



## รายงานวิจัยฉบับสมบูรณ์

โครงการ การเข้าเซลล์ของไวรัสไข้เลือดออกโดยอาศัยและไม่อาศัยตัว  
ตอบรับบนผิวเซลล์

โดย Visiting Prof. Duncan R. Smith และคณะ

พฤษภาคม 2547

สัญญาเลขที่ BGJ4680018

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โครงการ การเข้าเชลล์ของไวรัสไข้เลือดออกโดยอาศัยและไม่ออาศัยตัว  
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คณะผู้วิจัย

สังกัด

1. Visiting Prof. Duncan R. Smith สถาบันอณูชีววิทยาและพันธุศาสตร์  
มหาวิทยาลัยมหิดล
2. นางสาวสุมารี จินดา darmangkon สถาบันอณูชีววิทยาและพันธุศาสตร์  
มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

สัญญาเลขที่ BGJ4680018

โครงการ “การเข้าเซลล์ของไวรัสไข้เลือดออกโดยอาศัยและไม่อาศัยตัวตอบรับบนผิวเซลล์”  
รายประจำปี

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## 1. Executive summary

### Objective

The objective of this grant was to obtain a functional characterization of a preliminarily identified dengue virus binding protein expressed on the surface of the liver cell line HepG2, as well as to provide the first imaging study on dengue virus internalization in a true target cell. Both of these objectives have been obtained and two papers describing aspects of the work have been either published or accepted for publication.

### Work of Project and Research

Using a combination of virus overlay protein binding assay (VOPBA) and mass spectroscopy, a candidate dengue virus binding protein (GRP78) was identified. The ability of this protein to function as a dengue virus receptor for dengue serotype 2 was established by fluorescent immunocytochemistry and functional inhibition with specific antibodies directed against GRP78. Further work showed that this receptor protein was specific for dengue virus serotype 2, and that VOPBA analysis revealed significant serotype heterogeneity of binding on liver cell membrane proteins. The entry of the virus into HepG2 cells was analyzed by electron microscopy on both target and non-target cells but no significant differences of entry modality were observed.

## 2. Results in 1 Year

### 2.1 Objective

Despite being a critical determinant of tissue tropism and thus of pathogenicity, the nature of the dengue virus (Family *Flaviviridae*, Genus *Flavivirus*, species *Dengue virus*) cellular receptor or receptors remains largely unknown. It is known that in cases of secondary dengue infection, where the virus becomes complexed with cross reacting but non-neutralizing antibodies from a primary infection with a different serotype the Fc receptor is used by the virus to gain entry to monocytes. However the identification of proteins used in primary infections of the dengue virus has been less successful, although several groups have identified potential virus binding proteins using the virus overlay protein binding assay (VOPBA) technique, and recently glycosaminoglycans, an LPS/CD14-associated binding protein and DC-SIGN have all been implicated as elements mediating dengue virus entry into cells. It is clear however that receptor usage is both cell type and serotype specific. Given that the liver is an established target organ, we sought to identify any protein or proteins that may be acting as a receptor on liver cells (HepG2) by a combination of VOPBA and mass spectrometry fingerprinting.

### 2.2 Work of Project

#### Virus and cells

Dengue serotype 2 (strain 16681) was a kind gift of Siritorn Butrapet (Center for Vaccine Development, Mahidol University, Thailand). Virus was propagated in Vero (African Green Monkey Kidney) cells. Vero cells were cultured at 37 °C, 2% CO<sub>2</sub> in Dulbecco's Modified Eagle's Medium (DMEM; HyClone, Logan, Utah, USA) supplemented with 5% heat inactivated fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, USA) and 100 units/ml of penicillin and 100 g/ml streptomycin. Virus was purified through a 30/60% sucrose step gradient in TNE buffer (10 mM Tris-HCl, pH 7.5, 140mM NaCl, 1mM EDTA). The human hepatoma cell line HepG2 was cultured at 37°C, 10% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, Utah, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, USA) and 100 units of penicillin and 100 g streptomycin.

#### Dengue virus production in HepG2 cells

HepG2 cells were grown in 75cm<sup>2</sup> tissue culture flasks containing 20ml of growth medium. Prior to confluence, when the cell number reached  $1.0 \times 10^7$  the culture media was discarded and replaced with 3mls of BA-1 (1 x Medium 199/Earle's balanced salts; 0.05M Tris-HCl pH 7.6, 1% BSA Fraction V, 7.5% NaHCO<sub>3</sub>, 100 units penicillin-streptomycin per ml) containing the dengue virus at a MOI of 1. Viral absorption was allowed to proceed for 90 minutes at 37°C with constant agitation. After this period cell/virus mixture was treated with acid glycine at pH 3.0 for 1 minute to inactivate any uninternalized viruses [19] and cells washed three times with PBS. Fresh culture media was added to the cells and the cells incubated under standard conditions. Aliquots of growth media were taken at hourly intervals between 10 and 24 hours and analyzed for viral titer in duplicate. Experiment was undertaken in duplicate. Data was analyzed using the GraphPad Prism program (GraphPad Software Inc, San Diego, Ca, USA).

### Cell membrane preparation

Cell membrane preparations were prepared essentially as described by Martinez-Barragan and del Angel [23] although with minor modifications. Briefly, exponentially growing HepG2 cells were harvested by scraping from 100mm x 20mm culture plates in TBS (50mM Tris HCl, pH 7.6, 150mM NaCl) and pelleted by centrifugation at 600 x g for 3 minutes followed by resuspension in 5 volumes ice cold buffer M (100mM Tris-HCl, pH 8.0, 2 mM MgCl<sub>2</sub>, 1mM EDTA, 0.2% Triton X-100, 1 mM PMSF). Cells were lysed by vortexing and solution was centrifuged at 600 x g to remove nuclei and cell debris. The supernatant was then centrifuged at 6000 x g to remove membranous organelles and then at 20,000 x g to pellet the membrane proteins. The pellet was resuspended in buffer M, and protein concentration determined by the Bradford method [6].

