BC038952.2 | Chromosome 1 open reading frame 37 | ↑ |
| AC008870 | Chromosome 16 clone CTD-2196E14 | ↓ |
| AC064869 | BACclone RP11-534J18 from 2 | ↑ |
| AK097499 | cDNA FLJ40180 fis, clone TESTI2018035 | ↓ |

was shown to be sharply downregulated at day 2 post-infection, but to increase over the next 2 days.

## DISCUSSION

Several lines of evidence including clinical studies [Bhamarapravati, 1989; Nguyen et al., 1997; Mohan et al., 2000; Wahid et al., 2000; Huerre et al., 2001; Pancharoen et al., 2002; Souza et al., 2004; Subramanian et al., 2005], a high frequency of hepatomegaly [Bhamarapravati, 1989], liver dysfunction [Kuo et al., 1992; Nguyen et al., 1997; Mohan et al., 2000; Wahid et al., 2000; Pancharoen et al., 2002; Souza et al., 2004; Wichmann et al., 2004; Kularatne et al., 2005], the demonstration of the dengue viral genome [Rosen et al., 1999; Huerre et al., 2001], antigens [Couvelard et al.,

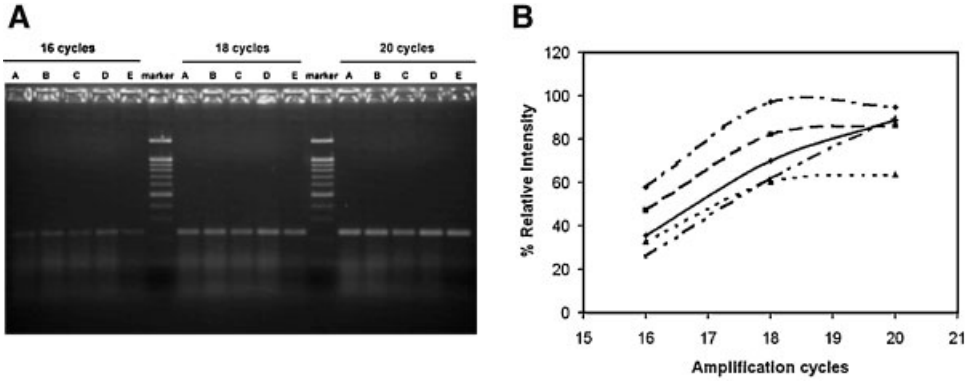


Fig. 2. Amplification curves of RiboS4 gene from semi-quantitative RT-PCR reactions. **A**: RT-PCR products of RiboS4 from different cycle conditions of RNA extracted from mock-infected (**Lanes A**,**E**) and infected (**Lanes A**,**C**,**E**) HepG2 cells at 24 (**lanes A**,**B**), 48 (lane C), and 72 hr (**lanes D**,**E**) post-infection/mock infection. **B**: Quantitation of RT-PCR products from **panel A**. Solid line, amplification of RNA from mock infection at 24 hr; dashed line, amplification of RNA from dengue-infected sample at 24 hr post-infection; dotted line, amplification of RNA from dengue-infected sample at 48 hr post-infection; dashed line with one dot, amplification of RNA from dengue-infected sample at 72 hr post-infection; dashed line with two dots, amplification of RNA from mock-infected sample at 72 hr post-infection.

