FINAL REPORT

ชื่อโครงการ (ภาษาไทย) การค้นหาและการแยกลักษณะของตัวตอบรับของเชื้อไวรัส

ไข้เลือดออกโดยใช้เทคนิค affinity chromatography

Identification and characterization of dengue virus receptors using affinity chromatography

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ชื่อโครงการ (ภาษาไทย) การค้นหาและการแยกลักษณะของตัวตอบรับของเชื้อไวรัสไข้เลือดออกโดยใช้เทคนิค affinity chromatography

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การค้นหาและการแยกลักษณะของตัวตอบรับของเชื้อไวรัสไข้เลือดออกโดยใช้เทคนิค affinity chromatography

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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: Papers

1. Panyasrivanit, M., Khakpoor, A., Wikan, N. and Smith, D.R. Linking dengue virus entry and translation/replication through amphisomes. Autophagy 5: 434-435, 2009. (Impact factor 5.479)

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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.

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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

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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.

0029_R01

Basic Virology

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

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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]

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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

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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

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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

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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

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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

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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

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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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Identification and characterization of dengue virus receptors

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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

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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

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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

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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

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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

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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 1×10^6, 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

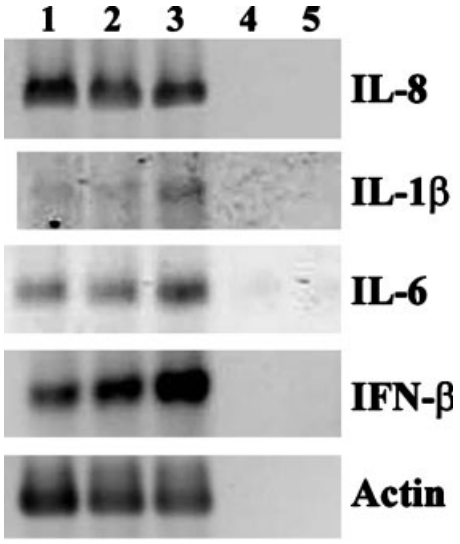

Fig. 1. Induction of cytokine gene expression in response to LPS treatment. HepG2 cells were either not treated (**lane 1**) or treated with 1 μg/ml LPS for 24 (**Lane 2**) or 48 (**lane 3**) hr after which the levels of mRNA for IL-8, IL-1β, IL-6, and IFN-β were determined semi-quantitatively by RT-PCR 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 1×10^6 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

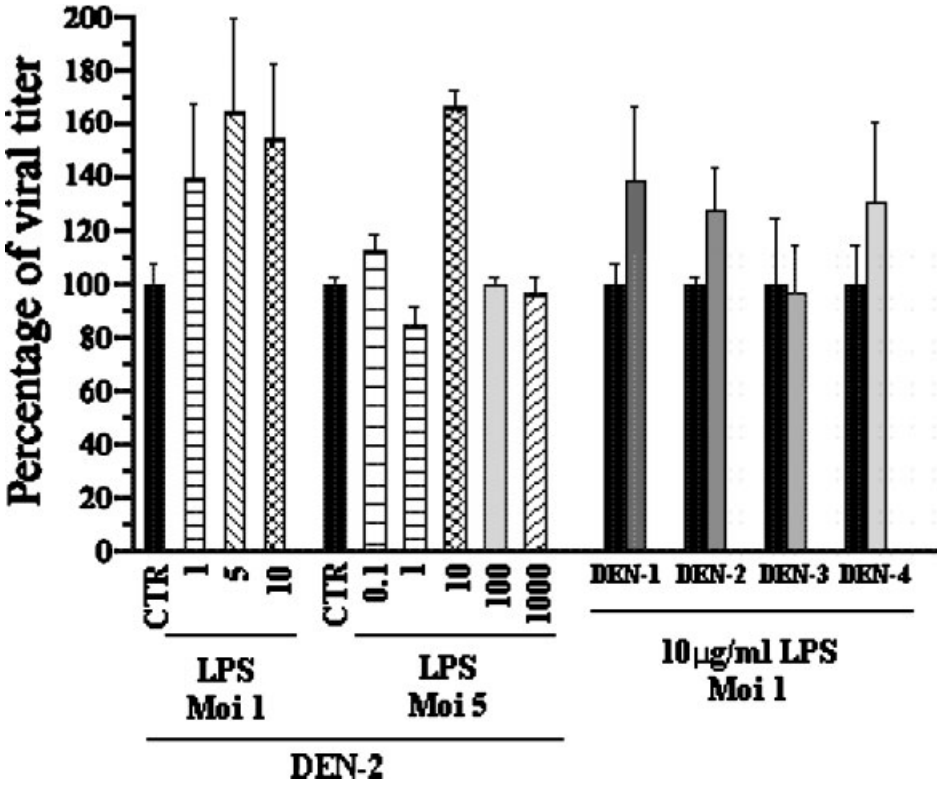

Fig. 2. HepG2 cells were pre-treated with varying concentrations of LPS for 24 hr before infection with dengue serotypes 1 to 4 as indicated. Supernatants were assayed after one and a half replication cycles. Results are expressed as a percentage of virus titer of untreated HepG2 cells (CTR, solid bars) 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

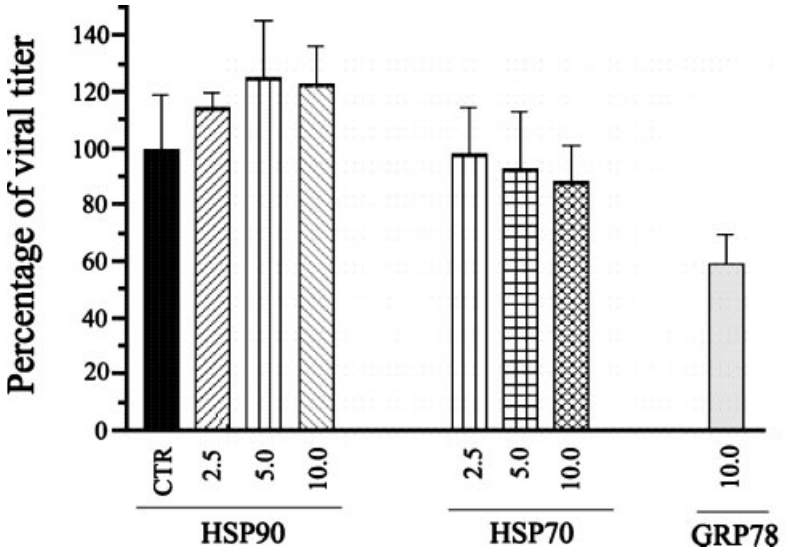

Fig. 3. HepG2 cells were pre-treated with 2.5, 5, or 10 μg/ml of antibodies directed against hsp90, hsp70, or GRP78 for 1 hr before infection with dengue serotypes 2 at an MOI of 5. Supernatants were assayed by standard plaque assay after one and a half replication cycles. Results are expressed as a 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

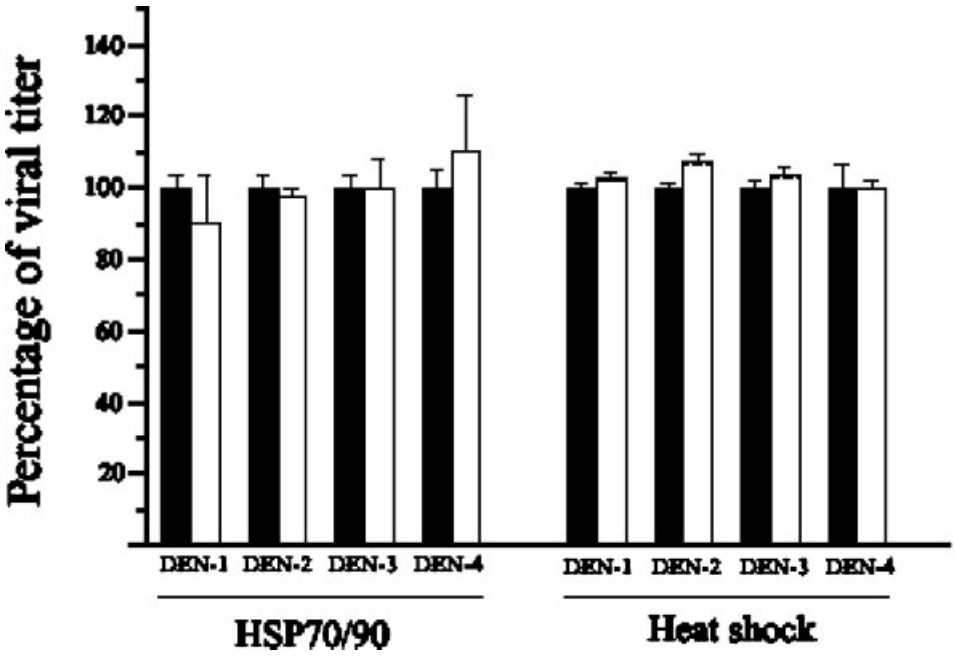

Fig. 4. Hep G2 cells were either pre-treated with 10 μg/ml of a combination of antibodies against hsp70 and hsp90 or heat shocked for 30 min at 42°C before being infected separately with dengue virus serotypes 1 to 4 at an MOI of 5 (antibody pre-treatment) or 1 (heat shock). Supernatants were assayed 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

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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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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

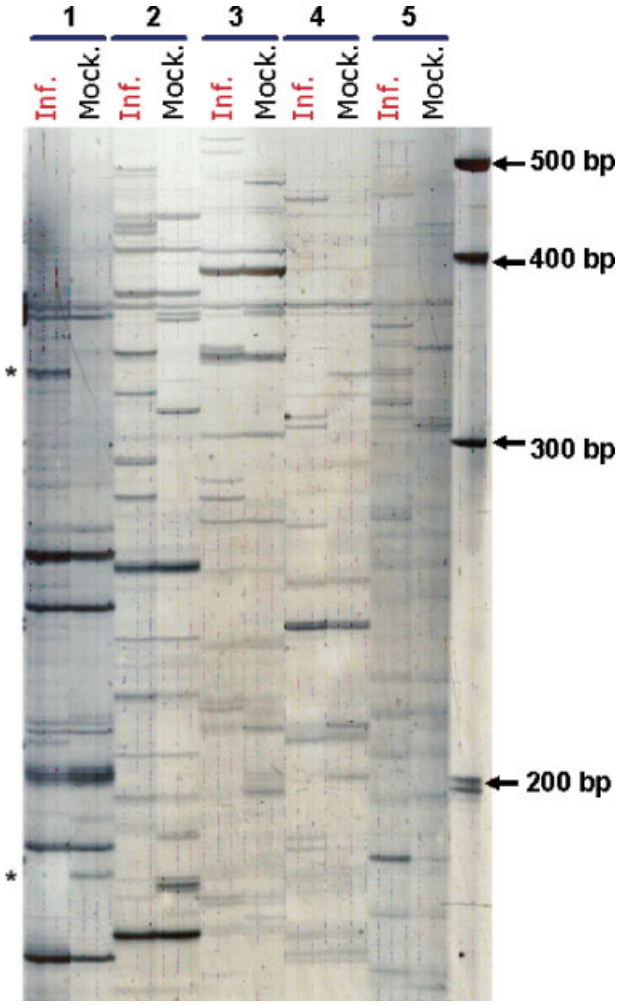

Fig. 1. cDNA-AFLP gel from five different primer combinations (*Eco*RI primer: E_AAC plus *Mse*I primer; 1: M_CCA, 2: M_CAg, 3: M_CAC, 4: M_CAT and 5: M_CAA). One microgram RNA was used for each cDNA-AFLP reaction. Subsequent to adaptor ligation, equal amounts of sample were used in all the following 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.,

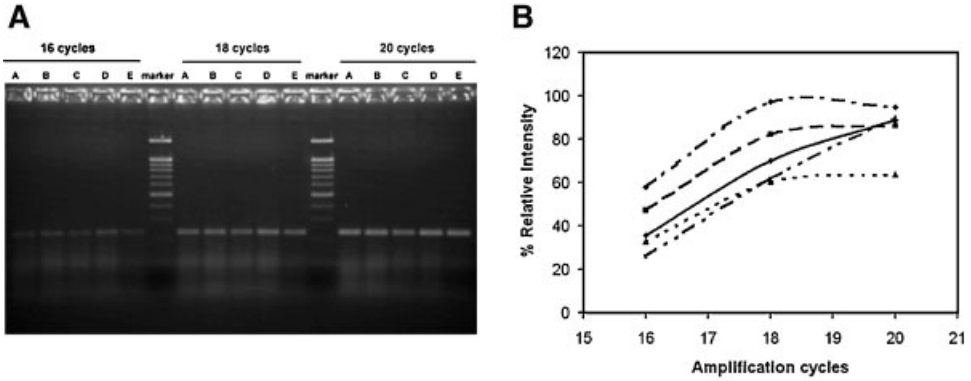

Fig. 2. Amplification curves of RiboS4 gene from semi-quantitative RT-PCR reactions. **A**: RT-PCR products of RiboS4 from different cycle conditions of RNA extracted from mock-infected (**Lanes A**,**E**) and infected (**Lanes A**,**C**,**E**) HepG2 cells at 24 (**lanes A**,**B**), 48 (lane C), and 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.

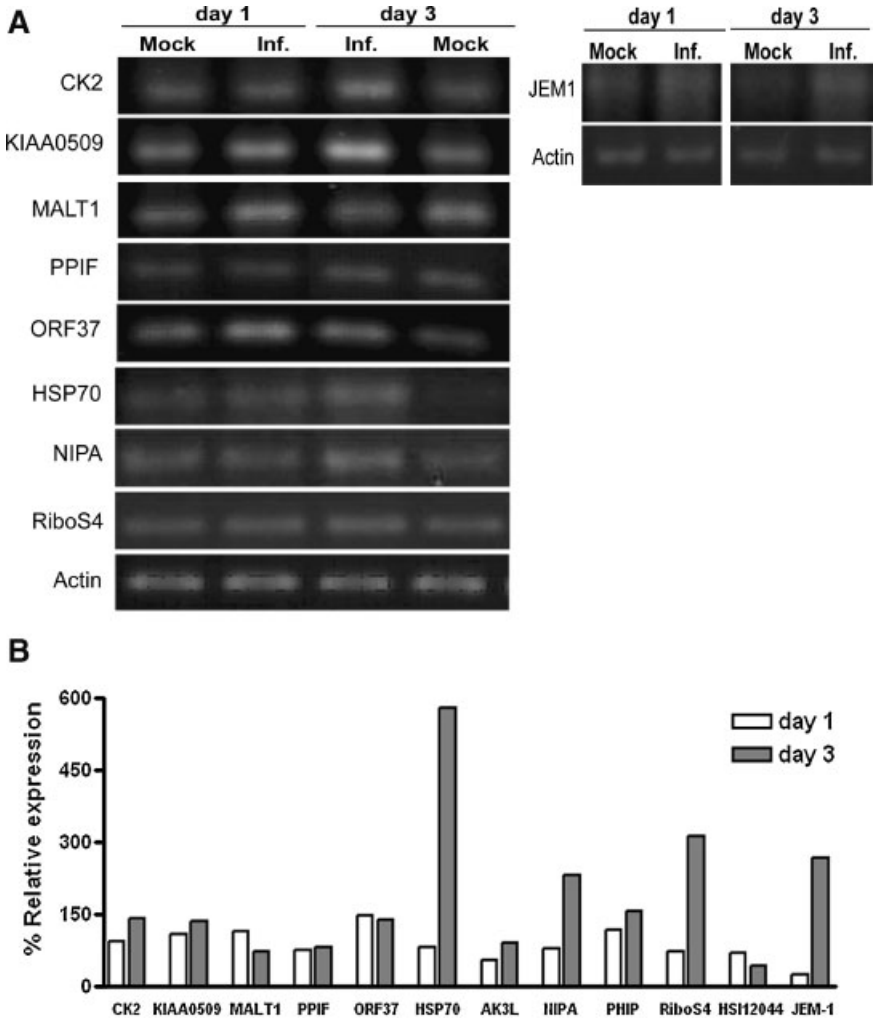

Fig. 3. Expression profiles of five selected transcripts identified through the cDNA-AFLP analysis. **A**: Semi-quantitative RT-PCR analysis of five selected gene transcripts and actin at days 1 and 3 post-infection. Each sample was analyzed in parallel with a mock-infected control sample. **B**: The 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.

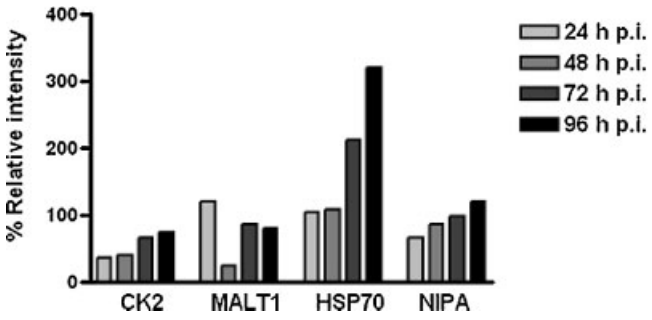

Fig. 4. Expression profiles of four selected transcripts. Semi-quantitative RT-PCR was used to establish the expression profile for four selected transcripts over 4 days post-infection. Relative intensity was calculated as a percentage increase over mock-infected control samples after normalization with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

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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

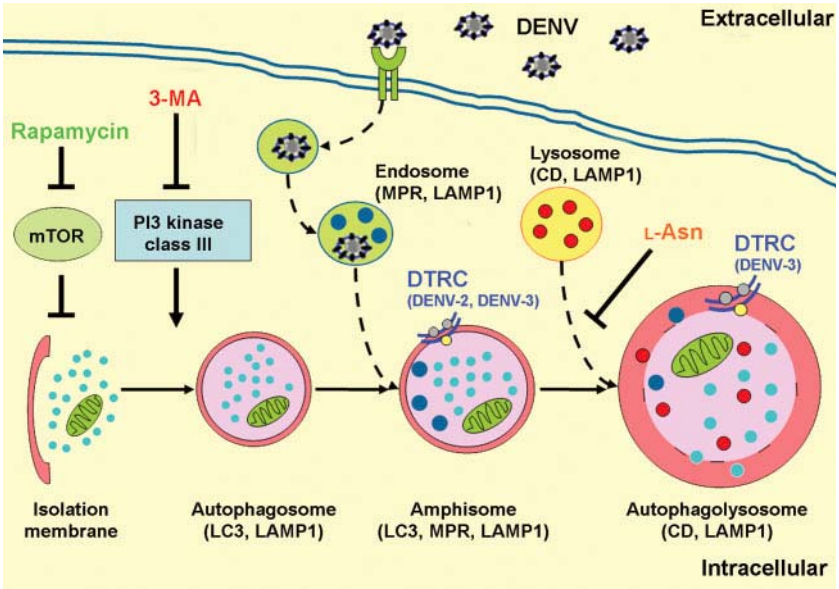

Fig. 1. Autophagy and DENV. A schematic representation of the formation of autophagic vesicles in relation to our proposed model of DENV entry and translation/replication. The actions of the main biochemical autophagy modulators are indicated, as are the positions of markers used in this and our previous 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;

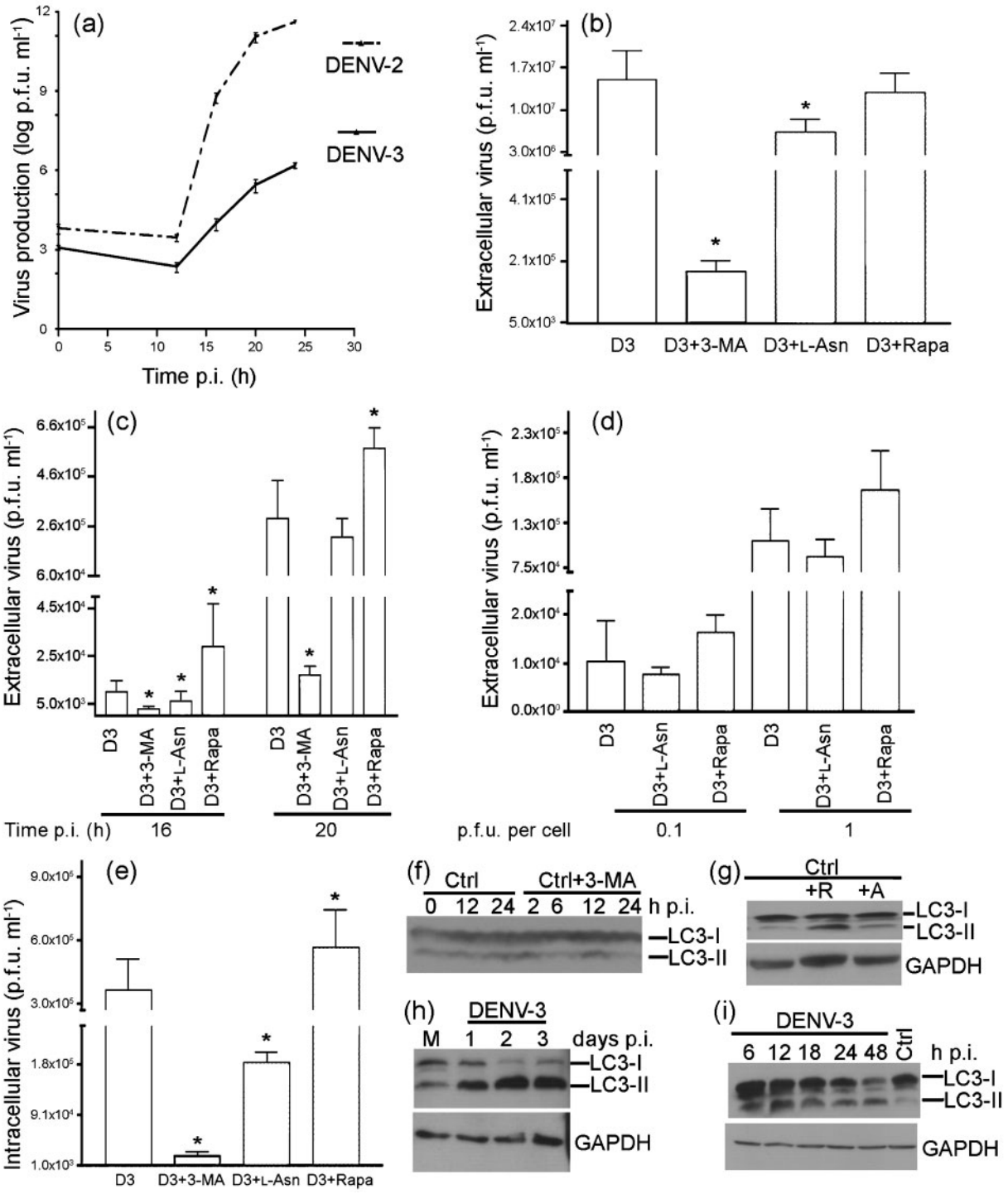

Fig. 2. Interaction of DENV-3 and autophagy. (a) DENV-2 and DENV-3 virus production at 10 p.f.u. per cell. (b, c) Extracellular DENV-3 production at 24 h (b) and at 16 and 20 h (c) p.i. at 10 p.f.u. per cell in the presence or absence of autophagy modulators. (d, e) Extracellular (d) and intracellular (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.

Panyasrivanit *et al.*, 2009) by using Western blotting to detect the increased formation of the autophagy-associated form of LC3, LC3-II, in response to DENV-3 infection. To verify the action of 3-MA, L-Asn and rapamycin on autophagy in control cells, we confirmed the reduction of LC3-II in response to 3-MA (Fig. 2f), as well as the increase of LC3-II in response to both rapamycin and L-Asn (Fig. 2g). In L-Asn-treated cells, the normal degradation of LC3-II that occurs upon fusion of autophagosomes with lysosomes is prevented as fusion with lysosomes is inhibited, resulting in accumulation of LC3-II (Eskelinen, 2005; Mizushima & Yoshimori, 2007). In response to DENV-3 infection, a significant increase in LC3-II was observed for up to 3 days p.i. (Fig. 2h) and examination of earlier time points showed that increased LC3-II was detectable as early as 6 h p.i. (Fig. 2i).

To confirm the induction of autophagy by DENV-3 infection, confocal microscopy was used to detect increased co-localization of LC3 and LAMP1. In this analysis, cells

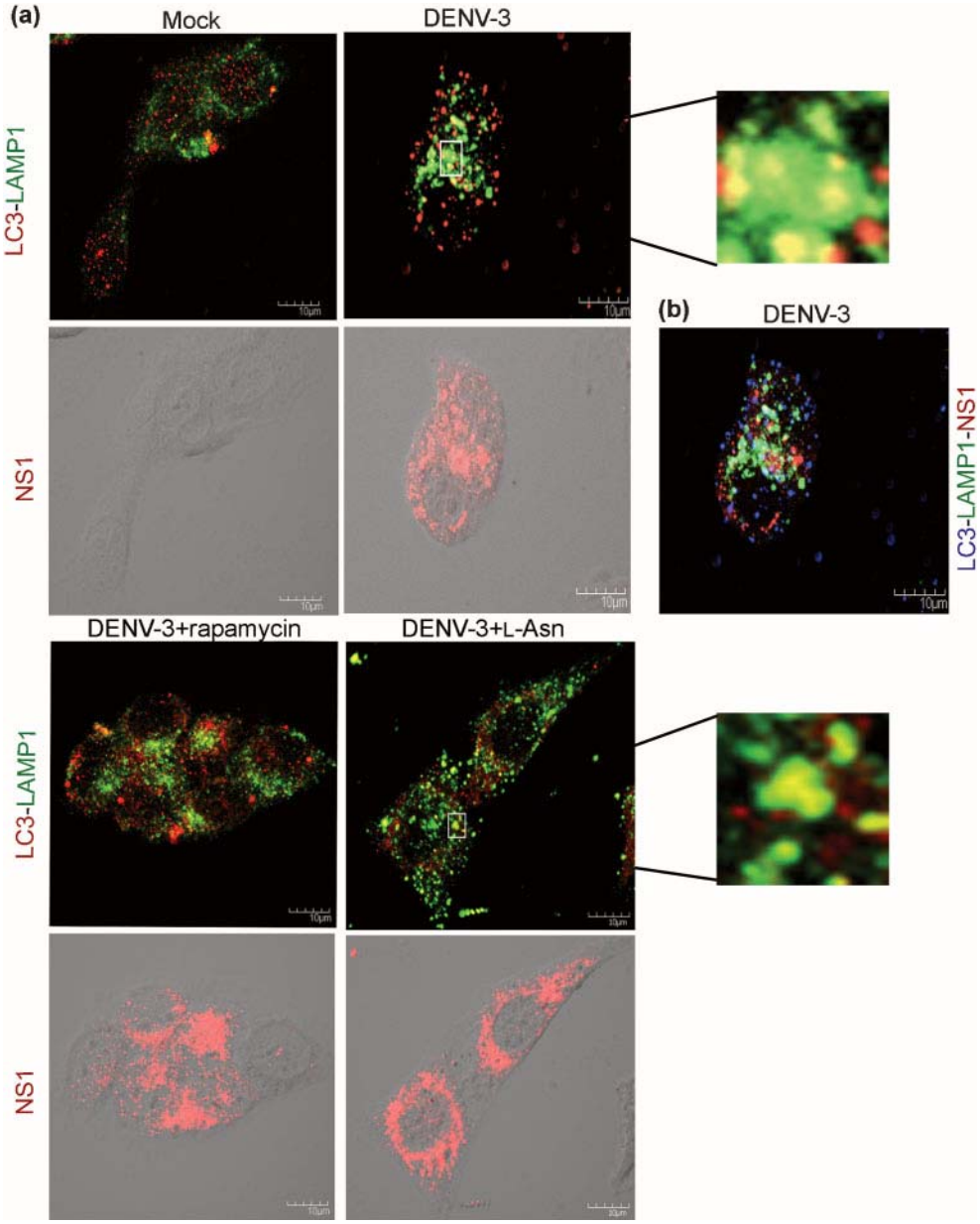

Fig. 3. Induction of autophagy in response to DENV-3 infection. (a) HepG2 cells were grown on glass coverslips and either mock-infected or infected with DENV-3 either directly or in the presence of rapamycin or L-Asn. Cells were examined simultaneously for the localization of LC3 (far-red), LAMP1 (green) and separately for NS1 (red) (a) or simultaneously for LC3 (blue), LAMP1 (green) and NS1 (red) in infected cells only (b).