### Western blotting and Viral overlay protein binding assay (VOPBA)

Membrane proteins (80 µg) were separated by electrophoresis through an 8% SDS-PAGE gel and transferred to nitrocellulose membranes using the WetBlot technique (Bio-Rad, Richmond, CA, USA). After transfer was complete the membrane was blocked with 5% Skim milk in TBS at room temperature of 1 hour with constant agitation. For western blots the membranes were incubated with a 1:500 dilution of either anti-human GRP 78 goat polyclonal antibody N-20 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or anti-human GRP 78 rabbit polyclonal antibody H-129 (Santa Cruz Biotechnology Inc.) for 2 hours at room temperature. Following rinsing three times with TBS membranes were incubated with a 1:2000 dilution of either peroxidase conjugated anti-goat IgG or peroxidase conjugated anti-rabbit IgG (Pierce, Rockford, IL, USA) for 1 hour at room temperature. Signal was developed using the ECL Plus Western Blotting Analysis kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). For viral overlay the membranes were incubated with 1 x 10<sup>7</sup> pfu dengue serotype 2 virus in 1% skim milk in TBS. Hybridization was undertaken at 4°C for 2 hours followed by washing three times with TBS at room temperature. Immunoblotting was undertaken with a pan specific anti-dengue virus monoclonal antibody produced by hybridoma cell line HB-114, (a kind gift of Siritorn Butrapet) at a dilution of 1:100 in 5% skim milk in TBS. Incubation was undertaken at room temperature for 2 hours followed by washing three times with TBS. Following this, membranes were incubated at room temperature for 1 hours with HRP-conjugated rabbit anti-mouse IgG (Sigma Chemical Co., St Louis, MO, USA) at a dilution of 1: 3000 in 5% skim milk in TBS. Signal was developed as for western blotting.

### Mass spectrometry

Mass spectrometry was undertaken commercially by the Australian Proteome Analysis Facility (APAF). Bands were subjected to tryptic digestion for 16 hours followed by Matrix assisted laser desorption ionization (MALDI) mass spectrometry was performed with a Micromass Maldi Time of Flight (MALDI-TOF) Mass Spectrometer. Spectra were acquired in the mass range 750-3500 Da. Spectra were searched against Human using ProteinLynx on MassLynx by APAF.

### Inhibition of Infection

HepG2 cells ( $1 \times 10^6$ ) were pre-incubated with either anti-human GRP 78 goat polyclonal antibody N-20 (Santa Cruz Biotechnology Inc.), anti-human GRP 78 rabbit polyclonal antibody H-129 (Santa Cruz Biotechnology Inc.) or anti-human cytochrome C mouse monoclonal antibody A-8 (Santa Cruz Biotechnology, Inc.) for 1 hour at 37°C in 1 ml of serum -free medium. The cells were then washed twice with PBS and incubated with dengue serotype 2 at a multiplicity of infection (MOI) of 0.1 for 90 minutes at 37°C. Cells were treated with acid glycine pH 3.0 to inactivate un-internalized viruses [19], then allowed to grow under standard conditions for 24 hours before viral titer was assayed by plaque titration on Vero cells.

### Immunohistochemistry for cell surface expression of GRP78

HepG2 cells were grown on glass slides for 1 day and subsequently fixed with cold acetone for 10 minutes. Endogenous peroxidases were quenched by incubation with 5% H<sub>2</sub>O<sub>2</sub> for 5 minutes at room temperature. Cells were blocked with PBS containing 0.02% calf serum for 5 minutes at room temperature. Slides were then incubated with a 1:20 dilution in PBS of either anti-human GRP 78 goat polyclonal antibody N-20 (Santa Cruz Biotechnology Inc.), anti-human GRP 78 rabbit polyclonal antibody H-129 (Santa Cruz Biotechnology Inc.) or anti-human cytochrome C mouse monoclonal antibody A-8 (Santa Cruz Biotechnology, Inc.) for 2 hours at room temperature. After 2 washes with PBS slides were incubated with a 1:50 dilution of either peroxidase conjugated anti-goat IgG or peroxidase conjugated anti-rabbit IgG (Pierce, Rockford, IL, USA) for 1 hour at room temperature. Subsequent to washing with PBS slides were incubated with chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB; Dako Carpinteria, CA, USA) and incubated for 10 minutes at room temperature. The cells were counterstained with hematoxylin and viewed under a light microscope.

### Immunofluorescence of dengue virus 2 binding

HepG2 cells were grown on glass slides for 1 day and fixed in 1% formaldehyde for 15 minutes at room temperature. Cells were incubated with 10  $\mu$ g of either anti-human GRP 78 goat polyclonal antibody N-20 (Santa Cruz Biotechnology Inc.), anti-human GRP 78 rabbit polyclonal antibody H-129 (Santa Cruz Biotechnology Inc.) or anti-human cytochrome C mouse monoclonal antibody A-8 (Santa Cruz Biotechnology, Inc.) for 1 hour at room temperature. After incubation, slides were washed twice with PBS and dengue virus serotype 2 added at a ratio of 100 virus per cell and slides incubated for 2 hours at room temperature. After washing twice with PBS, slides were further incubated with a pan specific anti-dengue virus monoclonal antibody produced by hybridoma cell line HB-114, (a kind gift of Siritorn Butrapet) for 1 hour at room temperature. The slides were washed twice with PBS and then incubated in the dark with a 1:50 dilution of a goat anti-mouse IgG antibody labeled with FITC (Molecular Probes, Eugene, Oregon, USA) for 30 minutes at room temperature. After washing slides twice with PBS the slides were air dried, mounted with glycerol and viewed under a fluorescent microscope.