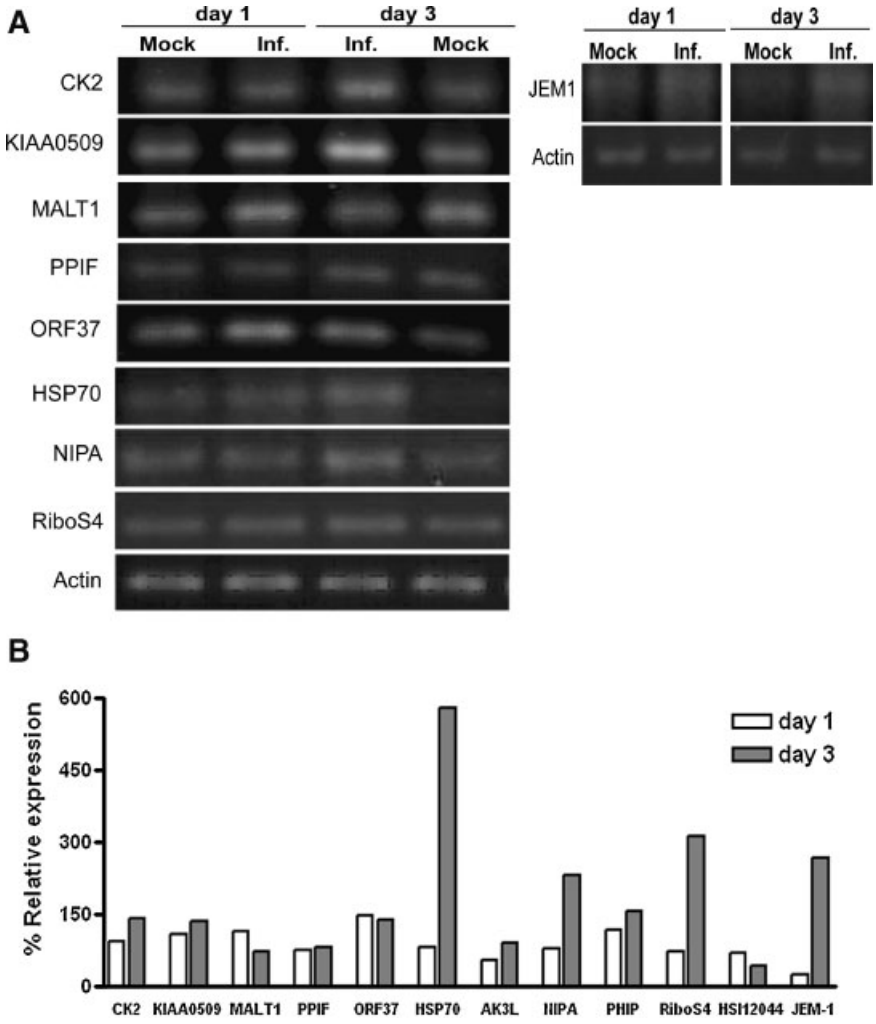


Fig. 3. Expression profiles of five selected transcripts identified through the cDNA-AFLP analysis. **A**: Semi-quantitative RT-PCR analysis of five selected gene transcripts and actin at days 1 and 3 post-infection. Each sample was analyzed in parallel with a mock-infected control sample. **B**: The relative expression levels of 12 selected transcripts at days 1 and 3 post-infection. Relative intensity was calculated as a percentage increase over mock-infected control after normalization with actin.

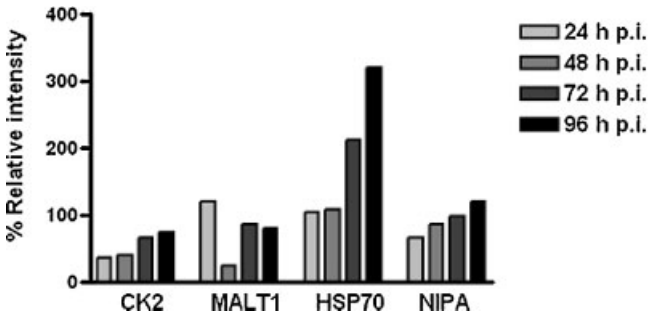


Fig. 4. Expression profiles of four selected transcripts. Semi-quantitative RT-PCR was used to establish the expression profile for four selected transcripts over 4 days post-infection. Relative intensity was calculated as a percentage increase over mock-infected control samples after normalization with actin.

1999; Huerre et al., 2001] as well as the virus itself [Rosen et al., 1989] in pathologic specimens have implicated the liver in the pathogenesis of dengue infections. In the absence of easily available or cheap human primary hepatocytes considerable use has been made of transformed hepatocytes, which are broadly susceptible to the dengue viruses [Lin et al., 2000; Thongtan et al., 2004] in an attempt to understand the interaction of the dengue viruses and the human host cell.

In infectious diseases, the disease pathogenesis is the outcome of the interaction between two genomes, those of the host and the pathogen [Kellam and Weiss, 2006]. In viral infectious diseases, the interplay between viruses and host cells can result in changes in the host transcriptome and so transcription profiling can reveal how the virus re-models the expression the transcriptome in the host-virus interaction. Analyses of the

alterations of the transcriptome in response to viral infection can be broadly categorized as "open architecture" or "closed architecture." Closed architecture analysis (DNA microarrays, macro arrays) rely upon the hybridization of the transcriptome to pre-characterized oligonucleotides or cDNA clones and as such the analysis is limited a pre-defined set of genes. Open architecture analyses require little or no sequence characterization, and are able to detect the response of both characterized and unknown genes. Studies investigating the alterations of the human transcriptome in defined cell systems in response to dengue virus infection have employed both open architecture [Warke et al., 2003; Liew and Chow, 2004] and closed architecture [Warke et al., 2003; Liew and Chow, 2006] systems. Previous open architecture analyses have investigated the alteration of the transcriptomes of both human endothelial cells and HUVEC cells in response to dengue virus infection [Warke et al., 2003; Liew and Chow, 2004] using DDRT-PCR with varying numbers of genes characterized. However, DDRT-PCR is reported to give a preponderance of 3′-untranslated sequences [Bachem et al., 1996] as well as to require the use of radioisotopes. The recently developed technique of cDNA-AFLP avoids these draw backs [Vos et al., 1995; Bachem et al., 1996], and is able to provide both quantitative and qualitative information about the nature of the alterations in the transcriptome [Cappelli et al., 2005; Vandeput et al., 2005].