Table 1. Summary of Pearson correlation coefficients of LC3, LAMP1, NS1, dsRNA and cathepsin D (CD) for DENV-3- and DENV-2-infected cells grown in the presence or absence of autophagy inhibitors

	Pearson correlation coefficient	
	DENV-3	DENV-2*
LC3–LAMP1	0.33 ± 0.04	0.34 ± 0.03
LC3–LAMP1 (rapamycin)	0.49 ± 0.01	0.55 ± 0.03
LC3–LAMP1 (L-Asn)	0.54 ± 0.05	0.46 ± 0.03
LC3–NS1	0.15 ± 0.08	0.44 ± 0.05
LC3–NS1 (L-Asn)	0.37 ± 0.03	0.51 ± 0.05
LC3–dsRNA	0.22 ± 0.03	0.35 ± 0.07
LC3–dsRNA (L-Asn)	0.47 ± 0.04	0.45 ± 0.06
CD–dsRNA	0.28 ± 0.05	0.19 ± 0.01
CD–dsRNA (L-Asn)	0.06 ± 0.05	–

*Data from Panyasrivanit *et al.* (2009).

were additionally stained with NS1 simultaneously to ensure that co-localization of LC3 and LAMP1 was assessed in infected cells. This experiment was undertaken in parallel with cells infected with DENV-3 in the presence of rapamycin or L-Asn and mock-infected cells. Results showed that co-localization between LC3 and LAMP1 significantly increased in DENV-3-infected cells (Pearson correlation coefficient 0.33, 95 % CI 0.29–0.37) compared with mock-infected cells (Pearson correlation coefficient 0.23, 95 % CI 0.21–0.25; $P=0.001$) (Fig. 3a). Significantly greater co-localization was seen in infected cells treated with rapamycin (Pearson correlation coefficient 0.49, 95 % CI 0.48–0.50; $P<0.001$ compared with DENV-3 infected cells) and in infected cells treated with L-Asn (Pearson correlation coefficient 0.54, 95 % CI 0.50–0.60; $P<0.001$ compared with DENV-3-infected cells). A degree of co-localization between LAMP1, LC3 and NS1 was observed in DENV-3-infected cells (Fig. 3b).

Both LC3-II analysis and confocal analysis of LC3–LAMP1 co-localization show that autophagy is induced in DENV-3-infected HepG2 cells. The degree of co-localization observed between LC3 and LAMP1 in DENV-3-infected cells was comparable to that previously observed in DENV-2-infected cells, as was the co-localization between LC3 and LAMP1 seen in either DENV-3- or DENV-2-infected cells treated with either rapamycin or L-Asn (Table 1 and Panyasrivanit *et al.*, 2009). We note that the level of LC3 and LAMP1 co-localization in mock-infected cells was slightly higher in this study than that determined previously (Panyasrivanit *et al.*, 2009), but it has been observed that the amount of LC3-II (the membrane-bound form which co-localizes with LAMP1) can fluctuate greatly when cells are cultured at different times, even when identical conditions are used (Mizushima & Yoshimori, 2007).

To determine whether the DENV-3 translation/replication complex is associated with autophagic vesicles, as seen with DENV-2 (Panyasrivanit *et al.*, 2009), infected cells were examined for the co-localization of LC3 and NS1 protein (Fig. 4a). A very low level of co-localization was observed between LC3 and NS1 in DENV-3-infected cells (Pearson correlation coefficient 0.15, 95 % CI 0.08–0.24), which is in contrast with results seen previously with DENV-2 (Table 1 and Panyasrivanit *et al.*, 2009). The level of co-localization between LC3 and NS1 was significantly increased when infection occurred in the presence of L-Asn (Pearson correlation coefficient 0.38, 95 % CI 0.35–0.41; $P<0.001$). The increase in co-localization of NS1 and LC3 in DENV-3-infected cells in the presence of L-Asn was coupled with increased levels of NS1 (Fig. 4b). A slight increase in NS1 levels was observed in DENV-2-infected cells in the presence of L-Asn compared with control infection (Fig. 4c). The low level of co-localization between NS1 and LC3 in DENV-3-infected cells and the increase in co-localization seen when lysosomal fusion is inhibited with L-Asn, suggests that, in contrast to DENV-2 where pre-lysosomal vacuoles (amphisomes or autophagosomes) are the site of translation/replication (Panyasrivanit *et al.*, 2009), post-lysosomal fusion vacuoles (autophagolysosomes) may play a role in DENV-3 infection (Fig. 1).

We therefore investigated whether autophagolysosomes were associated with NS1 in DENV-3-infected cells using cathepsin D, a constituent of mature autophagolysosomes (Eskelinen *et al.*, 2002). A similar level of co-localization between cathepsin D and NS1 was observed in DENV-3-infected cells as the level previously found between LC3 and NS1 (Fig. 4d; Pearson correlation coefficient 0.17, 95 % CI 0.12–0.22). Importantly, co-localization between Cathepsin D and NS1 was significantly reduced in the presence of L-Asn (Fig. 4d; Pearson correlation coefficient 0.084, 95 % CI 0.04–0.12; $P<0.05$). Therefore, both the increase in co-localization between LC3 and NS1 and the reduction of co-localization between cathepsin D and NS1 when DENV-3 infection occurs in the presence of L-Asn suggest that NS1 is divided between both pre- and post-lysosomal fusion autophagic vacuoles.

These results serve to differentiate between DENV-2 and DENV-3 infections. In DENV-2 infections, NS1 is predominantly associated with pre-lysosomal fusion vacuoles; treatment with L-Asn to inhibit lysosomal fusion and the formation of autophagolysosomes results in only a marginal and non-significant increase in co-localization between LC3 and NS1 (Panyasrivanit *et al.*, 2009) and only a marginal increase in the levels of NS1 (Fig. 4c). In contrast, in DENV-3-infected cells, NS1 is associated with both pre- and post-lysosomal fusion vacuoles and treatment with L-Asn results in a significant increase in the levels of NS1 (Fig. 4b), a significant increase in co-localization between LC3 and NS1 (Fig. 4a) and a decrease in co-localization between cathepsin D and NS1 (Fig. 4d).

To investigate the location of the DENV-3 replication complex on autophagic pre-lysosomal fusion vacuoles, the localization of dsRNA was investigated in relation to LC3

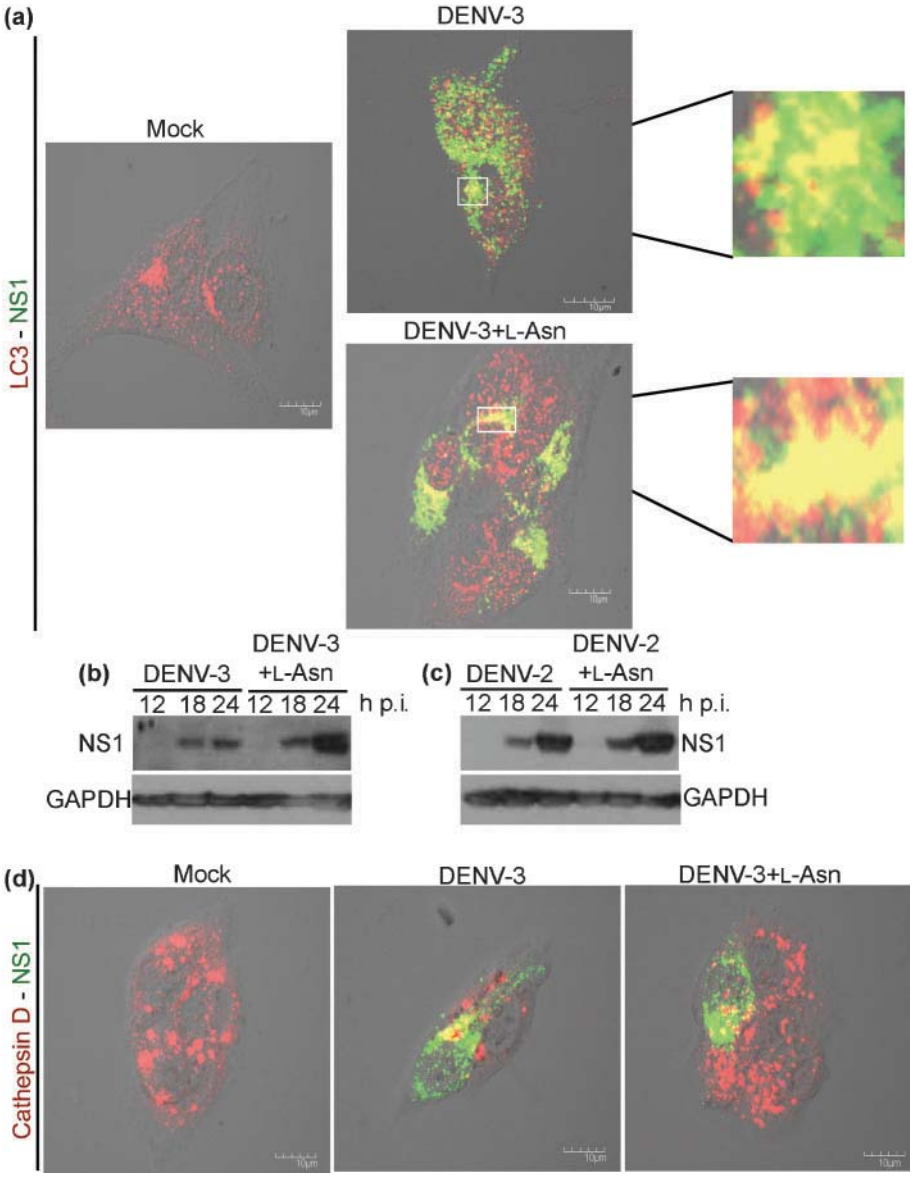

Fig. 4. NS1 in DENV-3-infected HepG2 cells. HepG2 cells grown on glass coverslips (a and d) or in culture (b and c) were either mock-infected or infected with DENV-3 (a, b and d) or DENV-2 (c) in the presence or absence of L-Asn. Cells were examined for co-localization of NS1 with LC3 (a) or NS1 with cathepsin D (d) or for the level of NS1 by Western blotting with GAPDH as a control (b and c). LC3 and cathepsin D are shown as red; NS1 is shown as green.

and to LC3 and MPR. Limited co-localization was observed between dsRNA and LC3 (Pearson correlation coefficient 0.22, 95 % CI 0.19–0.25) which significantly increased in the presence of L-Asn (Pearson correlation coefficient 0.48, 95 % CI 0.44–0.52; $P<0.001$) (Fig. 5a); triple localization of LC3, dsRNA and MPR identified the pre-lysosomal fusion structures as amphisomes (Fig. 5b), which is also seen in DENV-2 (Panyasrivanit *et al.*, 2009). As with DENV-2, ribosomal proteins were found to be co-localized with DENV-3 dsRNA (Fig. 5c), suggesting the capacity for translation exists at the replication complex on amphisomes.

To investigate the localization of the replication complex on post-lysosomal fusion vacuoles, the co-localization of cathepsin D and dsRNA was investigated at 24 h p.i. A degree of co-localization was observed between dsRNA and cathepsin D (Pearson correlation coefficient 0.28, 95 % CI 0.23–0.33); this decreased significantly when infection occurred in the presence of L-Asn (Pearson correlation coefficient 0.06, 95 % CI 0.01–0.11; $P<0.001$) (Fig. 6a), similar to the results seen for NS1. This again suggests that DENV-3 utilizes both pre- and post-lysosomal fusion vacuoles as translation/replication sites, in contrast with DENV-2, which has been proposed to use only pre-

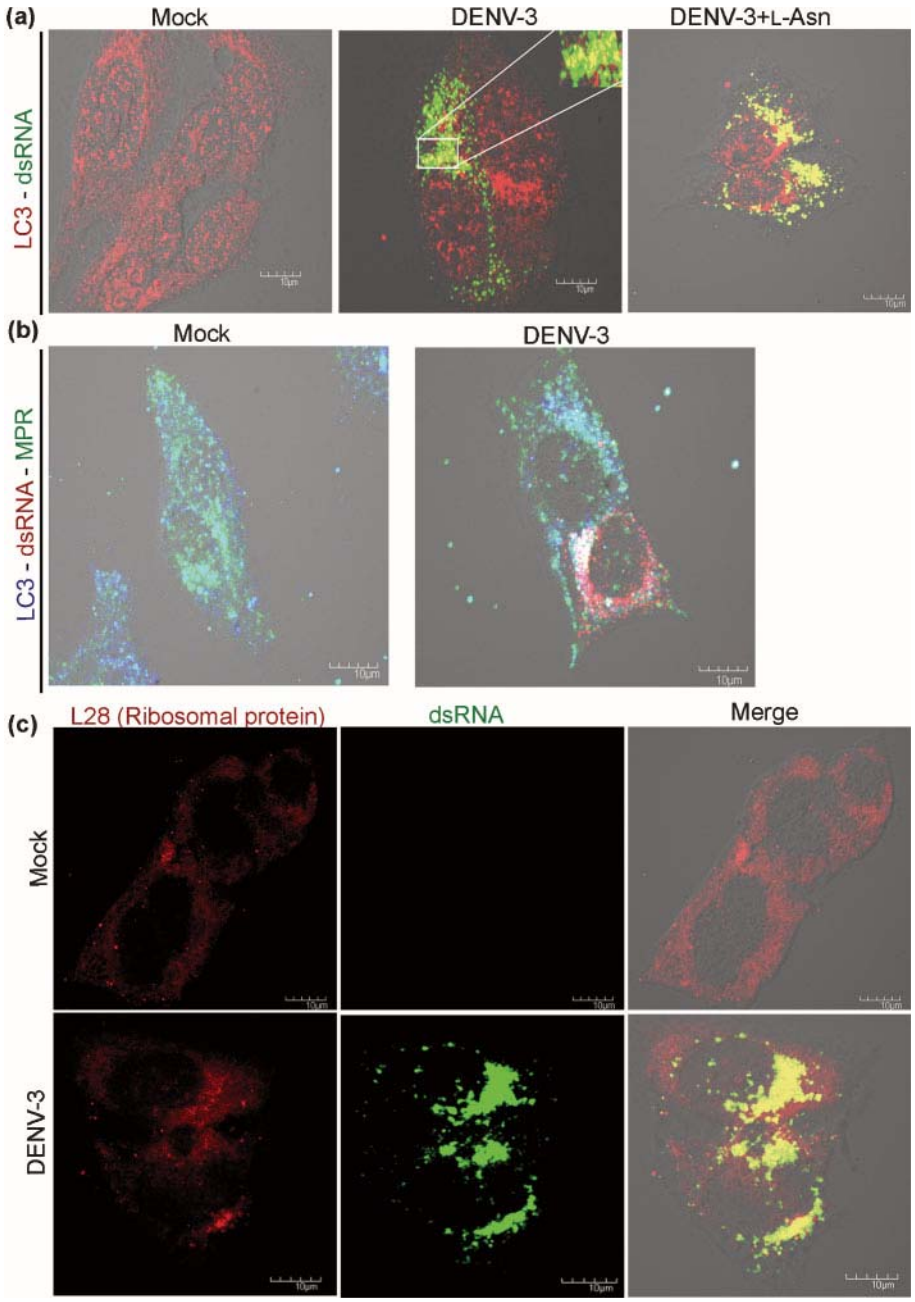

Fig. 5. Location of dsRNA in DENV-infected cells. HepG2 cells grown on glass coverslips were infected with DENV-3 in the presence or absence of L-Asn and examined for either the co-localization of LC3 (red) and dsRNA (green) (a), MPR (green), LC3 (far-red, false-coloured as blue in this figure) and dsRNA (red) (b) or ribosomal protein L28 (red) and dsRNA (green) (c).

lysosomal vacuoles. To provide further evidence to support this hypothesis, we investigated the co-localization of DENV-2 dsRNA and cathepsin D (Fig. 6a) and found, as expected, that DENV-2 dsRNA shows a significantly lower level of co-localization with cathepsin D (Pearson correlation coefficient 0.19, 95 % CI 0.18–0.20; $P=0.007$) compared with DENV-3 dsRNA. To provide further evidence, both earlier and later time points in the DENV replication cycle were examined. At 15 h p.i., co-localization between cathepsin D and dsRNA was significantly higher in DENV-3-infected samples (Pearson correlation coefficient 0.227, 95 % CI 0.19–0.27) than in DENV-2-infected samples (Pearson correlation coefficient 0.104, 95 % CI 0.07–0.13; $P<0.001$) (Fig. 6b). Similarly, at 36 h p.i., co-localization was higher in DENV-3-infected samples (Pearson correlation coefficient 0.229, 95 % CI 0.17–0.29) than in DENV-2-infected samples (Pearson correlation coefficient 0.093, 95 % CI 0.06–0.12; $P=0.003$) (Fig. 6b).

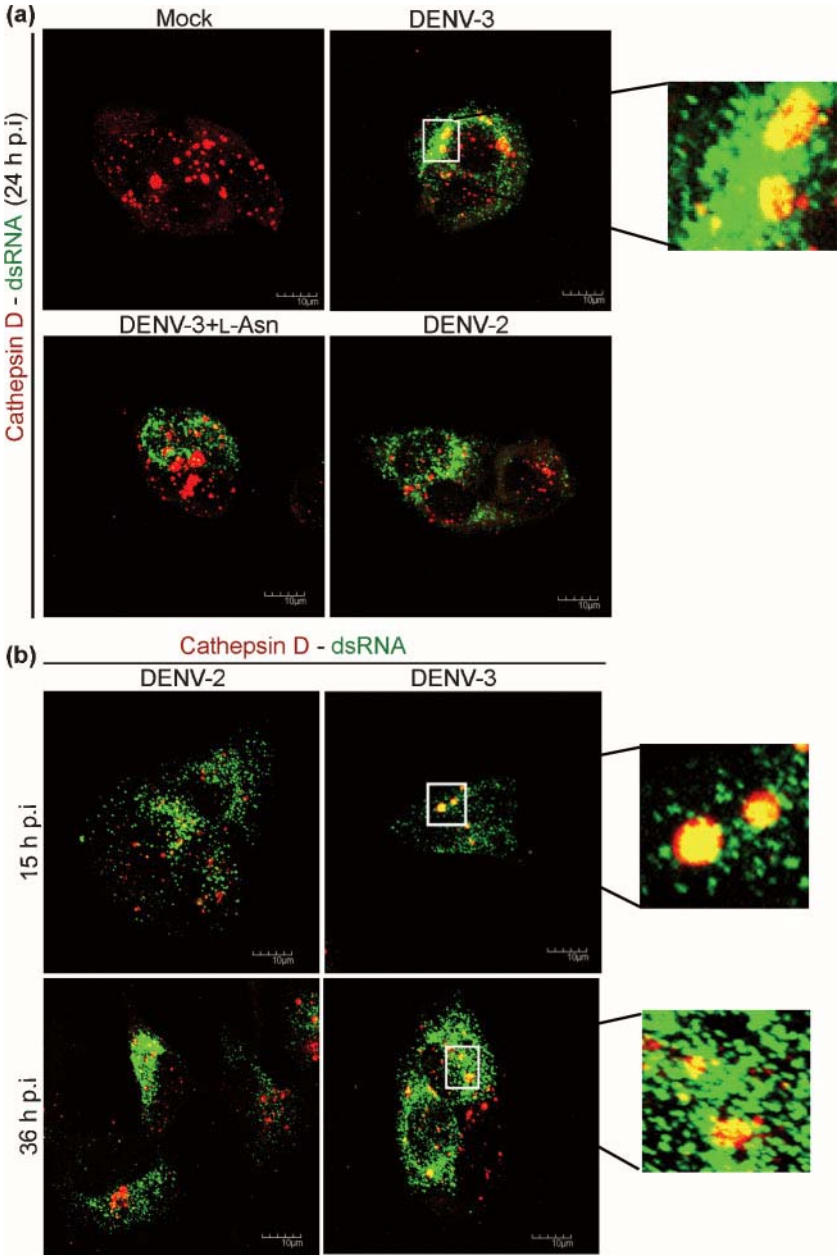

Fig. 6. Co-localization of cathepsin D and dsRNA in DENV-infected HepG2 cells. HepG2 cells grown on glass coverslips were infected with DENV-2 or DENV-3 in the presence or absence of L-Asn and examined for co-localization between cathepsin D (red) and dsRNA (green) at 24 h (a) and at 15 and 36 h (b) p.i.

We have previously shown that DENV-2 uses pre-lysosomal fusion amphisomes as a site for translation and replication, and that infection in the presence of L-Asn results in an increase of intracellular and extracellular virus, suggesting that lysosomal fusion results in loss of virus viability (Panyasrivanit *et al.*, 2009). As shown here, this is in contrast with DENV-3 which uses both amphisomes and autophagolysosomes as part of its replication strategy. Infection in the presence of L-Asn decreases both intracellular and extracellular virus yields, suggesting that interaction with the autophagolysosome is required for completion of the DENV-3 life cycle. However, while the interaction with the autophagolysosome is beneficial in terms of DENV-3 production, it is detrimental to the replication complexes, as demonstrated by the increase of NS1 in DENV-3 infections in the presence of L-Asn. Overall, our results are consistent with both DENV-2 and DENV-3 utilizing the endosomal–autophagosomal fusion pathway to gain entry into the cell and undertake translation and replication (Fig. 1). However, in detail, DENV-2 and DENV-3 employ significantly different translation/replication strategies.

Both this study and our previous study (Panyasrivanit *et al.*, 2009) have investigated the interaction between DENV and autophagy in liver cells. While the role of liver cells in the pathogenesis of the disease has been somewhat controversial, a significant body of work exists that supports the

involvement of the liver in dengue infections (Seneviratne *et al.*, 2006) and we have shown previously that human primary liver hepatocytes are able to be productively infected by DENV (Suksanpaisan *et al.*, 2007). However, studies on other cell types are urgently required, particularly cells of a monocyte/macrophage lineage, as these cells are the primary mediators of dengue infection.

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Co-localization of constituents of the dengue virus translation and replication machinery with amphisomes

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Infections with dengue virus (DENV) are a significant public health concern in tropical and subtropical regions. However, little detail is known about how DENV interacts with the host-cell machinery to facilitate its translation and replication. In DENV-infected HepG2 cells, an increase in the level of LC3-II (microtubule-associated protein 1 light chain 3 form II), the autophagosomal membrane-bound form of LC3, was observed, and LC3 was found to co-localize with dsRNA and DENV NS1 protein, as well as ribosomal protein L28, indicating the presence of at least some of the DENV translation/replication machinery on autophagic vacuoles. Inhibition of fusion of autophagic vacuoles with lysosomes resulted in an increase in both intracellular and extracellular virus, and co-localization observed between mannose-6-phosphate receptor (MPR) and dsRNA and between MPR and LC3 identified the autophagic vacuoles as amphisomes. Amphisomes are formed as a result of fusion between endosomal and autophagic vacuoles, and as such provide a direct link between virus entry and subsequent replication and translation.

INTRODUCTION

With an estimated 100 million infections per year worldwide, dengue virus (DENV), which is spread to humans by the bite of female *Aedes* mosquitoes, represents a significant public health threat in tropical and subtropical countries (Guzman & Kouri, 2002). The DENV complex comprises four antigenically distinct viruses termed DENV serotypes 1, 2, 3 and 4 (DENV-1 to -4), all of which can cause a wide spectrum of disease presentation, from a relatively mild febrile disease to a life-threatening haemorrhagic syndrome (Malavige *et al.*, 2004). DENV is an enveloped, positive-sense, single-stranded RNA virus of approximately 11 kb that encodes 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). Entry of DENV into a susceptible cell occurs primarily through receptor-mediated endocytosis via clathrin-coated pits (Krishnan *et al.*, 2007) and the virus is then trafficked to endosomes (Krishnan *et al.*, 2007; van der Schaar *et al.*, 2007) and fuses with the endosomal membranes (Heinz *et al.*, 2004) through a pH-dependent conformational change in the envelope protein (Modis *et al.*, 2004; Mukhopadhyay *et al.*, 2005). The fate of the released nucleocapsid is largely obscure, although it is known that, after uncoating of the virus genome, the host-cell translational machinery is utilized to synthesize a polyprotein precursor that is processed co- and post-translationally by a virus-encoded protease (NS3) and host signallases (Cahour *et al.*, 1992). Translation and subsequent replication of the DENV genome occur in tight association with intracellular membranous structures that are believed to be endoplasmic reticulum (ER)-derived (Clyde *et al.*, 2006; Miller & Krijnse-Locker, 2008; Salonen *et al.*, 2005), although the exact origin of these membranes remains unclear.

Autophagy is a lysosomal degradation pathway involved in the cellular turnover of macromolecules and organelles. It is conserved among eukaryotes and has been shown to be important for cellular development and remodelling (Levine & Klionsky, 2004; Meijer & Codogno, 2006), as well as being involved in a wide range of disease processes including cancer, neurodegeneration, innate and adaptive immunity, heart disease, liver disease and ageing (Lerena *et al.*, 2008; Mizushima *et al.*, 2008). Autophagy begins with the sequestration of an area of the cytoplasm within a crescent-shaped membrane called the isolation membrane, which probably arises from a pre-existing body (Hanada *et al.*, 2007; Kim *et al.*, 2002; Levine & Klionsky, 2004). This membrane has been suggested to originate from the ER (Dunn, 1990a) and/or from the *trans*-Golgi network (Kihara *et al.*, 2001). The isolation membrane then expands and matures into a large, characteristically double-membraned vesicle with a diameter of 500–1000 nm called an autophagosome (Dunn, 1990a). Among the key regulators

of this process are mTOR (a kinase target of rapamycin) and the beclin1–class III PI3k complex (Xie & Klionsky, 2007). The execution phase of autophagy is mediated primarily through two covalent conjugation pathways: the covalent linkage of Atg5 and Atg12 (Mizushima *et al.*, 1998, 2002; Ohsumi, 2001) and the covalent lipidation of Atg8 (called microtubule-associated protein 1 light chain 3, or LC3, in mammalian cells) by phosphatidylethanolamine (Kabeya *et al.*, 2000; Ohsumi, 2001). Lipidated LC3 eventually associates with the autophagy membranes (Kabeya *et al.*, 2000) and as such is the only creditable marker of autophagosomes in mammalian cells (Bampton *et al.*, 2005; Kimura *et al.*, 2007). Fusion of the autophagosome with endosomes forms structures called amphisomes (Gordon & Seglen, 1988), whilst subsequent fusion with lysosomes forms autophagolysosomes (Dunn, 1990b).

Recently, it has been shown that autophagy is induced upon DENV-2 infection of Huh7 cells (Lee *et al.*, 2008). Biochemical inhibition of autophagy resulted in a reduction in the number of virus progeny produced, and infection was significantly inhibited in Atg5-knockout MEF cells, suggesting that DENV subverts the autophagic process (Lee *et al.*, 2008). However, the details of how DENV affects the autophagic process remain unknown. Given that double-membrane vesicle structures have been associated with DENV replication (Miller & Krijnse-Locker, 2008) and that such double-membrane structures are a classic hallmark of autophagosomes (Dunn, 1990a), it is possible that, similar to poliovirus (Jackson *et al.*, 2005), equine arteritis virus (Pedersen *et al.*, 1999), coronavirus and mouse hepatitis virus (Lee & Iwasaki, 2008), DENV uses autophagic membranes as sites for virus replication.

METHODS

Cells, viruses and infection. The human hepatoma cell line HepG2 (ATCC HB-8065) was cultivated at 37 °C under 10 % CO_2 in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10 % heat-inactivated fetal bovine serum (Gibco) (DMEM/FBS) and 100 U penicillin/streptomycin ml^{-1} (PAA). For characterization of autophagy, HepG2 cells were seeded onto glass coverslips and grown for 24 h under standard conditions after which the growth medium was replaced with complete growth medium or growth medium supplemented with either 100 nM rapamycin (Sigma-Aldrich) or 100 nM rapamycin and 10 mM 3-methyladenine (3-MA; Sigma-Aldrich).

DENV-2 strain 16681 was propagated in the *Aedes albopictus*-derived cell line C6/36 (ATCC CRL-1660). The virus was partially purified by centrifugation to remove cell debris and stored at −80 °C.