### 2.3 Results

To preliminarily identify the nature of the molecules on HepG2 cells involved in binding dengue viruses, the virus overlay protein binding assay (VOPBA) methodology was utilized. Membrane proteins from HepG2 cells were isolated and separated by SDS-PAGE on two parallel 8% gels. One gel was stained with Coomassie brilliant blue R-250, while the other gel was transferred to nitrocellulose solid matrix support by wet electroblotting. The resulting filter was hybridized with  $1 \times 10^7$  pfu of dengue serotype 2. A major virus binding band of approximately 90 kDa was visualized by subsequent western blotting using a pan specific monoclonal antibody directed against dengue E protein. Several other minor virus binding bands were also observed. Binding of the virus was shown to be lost by pre-treating cells with trypsin prior to membrane extraction. The position equivalent to the major virus binding band was extracted from the duplicate gel and sent for commercial mass spectrometry fingerprint analysis. The only significant match (18 matched peptides with coverage of 34.61% and MOWSE score of  $1 \times 10^5$ ) was glucose regulated protein 78 (GRP 78; BiP). The entire experiment was duplicated independently, and the extracted band again sent for mass spectrometry fingerprint analysis. Again, GRP 78 (BiP) was identified as the only significant match.

Given that GRP 78 (BiP) has been primarily characterized as an endoplasmic reticulum resident chaperonin [21] we sought to confirm the expression of GRP 78 (BiP) on the surface of HepG2 cells via immunohistochemistry. Two polyclonal antibodies directed against human GRP 78 (BiP) were evaluated, one directed against an N-terminal epitope (GRP 78 N-20) and one against a C-terminal epitope (GRP 78 H-129). Both antibodies showed significant levels of expression on the surface of HepG2 cells in contrast to an irrelevant antibody directed against human cytochrome C (Figure 3).

To investigate the position of the major VOPBA binding band as compared to the position of GRP78 (BiP) in the gel system used, we undertook parallel western blotting and VOPBA with the two antibodies. A total of two major and one minor band were identified with the two antibodies in the western blots, suggesting that the membrane associated GRP78 (BiP) may have different forms. The VOPBA binding band was co-incident with the upper, minor band of GPR78 (BiP) detected with the C-terminal epitope antibody. A corresponding band was not observed with the N-terminal epitope antibody, but this may be below the sensitivity of this antibody.

To investigate the binding of the dengue virus onto HepG2 cells in the presence of anti-GRP78 (BiP) antibodies, cells were pre-incubated with both anti-GRP 78 (BiP) antibodies, as well as an irrelevant control antibody prior to incubation with dengue virus serotype 2. Subsequent to incubation with the dengue virus, cells were incubated with a pan-specific anti-dengue monoclonal antibody and a fluorescent secondary antibody. Visualization under a fluorescent microscope showed a significant reduction from the levels of fluorescence seen with either with no antibody pre-incubation or pre-incubation with an irrelevant antibody for the pre-incubation with the antibody directed against the N-terminus of GRP78 (BiP), and a more equivocal picture with pre-incubation with the antibody directed against the C-terminal of GRP78 (BiP).

Functional confirmation of a candidate protein as a viral receptor can be achieved through a plaque reduction assay. However, as there is no validated plaque assay for HepG2 cells which do not grow in monolayer, we sought to evaluate the effect of pre-

incubation of HepG2 cells with antibodies directed against the putative receptor by determining the level of mature infectious virus particles produced. To avoid complications arising through multiple rounds of infection we first determined a virus production profile for dengue serotype 2 which determined that infectious viruses were first detectable 17 hours after infection. Hence viral levels detected at 24 hours after infection would result from a single non-amplified round of viral production. Antibody mediated inhibition of infection was carried out using 10  $\mu$ g/ per reaction of the two anti-GRP 78 polyclonal antibodies as well as 10  $\mu$ g of an irrelevant antibody (directed against human cytochrome C). Experiment was undertaken in twice, once in triplicate with three assays of each virus titer, and once in triplicate with six assays of each virus titer. Results show an inhibition to about 60% of control levels with pre-incubation of the cells with the antibody directed against the N-terminus of GRP 78 (BiP) while pre-incubation of HepG2 cells with an antibody directed against the C-terminus of GRP 78 (BiP) shows an enhancement to approximately 180% of control levels. The enhancement of infection with the antibody directed against the C-terminus of GRP 78 (BiP) was shown to be concentration dependent.

#### **2.4 Work in next time**

Work will continue to identify additional receptor proteins in this and other cell lines.

#### **3. Out put**

- 3.1 S. Jindadamrongwech, C. Thepparat, and D. R. Smith Identification of GRP 78 (BiP) as a liver cell expressed receptor element for dengue virus serotype 2. Arch Virol 2004; 149:915-927.
- 3.2 S. Jindadamrong wech and D. R. Smith Virus overlay protein binding assay (VOPBA) reveals serotype specific heterogeneity of dengue virus binding proteins on HepG2 human liver cells. Intervirology, accepted on April 20, 2004

# Appendix

## Identification of GRP 78 (BiP) as a liver cell expressed receptor element for dengue virus serotype 2

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**Summary.** This study sought to identify receptor elements for dengue virus serotype 2 on human liver cells (HepG2) using the viral overlay protein binding assay (VOPBA) technique and Mass Spectrometry fingerprinting. A single major and several minor virus binding bands were observed, and mass spectrometry identified a candidate binding protein for the major binding band as GRP 78 (BiP). GRP78 expression on the cell surface was confirmed, and antibodies directed against both the N and C-terminus of GRP 78 (BiP) altered both the binding of the virus to the cell surface as well as the infectivity profile of HepG2 cells in response to dengue serotype 2 infection. GRP 78 (BiP), which has previously been identified as a co-receptor protein for coxsackievirus A9, is the first non-Fc receptor protein identified for the dengue virus, although GRP78 probably functions as part of a receptor complex.