To understand alterations in the liver cell transcriptome in response to dengue virus infection cDNA-AFLP was employed, and the transcriptome analyzed with a total of 73 primer combinations out of a theoretical total of 256 primer combination, resulting in a screening of nearly one third of the transcriptome ($73/256 = 28\%$). A total of 5,110 TDFs between 100 and 500 base pairs were scored and some 10% of these were shown to be differentially regulated upon dengue virus infection. Given that only 1/3 of the transcriptome was screened with the primer combinations used, this would suggest that the full transcriptome consisted of 15,000 TDFs of which 1,500 were transcriptionally regulated. If each message gave some 3–4 TDFs, this would suggest that the transcriptome consisted of 3–4,000 messages of which some 3–400 were transcriptionally regulated upon dengue virus infection, a figure in close agreement with the approximately 400 genes shown to be transcriptionally regulated in HUVEC cells in response to dengue virus infection when analyzed by micro-array analysis using the Affymetrix oligonucleotide GeneChips [Warke et al., 2003].

Although attempts were made to clone some 400 TDFs, only 65 TDFs were successfully cloned, and this stage clearly represented a major technical bottleneck. The reasons for the low success rate of cloning at this stage remain unclear. Partly responsible for the low total success was the fact that only 75% of the selected TDFs were reamplified, possibly as a result of low levels of eluted template or the inhibitory effect of polyacrylamide contamination in reamplification reaction [Siembieda and Lakatua, 1998]. A low frequency of cloning of amplified bands as well as the presence of small inserts further reduced the number of TDFs converted successfully to characterized transcripts.

Of the 65 successfully cloned TDFs, 28 matched with database sequences giving 27 unique matches. The remaining 37 TDFs without matches (as of October 10, 2006) suggest that a significant number of response genes remain to be identified. A total of 12 of the identified TDFs were scored originally as upregulated transcripts from the cDNA-AFLP gels and another 15 were scored as downregulated transcripts (Table III). None of these genes have, to date, been identified independently as being regulated in HepG2 cells by dengue virus infection, and conversely, no gene that has been identified in other studies as being regulated in HepG2 cells by dengue virus infection was identified in this study. Validation of the regulation of the identified genes was undertaken via semi-quantitative RT-PCR. While real time PCR would, ideally, offer a more accurate validation than semi-quantitative PCR, this technology is still not widely available in developing countries. However, the result from semi-quantative RT-PCR showed that not all the genes had expression profiles similar to those seen in the cDNA-AFLP gels (Table IV). From the 12 genes selected for validation, 2 genes were shown to be non-regulated transcripts and 10 genes were differentially expressed. The upregulated transcripts were CK2, KIAA509, HSP70, AK3L, NIPA, PHIP, RiboS4, and JEM-1, while MALT1 and HSI12044 were downregulated. Among these, only HSP70, NIPA, RiboS4, and JEM-1 showed more than twofold upregulation upon dengue virus infection. The discrepancy between the cDNA-AFLP profile and the more detailed semi-quantitative RT-PCR analysis remain unclear, but one possible explanation is that the TDF bands on the cDNA-AFLP gel may represent a mixture of TDFs which were not selected through on the cloning stage.

Out of the entire analysis four genes were seen to be regulated by more than twofold in response to dengue virus infection. *JEM-1* gene is a novel gene that encodes

TABLE IV. Comparison of Results From Differentially Expressed Transcripts Analyzed by cDNA-AFLP or Semi-Quantitative RT-PCR

| Gene | RT-PCR | cDNA-AFLP |
|---|---|---|
| *CK2* | Up | Up |
| *MALT1* | Down | Up |
| *PPIF* | None | Up |
| *ORF37* | None | UP |
| *KIAA0509* | Up | Down |
| *HSP70* | Up | Up |
| *AK3L* | Up | Up |
| *NIPA* | Up | Up |
| *RiboS4* | Up | Down |
| *HSI12044* | Down | Down |
| *PHIP* | Up | Down |
| *JEM-1* | Up | Up |

a 45 kDa nuclear product. Studies of JEM-1 have revealed a basic leucine zipper structure suggesting a role as a transcription factor [Duprez et al., 1997] and further study had shown that JEM-1 protein enhances AP-1 activity without directly interacting with either c-Jun or c-Fos proteins [Tong et al., 1999]. However, understanding the role of JEM-1 in dengue virus infection will clearly require further investigation.

NIPA (nuclear interaction partner of ALK) encodes a 60-kDa protein that is expressed in a broad range of human tissues and contains a classical nuclear translocation signal in its C terminus, which directs its nuclear localization [Ouyang et al., 2003]. Recently, NIPA was identified as a human F-box-containing protein and reported to play a role in controlling mitotic cell entry via the SCF complex [Bassermann et al., 2005]. The association of an increase in NIPA expression with virus infection has not been previously reported, but NIPA might play a role in the induction of cell cycle arrest upon dengue virus infection prior to the induction of apoptosis as has been observed by some authors [Su et al., 2001].