For infection of HepG2 cells, cells were grown to subconfluency and pre-treated for 3 h with 10 mM 3-MA in DMEM/FBS or for 1 h with 100 nM rapamycin or 30 mM L-asparagine (L-Asn; Sigma-Aldrich) in DMEM/FBS, or left untreated, and were then infected with DENV-2 at 10 p.f.u. per cell for 2 h in DMEM with or without an autophagy modulator as appropriate. After 2 h, normal growth medium (with or without autophagy modulator as appropriate) was added and cells were incubated under normal conditions until harvesting of the cells or medium. Virus titres were determined by standard plaque assay as described previously (Sithisarn *et al.*, 2003) and intracellular virus levels were determined as described elsewhere (Thepparit & Smith, 2004).

Indirect immunofluorescence. Approximately 3×10^4 HepG2 cells were seeded and grown on 1 cm^2 coverslips under standard conditions for 24 h, followed directly by infection with DENV-2 at 10 p.f.u. per cell for 2 h or pre-incubated for 3 h with 10 mM 3-MA in DMEM/FBS or for 1 h with 100 nM rapamycin or 30 mM L-Asn in DMEM/FBS before virus infection at 10 p.f.u. per cell. At various time points, cells were washed twice with PBS and then fixed in 100 % ice-cold methanol for 20 min. Cells were subsequently permeabilized with 0.3 % Triton X-100 in PBS for 10 min and then washed with

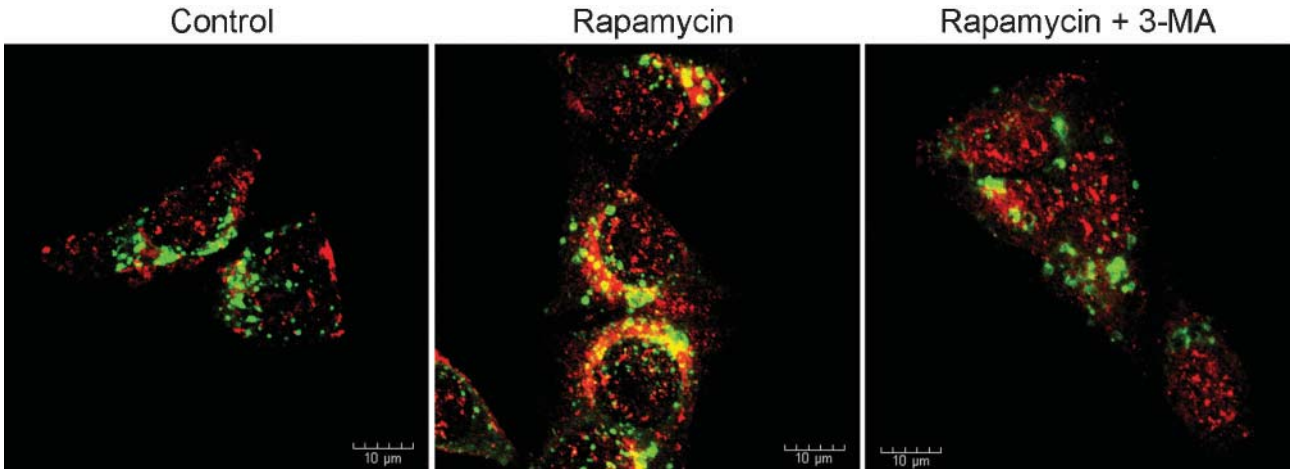

Fig. 1. Induction of autophagy in HepG2 cell. HepG2 cells were grown on glass coverslips and then incubated in complete medium (control) or in the presence of rapamycin or rapamycin and 3-MA for 15 min and subsequently incubated with appropriate primary and secondary antibodies to detect LC3 (red) and LAMP1 (green). Fluorescent signals were observed using an Olympus FluoView 1000 confocal microscope. At least 15 fields were examined, and representative, non-contrast-adjusted merged images are shown.

0.03 % Triton X-100 in PBS or permeabilized, washed and then blocked for 1 h at room temperature with 5 % FBS in 0.03 % Triton X-100 in PBS (for anti-NS1 antibody). The cells were then incubated with two or three primary antibodies at 4 °C overnight. Following incubation, cells were washed four times with 0.03 % Triton X-100 in PBS and incubated with two or three appropriate secondary antibodies for 1 h at room temperature. Subsequently, coverslips were washed with 0.03 % Triton X-100 in PBS six times and then mounted onto glass slides. The cells were observed under a confocal microscope.

The primary antibodies used were a rabbit polyclonal anti-MAP-LC3 antibody (Santa Cruz Biotechnology), a goat polyclonal anti-MAP-LC3 antibody (Santa Cruz Biotechnology), a mouse monoclonal anti-CD107a (LAMP1) antibody (BD Transduction), a rabbit polyclonal anti-LAMP1 antibody (Abcam), a mouse monoclonal anti-DENV NS1 antibody (Puttikhunt *et al.*, 2003), a mouse monoclonal anti-dsRNA antibody (J2; English and Scientific Consulting), a goat polyclonal anti-ribosomal protein L28 antibody (Santa Cruz Biotechnology) and a rabbit polyclonal anti-mannose-6-phosphate receptor antibody (Abcam).

Secondary antibodies used as appropriate were rhodamine Red X-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch), fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG

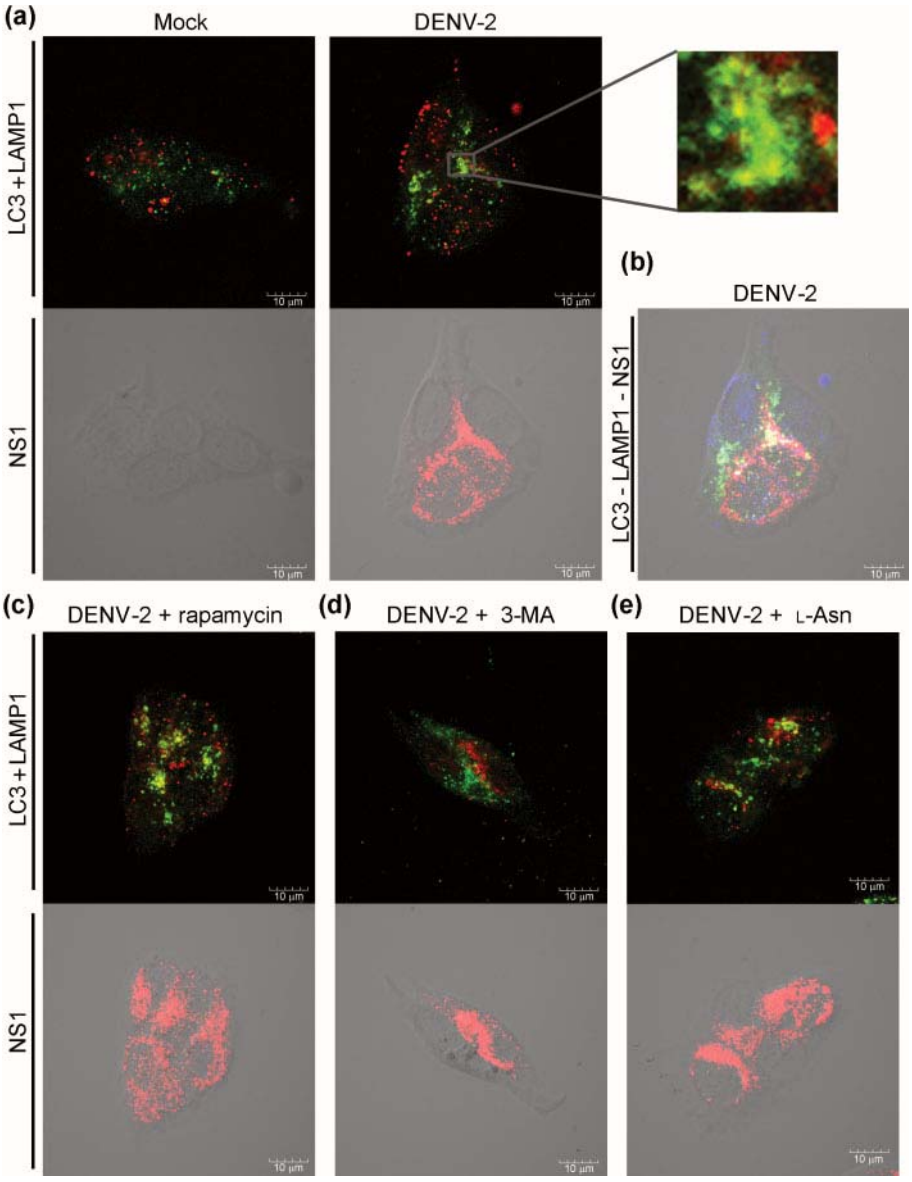

Fig. 2. Induction of autophagy in response to DENV-2 infection. HepG2 cells were grown on glass coverslips and either mock infected or infected with DENV-2 either directly (a, b) or in the presence of rapamycin (c), 3-MA (d) or L-Asn (e). Cells were examined simultaneously for the localization of LC3 (far red) and LAMP1 (green) and separately for NS1 (red) (a, c–e) or simultaneously for LC3 (blue), LAMP1 (green) and NS1 (red) (b), in uninfected or infected cells as indicated. Fluorescent signals were observed using an Olympus FluoView 1000 confocal microscope. At least 15 fields were examined. Images were merged for LC3 and LAMP1, with the NS1 signal from the same field shown merged with the bright-field view.

(Santa Cruz Biotechnology), Cy5-conjugated rabbit anti-goat IgG (Invitrogen), FITC-conjugated goat anti-mouse IgG (KPL) and Alexa Fluor 594-conjugated chicken anti-mouse IgG (Molecular Probes).

Fluorescence confocal microscope imaging and analysis. Fluorescently labelled samples were observed under an Olympus FluoView 1000 microscope equipped with Olympus FluoView software version 1.6. For samples stained with two primary antibodies, images were captured in the red (rhodamine-labelled secondary antibodies), far-red (Cy5-labelled secondary antibodies) or green (FITC-labelled secondary antibodies) channels. For samples stained with three primary antibodies, images were captured in the green (FITC-labelled secondary antibodies), red (Alexa Fluor 594-labelled secondary antibodies) and far-red (Cy5-labelled secondary antibodies) channels. Final images were a non-contrast-adjusted merge of two or three channels. Where three channels were merged, far-red images were shown as blue. Some images had a bright-field image included in the final merge. At least 15 fields from each coverslip were examined and a minimum of two independent experiments was undertaken for each condition. Representative images of selected fields are shown.

Image analysis was undertaken using the ImageJ analysis program (Abramoff *et al.*, 2004) using the PSC co-localization plug-in (French *et al.*, 2008) to calculate co-localization. At least 20 cells were analysed for each condition. Results are presented in terms of Pearson correlation coefficients, which represent the linear relationship of the signal intensity from the green and red channels of the analysed image. The program allowed masking of areas to be excluded from the analysis, and uninfected cells were masked prior to analysis. Statistical analysis of significance between datasets was undertaken by a paired sample test using SPSS (SPSS Inc.) using a value of $P<0.05$ for significance.

Western blot analysis. Total proteins from mock-infected or DENV-infected HepG2 cells were extracted and separated by PAGE before transfer to a solid support. Membranes were blocked with 5 % skimmed milk in TBS for 2 h at room temperature, followed by incubation with antibodies against LC3 in 5 % BSA in TBS or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in 5 % skimmed milk in TBS at 4 °C overnight. The membranes were then incubated with appropriate secondary antibodies in 5 % skimmed milk in TBS at room temperature for 1 h. The antibodies used were a 1 : 3000 dilution for rabbit polyclonal anti-LC3 antibody (Novus Biological) and a 1 : 800 dilution for mouse monoclonal anti-GAPDH antibody (Santa Cruz Biotechnology), followed by a 1 : 3000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce) or a 1 : 4000 dilution of horseradish peroxidase-conjugated anti-mouse IgG (Sigma Chemical Co.). Signals were developed using an ECL-Plus Western Blotting Analysis kit (GE Healthcare).

Statistical analysis. Virus production data were analysed using the GraphPad Prism program (GraphPad Software). Statistical analysis of significance was undertaken by a paired sample test using SPSS with a value of $P<0.05$ for significance.

RESULTS

Autophagy in HepG2 cells

Lee *et al.* (2008) showed that autophagy is induced in response to DENV infection, but the study was undertaken in Huh7 cells. This study therefore sought initially to establish that autophagy could be induced in HepG2 cells and that this pathway was susceptible to manipulation in these cells. To establish that the autophagy pathway is viable in HepG2 cells, the localization of LC3 (a specific marker of autophagic vacuoles) and LAMP1 (a marker of endosomal and lysosomal membranes) was examined in control cells, in cells treated with the autophagy inducer rapamycin (Noda & Ohsumi, 1998) and in cells treated

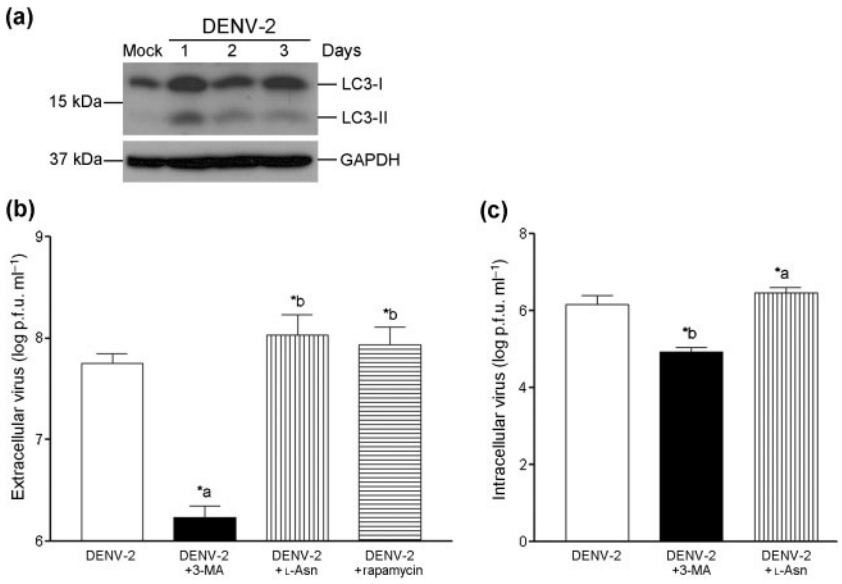

Fig. 3. Induction of autophagy in response to DENV-2 infection and effects of autophagy modulation. (a) Western blot analysis of LC3 and GAPDH expression in mock-infected or DENV-2-infected HepG2 cells on days 1–3 p.i. (b) Extracellular virus production of HepG2 cells infected with DENV-2 in the presence or absence of 3-MA, rapamycin or L-Asn. Virus yield is plotted as log virus titre ± SD. Data were derived from six independent replicates, with duplicate assays of each replicate. The statistical significance of virus output compared with the control is shown. *a, $P<0.001$; *b, $P=0.012$. (c) Intracellular virus production of HepG2 cells infected with DENV-2 in the presence or absence of 3-MA or L-Asn. Virus yield is plotted as log virus titre ± SD. Data were derived from six independent replicates, with duplicate assays of each replicate. The statistical significance of virus yield compared with the control is shown. *a, $P<0.001$; *b, $P=0.001$.

with both rapamycin and the PI3k kinase class III inhibitor 3-MA, which inhibits autophagy (Seglen & Gordon, 1982). Little if any co-localization was seen in control cells, whilst co-localization was observed in rapamycin-treated cells by 15 min after the addition of rapamycin. Co-localization of LAMP1 and LC3 in response to rapamycin treatment was completely abolished by 3-MA treatment (Fig. 1).

Induction of autophagy in response to DENV infection

To confirm that autophagy was induced in response to DENV-2 infection using strain 16681 as opposed to strain PL0146 as used by Lee *et al.* (2008), HepG2 cells were infected with DENV-2 strain 16681 at 10 p.f.u. per cell in parallel with samples treated with rapamycin, 3-MA and L-Asn, which inhibits fusion of lysosomes with autophagosomes and amphisomes (Gordon & Seglen, 1988). An m.o.i. of 10 had been determined separately to give infection rates of greater than 90 % at 24 h post-infection (p.i.) (data not shown). At 24 h p.i., samples were simultaneously stained with antibodies directed against LC3, LAMP1 and NS1. The co-localization of LC3 and LAMP1 was examined only in cells positive for NS1 staining to ensure that only DENV-infected cells were analysed.

The results showed an increased co-localization between LC3 and LAMP1 in response to DENV-2 infection [mean Pearson correlation coefficient 0.34, 95 % confidence interval (CI) 0.31–0.37] compared with mock-infected cells (mean Pearson correlation coefficient 0.14, 95 % CI 0.13–0.15; $P<0.001$; Fig. 2a). The level of co-localization between LC3 and LAMP1 in infected cells was significantly increased in the presence of the autophagy inducer rapamycin (mean Pearson correlation coefficient 0.55, 95 % CI 0.52–0.58; $P<0.001$; Fig. 2c) and abolished in the presence of 3-MA (Fig. 2d). An increase in the co-localization of LC3 and LAMP1 over and above that seen in infected cells was observed in cells infected in the presence of L-Asn (mean Pearson correlation coefficient 0.46, CI 0.43–0.49; $P<0.001$; Fig. 2e). Western blotting demonstrated the formation of the autophagy-associated form of LC3, LC3-II, in response to DENV infection (Fig. 3a). Virus yield at 24 h p.i. as determined by standard plaque assay was significantly reduced in response to 3-MA treatment ($P<0.001$) and increased by the presence of both rapamycin ($P=0.012$) and L-Asn ($P=0.012$), with all experiments undertaken six times independently, with duplicate assays of titre (Fig. 3b). The increase in virus yield seen in the presence of L-Asn suggested that fusion with lysosomes resulted in a degree of virus degradation. The yield of intracellular virus, determined as described elsewhere (Thepparit & Smith, 2004), was similarly increased in the presence of L-Asn ($P<0.001$) and decreased in the presence of 3-MA ($P=0.001$) (Fig. 3c).

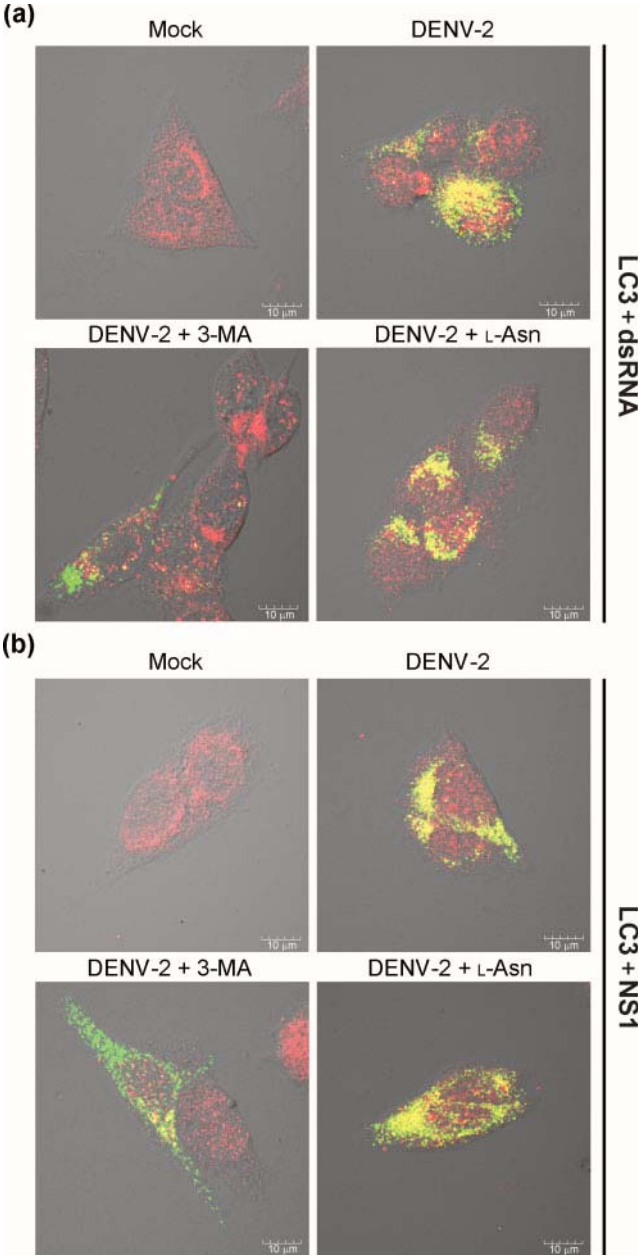

Fig. 4. Localization of the DENV replication complex. HepG2 cells were grown on glass coverslips and either mock infected or infected with DENV-2 either directly or in the presence of 3-MA or L-Asn, and examined for the localization of LC3 (red) and either dsRNA (green) (a) or NS1 (green) (b). Fluorescent signals were observed using an Olympus FluoView 1000 confocal microscope. At least 15 fields were examined, and representative, non-contrast-adjusted merged images are shown.

Autophagy and DENV replication and translation

To investigate whether parts of the replication/translation machinery of DENV co-localized with LC3, DENV-2-infected cells were examined for the localization of DENV

NS1 protein and dsRNA in relation to LC3. The location of dsRNA was detected using the antibody J2, which has previously been established as being able to detect flavivirus dsRNA (Weber *et al.*, 2006), whilst NS1 protein was detected using a well-characterized monoclonal antibody (Puttikhunt *et al.*, 2003).

At 24 h p.i., significant levels of co-localization were observed between dsRNA and LC3 (mean Pearson correlation coefficient 0.35, 95 % CI 0.28–0.42), which was largely eliminated by treatment with 3-MA, whilst L-Asn treatment significantly increased the co-localization between dsRNA and LC3 (mean Pearson correlation coefficient 0.45, 95 % CI 0.39–0.51; P=0.001; Fig. 4a). NS1 protein similarly co-localized with LC3 in DENV-2-infected cells (mean Pearson correlation coefficient 0.44, 95 % CI 0.39–0.49), and co-localization was again largely eliminated by treatment with 3-MA and increased when DENV-2-infected cells were treated with L-Asn (mean Pearson correlation coefficient 0.51, 95 % CI 0.46–0.56; Fig. 4b), again suggesting degradation of virus or viral proteins upon fusion of the autophagic vacuole with lysosomes. NS1 protein also co-localized with both LC3 and LAMP1 (Fig. 2b).

We further investigated the co-localization of ribosomal proteins with the presence of dsRNA to determine whether there was translational capacity at the site of RNA replication and found significant co-localization between dsRNA and ribosomal proteins using an antibody directed against ribosomal protein L28 (Fig. 5).

As a marker of both endosomes and lysosomes, LAMP1 co-localization is unable to discriminate between the formation of autophagolysosomes (fusion of autophagosomes with lysosomes) and amphisomes (fusion of autophagosomes with endosomes). We therefore investigated whether the endosomal marker mannose-6-phosphate receptor (MPR) co-localized with dsRNA. High levels of MPR signal were observed in mock-infected cells, and co-localization between MPR and LC3 was more evident than co-localization between LC3 and LAMP1 in mock-infected cells. This may reflect either a higher level of MPR in HepG2 cells or may simply result from differences in antibody avidity. However, clear co-localization between MPR and dsRNA (Fig. 6a) was observed, as well as increased co-localization between MPR and LC3 in infected cells (Fig. 6b), suggesting amphisomes as the site of DENV-2 replication and translation.

To confirm amphisomes as a site of at least part of the DENV replication complex, triple staining using antibodies directed against MPR, LC3 and dsRNA was undertaken in infected cells. The results (Fig. 7) showed co-localization of these three markers.

DISCUSSION

Whilst entry of DENV into target cells by receptor-mediated endocytosis into clathrin-coated pits and subsequent pH-dependent fusion of the virus structural envelope protein with membranes of the late endosomes

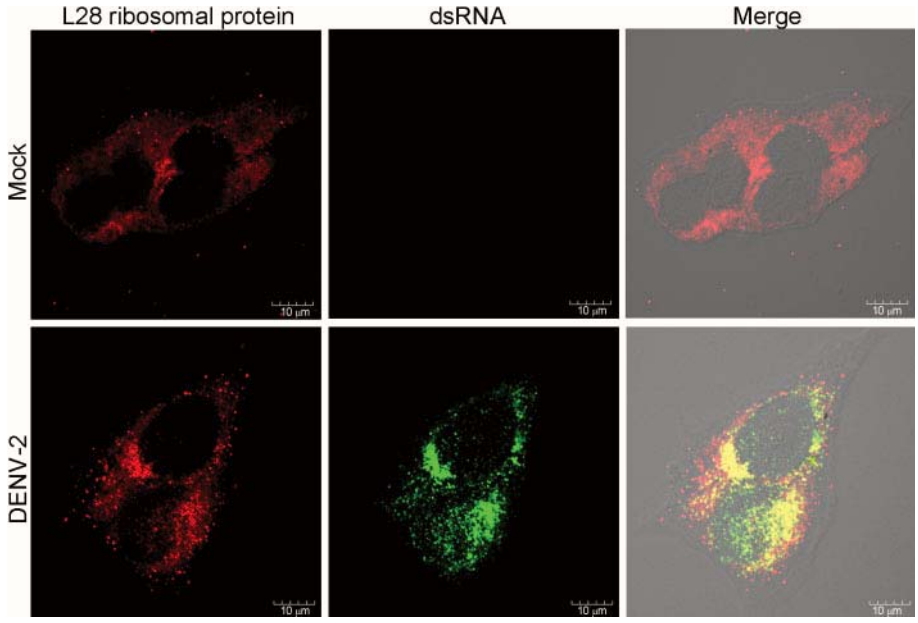

Fig. 5. Co-localization of ribosomes with DENV dsRNA. HepG2 cells were grown on glass coverslips and either mock infected or infected with DENV-2 and examined for the localization of L28 (red) and dsRNA (green). Fluorescent signals were observed using an Olympus FluoView 1000 confocal microscope. At least 15 fields were examined, and representative single-channel and merged non-contrast-adjusted images are shown.