### Introduction

Despite being a critical determinant of tissue tropism and thus of pathogenicity, the nature of the dengue virus (family *Flaviviridae*, genus *Flavivirus*, species *Dengue virus*) cellular receptor or receptors remains largely unknown. It is known that in cases of secondary dengue infection, where the virus becomes complexed with cross reacting but non-neutralizing antibodies from a primary infection with a different serotype the Fc receptor is used by the virus to gain entry to monocytes [12]. However the identification of proteins used in primary infections of the dengue virus has been less successful, although several groups have identified potential virus binding proteins using the virus overlay protein binding assay (VOPBA) technique [22, 23], and recently glycosaminoglycans [8], an LPS/CD14-associated binding protein [9] and DC-SIGN [27] have all been

implicated as elements mediating dengue virus entry into cells. It is clear however that receptor usage is both cell type and serotype specific [2]. While infection with one of the four dengue serotypes usually results in no symptoms or a relatively mild, self limiting febrile disease [7], in a number of cases the disease can progress to a severe form with significant hemorrhage and significant mortality may result [18]. In these cases the liver is frequently involved in the disease [24], and the virus has been recovered from the livers of fatal cases of dengue infection [25]. Given that the liver is an established target organ, we sought to identify any protein or proteins that may be acting as a receptor on liver cells (HepG2) by a combination of VOPBA and mass spectrometry fingerprinting.

## Materials and methods

### *Virus and cells*

Dengue serotype 2 (strain 16681) was a kind gift of Siritorn Butrapet (Center for Vaccine Development, Mahidol University, Thailand). Virus was propagated in Vero (African Green Monkey Kidney) cells. Vero cells were cultured at 37 °C, 2% CO<sub>2</sub> in Dulbecco's Modified Eagle's Medium (DMEM; HyClone, Logan, Utah, U.S.A.) supplemented with 5% heat inactivated fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, U.S.A.) and 100 units/ml of penicillin and 100 µg/ml streptomycin. Virus was purified through a 30/60% sucrose step gradient in TNE buffer (10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1 mM EDTA). The human hepatoma cell line HepG2 was cultured at 37 °C, 10% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, Utah, U.S.A.) supplemented with 10% heat inactivated fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, U.S.A.) and 100 units of penicillin and 100 µg streptomycin.

### *Dengue virus production in HepG2 cells*

HepG2 cells were grown in 75 cm<sup>2</sup> tissue culture flasks containing 20 ml of growth medium. Prior to confluence, when the cell number reached  $1.0 \times 10^7$  the culture media was discarded and replaced with 3 ml of BA-1 (1 × Medium 199/Earle's balanced salts; 0.05 M Tris-HCl pH 7.6, 1% BSA Fraction V, 7.5% NaHCO<sub>3</sub>, 100 units penicillin-streptomycin per ml) containing the dengue virus at a MOI of 1. Viral absorption was allowed to proceed for 90 min at 37 °C with constant agitation. After this period cell/virus mixture was treated with acid glycine at pH 3.0 for 1 min to inactivate any uninternalized viruses [19] and cells washed three times with PBS. Fresh culture media was added to the cells and the cells incubated under standard conditions. Aliquots of growth media were taken at hourly intervals between 10 and 24 h and analyzed for viral titer in duplicate. Experiment was undertaken in duplicate. Data was analyzed using the GraphPad Prism program (GraphPad Software Inc., San Diego, Ca, U.S.A.).

### *Cell membrane preparation*

Cell membrane preparations were prepared essentially as described by Martinez-Barragan and del Angel [22] although with minor modifications. Briefly, exponentially growing HepG2 cells were harvested by scraping from 100 mm × 20 mm culture plates in TBS (50 mM Tris HCl, pH 7.6, 150 mM NaCl) and pelleted by centrifugation at 600 × g for 3 min followed by resuspension in 5 volumes ice cold buffer M (100 mM Tris-HCl, pH 8.0, 2 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.2% Triton X-100, 1 mM PMSF). Cells were lysed by vortexing and solution was centrifuged at 600 × g to remove nuclei and cell debris. The supernatant was then centrifuged

at  $6000 \times g$  to remove membranous organelles and then at  $20,000 \times g$  to pellet the membrane proteins. The pellet was resuspended in buffer M, and protein concentration determined by the Bradford method [6].