The human ribosomal protein 4 (RiboS4) is unique amongst the components of the 40S subunit of the eukaryotic ribosome in that two functional genes, one on the X-chromosome and one on the Y-chromosome, encode non-identical isoforms [Fisher et al., 1990]. Interestingly RiboS4 as well as other ribosomal proteins have been shown to be over-expressed in response to increased proliferative activity of the cell [Chen and Ioannou, 1999; Ivanov et al., 2005] presumably as a result of an increase demand for protein biosynthesis, and as such the increased expression of RiboS4 in response to the increased protein biosynthesis under conditions of virus infection may be similar.

The *HSP70* gene encodes a heat shock cognate protein which belongs to the heat shock protein family, a family of highly conserved molecular chaperones with a broad intra-cellular location that assist the structure formation of proteins in vivo and participate in a number of normal, stress and pathological responses [Walter and Buchner, 2002]. These properties are exploited by a number of different viruses to assist the correct folding and trafficking of viral proteins during the virus life cycle [Mayer, 2005]. More recently, HSP70 has been implicated in forming a receptor complex for dengue virus infection into monocytes and macrophages [Reyes-del Valle et al., 2005]. However, as a stress protein it is possible that the increased expression of Hsp70 is due to the innate cellular response of the cell to virus infection [Bukrinsky and Zhao, 2004].

Overall, cDNA-AFLP analysis has proven applicable to determining the expression profile of dengue virus-infected HepG2 cells and in providing an estimate of the global alterations of the transcriptome of liver cells. The technique proved reasonably useful in characterizing the specific genes involved in the response to infection, although the low conversion factor from TDF to sequence characterized clone suggests that there is room for technical improvement. The majority of the genes characterized by specific transcription profiling were indeed regulated transcriptionally in response to infection, although often the degree of alteration in expression was minimal. Perhaps the strongest advantage of the technique over other methodologies, especially those employing a closed architecture analysis were in the identification of transcripts not in current databases. These genes when characterized further may give particular insights to the process of transcriptome re-modeling by dengue virus infection.

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# A role for autophagolysosomes in dengue virus 3 production in HepG2 cells

Atefeh Khakpoor, Mingkwan Panyasrivanit, Nitwara Wikan and Duncan R. Smith

Molecular Pathology Laboratory, Institute of Molecular Biology and Genetics, Mahidol University, Thailand

Correspondence
Duncan R. Smith
duncan_r_smith@hotmail.com

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We have recently proposed that amphisomes act as a site for translation and replication of dengue virus (DENV)-2 and that DENV-2 entry and replication are linked through an ongoing association with membranes of an endosomal–autophagosomal lineage. In this report, we present the results of an investigation into the interaction between DENV-3 and the autophagy machinery. Critically, treatment with the lysosomal fusion inhibitor L-asparagine differentiated the interaction of DENV-3 from that of DENV-2. Inhibition of fusion of autophagosomes and amphisomes with lysosomes resulted in decreased DENV-3 production, implying a role for the autophagolysosome in the DENV-3 life cycle. Evidence based upon the co-localization of LC3 and cathepsin D with double stranded RNA and NS1 protein, as assessed by confocal microscopy, support a model in which DENV-3 interacts with both amphisomes and autophagolysosomes. These results demonstrate that the interactions between DENV and the host cell autophagy machinery are complex and may be determined in part by virus-encoded factors.

## INTRODUCTION

The mosquito-borne dengue virus (DENV) is believed to cause 100 million new infections each year when female *Aedes* mosquitoes carrying the virus feed on immunologically susceptible humans (Guzman & Kouri, 2002). While the majority of these infections may either be asymptomatic or result in a relatively mild febrile disease, significant consequences can develop with the disease progressing to the life threatening dengue haemorrhagic fever or dengue shock syndrome (Halstead, 1988; Malavige *et al.*, 2004). DENV comprises four antigenically related but distinct viruses termed DENV-1 to DENV-4, with each virus comprising many distinct genotypes (Holmes & Twiddy, 2003). Despite a large number of studies, no direct link between a specific DENV genotype and the cause of the disease has yet been published, possibly as a consequence of an imperfect understanding of the interaction between specific DENV genotypes and susceptible human cells.

It has been shown that DENV enters both insect and mammalian cells through endocytosis into clathrin-coated pits (Acosta *et al.*, 2008; Krishnan *et al.*, 2007) and that subsequent uncoating of the virus occurs in the endosome (Krishnan *et al.*, 2007; van der Schaar *et al.*, 2007) in response to the low pH environment (Heinz *et al.*, 2004; Modis *et al.*, 2004; Mukhopadhyay *et al.*, 2005); however, the details of how and where DENV is translated and replicated remain poorly understood (Clyde *et al.*, 2006). Replication in both insect and mammalian cells is accompanied by the expansion of intracellular membranes (Clyde *et al.*, 2006; Ko *et al.*, 1979; Miller & Krijnse-Locker, 2008; Salonen *et al.*, 2005) but a causal link between membrane amplification and virus translation and replication remains to be elucidated.