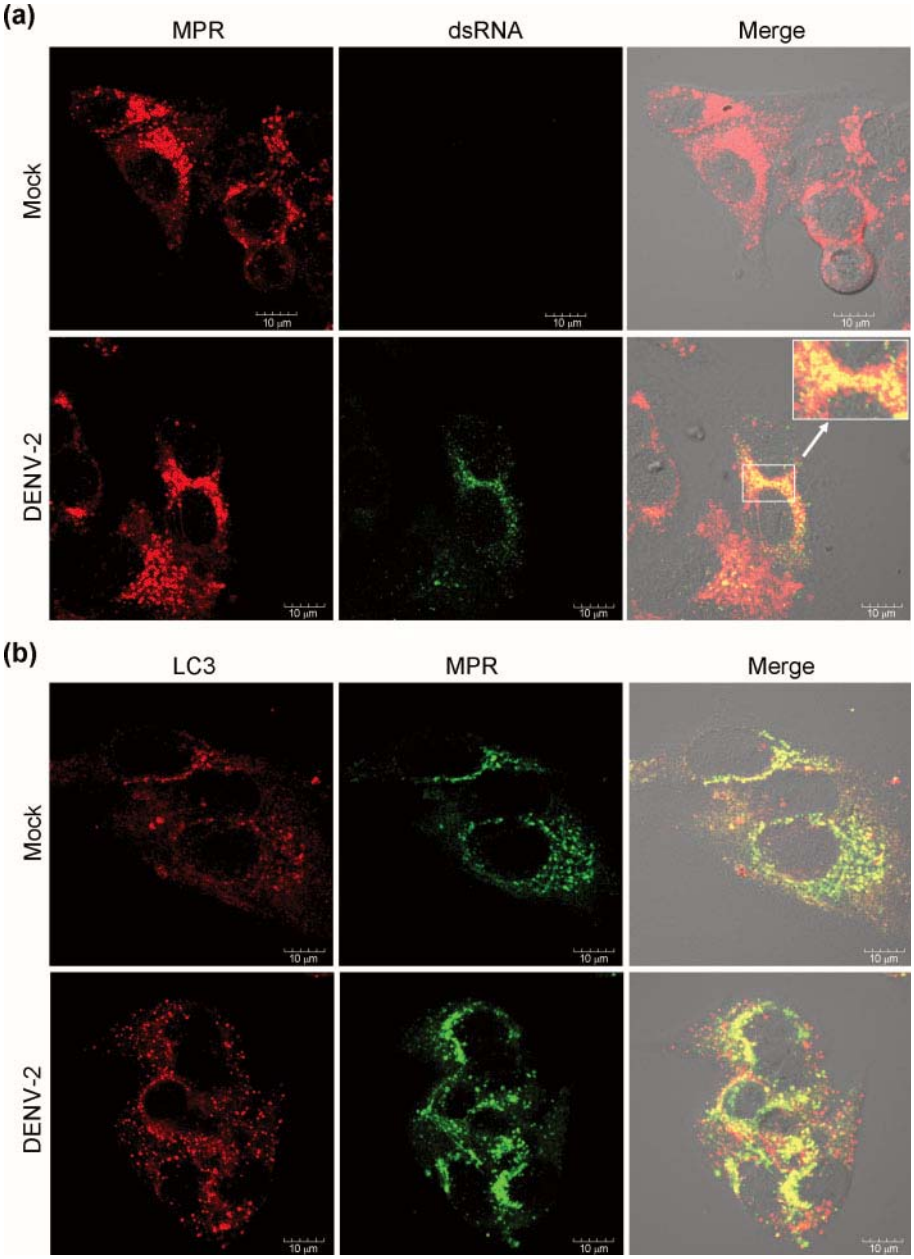

Fig. 6. Investigation of amphisomes as sites of DENV replication. HepG2 cells were grown on glass coverslips and either mock infected or infected with DENV-2 and examined for the localization of MPR (red) and dsRNA (green) (a) or MPR (green) and LC3 (red) (b). Fluorescent signals were observed using an Olympus FluoView 1000 confocal microscope. At least 15 fields were examined, and representative single-channel and merged non-contrast-adjusted images are shown.

has been well documented (Allison *et al.*, 1995; Mukhopadhyay *et al.*, 2005), subsequent events are less well characterized. It is currently believed that flavivirus genomes are released into and replicate in the cytoplasm in close association with intracellular membranous structures that possibly derive from the ER (Clyde *et al.*, 2006; Miller & Krijnse-Locker, 2008). Consistently, flavivirus infections characteristically result in significant proliferation of rough ER membranes, and the flavivirus replication complex has been partly correlated with these ER membranes (Boulton & Westaway, 1976) and with cytoplasmic vesicles and vacuoles (Mackenzie *et al.*, 1996), and it has been proposed that flaviviruses bud from ER membranes and transit the Golgi body before release from the cell (Clyde *et al.*, 2006; Yoshii *et al.*, 2004).

More recently, Lee *et al.* (2008) showed that autophagy is induced by DENV-2 infection in Huh7 cells, and that the

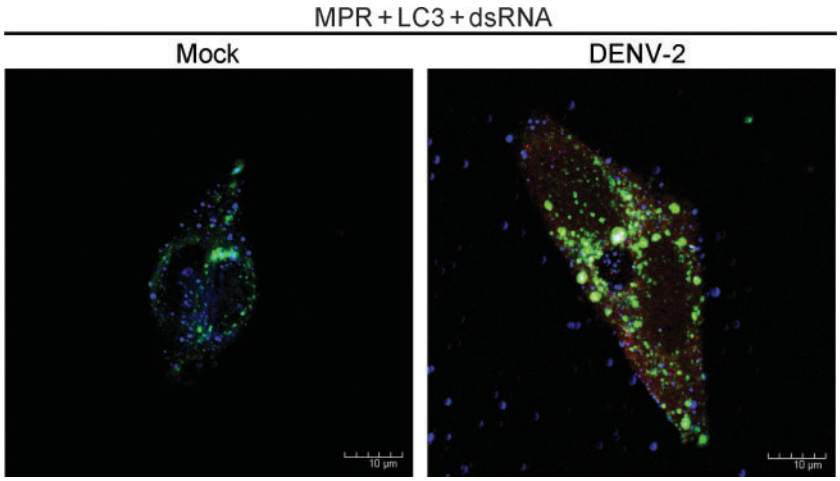

Fig. 7. Identification of amphisomes as sites of DENV replication. HepG2 cells were grown on glass coverslips and either mock infected or infected with DENV-2 and examined simultaneously for the localization of MPR (green), LC3 (blue) and dsRNA (red). The LC3 signal was originally collected in the far-red channel and is coloured blue for this figure. Co-localization of all three markers is shown as white.

induction of autophagy serves to enhance DENV replication. Inhibition of autophagy, either by biochemical treatment with 3-MA or by knockout of Atg5 (in MEF cells) serves to reduce the levels of infectious virus produced (Lee *et al.*, 2008). Similarly, as shown here, DENV-2 induced autophagy in HepG2 cells and biochemical inhibition of autophagy with 3-MA reduced virus yield, whilst induction of autophagy with rapamycin enhanced virus yield.

The process of autophagy initially generates double-membraned structures called autophagosomes (Dunn, 1990a), which are capable of fusing with endosomes to form amphisomes (Gordon & Seglen, 1988). Both autophagosomes and amphisomes can subsequently fuse with lysosomes to form autophagolysosomes, the primary degradative vesicle (Dunn, 1990b). Interestingly, however, the inhibition of lysosomal fusion with L-Asn served to increase both intracellular and extracellular virus yield, suggesting that lysosomal fusion of autophagic vacuoles to form autophagolysosomes has a deleterious effect on DENV replication. This consequently suggests that viable virus is produced prior to lysosomal fusion on either autophagosomes or amphisomes.

The co-localizations observed between LC3 and LAMP1, LC3 and dsRNA, LC3 and NS1, and between NS1, LC3 and LAMP1, as well as between ribosomal proteins and dsRNA, located the DENV translation/replication complex on autophagosomes, and the co-localization seen between MPR and dsRNA, MPR and LC3, and between MPR, LC3 and dsRNA would indicate that these structures are amphisomes, formed by the fusion of autophagosomes and endosomes. Given the localization of the DENV replication/translation complex on amphisomes, it is unsurprising that subsequent fusion with lysosomes (and their proteolytic contents) to form autophagolysosomes results in a decrease in the number of virus progeny.

The induction of autophagic structures and the location of part of the DENV replication/translation complex on these structures is consistent with a considerable body of work that locates the DENV replication complex on induced membranes and vacuoles (Boulton & Westaway, 1976; Mackenzie *et al.*, 1996) and explains why modulation of autophagy serves to modulate DENV output, as seen by Lee *et al.* (2008) and ourselves in this study. As amphisomes are formed by the fusion of endosomes and autophagosomes (Gordon & Seglen, 1988; Gordon *et al.*, 1992), the identification of these structures as the sites of at least part of the DENV replication/translation complex provides a basis for a unified model linking DENV entry and replication in terms of an ongoing and continual association with membranes of an endosomal–autophagic lineage.

ACKNOWLEDGEMENTS

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Autophagic Punctum

Linking dengue virus entry and translation/replication through amphisomes

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Key words: amphisome, autophagy, dengue, endosome, replication, translation

Amphisomes are preautolysosomal vacuoles formed upon the fusion of autophagosomes with endosomes, and as such represent a critical meeting point between endocytic and autophagic pathways. Dengue virus enters into susceptible cells by clathrin-mediated endocytosis, and colocalization of dengue markers with markers of both autophagic and endosomal vesicles demonstrates that amphisomes are a site of dengue virus replication and translation. This work links for the first time the processes of dengue virus entry and translation/replication, and allows for interpretation of the early part of the dengue virus life cycle in terms of a continual association with membranes of an endosomal-autophagosomal lineage.

Dengue virus (DENV) is the most significant arbovirus worldwide, with some 2.5 billion people living at risk of infection. It has been estimated that some 50 million to 100 million infections occur each year, and infection with dengue virus is the most common cause of hospitalizations among children in Southeast Asia. The disease has emerged worldwide throughout tropical and subtropical regions which are the habitat of the principal transmission vector, Aedes mosquitoes (principally *Aedes aegypti*, *A. albopictus* and *A. polynesiensis*), and dengue has emerged as a critical globally endemic disease. To date there is no specific treatment or vaccine.

DENV is a mosquito-borne single-stranded RNA virus that belongs to the genus Flavivirus (family Flaviviridae), a genus that includes many important human pathogenic viruses including yellow fever virus, West Nile virus, Japanese encephalitis virus and tick-borne encephalitis virus. Dengue virus is comprised of four antigenically-related serotypes called dengue serotypes 1, 2, 3 and 4 (DEN1 to DEN 4) and infection by any one of the four serotypes can cause either a relatively benign fever, namely dengue fever, or a more serious disease such as dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS).

Autophagy (macroautophagy) is a lysosomal degradative pathway primarily functioning to turn over macromolecules and organelles in eukaryotic cells. However, autophagy is also involved in a number of processes including the response to cellular starvation and defense against invading pathogens. In cases of virus infection, the interaction between the autophagy mechanism and the invading virus is thought to follow one of two routes: defense or subversion. In the case of autophagy as a cellular defense mechanism, autophagy is induced to clear the cell of the invading virus. However, some viruses have adapted to evade the autophagy mechanism, by downregulating the pathway (as occurs with herpes simplex virus). More recently it has become clear that some viruses have evolved to subvert the autophagy process by using the autophagic membranes as sites for viral replication (as occurs with poliovirus) and downregulation of autophagy results in a reduction of virus production.

Dengue virus induces autophagy in mammalian cells, and biochemical downregulation of autophagy results in a reduction in the amount of extracellular and intracellular virus produced. By colocalizing a dengue virus nonstructural protein (NS1) as well as double-stranded RNA (an essential part of the dengue replication complex) with LC3, we identified autophagic vacuoles as a site for at least a part of the dengue virus replication complex. In addition, extensive colocalization between L28 (a ribosomal protein) and dsRNA provide evidence of translational capacity, and provide a rational explanation as to why downregulation of autophagy results in reductions in extracellular and intracellular virus levels. Interestingly, inhibition of fusion between preautolysosomal vacuoles and lysosomes with L-asparagine results in a significant increase in extracellular and intracellular virus yield, suggesting that fusion of these vacuoles with lysosomes is detrimental to dengue virus production, and further suggesting that autophagic structures prior to lysosomal fusion are the critical site of dengue virus replication/translation. Autophagic vacuoles prior to lysosomal fusion include both autophagosomes and amphisomes, with amphisomes being formed by fusion of autophagosomes with endosomes. Colocalization of LC3, dsRNA and the endosomal marker mannose-6-phosphate receptor (MPR) provide evidence that the dengue virus replication/translation complex is associated with amphisomes.

Entry of DENV into mammalian cells through clathrin coated pit-mediated endocytosis and pH-mediated uncoating of the virus

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in endosomes has been well characterized, but subsequent events remain poorly understood. In particular, the process by which the released nucleocapsid is targeted to the previously presumed site of dengue virus replication (the endoplasmic reticulum) had not been addressed. In light of our results however, it is possible to reinterpret the early part of the dengue virus life cycle by proposing that virus entry and replication/translation occur in a tight and continuing association with membranes of an endosomal-autophagosomal lineage.

While the identification of amphisomes as a site for dengue virus replication/translation complex answers some questions, a great deal of further work remains to be done. In particular, while we have examined the interaction between DEN2 and the autophagic machinery, it is unclear as to whether all four dengue serotypes interact in the same manner. Moreover our study was undertaken in liver cells, which, as shown in much previous work, are involved in the pathology of dengue; however, they do not represent the critical cell type in dengue infections. More work will need to be undertaken to determine the nature of the interaction in other cell types (in particular cells of a monocytes/macrophage lineage) which may or may not be similar to that seen in liver cells.

In addition to the induction of autophagy, the dengue virus induces both ER stress and apoptosis in infected cells. Given that both of these processes have considerable crosstalk with the autophagy pathway, a reevaluation of those processes may be required. At this point, whereas entry of the virus and replication/translation have been linked, further attention to the latter stages of the dengue life cycle will be needed.

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Infection of Human Primary Hepatocytes With Dengue Virus Serotype 2

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While the impact of the dengue viruses on liver function is prominent as shown by hepatomegaly, liver enzyme abnormality, occasional fulminant hepatic failure and histological changes including hepatocellular necrosis, significant debate exists as to the possible involvement of the predominant cell type in the liver, hepatocytes, in the disease process. To address this issue purified human primary hepatocytes were exposed to dengue virus serotype 2 and the production of de novo viral progeny was established by standard plaque assay, RT-PCR and immunocytochemistry. To investigate the response of the primary hepatocytes to infection, the expression of a panel of 9 cytokine genes (IFN-β, TRAIL, MCP-1, IL-6, IL-1β, IL-8, MIP-1α, MIP-1β, and RANTES) was semi-quantitatively investigated by RT-PCR and up-regulation of TRAIL, MIP-1α, IFN-β, MIP-1β, IL-8, and RANTES was observed in response to infection. The induction of IL-8 in response to infection was accompanied by the secretion of IL-8 as verified by ELISA assay. The ability of hepatocytes to be infected with dengue virus serotype 2 in vitro support evidence implicating human hepatocytes as a target cell in cases of dengue virus infection, and provide the first experimental evidence to support the large number of clinical studies that implicate the liver as a critical target organ in severe cases of dengue infection. ***J. Med. Virol. 79:300–307, 2007.***

KEY WORDS: cytokine; flavivirus; IL-8; liver

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 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) in one open reading frame [Chang, 1997].

While 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; Nguyen et al., 1997; Mohan et al., 2000; Wahid et al., 2000], occasional fulminant hepatic failure [Subramanian et al., 2005] and histological changes including hepatocellular necrosis [Bhamarapravati, 1989] significant debate exists as to the possible involvement of the liver in the disease process. The liver is predominantly (80%) composed of hepatocytes, although other cells include Kupffer, endothelial, stellate and biliary cells. Kupffer cells have been previously shown to be infectable by the dengue viruses, and although the infection is not productive, cell death occurs [Marianneau et al., 1999]. While liver cell lines are broadly permissive to dengue infection [Lin et al., 2000b], the transformed nature of these cells makes interpretation of the significance of their infectablity problematic.

To address the question of whether primary human hepatocytes can be infected, we sought to infect viable

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human primary hepatocytes with dengue virus serotype 2 and to establish infection through the assay of progeny viruses, the detection of both dengue virus polarity strands as well as through indirect immunocytochemistry to detect the products of dengue virus translation. When productive dengue virus infection of human primary hepatocytes was established, the study further sought to determine the cellular response of the primary hepatocytes to infection by examining the expression of a panel of cytokine genes as well as the specific up-regulation and secretion of the chemotactic cytokine IL-8, a primary mediator of the inflammatory response [Remick, 2005].

MATERIALS AND METHODS

Cells and Viruses

Primary human hepatocytes from two different donors (batches 4F0011 and 5F0063; $\geq$95% hepatocytes) were purchased and maintained strictly as recommended by the manufacturer (Cambrex Bio Science Walkersville, Inc., Walkersville, MD) using the appropriate media and reagents purchased from the same manufacturer (HCM BulletKit, Cambrex Bio Science Walkersville, Inc.). Approximately 2.2×10^5 cells were seeded onto individual 12 mm diameter collagen coated PTFE membranes in 12 well polystyrene plates (Transwell-Col, Corning Incorporated, Corning, NY) and incubated under 5% CO_2 at 37°C overnight.

The human hepatoma cell line HepG2 was cultured in DMEM (HyClone, Logan, UT) containing 10% FCS (PAA, Pasching, Austria) 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were incubated at 37°C/10% CO_2. Dengue virus serotype 2 (strain 16681) was propagated in the *Aedes albopictus* derived cell line, C6/36. For infection, stock virus, prepared as described elsewhere [Suksanpaisan and Smith, 2003] was diluted in DMEM. Cells were incubated with virus at MOI of 5 for 1.5 hr following which the cells were washed twice and maintained in growth medium.

RNA Extraction and RT-PCR

Total RNA was extracted with Trizol reagent (Molecular research center, Inc., Cincinnati, OH) according to the manufacturer's instructions. cDNA was synthesized from total cellular RNA using Improm II™ reverse transcriptase (Promega, Madison, WI) and primer D2_R (Table I) for dengue virus positive strand or D1_F (Table I) for dengue virus negative strand or with oligo $(dT)_{15}$ primer (Promega) for cytokine genes. For PCR of dengue virus positive and negative strands, first strand cDNA was amplified for 35 cycles with D1_F and D2_R primers using Taq DNA polymerase (Promega). Cycling condition were as follows: denaturation at 94°C for 10 sec, annealing at 57°C for 30 sec, and extension at 72°C for 1 min. Cytokine genes and 18S RNA were amplified with 7 pmol each of specific primers pairs as shown in Table I in a total reaction volume of 10 μl. Cycling condition were as follows: denaturation at 94°C for 15 sec (for IFN-β, IL-1β, MIP1-α, MCP-1, IL-8, MIP1-β, TRAIL, RANTES, and 18S) or for 20 sec (IL-6), annealing at 55°C for 20 sec (for IFN-β, IL-1β, MIP1-α, MCP-1, IL-8, MIP1-β, TRAIL, IL-6, and 18S) or for 12 sec (RANTES) and extension at 72°C for 30 sec (for IFN-β, IL-1β, MIP1-α, MCP-1, IL-8, MIP1-β, TRAIL, and 18S) or for 18 sec (RANTES) or for 1 min (IL-6). Cytokine gene first strand cDNAs were amplified for 35 cycles while control 18S was amplified for 28 cycles. All PCR products were separated on 1.8% agarose gels and visualized by ethidium bromide staining.

TABLE I. Gene Specific Oligonucleotide Primers

Primer	Sequence	Product size	References
D1_F	5′-TCAATATGCTGAAACGCGCGAGAAACCG-3′	511	Lanciotti et al. [1992]
D2_R	5′-TTGCACCAACAGTCAATGTCTTCAGGTTC-3′		
IFN-β	5′-GATTCATCTAGCACTGGCTGG-3′	186	Li et al. [2005]
	5′-CTTCAGGTAATGCAGAATCC-3′		
IL-1β	5′-AAGCTTGGTGATGTCTGG-3′	330	Bosch et al. [2002]
	5′-TGAGAGGTGCTGATGTACCA-3′		
MIP-1α	5′-CGCCTGCTGCTTCAGCTACACCTCCCGGCAGA-3′	195	Dumoulin et al. [1999]
	5′-TGGACCCCTCAGGCACTCAGCTCCAGGTCGCT-3′		
MCP-1	5′-TTCTCAAACTGAAGCTCGCACTCTCGCC-3′	348	Nordskog et al. [2005]
	5′-TGTGGAGTGAGTGTTCAAGTCTTCGGAGTT-3′		
RANTES	5′-CCACATCAAGGAGTATTTCTACACC-3′	101	Lin et al. [2000a]
	5′-TCTTCTCTGGGTTGGCACACAC-3′		
IL-8	5′-AAGAGAGCTCTGTCTGGACC-3′	408	Bosch et al. [2002]
	5′-GATATTCTCTTGGCCCTTGG-3′		
MIP1-β	5′-GGAAGCTTCCTCGCAACTTTG-3′	200	Chiba et al. [2004]
	5′-GCTCAGGTGACCTTCCCTGAA-3′		
TRAIL	5′-CAATGACGAAGAGAGTATGA-3′	537	Matsuda et al. [2005]
	5′-CCCCCTTGATAGATGGAATA-3′		
IL-6	5′-CCACACAGACAGCCACTCACCTC-3′	313	Abdallah et al. [2005]
	5′-CTGGCTTGTTCCTCACTACTCTC-3′		
Actin	5′-GAAGATGACCCAGATCATGT-3′	330	
	5′-ATCTCTTGCTCGAAGTCCAG-3′		
18s RNA	5′-AAACGGCTACCACATCCAAG-3′	155	
	5′-CCTCCAATGGATCCTCGTTA-3′		

Indirect Immunofluorescence Microscopy

HepG2 cells were grown onto standard glass coverslips while human hepatocytes were grown on collagen type I coated coverslips (Becton Dickinson, Bedford, MA). Cells were infected with the dengue virus at MOI 5 for 1.5 hr and the cells were subsequently fixed at 0, 24, and 36 hr post-infection with 4% paraformaldehyde at room temperature for 30 min. The cells were then permeabilized by incubation with 0.3% Triton X-100 in PBS for 10 min and methanol for 5 min at room temperature followed by incubation with 5% BSA in PBS for 1 hr. Cells were subsequently incubated with either a 1:10 dilution of mouse anti-dengue monoclonal antibody HB114 [Henchal et al., 1982] or directly with a mouse anti-dengue NS1 protein monoclonal antibody [Puttikhunt et al., 2003] at 4°C overnight, follow by six wash with 0.03% Triton X-100 in PBS. Samples were then incubated with a 1:500 dilution of an anti-mouse IgG monoclonal antibody conjugated with Alexa594 (Molecular Probes, Eugene, OR) and 20μg/μl DAPI (4′-6-Diamidino-2-phenylindole) in 1% BSA (Molecular Probes) for 1 hr at room temperature, follow by six washes with 0.03% Triton X-100 in PBS. Coverslips were mounted on glass slides with Vectashield (Vector Laboratories, Burlingame, CA).

Human IL-8 ELISA

The concentration of IL-8 was determined using a commercial ELISA kit (ID Labs, Inc., ON, Canada). Culture medium from either control mock infected or cells infected with the dengue virus at MOI 5 was collected at 0, 12, 18, 24, and 36 hr post-infection. The assays were performed as recommend by the manufacturer, and IL-8 concentration was determined by spectrometry at 450 nm.

RESULTS

To establish if primary human hepatocytes are able to be productively infected with the dengue virus, approximately 2.2×10^5 primary hepatocytes were seeded onto individual 12 mm diameter collagen coated PTFE membranes in two 12 well polystyrene plates and incubated under 5% CO_2 at 37°C overnight. At the same time approximately 3×10^5 HepG2 cells were seeded into individual wells of a 12 well tissue culture plate and incubated overnight under 10% CO_2 at 37°C. Following overnight incubation, one well of HepG2 cells was trypsinized and cells counted and the well was shown to contain 3×10^5 cells. Primary hepatocytes and HepG2 cells were incubated with dengue virus serotype 2 at an MOI of 5 and cells incubated for a further 60 hr. Over this time assays of cell number showed that infected primary hepatocytes decreased from 1×10^5 to 1×10^4, or a drop of 90% (see Fig. 1a). Loss of viable cells occurred by the hepatocytes rounding up and detaching from the matrix support. In comparison mock infected primary hepatocytes declined from 7×10^4 to 4×10^4 over the same time period, a decline of some 40%, while infected HepG2 cells showed a 60% increase in cell number due to cell proliferation. Over the 60 hr of the experiment growth medium was sampled at 0, 6, 12, 18, 24, 36, 48, and 60 hr post-infection and levels of infectious progeny viruses established by standard plaque assay on LLC-MK_2 cells. Experiment was undertaken independently in duplicate with duplicate plaque assay of samples. By 60 hr a tenfold increase in virus titer over input was observed for both primary hepatocytes and HepG2 cells (Fig. 1b) strongly suggestive of de novo virus replication and productive infection. The genome of dengue is a positive sense RNA strand which can be translated directly [White and Fenner, 1994]. As part of the replication process an antisense (negative sense) strand is produced that is subsequently used as a template for further positive strand production [White and Fenner, 1994]. To establish the presence of both the positive (sense) and negative (antisense) strands of the dengue virus genome human primary hepatocytes and HepG2 cells were infected with dengue virus serotype 2 for 48 hr after which total RNA was extracted and cDNA was synthesized from total cellular RNA and subjected to RT-PCR using dengue specific primers (Table I) as well as primers directed against actin. Results (Fig. 1c) show the presence of both dengue polarity strands in the hepatocytes as well as in HepG2 cells.

As a final confirmation of productive dengue virus infection of primary hepatocytes, translation of the dengue genome was investigated through the detection of both structural and non-structural dengue proteins. While structural proteins may be present in the infected cell as a result of the original infecting virus, the presence of non-structural proteins would confirm dengue virus genome translation. For this reason, primary hepatocytes and HepG2 cells were grown separately on microscope cover slips overnight and cells were then infected with dengue virus serotype 2 at an MOI of 5 and the cells were incubated for a further 0, 24, or 36 hr after which they were subsequently fixed and incubated with either mouse anti-dengue monoclonal antibody HB114 [Henchal et al., 1982] or a mouse anti dengue NS1 monoclonal antibody [Puttikhunt et al., 2003] followed by a further incubation with an anti-mouse IgG monoclonal antibody conjugated with Alexa594. Samples were incubated further with DAPI before visualization under a fluorescent microscope. Results (Fig. 1d) show that both primary hepatocytes and HepG2 cells show the synthesis of E protein and NS1 protein by 24 hr post-infection. Nuclear staining with DAPI confirmed the presence of many fragmented nuclei in the primary hepatocytes and fragmentation patterns were consistent with the induction of cellular apoptosis (Fig. 2). Counting of multiple fields of cells double stained cells (either DAPI and NS1 or DAPI and E protein) at 24 hr gave cell infection rates of $67.74\% \pm 26.21$ for NS1 and $76.57\% \pm 18.94$ for E protein, suggesting that the majority of the primary hepatocytes were infected by 24 hr.

Having confirmed the productive infection of primary hepatocytes by the dengue virus the study sought to

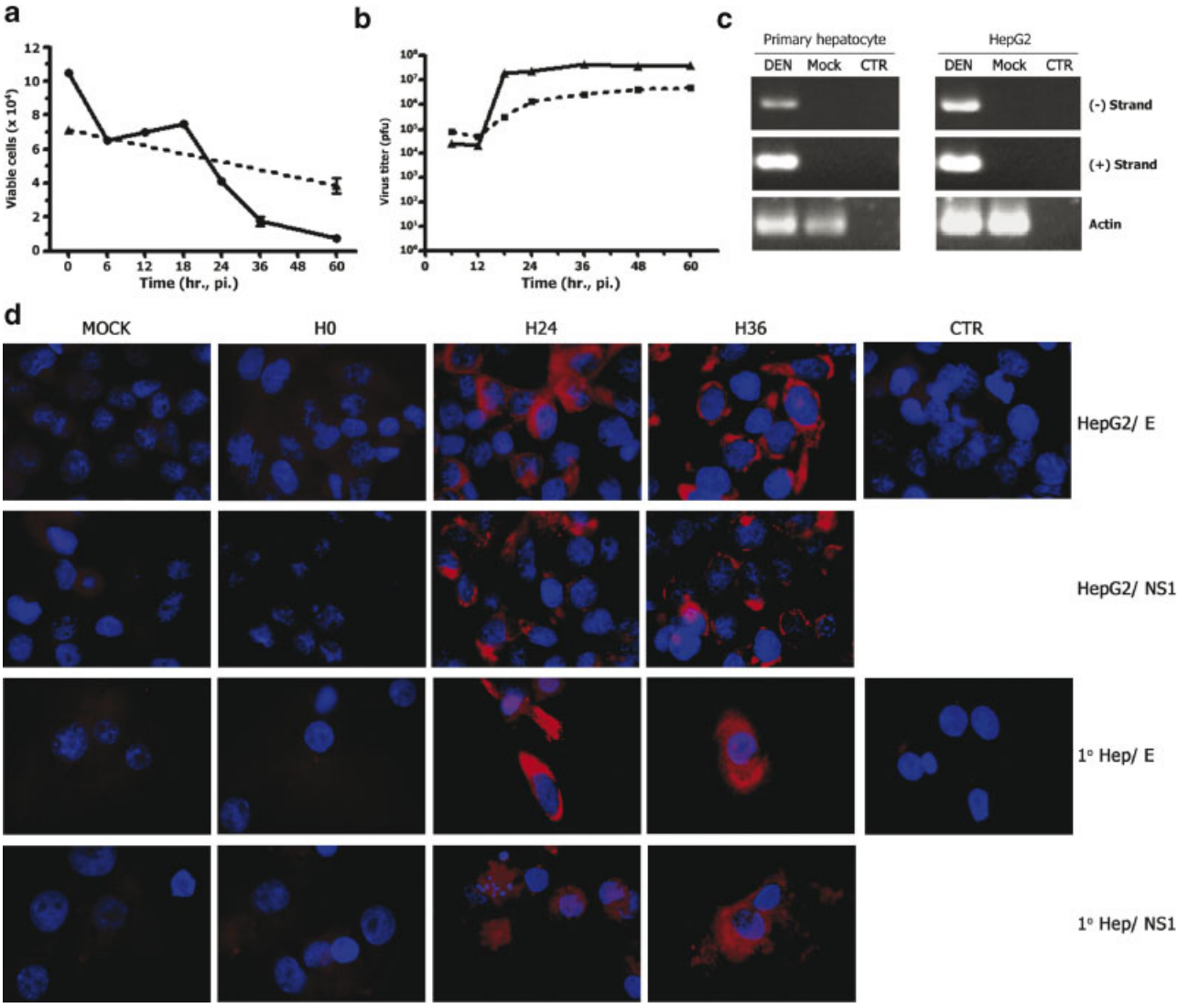

Fig. 1. Infection of human primary hepatocytes with the dengue virus. **Panel a**: Human primary hepatocytes were infected with the dengue virus at MOI 5 (solid line) or mock infected (broken line) and cell number counted for up to 60 hr post-infection. Error bars are s.e.m. of two independent experiments counted in quadruplet. **Panel b**: Human primary hepatocytes (broken line) or HepG2 cells (solid line) were infected with the dengue virus and media assayed by standard plaque assay for infectious progeny viruses. Experiment was undertaken in duplicate with duplicate assay of virus titers, error bars represent s.e.m. **Panel c**: RT-PCR analysis of dengue virus infected and mock infected human primary hepatocytes and HepG2 cells to detect positive (sense) and negative (anti-sense) dengue strands. CTR: control reaction with no template. **Panel d**: Merged images of immunoflourescent staining of dengue virus infected human primary hepatocytes (1° Hep) and HepG2 cells using monoclonal antibodies directed against either dengue virus E protein or dengue virus NS1 protein. Positive signal shows red, while DAPI staining shows nuclei as blue. CTR: no primary antibody.