#### *Western blotting and Viral overlay protein binding assay (VOPBA)*

Membrane proteins ( $80 \mu\text{g}$ ) were separated by electrophoresis through an 8% SDS-PAGE gel and transferred to nitrocellulose membranes using the WetBlot technique (Bio-Rad, Richmond, CA, U.S.A.). After transfer was complete the membrane was blocked with 5% skim milk in TBS at room temperature of 1 h with constant agitation. For western blots the membranes were incubated with a 1:500 dilution of either anti-human GRP 78 goat polyclonal antibody N-20 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.) or anti-human GRP 78 rabbit polyclonal antibody H-129 (Santa Cruz Biotechnology Inc.) for 2 h at room temperature. Following rinsing three times with TBS membranes were incubated with a 1:2000 dilution of either peroxidase conjugated anti-goat IgG or peroxidase conjugated anti-rabbit IgG (Pierce, Rockford, IL, U.S.A.) for 1 h at room temperature. Signal was developed using the ECL Plus Western Blotting Analysis kit (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). For viral overlay the membranes were incubated with  $1 \times 10^7$  pfu dengue serotype 2 virus in 1% skim milk in TBS. Hybridization was undertaken at  $4^\circ\text{C}$  for 2 h followed by washing three times with TBS at room temperature. Immunoblotting was undertaken with a pan specific anti-dengue virus monoclonal antibody produced by hybridoma cell line HB-114, (a kind gift of Siritorn Butrapet) at a dilution of 1:100 in 5% skim milk in TBS. Incubation was undertaken at room temperature for 2 h followed by washing three times with TBS. Following this, membranes were incubated at room temperature for 1 h with HRP-conjugated rabbit anti-mouse IgG (Sigma Chemical Co., St Louis, MO, U.S.A.) at a dilution of 1:3000 in 5% skim milk in TBS. Signal was developed as for western blotting.

#### *Mass spectrometry*

Mass spectrometry was undertaken commercially by the Australian Proteome Analysis Facility (APAF). Bands were subjected to tryptic digestion for 16 h followed by Matrix Assisted Laser Desorption Ionization (MALDI) mass spectrometry was performed with a Micromass Maldi Time of Flight (MALDI-TOF) Mass Spectrometer. Spectra were acquired in the mass range 750–3500 Da. Spectra were searched against Human using ProteinLynx on MassLynx by APAF.

#### *Inhibition of infection*

HepG2 cells ( $1 \times 10^6$ ) were pre-incubated with either anti-human GRP 78 goat polyclonal antibody N-20 (Santa Cruz Biotechnology Inc.), anti-human GRP 78 rabbit polyclonal antibody H-129 (Santa Cruz Biotechnology Inc.) or anti-human cytochrome C mouse monoclonal antibody A-8 (Santa Cruz Biotechnology Inc.) for 1 h at  $37^\circ\text{C}$  in 1 ml of serum-free medium. The cells were then washed twice with PBS and incubated with dengue serotype 2 at a multiplicity of infection (MOI) of 0.1 for 90 min at  $37^\circ\text{C}$ . Cells were treated with acid glycine pH 3.0 to inactivate un-internalized viruses [19], then allowed to grow under standard conditions for 24 h before viral titer was assayed by plaque titration on Vero cells.

#### *Immunohistochemistry for cell surface expression of GRP78*

HepG2 cells were grown on glass slides for 1 day and subsequently fixed with cold acetone for 10 min. Endogenous peroxidases were quenched by incubation with 5%  $\text{H}_2\text{O}_2$  for 5 min at room temperature. Cells were blocked with PBS containing 0.02% calf serum for 5 min

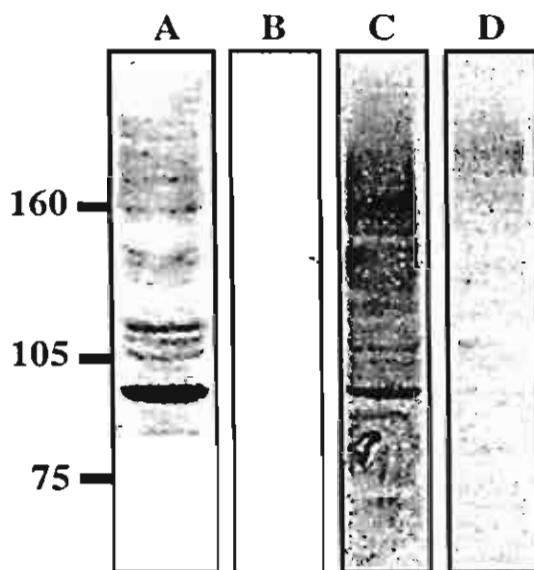
at room temperature. Slides were then incubated with a 1:20 dilution in PBS of either anti-human GRP 78 goat polyclonal antibody N-20 (Santa Cruz Biotechnology Inc.), anti-human GRP 78 rabbit polyclonal antibody H-129 (Santa Cruz Biotechnology Inc.) or anti-human cytochrome C mouse monoclonal antibody A-8 (Santa Cruz Biotechnology Inc.) for 2 h at room temperature. After 2 washes with PBS slides were incubated with a 1:50 dilution of either peroxidase conjugated anti-goat IgG or peroxidase conjugated anti-rabbit IgG (Pierce, Rockford, IL, U.S.A.) for 1 h at room temperature. Subsequent to washing with PBS slides were incubated with chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB; Dako Carpinteria, CA, U.S.A.) and incubated for 10 min at room temperature. The cells were counterstained with hematoxylin and viewed under a light microscope.