Several viruses have been shown to induce autophagy (Espert *et al.*, 2007; Lee & Iwasaki, 2008) which characteristically induces the formation of double- and single-membrane vesicles (Xie & Klionsky, 2007). Autophagy is an evolutionarily conserved lysosomal degradation pathway whose primary function is the catabolic salvage of cellular constituents from old organelles or aggregated proteins (Levine & Klionsky, 2004; Meijer & Codogno, 2006). Studies have shown that autophagy can be induced in response to a number of stimuli, such as nutrient depletion or starvation and bacterial and viral infection, as well as during ageing and in a number of disease conditions, including neurodegenerative diseases and cancer (Lerena *et al.*, 2008; Levine & Klionsky, 2004; Meijer & Codogno, 2006; Mizushima *et al.*, 2008). Induction of autophagy initially activates the formation of double-membraned autophagic vacuoles called autophagosomes (Dunn, 1990a) and it is believed that the membranes involved in autophagosome formation are sequestered from the endoplasmic reticulum (Dunn, 1990a) or trans-Golgi network (Kihara *et al.*, 2001). During maturation, autophagosomes can fuse with endosomes to form amphisomes (Gordon & Seglen, 1988) and both autophagosomes and amphisomes fuse with lysosomes in the final maturation step to form autophagolysosomes

(Dunn, 1990b), which represent the primary degradative vesicle (Xie & Klionsky, 2007). A schematic representation of autophagic vesicles is given in Fig. 1. Both autophagosomes and amphisomes are double-membrane vesicles, while autophagolysosomes are single-membrane vesicles.

While the process of autophagy is regulated by a large number of genes which were first identified in yeast (Xie & Klionsky, 2007), two conjugation pathways [the covalent linkage of Atg5 and Atg12 (Mizushima *et al.*, 1998, 2002; Ohsumi, 2001) and the covalent lipidation of Atg8 by phosphatidylethanolamine (Kabeya *et al.*, 2000; Ohsumi, 2001)] play a critical role in the formation of autophagic vesicles. Atg8 is also known as microtubule-associated protein 1 light chain 3 (LC3), and the formation and association of lipidated LC3 (the LC3-II form) with autophagic membranes (Kabeya *et al.*, 2000) is the only validated marker of autophagosome formation (Bampton *et al.*, 2005; Kimura *et al.*, 2007). The co-localization of LAMP1 (a marker of endosomal and lysosomal membranes) with LC3 is frequently used to identify autophagosomal vacuoles formed prior to lysosomal fusion (autophagosomes and amphisomes), as the subsequent fusion of autophagosomes with lysosomes results in the degradation of LC3 (Eskelinen, 2005; Mizushima & Yoshimori, 2007).

Recently, DENV-2 has been shown to induce autophagy (Lee *et al.*, 2008); modulation of autophagy with biochemical inhibitors such as 3MA, which inhibits the formation of autophagosomes (Seglen & Gordon, 1982), and activators of autophagy such as rapamycin, which induces autophagy by inhibiting the negative regulator of the autophagy pathway, the mammalian target of rapamycin (mTOR) (Noda & Ohsumi, 1998), result in modulation of DENV-2 virus yield (Lee *et al.*, 2008; Panyasrivanit *et al.*, 2009). Our previous study (Panyasrivanit *et al.*, 2009) located at least part of the DENV-2 translation/replication complex autophagosomal membranes, and infection in the presence of L-asparagine (L-Asn), which inhibits fusion of lysosomes with autophagosomes (Seglen *et al.*, 1996), resulted in an increase in virus output, indicating that amphisomes were the critical autophagic structure. This result was confirmed by co-localization experiments with mannose 6-phosphate receptor (MPR), an endosomal/amphisomal marker (Taylor *et al.*, 2005), and we have proposed that virus entry and translation–replication can be explained in terms of a continual association with endosomal–autophagosomal membranes (Panyasrivanit *et al.*, 2009; see also Fig. 1). However, to date, neither the study by Lee *et al.* (2008) nor our previous study (Panyasrivanit *et al.*, 2009) has investigated a DENV other than DENV-2. This study sought to address this issue and determined that significant differences exist in the way that DENV-3 interacts with the autophagy apparatus compared with DENV-2. This finding will have an impact on studies attempting to find intracellular targets aimed at disrupting DENV translation and replication.