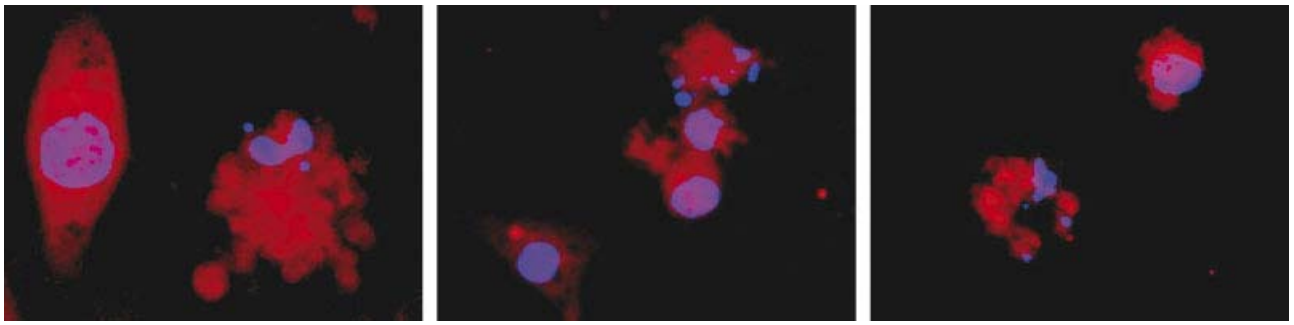

Fig. 2. Primary human hepatocyte nuclei fragmentation in response to dengue virus infection. Merged images of immunoflourescent staining of dengue virus infected human primary hepatocytes showing nuclear fragmentation using monoclonal antibodies directed against dengue virus NS1 protein and DAPI staining at 24 hr post-infection.

investigate the cellular response of the primary hepatocytes to dengue virus infection and multiple parallel wells of hepatocytes were infected with dengue virus serotype 2 at an MOI of 5 for 1.5 hr. At times of 0, 12, 18, 24, and 36 hr post-infection cells were harvested and RNA extracted and cDNA prepared. Semi-quantitative expression profiles for IFN-β, TRAIL, MCP-1, IL-6, IL-1β, IL-8, MIP-1α, MIP-1β, and RANTES were determined by RT-PCR using the primers given in Table I, together with 18S RNA levels as a control. All PCR products were separated on 1.8% agarose gels and visualized by ethidium bromide staining. The experiment was conducted in parallel with HepG2 cells, and undertaken twice independently.

Results (Fig. 3) show similar, but not identical induction profiles between primary hepatocytes and HepG2 cells. In particular a significant induction of IL-6 was observed in HepG2 cells, but no message for IL-6 was detectable in primary hepatocytes. Similarly, MCP-1 was observed to be expressed in hepatocytes, but not HepG2 cells. IL-1β was apparently down-regulated in primary hepatocytes, but induced in HepG2 cells. In both cell types a significant induction of TRAIL, MIP-1α, IFN-β, MIP-1β IL-8, and RANTES was observed. Quantitation of the signal intensities of the various PCR products against the 18S signal (Fig. 4) showed that in HepG2 cells a peak of induction was observed between 18 and 24 hr post-infection while contrast induction in primary hepatocytes was generally lower and without a significant peak, except for RANTES and IL-8 which showed a sharp increase in signal between 24 and 36 hr. Accurate quantitation of bands also revealed that the IL-1β profile was identical in both HepG2 cells and primary hepatocytes.

From the cytokine profile it was noted that IL-8, an important regulator of the acute inflammatory response [Remick, 2005], was up-regulated significantly and as IL-8 has been shown to be significantly higher in the serum of dengue hemorrhagic patients than in DF patients [Raghupathy et al., 1998] we sought to determine if the increase in the message was accompanied by the release of IL-8 protein and so primary hepatocytes were again infected at an MOI of 5 in parallel with HepG2 cells. Samples of the growth medium were sampled at 0, 12, 18, 24, and 36 hr post-infection and assayed for immunoreactive human IL-8 using the ELISA assay. Experiment was undertaken independently in duplicate with triplicate assay of each sample together with control (mock infected) cells. Results (Fig. 5) show that significant amounts of IL-8 are released from both primary hepatocytes and HepG2 cells in response to dengue virus infection. In contrast no IL-8 was detected in either mock infected human primary cells or mock infected HepG2 cells.

DISCUSSION

The involvement of the liver in the pathogenesis of dengue virus infections is the subject of some controversy. While several studies have shown the presence of high rates of hepatomegaly [Mohan et al., 2000] as well as significant disorder of the levels of serum liver

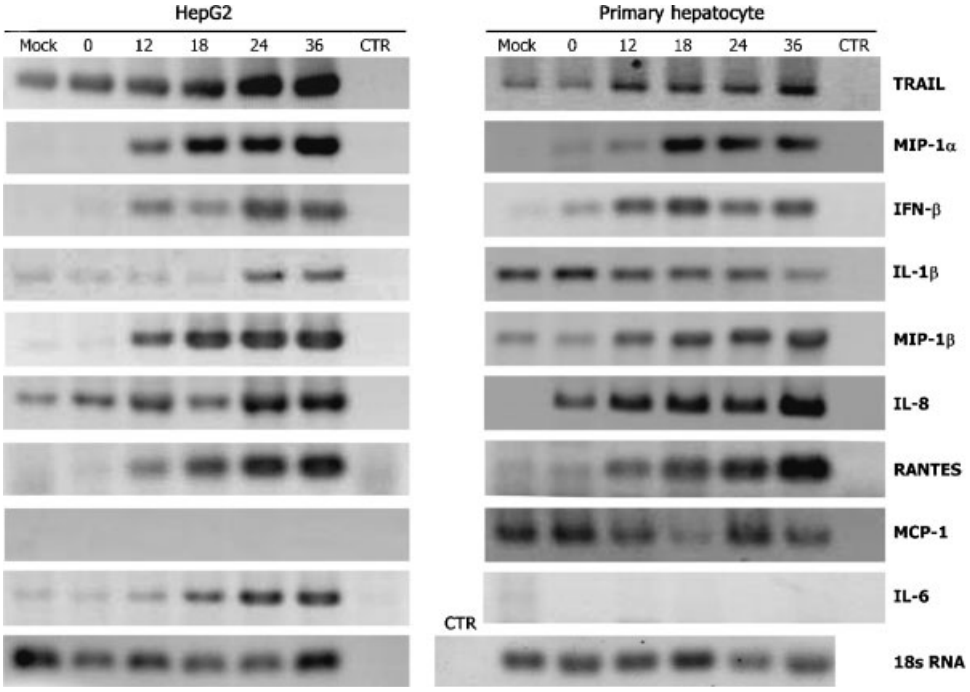

Fig. 3. Cytokine response of dengue virus infected human primary hepatocytes. Human primary hepatocytes or HepG2 cells were infected with the dengue virus at MOI 5 and the expression of nine cytokines (IFN-β, TRAIL, MCP-1, IL-6, IL-1β, IL-8, MIP-1α, MIP-1β, and RANTES) examined by RT-PCR for up to 36 hr post-infection. 18S ribosomal RNA was used as a control.

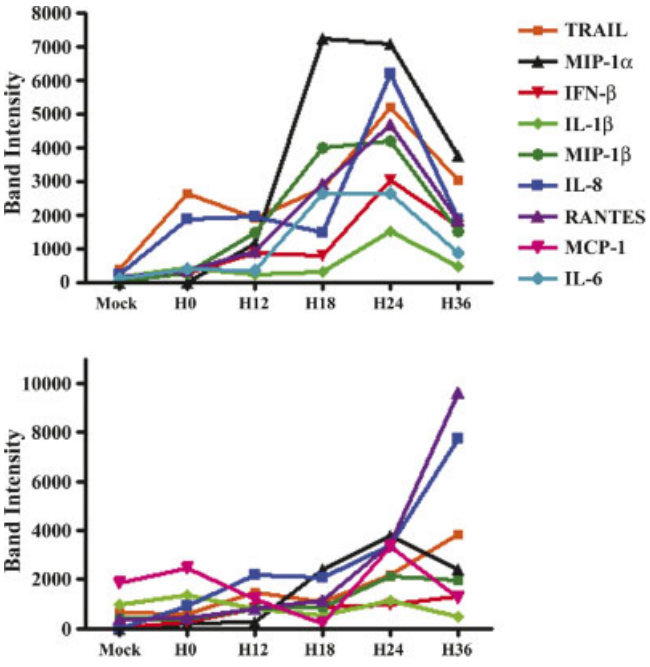

Fig. 4. Quantification of cytokine gene response in dengue infected HepG2 cells and primary hepatocytes. Band intensities of cytokine gene expression from Figure 2 were calculated relative to the 18S ribosomal intensity using the ImageJ program and plotted as a function of time. **Top**, HepG2; **bottom**, primary human hepatocytes.

enzymes [Kuo et al., 1992; Nguyen et al., 1997; Mohan et al., 2000; Wahid et al., 2000], whether these occur as a result from the direct infection of cells of the liver or result from a bystander effect possibly as a result of dysregulation of the host immune responses is unclear. Further uncertainty arises from the nature of the cell or cells in the liver affected by the infection process.

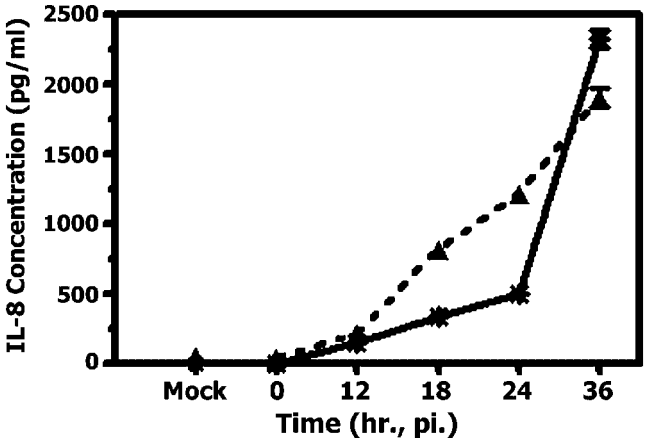

Fig. 5. Production of IL-8 from infected primary hepatocytes and HepG2 cells. Human primary hepatocytes and HepG2 cells were infected with dengue virus serotype 2 at MOI 5 and culture medium sampled over 36 hr post-infection and levels of immunoreactive human IL-8 assayed by ELISA assay. Solid line: HepG2 cells, broken line: human primary hepatocytes. Mock: Mock infected primary hepatocytes and HepG2 cells at 0 hr post-infection. Experiment was undertaken in duplicate with triplicate sample assay. Error bars represent s.e.m.

The direct involvement of the liver in the pathogenesis of dengue has been suggested by the demonstration of the dengue virus RNA by reverse transcription (RT)-PCR in liver tissue samples obtained from children with fatal DF [Rosen et al., 1999; Huerre et al., 2001], the demonstration of dengue virus antigens in hepatocytes [Couvelard et al., 1999; Huerre et al., 2001] as well as the recovery of the virus from liver samples from fatal cases of dengue [Rosen et al., 1989]. While earlier 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], this is contradicted by more recent studies that report only the involvement of Kupffer cells [Jessie et al., 2004]. Animal studies have similarly been contradictory. Using BALB/c mice injected interperitonealy with dengue serotype 2 obtained from a human patient, Paes et al. [2005] found results consistent with the involvement of hepatocytes including the detection of viral antigens in hepatocytes as well as morphological changes of hepatocytes and hepatocyte necrosis. In contrast to this, an earlier study by Chen et al. [2004] using immunocompetent C57BL/6 mice injected with a high titer inoculation of Dengue 2 strain 16681 (the same strain type as used in this study) found little if any direct involvement of hepatocytes and the authors concluded that liver injury primarily arose though the infiltration of activated lymphocytes, especially CD8+ T cells [Chen et al., 2004]. Other authors have suggested that CD4+ cytotoxic T cells (CTL's) may mediate liver damage through bystander lysis after activation by dengue virus infected Kupffer cells through the Fas/FasL pathway [Gagnon et al., 1999]. Antibodies directed against dengue virus proteins [Lin et al., 2002, 2003], or autoantibodies [Chaturvedi et al., 2001; Lin et al., 2001; Oishi et al., 2003] may also play a role in mediating liver damage through mechanisms independent of direct infection of hepatocytes. As such the question of whether hepatocytes can be directly infected is of critical importance in understanding the pathogenesis of dengue virus infections.

Using isolated purified human primary hepatocytes, this study has demonstrated that human primary hepatocytes in vitro are able to be productively infected with dengue virus serotype 2, and that infection results in a significant cytokine response. There was a marked and rapid loss of infected hepatocytes, significantly over and above the loss seen for uninfected hepatocytes and cells from infection experiments were observed to round up and detach from the matrix support. In addition, infected hepatocytes with fragmented nuclei were observed frequently and both observations are morphologically consistent with the induction of apoptosis in infected hepatocytes. Although the induction of apoptosis was not verified formally in this study, the induction of apoptosis in infected transformed hepatocytes has been well established [Marianneau et al., 1997, 1998; Thongtan et al., 2004] and evidence suggests that in transformed hepatocytes induction of apoptosis occurs through the up-regulation of TRAIL [Matsuda et al.,

2005]. TRAIL (TNF-related apoptosis-inducing ligand or Apo2 ligand) is a type II transmembrane protein that triggers apoptosis mainly in tumor cells [Ashkenazi and Dixit, 1999; Walczak et al., 1999] but has a minimal proapoptotic effect in most normal human cells in vitro and in TRAIL-treated animals [Wiley et al., 1995; Pitti et al., 1996; Gura, 1997; Rieger et al., 1998; French and Tschopp, 1999; Walczak et al., 1999]. TRAIL mRNA is constitutively present in many tissues, unlike the restricted expression of other proapoptotic members of the TNF family [Wiley et al., 1995; Pitti et al., 1996]. Interestingly though, in contrast to many other cell types hepatocytes are highly sensitive to the action of TRAIL [Jo et al., 2000], and as such while the induction of TRAIL seen in dengue infected transformed hepatocytes [Matsuda et al., 2005] may reflect the transformed nature of these cells, the induction of TRAIL seen here in dengue infected primary hepatocytes may well represent a bone fide pathway of apoptotic induction in dengue infected hepatocytes.

In addition to the transcriptional up-regulation of TRAIL, we also noted a significant up-regulation of IL-8 which was associated with the secretion of immunoreactive IL-8. IL-8 is a chemotactic cytokine responsible for inducing chemotaxis, which is the directed migration of cells to a site of inflammation and IL-8 is a chemoattractant for neutrophils, basophils, and lymphocytes [Mantovani et al., 1992]. Up-regulation of IL-8, together with RANTES, MIP-1α, MIP-1β, has been reported previously for dengue infected HepG2 cells [Medin et al., 2005] consistent with the results found here for both HepG2 and primary hepatocytes. IL-8 has been reported to be found at significantly higher levels in the serum of DHF patients with severe grade fever than in DF patients [Raghupathy et al., 1998].

As such, these results suggest that the role of the liver in severe DF infections may well be underestimated. It is likely that the combination of a significant cytokine response, coupled with the early induction of cell death will serve to limit the spread of the virus both by the direct removal of apoptotic cells by recruited phagocytic cells as well as the provision of a dead end host cell for free viruses [Marianneau et al., 1999]. In particular these results may serve to explain the discrepancy between the high proportion of liver abnormality as evidenced by liver enzyme levels [Kuo et al., 1992; Nguyen et al., 1997; Mohan et al., 2000; Wahid et al., 2000] and the low levels of liver damage seen in classical autopsy studies [Bhamarapravati, 1989; Subramanian et al., 2005]. Given that fatalities occur commonly 7–10 days after infection [Malavige et al., 2004] the evidence of significant hepatocyte loss may not be detectable without significantly more refined studies, leading to an underestimation of the effects of liver involvement in the pathobiology of dengue infections.

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Characterization of dengue virus entry into HepG2 cells

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Abstract

Background: Despite infections by the dengue virus being a significant problem in tropical and sub-tropical countries, the mechanism by which the dengue virus enters into mammalian cells remains poorly described.

Methods: A combination of biochemical inhibition, dominant negative transfection of Eps15 and siRNA mediated gene silencing was used to explore the entry mechanism of dengue into HepG2 cells.

Results: Results were consistent with entry via multiple pathways, specifically via clathrin coated pit mediated endocytosis and macropinocytosis, with clathrin mediated endocytosis being the predominant pathway.

Conclusion: We propose that entry of the dengue virus to mammalian cells can occur by multiple pathways, and this opens the possibility of the virus being directed to multiple cellular compartments. This would have significant implications in understanding the interaction of the dengue virus with the host cell machinery.

Background

While most animal viruses enter into cells by receptor mediated endocytosis in clathrin coated pits [1,2], evidence to date suggests that the normal mechanism of dengue virus entry into both insect and mammalian cells is by direct fusion of the virus with the cell membrane [3-6], although endocytosis of dengue viruses has been observed with neutralization escape mutants [5], in the presence of neutralizing antibodies [6] as well as in the non-target cell line Hela [7]. These results are somewhat contradictory to results with other flaviviruses, and both Japanese encephalitis virus (JEV) and West Nile virus have been shown to enter cells via clathrin coated pits [8-10]. However, the majority of the studies undertaken to date on the dengue viruses have been based upon electron microscopy ultrastructural studies [4-6] or non-target cells [7] and as such no comprehensive direct biochemical or genetic analysis of the entry mechanism of the dengue virus has yet been undertaken.

Methods

Cells and viruses

HepG2 and Vero cells were maintained as previously described [11-13] Dengue serotypes 1 (strain 16007), 2

(strain 16681), 3 (strain 16562) and 4 (strain 1036) were propagated in Vero cells and purified as described previously [11,12].

Cytotoxicity assessment by Annexin V staining

Confluent HepG2 cells were pretreated at 37°C with either 20 μM cytochalasin D or 30 μg/ml nystatin for 2 hr, or with 15 μg/ml chlorpromazine or 3 mM amiloride or 50 μM LY294002 or 0.2 μM wortmannin for 1.5 hr with 80% DMSO for 20 hours as a positive control. Cells were then trysinized and subsequently washed twice with cold-PBS. Cells were then washed with binding buffer (BD Biosciences Pharmingen, San Diego, CA) and resuspended in binding buffer. Annexin V-FITC (BD Biosciences Pharmingen, San Diego, CA) was added to the cell suspensions and samples were incubated in the dark for 15 min before analysis by flow cytometry (BD FACSCalibur # E6361).

Biochemical inhibition of dengue entry

Confluent HepG2 cells were pretreated at 37°C with chlorpromazine, amiloride, wortmannin or LY294002 for 30 min or with cytochalasin D or nystatin for 1 hr. The cells were infected with each dengue serotype in either in the presence or absence of the appropriate inhibitor at an MOI of 1, for 1 hr at 37°C. The extracellular viruses were then inactivated by acid glycine (pH3) treatment [14]. The infected cells were further grown for one propagation cycle minus two hours with the exact time dependent upon the dengue serotype as determined previously [12], and the number of infected cells determined by our adaptation of the standard plaque assay [15]. Experiments were undertaken independently in triplicate with duplicate plaque assay of samples.

Eps15 transfection, infection and indirect immunofluorescence microscopy

Plasmid constructs of dominant negative (DIII and EH29) and control (D3Δ2) Eps15 were kindly provided by A. Benmerah (Department of Infectious Diseases, Institut Cochin, Paris, France). Transfections of HepG2 cells were undertaken using lipofectamine2000 (Invitrogen, OR., USA). Briefly, 3 μg of the appropriate plasmid DNA was complexed with 4 μl of lipofectamine2000 for 30 min at room temperature and then added to HepG2 cells pregrown to 70–80% confluency on glass coverslips. The cell/complex mix was incubated at 37°C, 10% CO_2 for 2 days. Transfection efficiencies routinely exceeded 70% efficiency as determined by counting of multiple fields. Transfected cells were subsequently infected with dengue virus serotype 1, 2, 3 or 4 at an MOI of 20 for 1.5 hr followed by acid glycine (pH3) treatment to inactivate uninternalized viruses [14] and incubated for 15 hr at 37°C, 10% CO_2. A further set of cells were serum starved for 30 min and incubated with 5 μg transferrin conjugated with Alexa 594 (Molecular Probes, OR) at 37°C for 30 min follow by acid glycine treatment.

Both dengue infected and transferrin treated transfected cells were fixed with 4% paraformaldehyde for 30 min at room temperature. Transferrin control cells were directly mounted with Vectashield (Vector Laboratories, Inc., CA) while dengue virus infected cells were further permeabilized with 0.3% TritonX-100 and methanol. Nonspecific binding was blocked by incubation with 5% BSA for 1 hr at room temperature. Cells were incubated with an anti-dengue E protein monoclonal antibody, HB-114 [16] at 4°C overnight. After six washes with 0.03% TritonX-100 in PBS cells were incubated with a chicken anti-mouse IgG conjugated with Alexa 594 (Molecular Probes, OR) for 1 hr at room temperature and subsequently washed with six washes of 0.03% TritonX-100 in PBS before mounting with Vectashield (Vector Laboratories, Inc., CA).

siRNA design and generation

Target sites on clathrin heavy chain (GenBank accession number NM_004859) and the green fluorescent protein (GFP; GenBank accession number U50974) were determined using the on-line tool from Ambion, Austin, TX http://www.ambion.com/techlib/misc/siRNA_finder.html and the selected sequences were subjected to siRNA template design to generate DNA oligonucleotide sequences for use with the Silencer™ siRNA Construction kit (Ambion). Six templates for siRNA generation were selected:

siCHC1: 298-AACCCAGCAACATTGGCTTC-318

siCHC2: 411-AAGTAATCCAATTCGAAGACC-431

siCHC3: 484-AAAGCTGGGAAAACTCTTCAG-504

siCHC4: 1951-AATAATCGCCCATCTGAAGGT-1971

siCHC5:3544-AATGAACCTGCGGTCTGGAGT-3564

siGFP: 295-AAAGATGACGGGAACTACAAG-315

Numbering indicates the corresponding position of the selected 21 nucleotide sequence in the open reading frame of NM_004859 (siCHC1 to siCHC5) or U50974 (siGFP). All sequences were searched against the NCBI's database to confirm specificity. Sense and antisense DNA templates were chemically synthesized (BioBasic, Canada) and following the kit instructions based on *in-vitro* transcription, the siRNAs were produced and quantified by spectrophotometry.

siRNA transfection

HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum without antibiotics. Reverse transfections according to the manufacturers protocols were performed with Lipofectamine™RNAiMAX (Invitrogen, Carlsbad, CA) by mixing the respective siRNA and 1.2 μl of Lipofectamine™RNAiMAX and adding to a single well of a 24 well plate. After 20 minutes of incubation at room temperature, a suspension of 5×10^4 HepG2 cells was added and cell: complex mixtures incubated under standard conditions. Mock transfections (lipofectamine only) were performed in parallel. All transfections were undertaken in a final volume of 600 μl with siRNA at a final concentration at 50 nM. Transfections were harvested at 1 to 4 days post-transfection.

RNA extraction and RT-PCR analysis

Transfected cells from a single well of a 24-well plate were homogenized in 0.5 ml Trizol reagent (Molecular Research Center, Cincinnati, OH) and RNA purified as recommended by the manufacturer. For the RT-PCR analysis, an oligo$(dT)_{17}$ primer was used to synthesize first strand cDNA using ImpromII™ reverse transcriptase (Promega, Madison, WI). The cDNA was then amplified in a multiplex reaction with 2 specific primer pairs for CHC (CHCf: 5'-AAGCTCATCTTTGGGCAGAA-3'; CHCr: 5-GAGACAGCACCATCAGCAAA-3') and GAPDH (GAPDHf: TTGGTATCGTGGAAGGACTCA-3'; GAPDHr: 5'-ACCACCTGGTGCTCAGTGTAG-3') as an internal control. Expected products were 343 bp (GAPDH) and 222 bp (CHC) Cycle conditions were 94°C for 3 minutes followed by 20 cycles of 94°C for 30 seconds, 58°C for 45 seconds and 72°C for 45 seconds followed by a final extension of 72°C for 7 minutes. PCR products were analyzed on 1.8% agarose gels containing ethidium bromide.

Infection of siRNA silenced HepG2 cells

HepG2 cells (5×10^4) were grown on coverslips in single wells of a 24 well plate and transfected as above with either siRNAs as stated or mock transfected. At day 3 post transfection cells were infected with dengue virus serotype 2 MOI 20 for 2 hours followed by an acid glycine wash and subsequently incubated for 15 hours under standard conditions. Dengue virus E protein was detected as described above except that cells were also stained with DAPI. Parallel non-infected samples were incubated with transferrin as described above and were additionally stained with DAPI. Where biochemical inhibition was used in conjunction with siRNA silencing, samples were treated with 0.2 μM wortmannin for 30 minutes immediately preceding dengue virus infection.

Results

Effect of endocytosis inhibitors on dengue virus entry

To investigate the mechanism of dengue virus internalization into HepG2 cells, cells were pre-treated with cytochalasin D, amiloride, LY294002 or wortmannin to inhibit macropinocytosis, nystatin to inhibit caveolae mediated entry or chlorpromazine to inhibit clathrin-coated pit mediated. Prior to the infection experiment cells were incubated with a range of concentrations of the inhibitors to assess cytotoxicity. Cytotoxicity was initially assessed by cellular morphological changes under light microscopy (data not shown). Working concentrations (the highest concentration without apparent cytotoxicity) were established as: cytochalasin D at 20 μM, amiloride at 3 mM, LY294002 at 50 μM, wortmannin at 0.2 μM, nystatin 20 μM at 30 μM and chlorpromazine at 15 μM (HepG2). The lack of cytotoxicity at these concentrations was confirmed by Annexin V staining and flow cytometry (Figure 1).

To determine the effects of the various inhibitors on dengue virus entry, cells were pre-incubated for 1 hr with cytochalasin D or nystatin and for 30 min with chlorpromazine, amiloride, wortmannin or LY294002 at the concentrations determined above, following which the cells were incubated separately with all four dengue virus serotypes individually at an MOI of 1 for 1 hr following which the virus: cell mixtures were treated with acid glycine pH3 to inactivate any uninternalized viruses [14]. Cells were incubated under optimal growth conditions for a time equivalent to one virus replication cycle minus 2 hr based on our previous data for each serotype in HepG2 cells [12] following which the cells were briefly trypsinized, serially diluted and plated onto pre-grown cell monolayers and overlaid with agarose/nutrient medium as previously described for this adaptation of the standard plaque assay [15]. All experiments were undertaken independently in triplicate with duplicate assay of infected cell number.