#### *Immunofluorescence of dengue virus 2 binding*

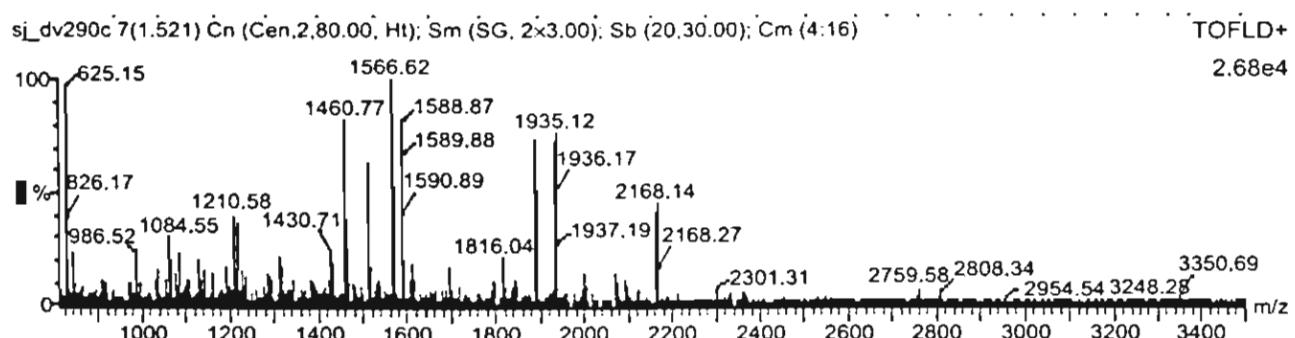
HepG2 cells were grown on glass slides for 1 day and fixed in 1% formaldehyde for 15 min at room temperature. Cells were incubated with 10 µg of either anti-human GRP 78 goat polyclonal antibody N-20 (Santa Cruz Biotechnology Inc.), anti-human GRP 78 rabbit polyclonal antibody H-129 (Santa Cruz Biotechnology Inc.) or anti-human cytochrome C mouse monoclonal antibody A-8 (Santa Cruz Biotechnology Inc.) for 1 h at room temperature. After incubation, slides were washed twice with PBS and dengue virus serotype 2 added at a ratio of 100 virus per cell and slides incubated for 2 h at room temperature. After washing twice with PBS, slides were further incubated with a pan specific anti-dengue virus monoclonal antibody produced by hybridoma cell line HB-114, (a kind gift of Siritorn Butrapet) for 1 h at room temperature. The slides were washed twice with PBS and then incubated in the dark with a 1:50 dilution of a goat anti-mouse IgG antibody labeled with FITC (Molecular Probes, Eugene, Oregon, U.S.A.) for 30 min at room temperature. After washing slides twice with PBS the slides were air dried, mounted with glycerol and viewed under a fluorescent microscope.

## Results

To preliminarily identify the nature of the molecules on HepG2 cells involved in binding dengue viruses, the virus overlay protein binding assay (VOPBA) methodology was utilized. Membrane proteins from HepG2 cells were isolated and separated by SDS-PAGE on two parallel 8% gels. One gel was stained with Coomassie brilliant blue R-250, while the other gel was transferred to nitrocellulose solid matrix support by wet electroblotting. The resulting filter was hybridized with  $1 \times 10^7$  pfu of dengue serotype 2. A major virus binding band of approximately 90 kDa was visualized by subsequent western blotting using a pan specific monoclonal antibody directed against dengue E protein (Fig. 1). Several other minor virus binding bands were also observed (Fig. 1). Binding of the virus was shown to be lost by pre-treating cells with trypsin prior to membrane extraction (Fig. 1). The position equivalent to the major virus binding band was extracted from the duplicate gel and sent for commercial mass spectrometry fingerprint analysis (Fig. 2). The only significant match (18 matched peptides with coverage of 34.61% and MOWSE score of  $1 \times 10^5$ ) was glucose regulated protein 78 GRP 78 (BiP). The entire experiment was duplicated independently, and the extracted band again sent for mass spectrometry fingerprint analysis. Again, GRP 78 (BiP) was identified as the only significant match.