## METHODS

**Cells, viruses and infection.** The human hepatoma cell line HepG2 (ATCC no. HB-8065) was cultivated at 37 °C as described previously (Panyasrivanit *et al.*, 2009; Thepparit *et al.*, 2004; Thepparit & Smith, 2004). DENV-3 (strain 16562) and DENV-2 (strain 16681) were propagated in the *Aedes albopictus*-derived cell line C6/36 (ATCC no. CRL-1660). Viruses were partially purified by centrifugation to remove cellular debris and stored frozen at −80 °C. Virus titre was determined by plaque assay on monolayers of the rhesus monkey kidney cell line LLC-MK2 as described previously (Sithisarn *et al.*, 2003).

Cells were appropriately treated with 3-methyladenine (3-MA), L-Asn and rapamycin (all Sigma-Aldrich) as described previously

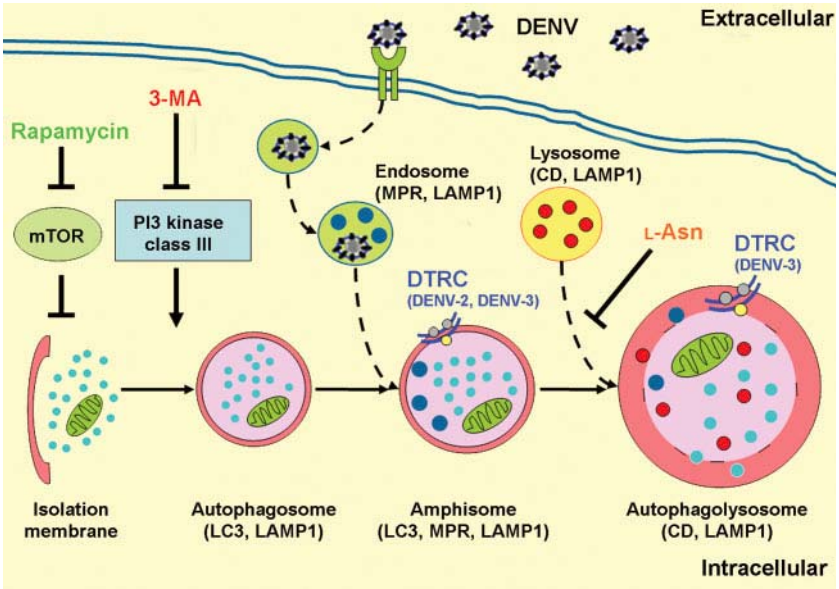


**Fig. 1.** Autophagy and DENV. A schematic representation of the formation of autophagic vesicles in relation to our proposed model of DENV entry and translation/replication. The actions of the main biochemical autophagy modulators are indicated, as are the positions of markers used in this and our previous study (Panyasrivanit *et al.*, 2009). The location of the DENV translation/replication complexes (DTRC) for DENV-2 and DENV-3 are indicated.

(Panyasrivanit *et al.*, 2009). Cells were infected with DENV-2 or DENV-3 in the presence or absence of appropriate inhibitor at 10 p.f.u. per cell for 2 h in Dulbecco's modified Eagle's medium and grown with or without biochemical treatment, as appropriate, for the times indicated.

**Indirect immunofluorescence.** Approximately 30 000 HepG2 cells were seeded onto glass coverslips and grown for 24 h under standard conditions. Coverslips were then directly infected for 2 h with DENV-3 or DENV-2 at 10 p.f.u. per cell or pretreated with autophagy modulators, as above, before infection. Cells were then grown for the times indicated in the presence or absence of an autophagy modulator as appropriate. Subsequently, cells were processed and incubated with two or three primary antibodies followed by incubation with two or three secondary antibodies, as described previously (Panyasrivanit *et al.*, 2009).

Primary antibodies used were a rabbit polyclonal anti-MAP-LC3 antibody (sc-28266, Santa Cruz Biotechnology) or goat polyclonal anti-MAP-LC3 antibody (sc-16756, Santa Cruz Biotechnology), a rabbit polyclonal anti-LAMP1 antibody (ab24170, Abcam) a rabbit polyclonal anti-MPR antibody (ab32815, Abcam), a mouse monoclonal anti-dengue NS1 antibody (Puttikhunt *et al.*, 2003), a mouse monoclonal anti-double-stranded (ds)RNA antibody (J2, English & Scientific Consulting), a goat polyclonal anti-ribosomal protein L28 (sc-14151, Santa Cruz Biotechnology) and a rabbit polyclonal anti-cathepsin D antibody (Ab-2) (IM 16, Calbiochem).

Secondary antibodies used were a Rhodamine Red X-conjugated goat anti-rabbit IgG (111-295-144, Jackson ImmunoResearch laboratories), Cy5-conjugated rabbit anti-goat IgG (81-1616; Invitrogen), an FITC-conjugated donkey anti-rabbit IgG (sc-2090, Santa Cruz Biotechnology), an FITC-conjugated goat anti-mouse IgG (02-18-06; KPL), an Alexa Fluor 594-conjugated chicken anti-mouse IgG (A21201, Molecular Probes) and an Alexa Fluor 647-conjugated donkey anti-rabbit IgG (A31573, Molecular Probes).