Results (Figure 2) show that inhibition of caveolae mediated endocytosis with nystatin results in a relatively uniform increase in the number of dengue virus infected cells for all serotypes, while inhibition of clathrin coated pit mediated endocytosis with chlorpromazine results in a significant reduction in the number of dengue infected cells for all four serotypes although the magnitude of the effect is variable. Inhibition of macropinocytosis with cytochalasin D, amiloride, LY294002 or wortmannin showed a broad range of effects depending upon the specific inhibitor used, as well as to some extent the serotype of the dengue virus (Figure 2).

Given that this study as well as our previous studies investigating dengue virus entry into HepG2 cells [11-13,15,17-20] have routinely employed acid glycine

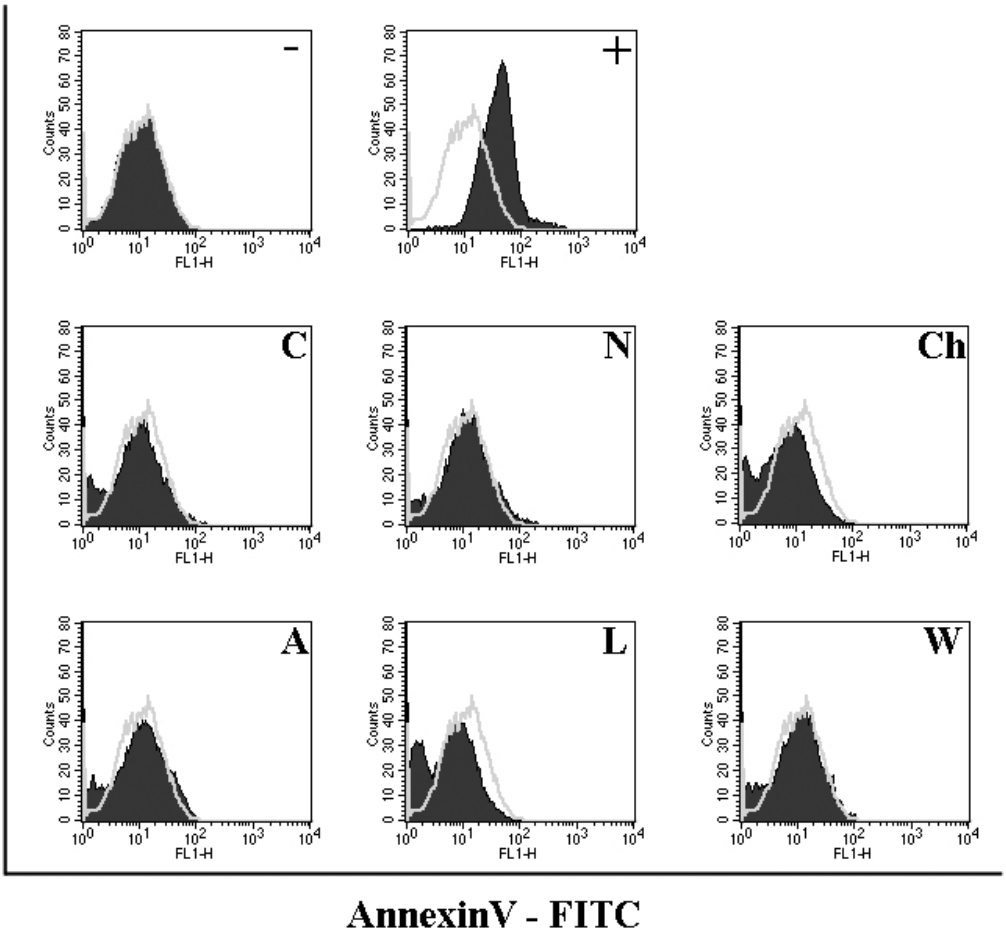

Figure 1
Cytotoxicity assessement of biochemical inhibitors. Flow cytometry histograms of HepG2 cells treated with working concentrations of cytochalasin D (C); amiloride (A); nystatin (N); chlorpromazine (Ch) LY294002 (L); wortmannin (W) or treated with 80% DMSO (+) or untreated (-).

washes [14] to inactivate uninternalized viruses, we sought to determine whether this would materially affect the inhibition studies. The effect of acid glycine treatment was assessed by pre-incubating HepG2 cells with one the inhibitors (15 μM chlorpromazine) or with medium alone as a control. Cells were then infected with dengue virus serotype 2 at MOI of 1 in presence or absence of the inhibitor following which cells were either treated with acid glycine (pH3) or subjected to three washes with PBS. Numbers of infected cells were determined by our adaptation of standard plaque assay [15]. Results showed that washes with acid glycine did not increase virus entry due to promoting viral fusion at the cell surface (Figure 3).

Dominant negative inhibition of clathrin coated pit endocytosis

Given that biochemical inhibitors can cause broad spectrum effects, we further sought to specifically knock out clathrin-dependent endocytosis using over-expressing dominant negative mutants of Eps-15 [21-23] which are able to effectively inhibit clathrin-mediated endoctyosis without affecting non-clathrin pathways [24]. HepG2 cells were transfected with either control (D3Δ2) or dominant negative mutants (DIII and EH29) of the Eps15 protein fused to GFP as well as the vector containing GFP only. Transfection with Lipofectamine2000 routinely resulted in transfection efficiencies of greater than 70% (data not shown). Transfected cultures were either infected with each of the four dengue virus serotype indi-

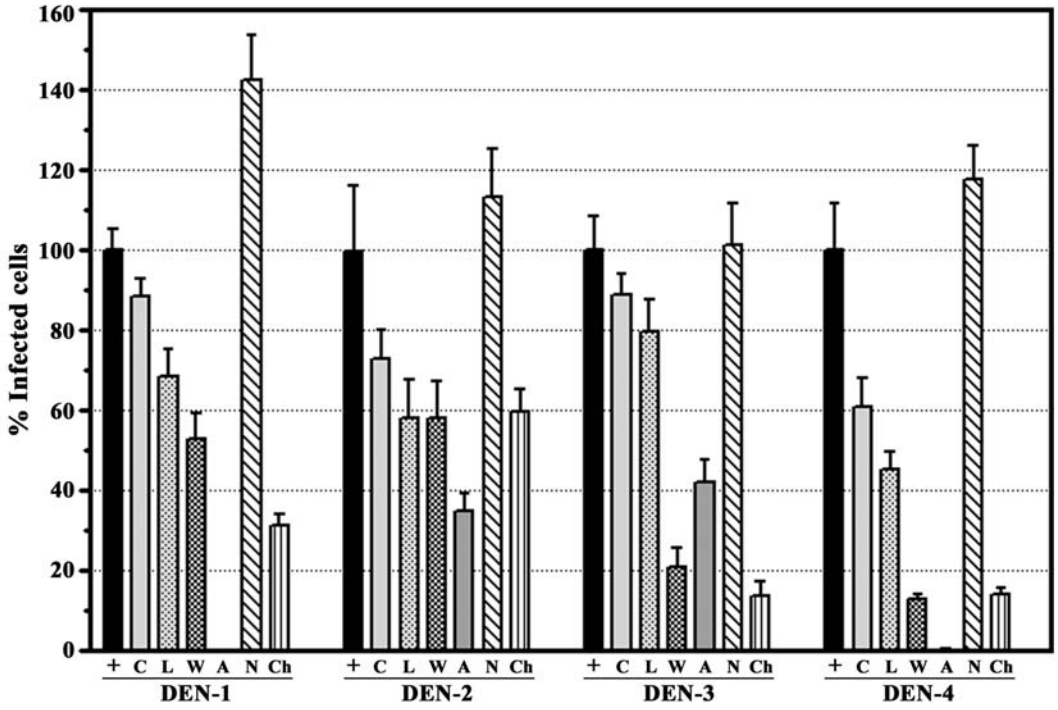

Figure 2
Effects of biochemical inhibitors of endocytosis on dengue virus entry. HepG2 cells were pre-incubated with cytochalasin D (C); amiloride (A); nystatin (N); chlorpromazine (Ch) LY294002 (L); wortmannin (W) or not pre-incubated (+) and subsequently infected with each serotype of the dengue viruses in the presence or absence of the respective treatment. Results are shown as a percentage of infected cells compared to control (100%). Error bar represent SEM of three independent experiments assayed in duplicate.

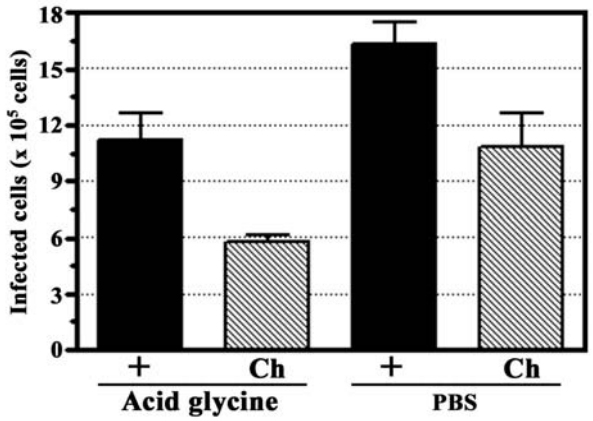

Figure 3
Effect of acid glycine wash. HepG2 cells were infected in the presence or absence of chlorpromazine (Ch) with our without an acid glycine wash. Results are shown as number of infected cells. Error bars represent SEM of three independent experiments assayed in duplicate.

vidually at MOI of 20, or incubated with Alexa 594 conjugated-transferrin before incubation and fixation. Visualization of signal was undertaken by incubating dengue infected samples with a primary monoclonal antibody directed against dengue E protein followed by incubation with a chicken anti-mouse IgG conjugated with Alexa 594.

Results show that both dominant negative Eps15 mutants (DIII and EH29) significantly excluded the entry of transferrin (Figure 4). However, while the two mutants predominantly excluded entry of all four dengue serotypes (Figure 4), numerous examples of dengue virus entry in the presence of expression of the dominant negative mutants were observed (Figure 5). Quantitation by counting multiple fields (n > 30) suggested that 15- to 20% of cells expressing either of the mutants were positive for dengue virus entry for each serotype. In contrast, only scattered cells were seen to be potentially positive for transferrin entry in the presence of the mutants, and these were possibly due to cells overlaying each other.

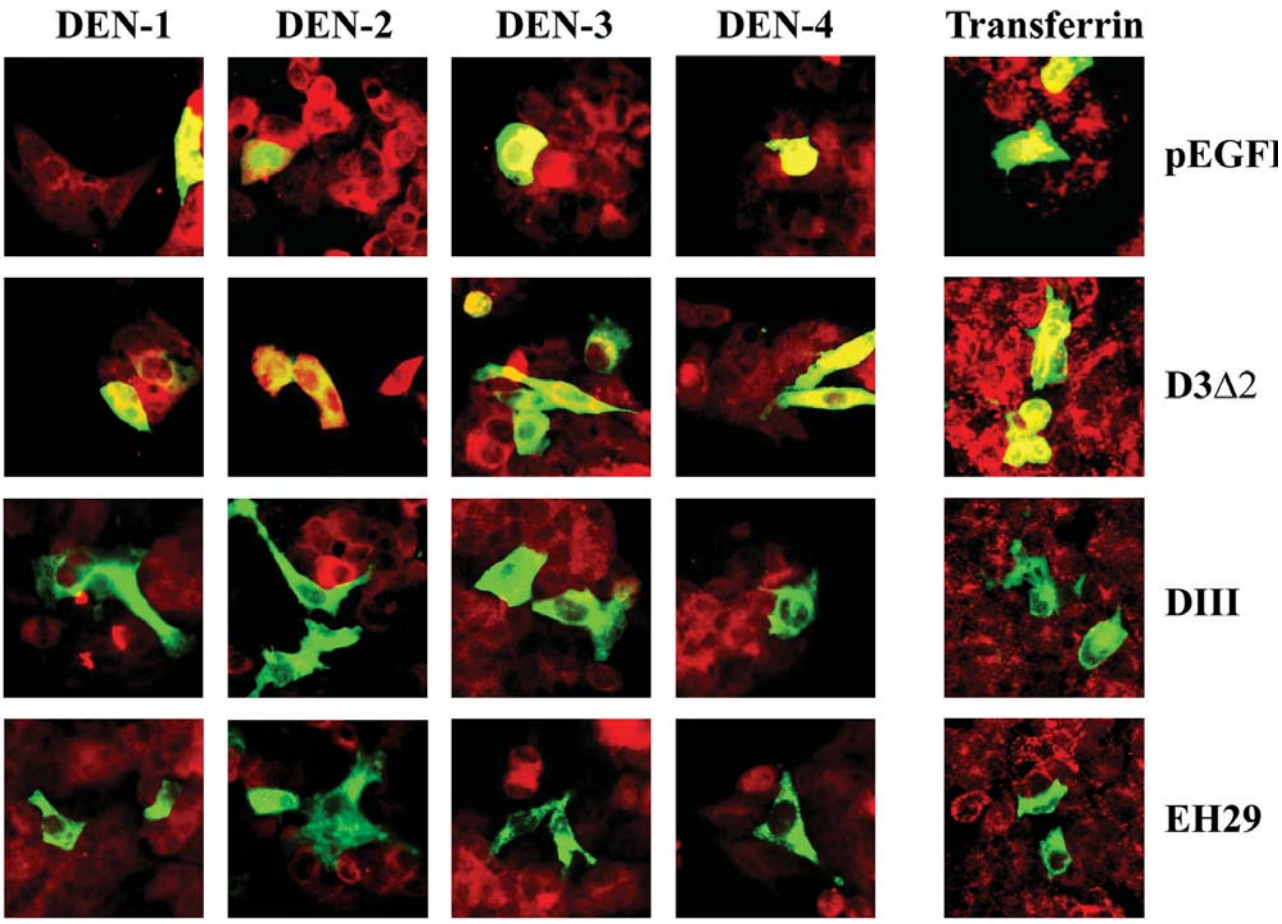

Figure 4
Immunofluorescence of dengue infection and dominant negative mutant of Eps15. HepG2 cell were transfected with either of two dominant negative Eps15 mutants (DIII or EH29) or a wild type Eps15 clone (D3Δ2) all fused to GFP or pEGFP as control, followed by infection with each dengue virus serotype individually or Alexa 594 conjugated-transferrin. Dengue infected samples were subsequently incubated with a mouse monoclonal antibody directed against dengue E protein and an Alexa 594 conjugated chicken anti-mouse IgG antibody. Signal from Alexa 594 (red) and GFP (green) were observed under a fluorescent microscope. Merged images are shown.

siRNA mediated inhibition of clathrin heavy chain expression

Given the significant entry of the dengue virus in the presence of over-expressing dominant negative mutants of Eps15, it is possible that either entry was occurring via multiple pathways, or the Eps15 mutants were not completely inhibiting clathrin mediated entry. To further explore this, RNA interference was used to down regulate the expression of clathrin heavy chain, an integral part of the clathrin vesicle [25]. Five different siRNAs (siCHCs) against human clathrin heavy chain (GenBank accession number NM_004859) were generated using in vitro transcription together with 1 siRNA targeted to the green fluorescent protein (GFP; GenBank accession number U50974) for use as a control. To confirm all siRNAs were double-stranded, an aliquot of each siRNA was treated with RNaseIII which digests double-stranded RNA or RNaseA which digests single-stranded RNA. All siRNA constructs were confirmed to be off the appropriate size and to consist of dsRNA (data not shown).

To optimize the silencing of the expression of the clathrin heavy chain, the 5 different siCHCs were transfected into HepG2 cells in parallel with transfections of siGFP and lipofectamine alone (mock). On days 1 to 4 days post-transfection, cells were harvested and RNA extracted. Multiplex RT-PCR was undertaken to detect messages from GAPDH and clathrin heavy chain (CHC) simultaneously

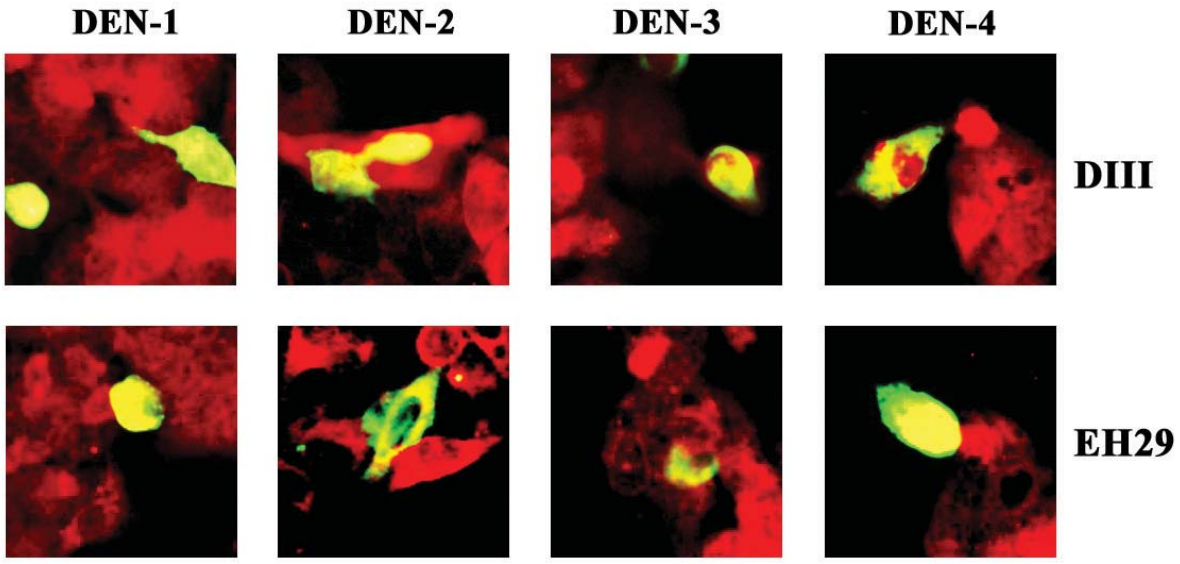

Figure 5
Entry of the dengue virus in the presence of dominant negative mutants of Eps15. Examples of cells positive for both dominant negative mutants of Eps15 (DIII or EH29)-GFP (green) and dengue virus infection (red). Merged images are shown.

and results analyzed by agarose gel electrophoresis. Experiments were undertaken independently in triplicate.

Results showed a constant signal for GAPDH and the clathrin heavy chain (CHC) for mock and siGFP transfection (Figure 6). Message for clathrin heavy chain was seen to be significantly reduced for all transfections, with the greatest signal reduction being seen on day 3 post infection and for siCHC3 and siCHC5 (Figure 6).

Dengue virus serotype 2 infection of clathrin heavy chain silenced HepG2 cells

Optimal silencing of clathrin heavy chain expression was noted at day 3 post transfection with siRNA constructs siCHC3 and siCHC5. These two siRNAs were again transfected into HepG2 cells as above in parallel with transfections of siGFP and mock (transfection agent only) and on day 3 post transfection cells were infected with dengue virus serotype 2 and an MOI of 20 and cells allowed to grow for 15 hours (the time for one replication cycle of dengue serotype 2 minus two hours) under optimal conditions. At 15 hours cells were either analyzed by microscopy or by our adaptation of the standard plaque assay [15] to determine the number of infected cells. Both microscopy and determination of infected cell number were undertaken independently in triplicate.

Consistent with our results with transfections of dominant negative constructs of Eps15, a significant reduction of transferrin entry was seen with siCHC transfections, but not with mock or siGFP (Figure 7). Dengue virus serotype 2 entry was observed for mock and siGFP transfections (Figure 7). While transfections of siCHC constructs again reduced dengue virus entry significantly, entry of the virus was still observed. Levels of entry of the dengue virus serotype 2 in siCHC transfected cells was again observed to be on the order of 15 to 20% of cells as determined by counting multiple microscope fields ($n > 30$).

Results of dengue virus entry as seen by our adaptation of the standard plaque assay [15] were consistent with the results observed by microscopy. We observed a slight reduction of dengue virus entry in siGFP transfected cells as compared to wild type (mock transfected) suggesting that transfection of even irrelevant siRNAs may marginally affect the viability of the HepG2 cells. A significant reduction in the number of dengue virus infected cells was observed with the siCHC transfections (Figure 8, Panel A), with the number of infected cells again being some 20% of wild type (mock transfected) cells.

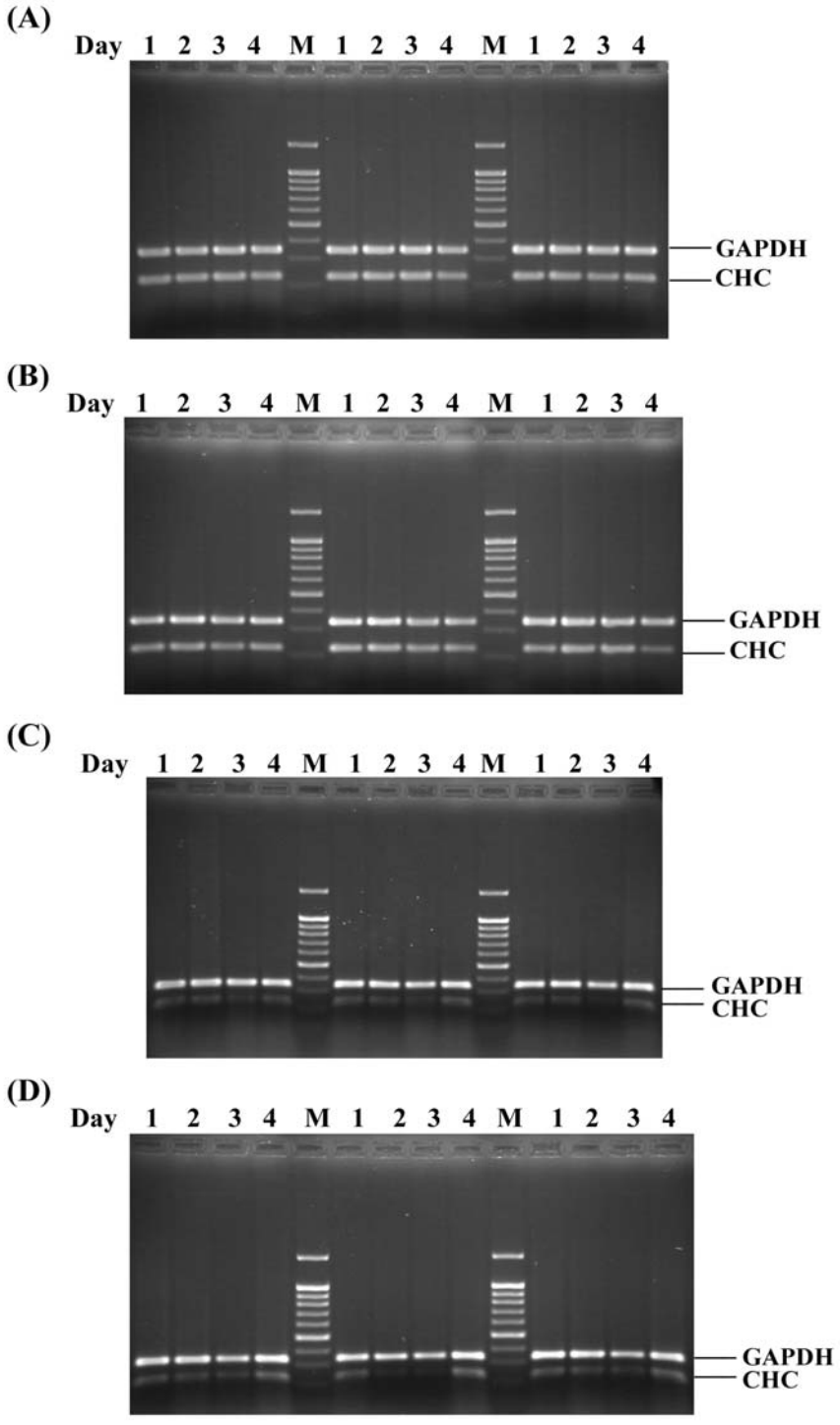

Figure 6
Silencing of Clathrin heavy chain in HepG2 cells. Multiplex RT-PCR products for GAPDH or clathrin heavy chain (CHC) of HepG2 cells either mock transfected (A); transfected with siGFP (B); transfected with siCHC3 (C) or transfected with siCHC5 (D). Samples represent day 1 to 4 post transfection and transfections were undertaken independently in triplicate.

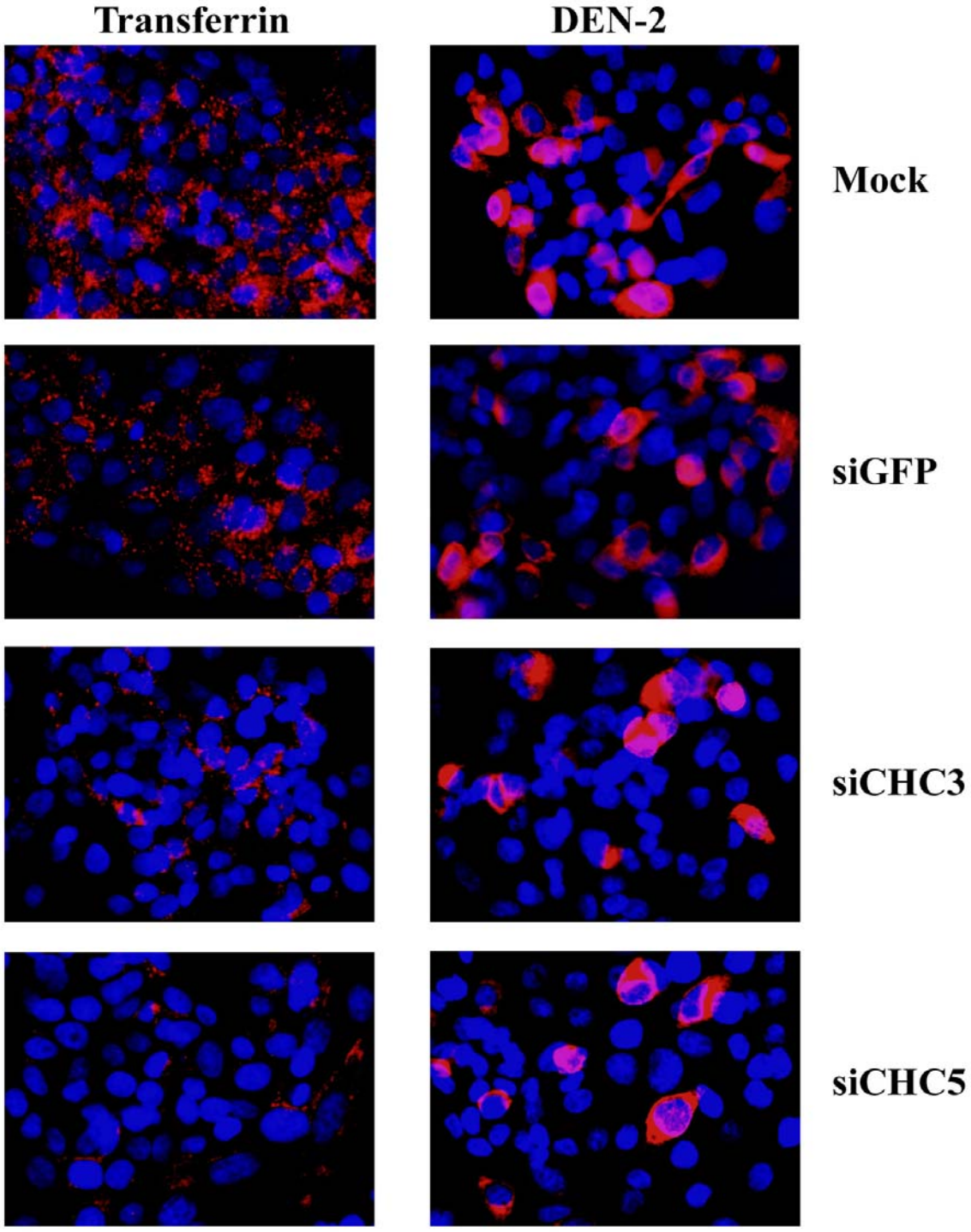

Figure 7
Dengue serotype 2 infection of siRNA silenced HepG2 cells. Indirect immunoflourescence of HepG2 cells either mock transfected or transfected with siGFP, siCHC3 or siCHC5 and subsequently either infected with the dengue virus (red) or incubated with transferrin (red). Cells were additionally stained with DAPI (blue) to show nuclei. Merged images are shown.