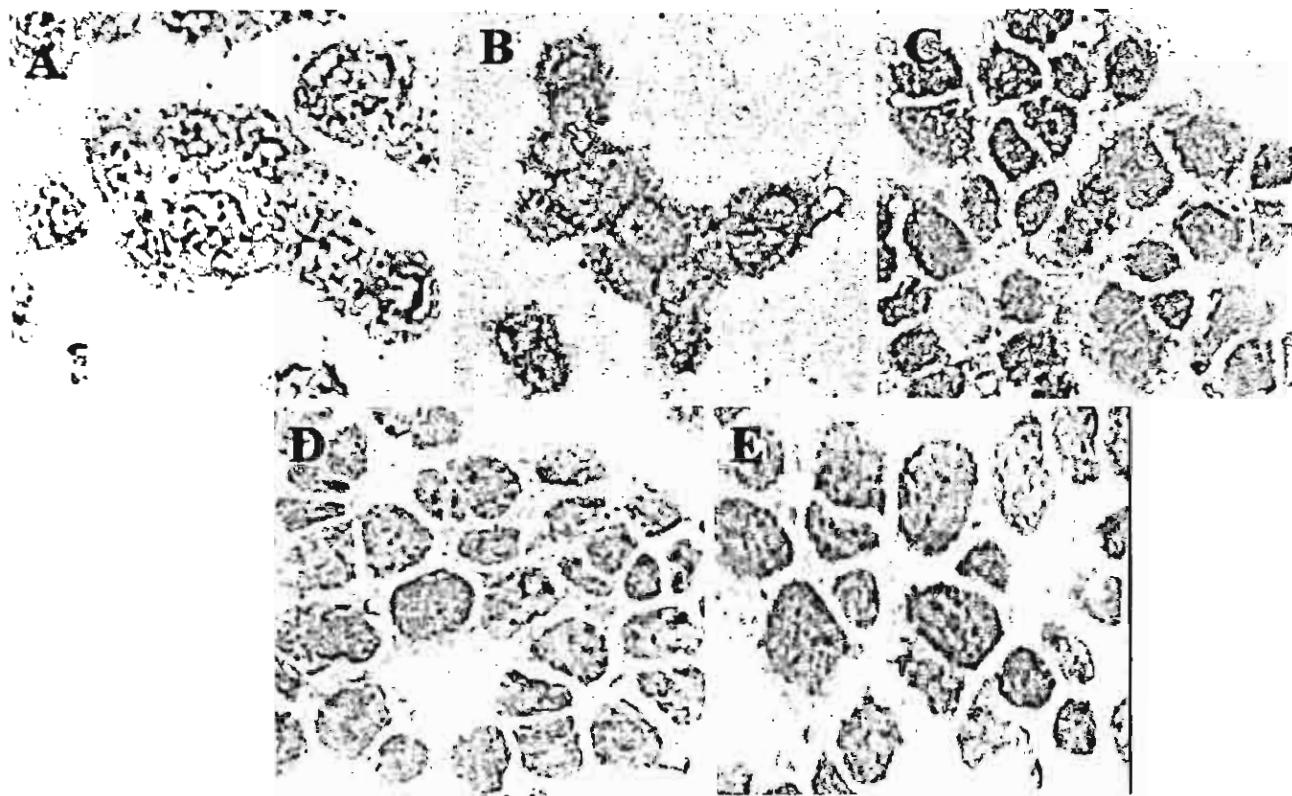


**Fig. 1.** VOPBA analysis of dengue virus serotype 2 on HepG2 cell membranes. Cell membranes were prepared from HepG2 and separated by SDS-PAGE. Proteins were transferred to solid matrix support and incubated with dengue virus serotype 2. Position of binding of dengue serotype 2 was detected by using a pan-specific monoclonal antibody directed against the dengue virus E protein (VOPBA). Panels A and C are independent experiments. Negative control (B) was obtained by incubating filter with no virus. The major binding band of approximately 90 kDa was established to be trypsin sensitive by pre-treating intact HepG2 cells with trypsin prior to membrane extraction (D). Position of protein marker bands are shown in kDa



**Fig. 2.** Spectra received from APAF (see Materials and methods) of MALDI-TOF analysis of dengue virus binding band from VOPBA experiment

Given that GRP 78 (BiP) has been primarily characterized as an endoplasmic reticulum resident chaperonin [21] we sought to confirm the expression of GRP 78 (BiP) on the surface of HepG2 cells via immunohistochemistry. Two polyclonal antibodies directed against human GRP 78 (BiP) were evaluated, one directed

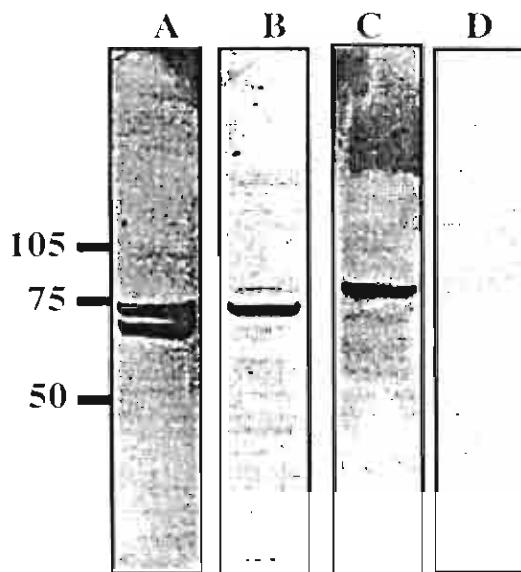


**Fig. 3.** Expression of GRP78 on the cell surface of HepG2 cells. HepG2 cells were grown on glass slides and reacted with polyclonal antibodies against the N-terminus (A and no primary antibody control, D) or C-terminus (B and no primary antibody control, E) of GRP 78 (BiP) as well as an irrelevant antibody (against human cytochrome C, C). Positive signal stains brown. Original magnification  $\times 400$

against an N-terminal epitope (GRP 78 N-20) and one against a C-terminal epitope (GRP 78 H-129). Both antibodies showed significant levels of expression on the surface of HepG2 cells (Fig. 3) in contrast to an irrelevant antibody directed against human cytochrome C (Fig. 3).

To investigate the position of the major VOPBA binding band as compared to the position of GRP78 (BiP) in the gel system used, we undertook parallel western blotting and VOPBA with the two antibodies. A total of two major and one minor band were identified with the two antibodies in the western blots, suggesting that the membrane associated GRP78 (BiP) may have different forms (Fig. 4). The VOPBA binding band was co-incident with the upper, minor band of GRP78 (BiP) detected with the C-terminal epitope antibody. A corresponding band was not observed with the N-terminal epitope antibody, but this may be below the sensitivity of this antibody.