**Fluorescence confocal microscope imaging.** Fluorescent confocal microscope images were captured using the Olympus FluoView 1000 (Olympus Corporation, Shinjuku-ku, Tokyo) equipped with Olympus FluoView software v. 1.6 exactly as described previously (Panyasrivanit *et al.*, 2009). Image analysis and calculation of Pearson correlation coefficients and confidence intervals (CIs) were carried out as described previously (Panyasrivanit *et al.*, 2009).

**Western blot analysis.** Total protein of either mock-infected or DENV-3- or DENV-2-infected HepG2 cells in the presence or absence of autophagy modulators was extracted at appropriate time points. Proteins were separated by SDS-PAGE and transferred to solid membranes. Membranes were then incubated with 5 % skimmed milk in Tris-buffered saline (TBS) for 2 h at room temperature. Membranes were incubated with a 1 : 50 dilution of a mouse monoclonal anti-dengue NS1 antibody, a 1 : 1000 dilution of a rabbit polyclonal anti-MAP-LC3B antibody (ab48394; Abcam) or a 1 : 800 dilution of a mouse monoclonal antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sc-32233, Santa Cruz Biotechnology) in 5 % skimmed milk in TBS. Membranes were then incubated with a 1 : 4000 dilution of horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (A9044, Sigma) or a 1 : 3000 dilution of HRP-conjugated goat anti-rabbit IgG antibody (31460, Pierce) in 5 % skimmed milk in TBS for 1 h at room temperature. Signals were developed by using the ECL Plus Western blotting Analysis kit (GE Healthcare).

**Virus titration assays.** Extracellular and intracellular virus titres were analysed by standard plaque assay as described previously (Sithisarn *et al.*, 2003; Thepparit & Smith, 2004), using either six or three (as indicated) independent replicates with duplicate assays for extracellular virus titre and three independent replicates with duplicate assays for intracellular virus titre.

**Statistical analysis.** Virus production data were analysed using the Graph Pad prism program (GraphPad Software). Statistical analysis of significance was undertaken by Paired sample test using SPSS (SPSS Inc.).

## RESULTS AND DISCUSSION

While we have shown previously that the virus production kinetics of DENV-2 and DENV-3 in liver cells are essentially identical at an m.o.i. of 1 (Thepparit *et al.*, 2004), previous experiments on DENV-2 and autophagy (Panyasrivanit *et al.*, 2009) were undertaken at 10 p.f.u. per cell. To confirm that the different virus : cell ratio did not alter the kinetics of virus production, virus production profiles of DENV-2 and DENV-3 at 10 p.f.u. per cell were determined. This showed that while the absolute levels of virus produced were different, as noted previously for an m.o.i. of 1 (Thepparit *et al.*, 2004), the time for *de novo* extracellular virus production did not differ between DENV-2 and DENV-3 (Fig. 2a).

To assess whether modulation of autophagy with biochemical inhibitors altered levels of extracellular DENV-3, HepG2 cells were infected with DENV-3 at 10 p.f.u. per cell in the presence or absence of 3-MA, L-Asn and rapamycin. Extracellular virus production at 24 h post-infection (p.i.) was assessed by standard plaque assay and the experiment was performed independently in parallel six times, with duplicate assays of virus titre. Infection in the presence of rapamycin, an autophagy inducer, did not alter DENV-3 output compared to the control (DENV-3 only) (Fig. 2b) but examination of earlier time points (16 and 20 h) showed significantly increased virus production in the presence of rapamycin compared with control infection ($P<0.001$ and $P=0.016$, respectively) (Fig. 2c). Infection in the presence of 3-MA showed a significant reduction in virus output compared with control infections at all time points examined ($P=0.013$, $P=0.007$ and $P<0.001$ for 16, 20 and 24 h p.i., respectively) (Fig. 2b, c).

Infection in the presence of L-Asn resulted in a significantly decreased extracellular virus production when assessed at 24 h (Fig. 2b; $P<0.001$) and 16 h (Fig. 2c; $P=0.01$) p.i., and a reduced virus output when assessed at 20 h p.i. (Fig. 2c). Similar, but not statistically significant, results were observed at infection ratios of 0.1 and 1 p.f.u. per cell (Fig. 2d).

The reduction in DENV-3 titre seen in the presence of L-Asn is in contrast with the increased production of DENV-2 when the infection is undertaken in the presence of L-Asn (Panyasrivanit *et al.*, 2009). Similarly, intracellular virus yield was also reduced in the presence of L-Asn ($P=0.024$; Fig. 2e), suggesting that DENV-3 interacts with the autophagy machinery in a manner distinct from that of DENV-2.