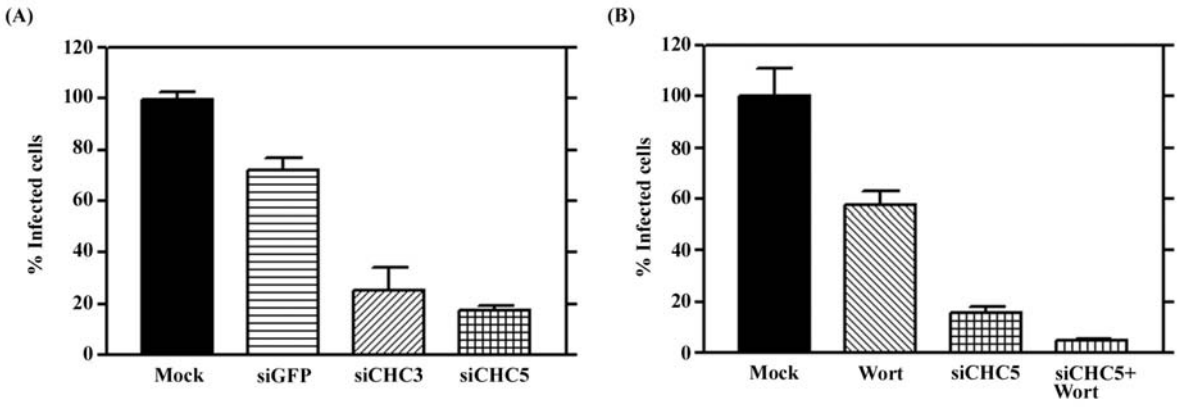

Figure 8
Infection of siRNA silenced HepG2 cells. A. HepG2 cells either mock transfected (mock) or transfected with siGFP, siCHC3 or siCHC5 were infected with dengue virus serotype 2 at MOI 20 and the number of infected cells determined by our adaptation of the standard plaque assay [15]. Results are expressed as a percentage of mock infection and error bars represent the SEM of three independent experiments assayed in duplicate. B. HepG2 cells were either mock transfected or transfected with siCHC5 and either treated or not treated with wortmannin prior to infection with the dengue virus serotype 2 at MOI 20. Results are expressed as a percentage of mock infection and error bars represent the SEM of three independent experiments assayed in duplicate.

dependent endocytosis

To determine whether the approximately 20% virus entry seen in cells in which clathrin mediated endocytosis has been inhibited is a result of background, or the result of dengue virus entry by macropinocytosis, we sought to simultaneously inhibit both pathways, clathrin mediated endocytosis through siRNA mediated RNA inhibition and macropinocytosis through biochemical inhibition using wortmannin.

Cells were therefore transfected with siCHC5 to silence clathrin heavy chain or mock transfected and on day 3 post-transfection were either treated or not treated with wortmannin for 1 hour before being either incubated with transferrin or infected with dengue serotype 2 at an MOI of 20. Following acid glycine treatment of dengue infected cells, cells were incubated for 15 hours before being either examined by microscopy or the number of infected cells determined by our adaptation of the standard plaque assay [15]. Experiments were all undertaken independently in triplicate.

Results (Figure 9) show that transfection with siCHC5, but not treatment with wortmannin significantly excluded transferrin and a similar level of transferrin exclusion was seen between cells treated with siCHC5 alone and cells treated with siCHC5 and wortmannin in combination. HepG2 cells treated with either siCHC5 or wortmannin both showed a reduction in levels of dengue infected cells as compared to control, but a significant number of dengue infected cells was observed in each case. The combination of siCHC5 and wortmannin treatment resulted in the infection of rare, single scattered cells (Figure 8, Panel B).

Results from our adaptation of the plaque assay (Figure 7, Panel B) provided consistent data, with a significant reduction in the number of dengue serotype infected cells seen in the cells treated with a combination of siCHC5 transfection and wortmannin as compared to the siCHC5 transfected cells alone.

Discussion

Despite flaviviral infections representing a significant world wide public health threat, little advance has been made in dissecting out the mechanism by which flaviviruses enter into either mammalian or insect cells. Studies on Japanese encephalitis virus and West Nile virus with either Vero (African Green monkey cells) or C6/36 cells have suggested that these two viruses enter by clathrin coated pit mediated endocytosis [8-10]. With the dengue virus however, data to date, which has been predominantly generated through electron microscopy studies [4-6], has suggested that direct fusion with the plasma membrane is the standard mode of entry of the dengue viruses.

Recently Chu et al., [9] provided evidence that West Nile virus enters into Vero cells via clathrin mediated endocy-

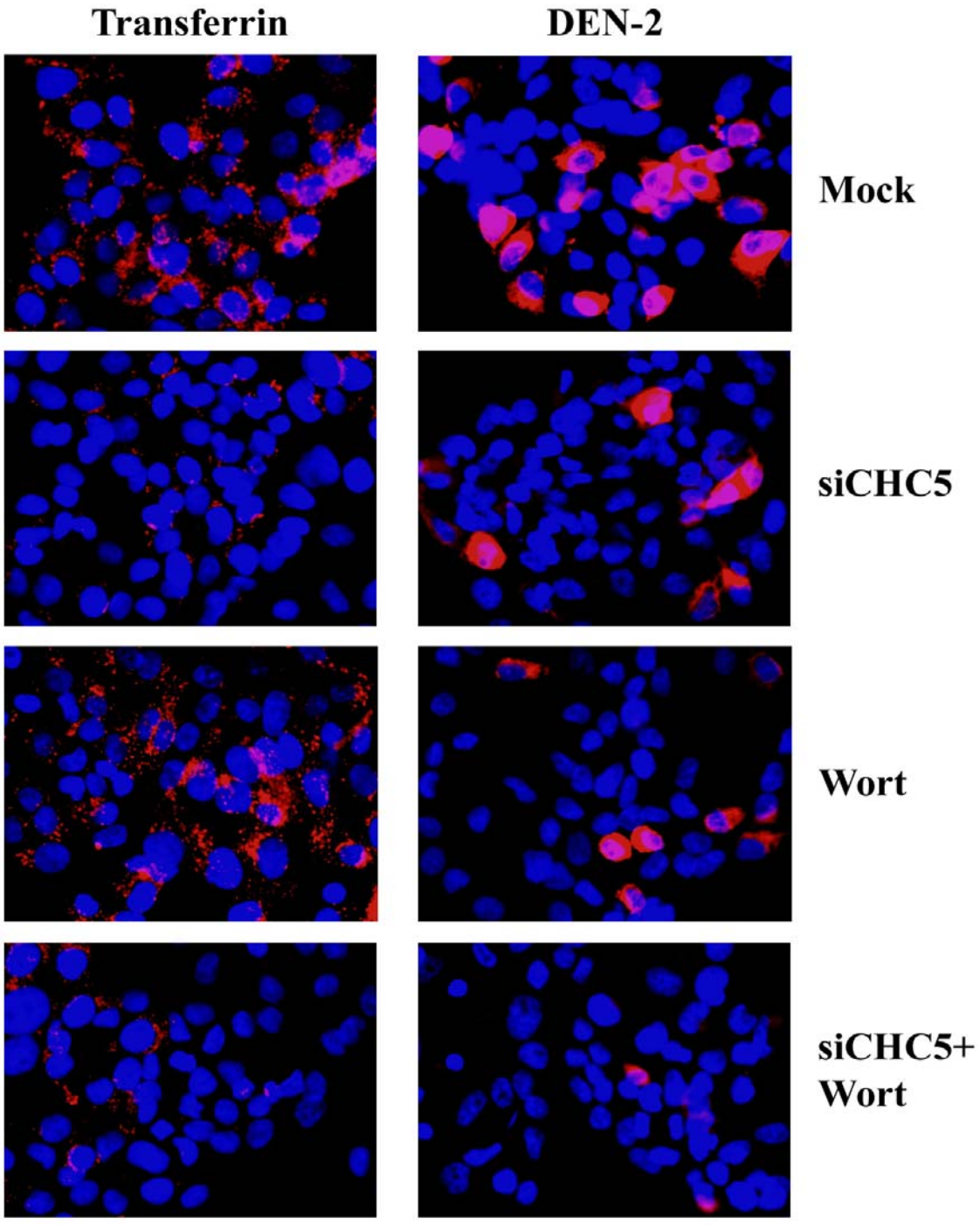

Figure 9
Simultaneous inhibition of macropinocytosis and clathrin mediated endocytosis in HepG2 cells and infection with dengue virus serotype 2. Indirect immunoflourescence of HepG2 cells either mock transfected or transfected with siCHC5 and subsequently either treated or not treated with wortmannin prior to infection with the dengue virus (red) at MOI 20 or incubated with transferrin (red). Cells were additionally stained with DAPI (blue) to show nuclei. Merged images are shown.

tosis [9]. The authors however noted that pre-treatment of Vero cells with cytochalasin D (an inhibitor of macropinocytosis) resulted in an inhibition of infection and the authors proposed that this was possibly due to an effect upon virus trafficking due to cytochalasin D mediated depolymerization of actin filaments [9]. In light of the results seen here it is possible that West Nile virus also enters via multiple pathways and the reduction seen in West Nile virus entry in the presence of cytochalasin D is a consequence of ablation of the macropinocytosis pathway rather than a consequence of altering virus trafficking. Further support for this is seen that reduction of West Nile virus entry in the presence of an Eps15 dominant negative mutant is some 80%, giving some 20% virus entry in the presence of the dominant negative mutant [9] – a figure comparable with the data presented here for the dengue virus.

Interestingly Chu and colleagues also investigated the entry of West Nile Virus into the aedes albopictus cell line C6/36 using the same dominant negative mutant of Eps15 [8] and similarly saw 15 to 20% entry of the virus in the presence of the mutant suggesting that West Nile virus may similarly enter into cells of both an insect and a mammalian origin by multiple pathways.

More recently Krishnan and colleagues have investigated the entry of the dengue virus into HeLa cells [7]. This study also used dominant negative mutants of Eps15 to ablate clathrin mediated endocytosis, and similarly concluded that the dengue virus entered by clathrin coated pit mediated endocytosis. Similar to Chu and Ng [9] however, some 20% virus entry as compared to wild type levels was observed, again giving the possibility that alternate pathways are responsible for the entry of some dengue virus into cells, and indeed, all four studies, this study and those of Chu and Ng [9], Chu and colleagues [8] and Krishnan and colleagues [7] suggest that ablation of clathrin coated pit mediated endocytosis only reduces virus entry by 80%.

Our data suggests that the remaining 20% virus entry observed is not the results of incomplete ablation of clathrin mediated endocytosis but represents virus entry by a viable, independent pathway, macropinocytosis. Entry of the dengue viruses (and more possibly flaviviruses in general) by multiple pathways as shown here raises some interesting questions, particularly with respect to the initial flavivirus: host cell interaction and may require a significant re-evaluation of our understanding of flavivirus entry into host cells.

Conclusion

Consistently, inhibition of clathrin mediated endocytosis using dominant negative mutants of Eps 15 results in a reduction of dengue virus entry of approximately 80% as shown by this study and others [7-9]. Our data shows that the incomplete ablation of virus entry is not a result of incomplete knock down of clathrin mediated endocytosis, but rather reflects entry via an alternate pathway.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LS undertook the biochemical inhibition studies and Eps15 transfections and analyzed and interpreted the data. TS undertook the siRNA mediated silencing studies and analyzed and interpreted the data. DRS was responsible for design and implementation of the study as well as drafting the manuscript. All authors read and approved the final version.

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Research article

siRNA-MEDIATED SILENCING OF THE 37/67-kDa HIGH AFFINITY LAMININ RECEPTOR IN Hep3B CELLS INDUCES APOPTOSIS

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Abstract: The laminin-binding protein, variously called the 37/67-kDa high affinity laminin receptor or p40, mediates the attachment of normal cells to the laminin network, and also has a role as a ribosomal protein. Over-expression of this protein has been strongly correlated with the metastatic phenotype. However, few studies have investigated the cellular consequence of the ablation of this gene's expression. To address this issue, the expression of the 37/67-kDa high affinity laminin receptor was knocked out with several siRNA constructs via RNA interference in transformed liver (Hep3B) cells. In each case where the message was specifically ablated, apoptosis was induced, as determined by annexin V/propidium iodide staining, and by double staining with annexin V and an antibody directed against the 37/67-kDa high affinity laminin receptor. These results suggest that this protein plays a critical role in maintaining cell viability.

Key words: siRNA, RNA interference, Laminin receptor, p40, Ribosomal, Liver, Silencing, LAMR1

INTRODUCTION

The multifunctional protein which we here designate the 37LBP/67LR protein has been variously called the 37/67-kDa high affinity laminin receptor protein, the 37-kDa laminin-binding protein (37LBP), the laminin receptor precursor

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Abbreviations used: GAPDH – glyceraldehyde 3-phosphate dehydrogenase; LBP-p40 – laminin-binding protein precursor p40; LRP – laminin receptor precursor; PrP^c – cellular prion protein; siRNA – small interfering RNAs; 37LBP – 37-kDa laminin binding protein; 67LR – 67-kDa laminin receptor

(LRP), the 67-kDa laminin receptor (67LR), LAMR1, and the laminin-binding protein precursor p40 (LBP-p40). It was initially identified as a 67-kDa protein through its high affinity interaction with laminin [1-3], a predominant glycoprotein component of the extracellular matrix that mediates cell attachment, movement, growth and differentiation. The screening of human cDNA libraries using antibodies directed against the purified protein enabled the isolation of a full-length cDNA encoding a protein with a calculated molecular mass of 32 kDa and an apparent molecular weight of 37 kDa, after *in vitro* translation of hybrid-selected mRNA and SDS-PAGE analysis [4]. Highly homologous cDNAs were subsequently isolated from human colon cancer cell lines [5] and obtained in a study on the structure and sequence determination of the rat 40S ribosomal subunit [6]. LPB-p40 was localized on 40S ribosomes [7] and in the nucleus [8], while 67LR was located on the cell surface, where, in addition to its role as a high affinity laminin receptor, it was shown to function as the receptor for elastin [9] and as a positional marker for the differentiation of the fetal eye organ [10]. 37-kDa LRP was identified as a PrP^c accomplice [11], and 37LBP/67LR acts as a receptor for PrP^c [12] and infectious prions [13], as extensively reviewed elsewhere [14-16]. Additionally, 37LBP/67LR acts as a receptor for a number of viruses, including sindbis [17], dengue [18], and the adeno-associated virus serotypes 8, 2, 3, and 9 [19]. Although expression of the mature 67-kDa form of the protein was detected on many normal cells, the immature, 37-kDa form was identified as an oncofetal antigen [20, 21], and its over-expression directly correlated with the increased invasiveness and metastatic potential of a number of different tumours (reviewed in [22]). Although a clear precursor-product relationship between the 37-kDa (or 40-kDa) and 67-kDa forms was established [23], the exact mechanism by which the 37-kDa form gives rise to the 67-kDa form has yet to be established, although post-translational modification involving acylation [23], specifically via palmitoylation [24], has been implicated. Inhibiting 37LBP/67LR with a specific immunoreactive polyclonal antibody inhibited the attachment of a human fibrosarcoma cell line in a dose-dependent manner, and inhibited the formation of pulmonary metastases in a mouse model system [25]. Down-regulation of 37LBP using antisense cDNA constructs was shown to induce apoptosis in HeLa cells [26]. Down-regulation of 37LBP/67LR expression using an siRNA approach resulted in a reduction in PrP^{sc} propagation in Scrapie-infected neuronal cells [27]. More recently, 37LBP/67LR expression was knocked down in the mouse brain using an antisense-LRP RNA approach [28]. One anecdotal report suggested that inhibiting 37LBP/67LR using small interfering RNAs (siRNAs) may induce apoptosis in several cell types [29]. Given the multifunctional nature of this protein, we sought to formally verify whether siRNA-mediated knock-down of expression resulted in the induction of apoptosis in transformed liver cells.

MATERIALS AND METHODS

Cell culture

The human hepatoma cell line Hep3B [30] was cultivated at 37ºC under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, Utah) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL, Gaitherburg, MD) and 100 U of penicillin-streptomycin (HyClone) per ml.

siRNA design and generation

The target sites on the human 37LBP/67LR (GenBank accession number NM_002295) and the green fluorescent protein (GFP; GenBank accession number U50974) were determined using the online tool from Ambion, Austin, TX (http://www.ambion.com/techlib/misc/siRNA_finder.html). The selected sequences were subjected to siRNA template design to generate DNA oligonucleotide sequences for use with the Silencer™ siRNA Construction kit (Ambion). Six templates for siRNA generation were selected:
siLRP1: 5'-AATTTCAGGGTGAATGGACTG-3' (nt 762-782);
siLRP2: 5'-AAATTTTCACAATGTCCGGAG-3' (nt 75-95);
siLRP3: 5'-AAATCTCAAGAGGACCTGGGA-3' (nt 232-252);
siLRP4: 5'-AACCTTCACTAACCAGATCCA-3' (nt 403-423);
siLRP5: 5'-AACAACAAGGGAGCTCACTCA-3' (nt 575-595);
siGFP: 5'-AAAGATGACGGGAACTACAAG-3' (nt 295-315).
The numbering indicates the corresponding position of the selected 21-nucleotide sequence in the open-reading frame of NM_002295 (siLRP1 to siLRP5) or U50974 (siGFP). All the sequences were searched against the NCBI database to confirm specificity to human 37LBP/67LR or GFP. Sense and antisense DNA templates were chemically synthesized (BioBasic, Canada), and following the kit instructions based on *in vitro* transcription, the siRNAs were produced and quantified by spectrophotometry. To confirm that the generated siRNAs were double-stranded, an aliquot of each siRNA was digested individually with RNaseIII or RNaseA. For RNaseIII treatment, 3 µg of siRNA, 1x $MnCl_2$, 1x ShortCut reaction buffer (50 mM Tris-HCl, 1mM DTT, pH 7.5) and 3 µl of ShortCut® RNaseIII (New England Biolabs, Inc. Ipswich, MA) were combined in a total volume of 20 µl, and incubated at 37ºC for 20 min. The reaction was stopped with the addition of EDTA. For the RNaseA treatment, 3 µg of siRNA, 1x RNaseA buffer (300 mM NaOAC, 10 mM Tris-HCl, pH 7.5 and 5 mM EDTA) and 0.01 µg/µl of RNaseA were mixed in a total volume of 20 µl, and the reaction mixture was incubated at 37ºC for 5 min before being terminated on ice. All the samples were analyzed by gel electrophoresis.

siRNA labeling

GAPDH-siRNA provided in the *Silencer*™ siRNA Labeling kit (Ambion) was end-labeled with the provided Cy3 by mixing the GAPDH-siRNA with the provided 10x labeling buffer and Cy3 labeling reagent. The reaction mixture was then incubated in the dark at 37ºC for 1 h, and excess label was removed via

ethanol precipitation by the addition of 0.1 volumes of 5 M NaCl and 2.5 volumes of 100% ethanol, with incubation at -20ºC for 1 h. The Cy3-labeled GAPDH-siRNA was pelleted by centrifugation at 10,000 g for 20 min, and the pellet was washed with 70% ethanol. Finally, the pellet was air-dried and dissolved in nuclease-free water. The concentration and base:dye ratio of the labeled siRNA was measured by spectrophotometry.

siRNA transfection

Hep3B cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum without antibiotics. Reverse transfections were performed with Lipofectamine™RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols, by mixing the respective siRNA and 1.2 µl of Lipofectamine™RNAiMAX, and adding to a single well of a 24-well plate. After 20 min of incubation at room temperature, a suspension of $5x10^4$ Hep3B cells was added, and the cell:complex mixtures were incubated under standard conditions. Mock transfections (lipofectamine only) were performed in parallel. To assess transfection efficiency using Cy3-labeled GAPDH-siRNA, a glass cover slip was placed in the well prior to the transfection mix. All the transfections were undertaken in a final volume of 600 µl with siRNA at a final concentration of 50 nM. The cells transfected with Cy3-labeled GAPDH-siRNA were analyzed via fluorescent microscopy at 24 h post-transfection, while other transfections were harvested 1 to 4 days post-transfection. For the analysis of apoptosis, a total of $1x10^5$ Hep3B cells were reverse-transfected in a 6-well plate using the same final siRNA concentration and 2.4 µl of the transfection agent. All the transfections were undertaken independently in triplicate.

Fluorescent microscopy

After 24 h, the cells transfected with Cy3-labeled GAPDH-siRNA were fixed with 4% paraformaldehyde in PBS at room temperature for 30 min, and then permeabilized by incubation with 0.3% TritonX-100 in PBS for 5 min at room temperature. The coverslips were incubated with 1:500 DAPI in 0.3% TritonX-100/PBS for 10 min at room temperature, followed by two washes with PBS. They were then mounted on glass slides with Vectashield (Vector Laboratories, Inc.) mounting medium. The fluorescent signal was visualized under an Olympus BX61 fluorescent microscope.

RNA extraction and RT-PCR analysis

Transfected cells from a single well of a 24-well plate were homogenized in 0.5 ml Trizol reagent (Molecular Research Center, Cincinnati, OH) and allowed to stand at room temperature for 5 min. The cell lysate was vigorously shaken for 15 s in the presence of 0.1 ml chloroform, and allowed to stand at room temperature for 3 min, followed by centrifugation at 12,000 *g* and 4ºC for 15 min. The aqueous phase solution was transferred to a new tube and precipitated with 0.25 ml isopropanol at room temperature for 10 min, followed

by centrifugation at 12,000 *g* and 4ºC for 10 min. The RNA pellets were washed with 75% ethanol with subsequent centrifugation at 7,500 *g* and 4ºC for 5 min. They were air-dried. Finally, the RNA was dissolved in DEPC-treated water. For the RT-PCR analysis, an oligo$(dT)_{17}$ primer was used to synthesize the first strand cDNA using ImpromII™ reverse transcriptase (Promega, Madison, WI). The cDNA was then amplified in a multiplex reaction with 2 specific primer pairs for 37LBP/67LR (LRPf: 5'-TCACTCAGTGGGTTTGATGTG-3'; LRPr: 5'-TTCAGACCAGTCTGCAACCTC-3'), with GAPDH (GAPDHf: 5'-TTG GTATCGTGGAAGGACTCA-3'; GAPDHr: 5'-ACCACCTGGTGCTCAG TGTAG-3') as an internal control. The expected products were 343 bp (GAPDH) and 247 bp (37LBP/67LR). The cycle conditions were 94ºC for 3 min, followed by 20 cycles of 94ºC for 30 s, 58ºC for 45 s and 72ºC for 45 s, followed by a final extension of 72ºC for 7 min. The PCR products were analyzed on 1.8% agarose gels containing ethidium bromide.

Protein extraction and Western blot analysis

A total of $2.5x10^5$ Hep3B cells were reverse transfected in 6-well plates using a final siRNA concentration of 50 nM and 6 µl of transfection agent. On days 3 and 4 post-transfection, the transfected cells were harvested by scraping from the tissue culture plates, and transferred into 1.5-ml tubes followed by centrifugation at 1,500 rpm for 5 min to pellet the cells. The culture medium was then discarded, and the cell pellets were resuspended in ice-cold 1x PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4). After centrifugation at 1,500 rpm for 5 min, 1x PBS was removed, and the cells were lysed by vigorous vortexing in RIPA lysis buffer (1x PBS with 1% Nonidet P-40, 0.5% Sodium Deoxycholate and 0.1% SDS), followed by sonication for 15 min on ice. Finally, the supernatant was collected after centrifugation at 12,000 rpm for 15 min at 4ºC, and the protein concentration was determined using the Bradford assay (Bio-Rad). A total of 60 µg of total proteins was subjected to electrophoresis through 12% sodium dodecyl sulfate-polyacylamide gels in Tris-glycine buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% SDS) at a constant voltage of 100 volts at room temperature. The separated samples were then transferred to nitrocellulose membranes in a transfer buffer (15.6 mM Tris Base, 120 mM glycine) at a constant voltage of 30 volts at 4ºC for 16 h. The membrane containing the transferred proteins was blocked with 5% skimmed milk in TBS (20 mM Tris-HCl, pH 7.5, 140 mM NaCl) at room temperature for 1 h, and incubated with a mixture of a goat polyclonal antibody against the 37/67-kDa high-affinity laminin receptor (SC-21534, Santa Cruz Biotechnology, Inc., Santa Cruz CA) at a dilution of 1:500, and with a 1:500 dilution of goat polyclonal antibody against actin (SC-1616, Santa Cruz Biotechnology Inc.) in 5% skimmed milk in TBS at room temperature for 2 h. After three washes with TBS-T, the membrane was further incubated with a 1:3000 dilution of HRP-conjugated rabbit anti-goat IgG (31402, Pierce,

Rockford Il.) in 5% skimmed milk in TBS for 1 h at room temperature. The signal was developed using an ECL Plus™ Western blotting detection kit (Amersham Biosciences), followed by exposure to autoradiography film.

Flow cytometry analysis

Transfected cells in a single well of a 12-well plate were collected by treatment with trypsin (0.25% trypsin/1 mM EDTA in Hank's balance salt solutions) for 3 min at 37ºC, and transferred to a new tube. The collected cells were pelleted by centrifugation at 2,000 rpm for 3 min, and the supernatant was discarded. An ApoAlert® AnnexinV Apoptosis kit (Clontech, PaloAlto, CA) was used to assess the level of apoptosis in the LRP-silenced Hep3B cells. Following the manufacturer's protocols, the cell pellets were resuspended in 400 µl 1x Binding buffer and incubated for 15 min with 5 µl FITC-conjugated annexinV and 10 µl propidium iodide at room temperature in the dark. Finally, the cells were analyzed by flow cytometry using a FacsScan equipped with Cell Quest software (Becton-Dickinson). For double staining with an antibody against LRP and annexinV, the cell pellets were washed with 200 µl ice-cooled FACS buffer (1x PBS with 2% FBS, freshly prepared) followed by centrifugation at 2,000 rpm for 3 min. Then they were incubated with a 1:50 dilution of rabbit polyclonal antibody against the 37/67-kDa high-affinity laminin receptor (SC-20979, Santa Cruz Biotechnology Inc.) for 1 h on ice. The cells were then washed twice in FACS buffer and labeled with PE-conjugated donkey anti-rabbit secondary antibody (711-116-152; Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) diluted 1:100 in FACS buffer for 45 min on ice in the dark. After two washes, the cells were resuspended in 400 µl FACS buffer, incubated with 5 µl FITC-conjugated annexinV for 15 min in the dark, and subjected to flow cytometry analysis.

RESULTS AND DISCUSSION

Small interfering RNAs (siRNAs) are double-stranded RNAs (dsRNAs) of approximately 19 to 21 bp in length that specifically induce the degradation of cellular mRNAs containing complementary nucleotide sequences [31] by activating the cellular RNA interference (RNAi) pathway [32]. As such, they can be used to specifically ablate the expression of single target genes. To down-regulate the expression of 37LBP/67LR, 5 different siRNAs against the human 37LBP/67LR gene (GenBank accession number NM_002295) were generated using *in vitro* transcription, together with 1 siRNA targeted to the green fluorescent protein gene (GFP; GenBank accession number U50974) for use as a control. To confirm that all the siRNAs were double-stranded, an aliquot of each siRNA was treated with RNaseIII, which digests double-stranded RNA, or RNaseA, which digests single-stranded RNA. All the siRNA constructs were confirmed to be of the appropriate size and to consist of dsRNA. Examples (siLRP2 and siLRP4) of the RNase treatment are shown in Fig. 1A. The optimal siRNA transfection conditions using lipofectamine were established using

commercially available siRNAs directed against GAPDH and subsequently labeled with Cy3. The optimal transfection conditions routinely resulted in transfection efficiencies of 80 to 90% (Fig. 1B).