We next established whether DENV-3 infection induces autophagy as shown with DENV-2 (Lee *et al.*, 2008;

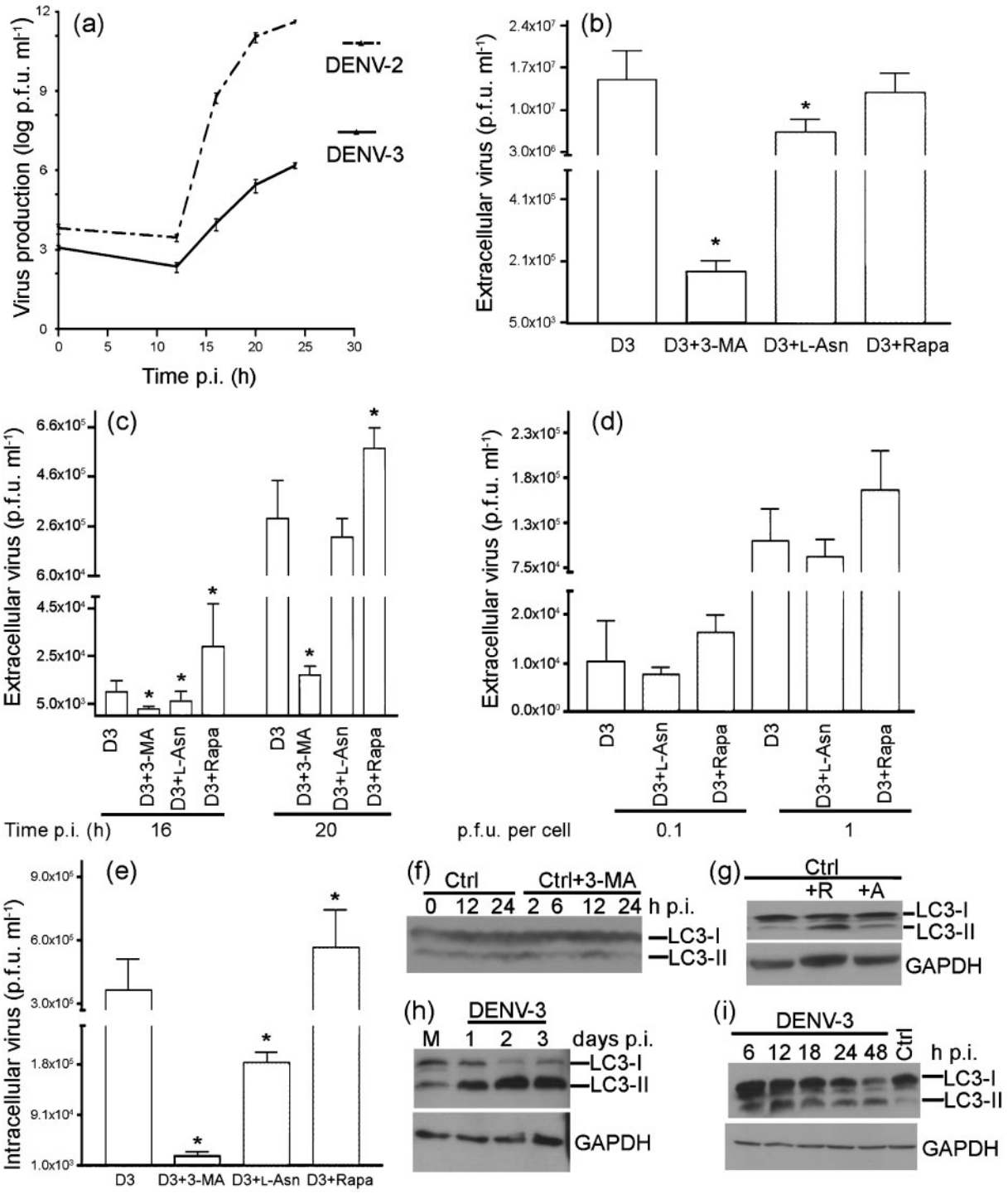


**Fig. 2.** Interaction of DENV-3 and autophagy. (a) DENV-2 and DENV-3 virus production at 10 p.f.u. per cell. (b, c) Extracellular DENV-3 production at 24 h (b) and at 16 and 20 h (c) p.i. at 10 p.f.u. per cell in the presence or absence of autophagy modulators. (d, e) Extracellular (d) and intracellular (e) DENV-3 production at 24 h p.i. at 0.1 and 1 p.f.u. per cell in the presence or absence of autophagy modulators. In (a)–(e), the mean virus titres (± SD) are shown. Extracellular virus data are derived from six (b) or three (a, c, d and e) independent replicates, with duplicate assays of each replicate; intracellular data are derived from three independent replicates assayed in duplicate. Statistical significance of virus output compared with the control is indicated by an asterisk (*$P<0.001$). (f, g) Western blot analysis of LC3 and GAPDH expression in control cells (Ctrl) either untreated or treated with 3-MA, rapamycin (+R) or L-Asn (+A). (h, i) Western blot analysis of LC3 and GAPDH expression in either mock-infected (M), control (Ctrl) or DENV-3-infected cells.