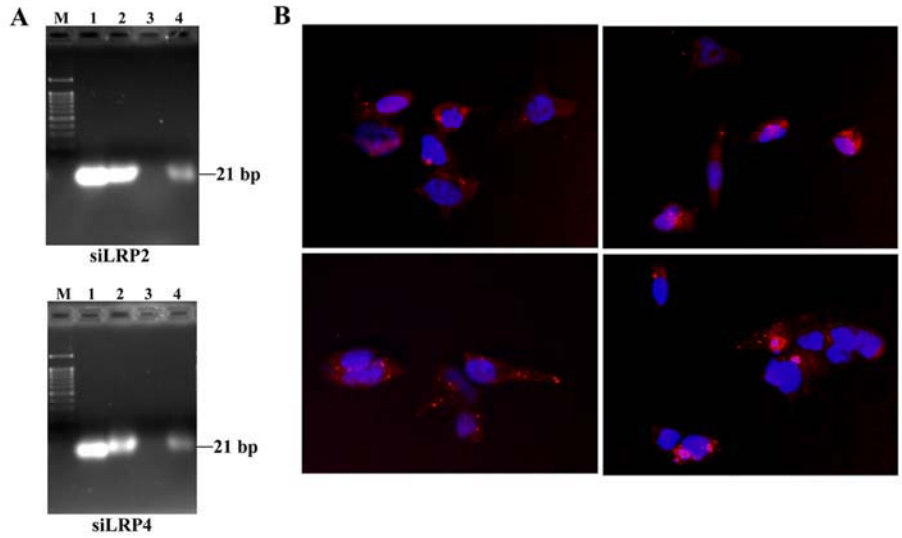

Fig. 1. siRNA analysis and transfection. A – Examples (siLRP2 and siLRP4) of RNAaseA- and RNaseIII-treated siRNAs. In both, lane M: 100-bp ladder, lane 1: dsDNA 21 bp, lane 2: siRNA, lane 3: RNaseIII-treated siRNA, lane 4: RNaseA-treated siRNA. B – Merged images of Hep3B cells transfected with Cy3-labeled siGAPDH (red signal). The nuclei are stained with DAPI (blue). Four representative individual fields are shown.

Silencing human 37LBP/67LR in cultured Hep3B cells

To silence the expression of 37LBP/67LR, 5 different siLRPs were transfected into Hep3B cells in parallel with transfections of siGFP and lipofectamine alone (mock control). On days 1 to 4 post-transfection, the cells were harvested and the RNA extracted. Multiplex RT-PCR was done to detect messages from GAPDH and 37LBP/67LR simultaneously, and the results were analyzed by agarose gel electrophoresis. The experiments were done independently in triplicate. The results (Fig. 2) showed a constant signal for GAPDH and 37LBP/67LR for the mock, siGFP and siLRP1 transfections. By contrast, a significant reduction in the level of expression for both genes was observed in the siLRP2 to siLRP5 transfections by day 3 to 4 post-transfection, with transfections for siLRP2 and siLRP4 showing a complete silencing of both genes, suggesting that cell death was occurring in response to the silencing of 37LBP/67LR.

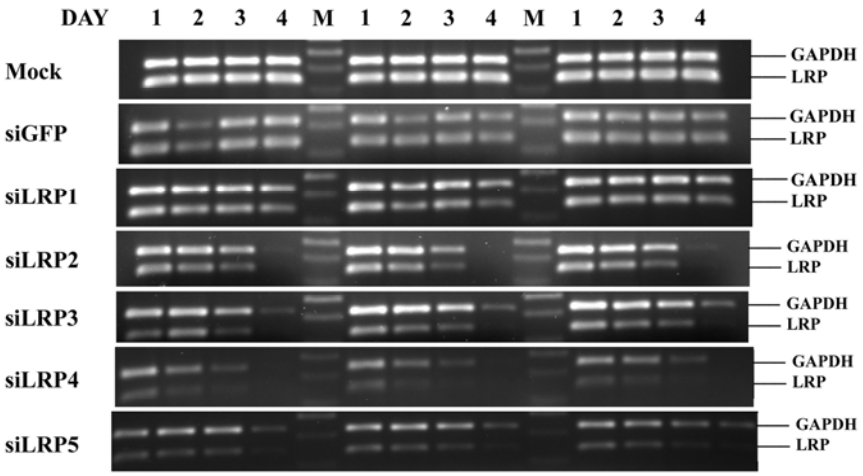

Fig. 2. siLRP-silencing profiles. Multiplex PCR products of GAPDH (upper band) and LRP (lower band), respectively (top to bottom) from mock, siGFP-, siLRP1-, siLRP2-, siLRP3-, siLRP4- and siLRP5-transfected Hep3B cells, from days 1 to 4 post-transfection. M: 100-bp ladder. The transfections were done independently in triplicate.

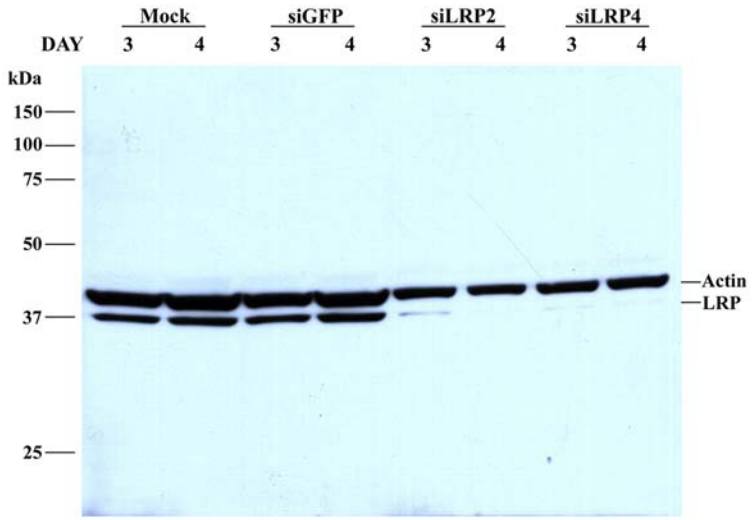

Fig. 3. Western blot analysis of the expression of 37LBP/67LR and actin. The expression levels of 37LBP/67LR and actin were simultaneously assessed by Western blot analysis for mock, siGFP-, siLRP2- and siLRP4-transfected Hep3B cells on days 3 and 4 post-transfection. The molecular weight in kDa is indicated. A representative gel of a duplicate experiment is shown.

Down-regulation of the 37LBP/67LR protein on days 3 and 4 post-transfection for mock, siGFP, siLRP2 and siLRP4 was investigated by Western blot analysis using a mixture of antibodies directed against actin and 37LBP/67LR. The experiment was done independently in duplicate. The results (Fig. 3) show a very significant reduction in the level of 37LBP/67LR protein in the cells transfected with siLRP2 and siLRP4 compared to the level seen in mock and siGFP-transfected cells. Notably, the level of actin was also reduced in siLRP2- and siLRP4-transfected cells as compared to mock and siGFP-transfected cells, again consistent with a loss of cells as a result of transfection with siLRP2 and siLRP4.

Observation of apoptosis in 37LBP/67LR knock-down Hep3B cells

To investigate whether silencing 37LBP/67LR was triggering apoptosis, both siLRP2 and siLRP4 were again transfected into Hep3B cells in parallel with a mock transfection as a control. On days 1 to 4 post-transfection, the samples were again harvested, and this time double-labeled with FITC-labeled annexinV and propidium iodide followed by analysis by flow cytometry (Fig. 4). The experiment was done independently in triplicate. The results showed a significant increase in Annexin V/propidium iodide-positive cells in cultures transfected with either siLRP2 or siLRP4 as compared to the mock transfected cultures, confirming that silencing 37LBP/67LR induces apoptosis in Hep3B cells. To confirm that the increase in the amount of apoptotic cells was associated with a loss in 37LBP/67LR protein, mock, siGFP-, siLRP2- and siLRP4-transfections were again undertaken, and this time, the cells were analyzed by flow cytometry on days 1 to 4 post-transfection by double staining with annexinV and an antibody directed against the 37LBP/67LR protein. This experiment was done independently in triplicate. The results (Fig. 5) show a significant increase in the percentage of AnnexinV+/37LBP/67LR- cells in the siLRP2 and siLRP4 transfections as compared to the mock and siGFP transfections, confirming that the cells undergoing apoptosis are those that have had the 37LBP/67LR protein down-regulated.

Besides its normal roles in mediating cellular adhesion and functioning as a member of the ribosomal translational machinery, the 37LBP/67LR protein has been implicated in a number of a pathological processes including metastasis (reviewed in [22]). It also functions as a receptor protein for a number of pathogenic agents including the prion protein [12] and several viruses [17-19]. As such, modulating the expression of 37LBP/67LR is an attractive prophylactic or therapeutic target. However, the induction of apoptosis in response to complete silencing of 37LBP/67LR, as shown here and previously for HeLa cells [26], suggests that this may not be a viable approach without specific targeting to avoid inducing apoptosis in non-tumorigenic cells. However, it should be noted that the induction of apoptosis in response to the down-regulation of 37LBP/67LR is limited to a small number of cell lines investigated, and this may not be the case for all cell types. In particular, the down-regulation

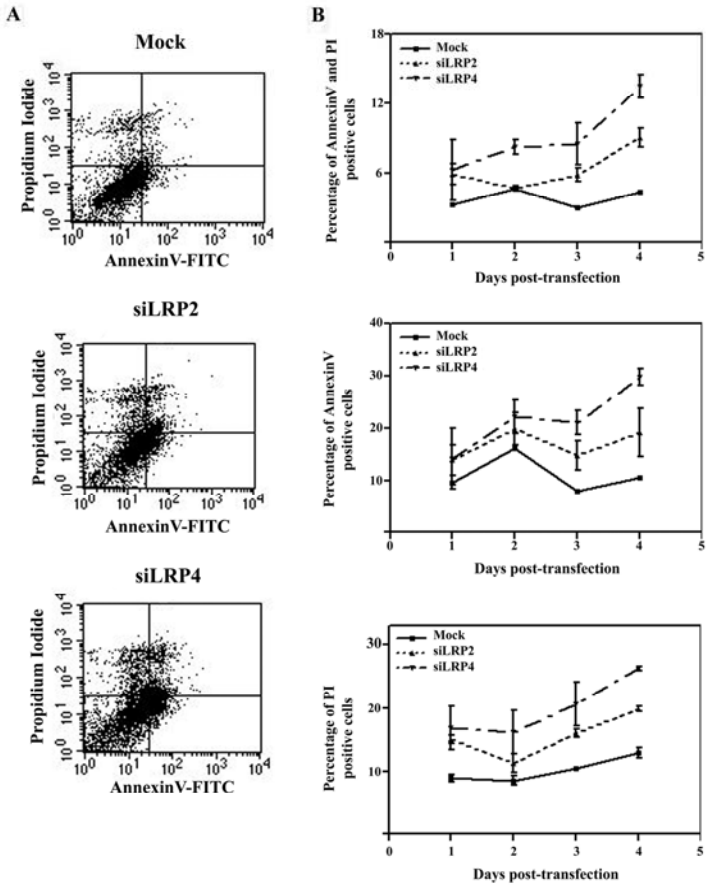

Fig. 4. Apoptosis detection by flow cytometry. A – Scattergrams of Hep3B cells 4 days after mock, siLRP2- or siLRP4-transfection, with double staining with annexinV and propidium iodide and analysis by flow cytometry. B – The results over days 1 through 4 of the flow cytometry analysis of Hep3B cells transfected with siLRP2 (dotted line) or siLRP4 (dashed line with one dot), or mock transfected (solid line), and stained with annexinV and propidium iodide. The graphs show the percentage of positive cells against the time point. The error bars represent the SEM of three experiments.

of 37LBP/67LR in the mouse brain is without apparent phenotypic abnormalities [28]. Given the high degree of over-expression of 37LBP/67LR in metastatic tumours [29], it is tempting to speculate that the expression of 37LBP/67LR is involved in maintaining a non-apoptotic state. As such, therapies that specifically target the cell surface-expressed protein [25], or that use viruses such as Sindbis, with 37LBP/67LR as their specific receptor protein [17], as the therapeutic agent [33] may well provide novel strategies for suppressing the growth of metastatic tumors.

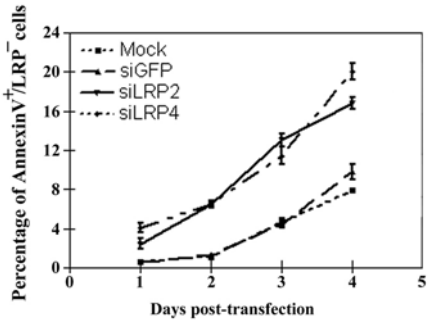

Fig. 5. Flow cytometric analysis of AnnexinV+/37LBP/67LR- cells. Flow cytometry was used to assess the percentage on days 1 through 4 of AnnexinV+/37LBP/67LR- cells of experimental populations that had been mock transfected, or transfected with siGFP, siLRP2 or siLRP4, and doubly stained with Annexin V and an antibody directed against the human 37LBP/67LR protein.

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Short communication

Identification of dengue virus binding proteins using affinity chromatography

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ABSTRACT

Several studies have identified putative dengue virus receptors using virus overlay protein binding assays (VOPBA) with some apparent success. Given that this technique relies upon the use of electrophoresis of proteins through polyacrylamide gels with varying amounts of protein denaturation, the physiological relevance of the proteins isolated is open to question. To address this issue a Sepharose 4B–dengue virus serotype 2-affinity column was constructed to selectively bind dengue virus binding proteins from HepG2 (liver) cell membrane preparations. Results show that GRP78, but not the 37/67 kDa high affinity laminin receptor, was specifically bound by the column. This result is consistent with earlier work and shows that while affinity chromatography may provide a useful adjunct to VOPBA based studies particularly in cases where proteins maybe sensitive to denaturation, proteins isolated by VOPBA can be physiologically relevant.

Dengue viruses are enveloped nucleocapsid spherical viruses of 40–50 nm in diameter that belong to the family *Flaviviridae*, genus *Flavivirus*, a genus that contains a number of important human pathogens (Leyssen et al., 2000). There are four antigenically distinct, but closely related, dengue virus serotypes (DEN-1, DEN-2, DEN-3, and DEN-4) with numerous virus strains found worldwide. Infection with any one of four dengue serotypes can causes either an asymptomatic infection or a spectrum symptomatic illness, ranging from an undifferentiated fever, dengue fever (DF), through to dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS).

The mature dengue virus virion contains three structural proteins: the core or capsid protein (C), a membrane-associated protein (M), and the envelope protein (E) and in order to infect target cells, the dengue virus utilizes its envelope glycoprotein or E protein, which contains the component responsible for target cell binding and fusion (Klasse et al., 1998; Modis et al., 2004), to interact with a specific receptor on the target cell surface.

Studies have suggested that monocytes, macrophages, B lymphocytes, T lymphocytes, hepatocytes, endothelial cells, epithelial cells, dendritic cells and fibroblasts are all potential targets for dengue virus infection and replication (Diamond et al., 2000; Kurane et al., 1990; Lin et al., 2000; Mentor and Kurane, 1997; Moreno-Altamirano et al., 2002; Palucka, 2000; Suksanpaisan et al., 2007; Tassaneetrithep et al., 2003; Wei et al., 2003) and dengue viral antigens have been detected in tissues including liver, spleen, lymph node, thymus, kidney, lung, and skin (Bhoopat et al., 1996; Jessie et al., 2004; Rosen et al., 1999). The liver seems to be a major target organ for dengue virus infection in humans since hepatomegaly, liver dysfunction, and pathologic findings including centrilobular necrosis, fatty change, Kupffer cell hyperplasia, acidophilic bodies, and monocyte infiltration of the portal tract have been detected in the livers of DHF/DSS patients (Bhamarapravati, 1989; Bhamarapravati et al., 1967; Burke, 1968; Wahid et al., 2000). The abnormal elevation of liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels observed in DHF patients is also suggestive of liver dysfunction (Nguyen et al., 1997; Wahid et al., 2000). In addition both dengue virus antigens (Huerre et al., 2001) as well as dengue virus RNA (Rosen et al., 1999) have been documented in liver specimens from fatal DHF cases (Rosen et al., 1999). The virus itself has been recovered from the liver of fatal cases of dengue infections (Rosen et al., 1989) and recently the productive infection of primary human hepatocytes with dengue virus serotype 2 has been documented (Suksanpaisan et al., 2007).

Two proteins, the 37/67-kDa high affinity laminin receptor (Thepparit and Smith, 2004) and GRP78 (Jindadamrongwech et al., 2004) have been identified as receptors on HepG2 (liver) cells for dengue virus serotypes 1 and 2 entry, respectively, although evidence suggests that GRP78 may not be the major receptor protein for dengue serotype 2 infection in HepG2 cells (Jindadamrongwech et al., 2004). Both of these proteins were identified initially through the use of virus overlay protein binding assays (VOPBA) a technique that relies on complete or partial denaturation of the proteins during the analysis, and as such can lead to doubts about the physiological relevance of the proteins isolated. To determine if VOPBA

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can indeed isolate physiologically relevant proteins a dengue virus serotype 2–Sepharose 4B affinity column was constructed to isolate binding proteins from membrane preparations of HepG2 (liver) cells. To ensure no contaminating cellular proteins were present in dengue virus preparations, dengue virus was initially concentrated through PEG precipitation and purified by discontinuous sucrose gradient centrifugation as described previously (Suksanpaisan and Smith, 2003). To construct the dengue virus serotype 2–Sepharose 4B affinity column, CNBr-activated Sepharose 4B freeze-dried powder (Amersham Biosciences, Piscataway, NJ) was prepared as recommended by the manufacturer. Subsequently 1.44×10^7 pfu of purified dengue virus serotype 2 (strain 16681) propagated in C6/36 cells was suspended in 2 ml of coupling buffer (0.1 M $NaHCO_3$ pH 8.3 containing 0.5 M NaCl). An aliquot of 30 μl of the dengue virus serotype 2-coupling solution was kept before the coupling solution was mixed with 0.4 g (approximately 1.5 ml of column volume) of the activated Sepharose 4B. The DEN-2–Sepharose 4B mixture was incubated at 4 °C for 6 h with gentle agitation. A further 30 μl of the DEN-2–Sepharose 4B coupling solution was removed at this point and coupling efficiency determined by quantitating the level of infectious virus in standard plaque assays (Sithisarn et al., 2003). Virus titers of 1.2×10^7 pfu/ml before coupling, and 4.4×10^5 pfu/ml after coupling suggested a coupling efficiency of approximately 90%. The amount of ligand (approximately 50 μg as assessed by Bradford assay) significantly lower than that recommended by the manufacturer was chosen to reduce non-specific protein–protein interactions. Under the coupling conditions used (pH, time, and temperature), approximately 20% natural loss of infectivity would be expected as determined previously (Sithisarn et al., 2003). Excess, unbound DEN-2 virus was washed away with 5 column volumes of coupling buffer, and any remaining active groups of CNBr-activated Sepharose 4B were blocked by adding 2 ml of 0.1 M Tris–HCl pH 8.0 and incubating at 4 °C for 6 h with gentle shaking. The DEN-2–Sepharose 4B column was washed with three cycles of alternating pH buffers (0.1 M sodium acetate pH 4.0 containing 0.5 M NaCl and 0.1 M Tris–HCl pH 8.0 containing 0.5 M NaCl) with 5 column volumes of each buffer. The column was further washed with 10 column volumes of binding buffer (0.1 M Tris–HCl pH 8.0) before use.

To prepare HepG2 membrane proteins, confluent HepG2 cells cultured as described elsewhere (Jindadamrongwech and Smith, 2004; Jindadamrongwech et al., 2004; Thepparit and Smith, 2004) were washed twice with PBS and the cells were detached by scraping in PBS. Cells were harvested by centrifugation at 1200 × *g* for 5 min at 4 °C in an Eppendorf tube. After discarding the supernatant, the cell pellet was resuspended in ice-cold modified buffer M (100 mM NaCl, 20 mM Tris–HCl pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, 0.2% Triton X-100, and 1× protease inhibitor cocktail) and lysed by vigorous vortexing. Subsequently organelle fractionation was performed by sequential differential velocity centrifugation as described previously (Jindadamrongwech and Smith, 2004; Jindadamrongwech et al., 2004; Thepparit and Smith, 2004). Briefly, nuclei and debris were removed by centrifugation at 600 × *g* for 3 min at 4 °C. The supernatant was further centrifuged at 6000 × *g* for 5 min at 4 °C to pellet membranous organelles and finally the supernatant was centrifuged at 24,000 × *g* for 10 min at 4 °C and from this step membrane proteins were pelleted and resuspended in modified buffer M. The proteins concentration was determined by the Bradford method (Bradford, 1976).

A total of 500 μg of HepG2 membrane proteins were dissolved in 2 ml of binding buffer, added onto the DEN-2–Sepharose 4B column, and incubated at 4 °C for 9 h with gentle agitation and unbound fractions were collected by washing the columns with 10 column volumes of binding buffer. Proteins were subsequently eluted from the column using 1 column volume of a step gradient of binding buffer containing NaCl concentrations of 0, 0.1, 0.5 and 1 M NaCl. Protein concentration of eluted fractions was too low to be measured directly by the Bradford method (Bradford, 1976) so fractions from the affinity column were concentrated by trichloroacetic acid (TCA) precipitation by adding bovine serum albumin fraction V (Research Organics, Cleveland, OH) to a final concentration of 5 μg/ml and TCA to 10% (v/v). The protein precipitates were placed on ice for 30 min and subsequently pelleted by centrifugation at 10,000 × *g* for 5 min at 4 °C. The pellet was finally resuspended in 0.1 M NaOH. Proteins were mixed with 1/5th volume of 5× loading buffer (62.5 mM Tris–HCl pH 6.8, 4% (w/v) SDS, 17.4% (v/v) glycerol, 100 mM DTT, and 0.1% (w/v) bromophenol blue), heated to 95 °C for 5 min and loaded onto 10% SDS-PAGE gels. After electrophoresis samples were transferred to solid matrix support (Schleicher & Schuell) before transfer using the Wet-Blot Electrophoretic Transfer cell (Bio-Rad, Hercules, CA). Nitrocellulose membrane was blocked with 5% skim milk in TBS for 1 h at room temperature and incubated with a 1:500 dilution of a rabbit polyclonal antibody directed against human GRP78 (H-129, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) followed by incubation with a 1:2000 dilution of a horseradish peroxidase conjugated goat anti-rabbit IgG (31460 Pierce, Rockford, IL). Signal was developed using the ECL Plus western blotting analysis kit (Amersham Pharmacia Biotech, Piscataway, NJ). Western blot was run in parallel with a control nitrocellulose membrane with 150 μg membrane proteins transferred after electrophoresis through an identical gel system. Results (Fig. 1) show that a band of approximately 78 kDa was detected by the anti-GRP78 antibody in both the control strip and fractions E3 and E4 corresponding to elution with 0.5 and 1.0 M NaCl. To confirm proteins were still able to bind dengue serotype 2, filter was split between lanes E2 and E3 (to minimize amount of virus used) and used in a VOPBA reaction as described previously (Jindadamrongwech and Smith, 2004; Jindadamrongwech et al., 2004). Binding of the virus to the membrane fractions was consistent with previous reports (data not shown).

Filters were subsequently stripped and incubated with a 1:500 dilution of a goat polyclonal directed against the human 37/67 kDa high affinity laminin receptor (SC-21534, Santa Cruz Biotechnology, Inc.) for 2 h at room temperature followed by incubation with

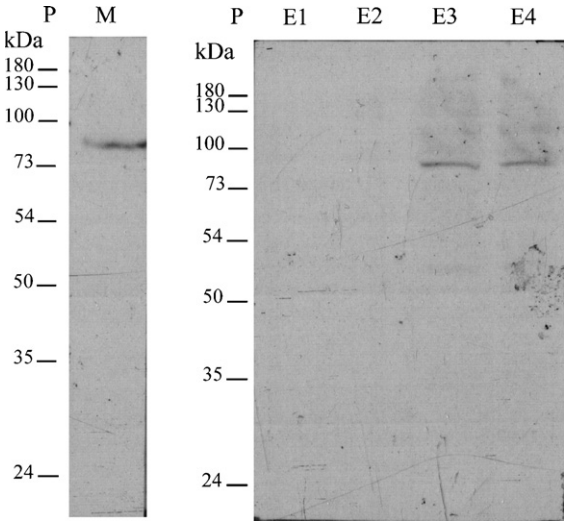

Fig. 1. Western blot of GRP78 after selection on a dengue serotype 2-affinity column. Nitrocellulose membranes with control membrane fraction (M) or elution fractions (E1, E2, E3, and E4) of a step gradient containing 0, 0.1, 0.5, and 1 M NaCl, were incubated with a polyclonal antibody directed against human GRP78 followed by a horseradish peroxidase labeled secondary antibody. Prestained protein marker (P) was used as a molecular mass marker as indicated.

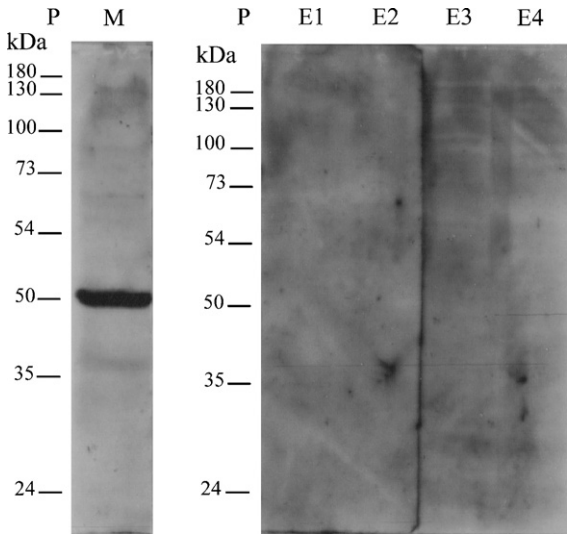

Fig. 2. Western blot of the 37/67 kDa high affinity laminin receptor after selection on a dengue serotype 2-affinity column. Filters used in Fig. 1 were stripped and re-probed with an antibody directed against the 37/67 kDa high affinity laminin receptor protein, followed by a horseradish peroxidase labeled secondary antibody. Prestained protein marker (P) was used as a molecular mass marker as indicated.

a horseradish peroxidase conjugated rabbit anti-goat IgG (31402, Pierce, Rockford, IL). Signal was developed as described previously. A single band of approximately 52 kDa was detected in the control strip with the antibody directed against the 37/67 kDa high affinity laminin binding protein while no band was seen in the NaCl eluted fractions (Fig. 2).

Virus overlay protein binding assays have previously identified GRP78 as a dengue virus serotype 2 receptor protein (Jindadamrongwech et al., 2004) and the 37/67 kDa high affinity laminin binding protein as a dengue virus serotype 1 receptor protein (Thepparit and Smith, 2004) expressed on the surface of HepG2 (liver) cells and shown that the interaction between the dengue virus and its cognate receptor is serotype specific (Jindadamrongwech et al., 2004; Thepparit and Smith, 2004). In this study affinity column chromatography was used to probe the interaction between dengue virus serotype 2 and HepG2 membrane proteins and results have again shown that there is a specific interaction between GRP78 and dengue virus serotype 2. As importantly, affinity chromatography again confirms the lack of an interaction between dengue virus serotype 2 and the 37/67 kDa high affinity laminin binding protein as proposed earlier (Thepparit and Smith, 2004) although other authors have proposed such an interaction occurs (Tio et al., 2005). This result suggests that while proteins maybe denatured as part of the virus overlay protein binding assay, the technique is still capable of selecting physiologically relevant binding molecules, possibly as a result of partial renaturation of the proteins during the overlay process. While column chromatography has been used by other to isolate putative dengue virus receptor proteins (Mercado-Curiel et al., 2006; Reyes-del Valle et al., 2005; Reyes-del Valle and del Angel, 2004) these studies have used significantly higher ligand concentrations (up to 10-fold higher) and as such have a much greater likelihood of isolating proteins through non-specific protein–protein interactions. The study here shows both that significantly lower ligand concentrations can be used to isolate dengue virus interacting proteins, and that the methodology is capable of discriminating serotype specific interactions. In contrast, previous studies (Mercado-Curiel et al., 2006; Reyes-del Valle et al., 2005; Reyes-del Valle and del Angel, 2004) have seen similar results for all four dengue serotypes. These results show that affinity chromatography can be used to investigate subtle interactions between the dengue viruses and their respective receptor or binding proteins and as such may play a role in future studies.

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