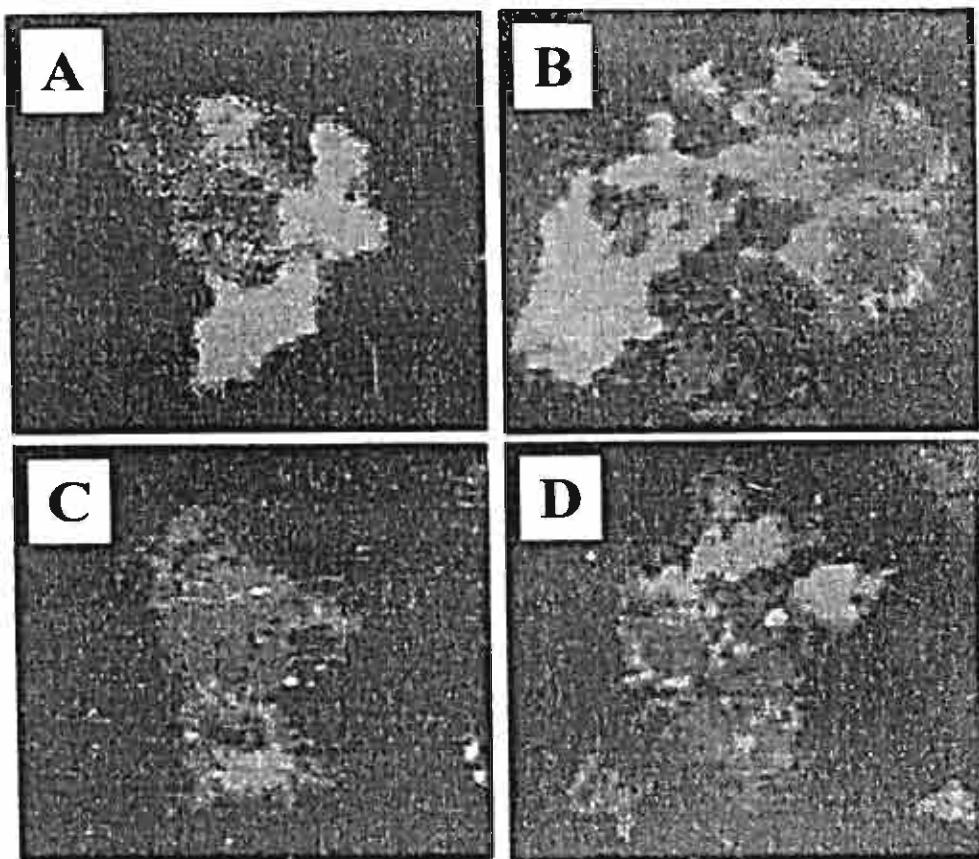
To investigate the binding of the dengue virus onto HepG2 cells in the presence of anti-GRP78 (BiP) antibodies, cells were pre-incubated with both



**Fig. 4.** Western blot and VOPBA analysis. HepG2 cell membrane proteins were extracted, separated by SDS-PAGE and transferred to solid matrix support. Filters were probed with polyclonal antibodies directed against either the N-terminus (A) or C-terminus (B) of GRP 78 (BiP), or incubated with dengue serotype 2 followed by detection of virus binding by incubation with a pan-specific monoclonal antibody directed against the dengue virus E protein (VOPBA; C). Negative control for VOPBA was obtained by incubating the filter with no virus (D). Position of protein marker bands are shown in kDa

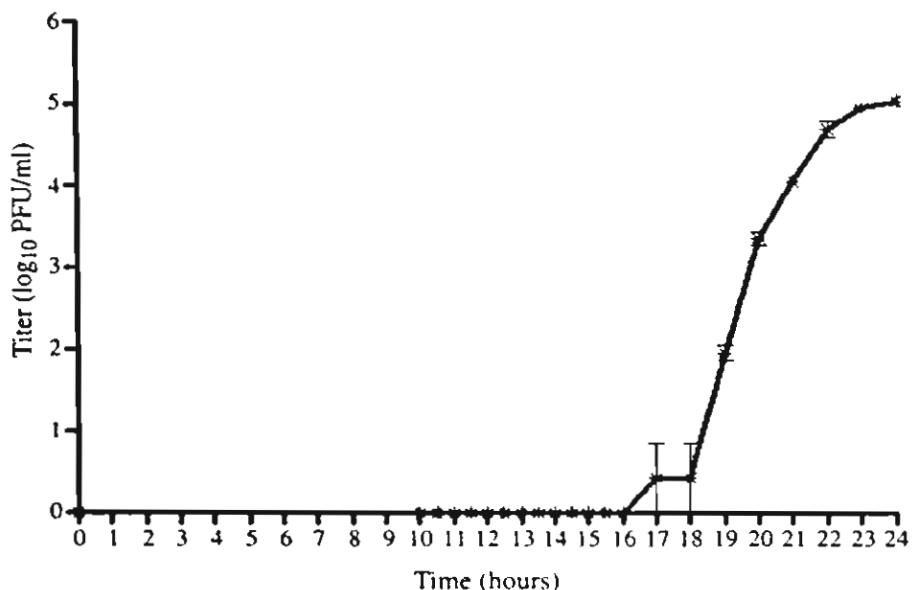
anti-GRP 78 (BiP) antibodies, as well as an irrelevant control antibody prior to incubation with dengue virus serotype 2. Subsequent to incubation with the dengue virus, cells were incubated with a pan-specific anti-dengue monoclonal antibody and a fluorescent secondary antibody. Visualization under a fluorescent microscope (Fig. 5) showed a significant reduction from the levels of fluorescence seen with either with no antibody pre-incubation or pre-incubation with an irrelevant antibody for the pre-incubation with the antibody directed against the N-terminus of GRP78 (BiP), and a more equivocal picture with pre-incubation with the antibody directed against the C-terminal of GRP78 (BiP).

Functional confirmation of a candidate protein as a viral receptor can be achieved through a plaque reduction assay [29]. However, as there is no validated plaque assay for HepG2 cells which do not grow in monolayer, we sought to evaluate the effect of pre-incubation of HepG2 cells with antibodies directed against the putative receptor by determining the level of mature infectious virus particles produced. To avoid complications arising through multiple rounds of infection we first determined a virus production profile for dengue serotype 2 (Fig. 6) which determined that infectious viruses were first detectable 17 h after infection. Hence viral levels detected at 24 h after infection would result from a single non-amplified round of viral production. Antibody mediated inhibition



**Fig. 5.** Fluorescence microscopy of dengue virus serotype 2 binding to HepG2 cells. HepG2 cells were grown on glass slides and incubated with the dengue virus after pre-incubation with antibodies directed against the N-terminus (C) or C-terminus (D) of GRP78 (BiP) or an irrelevant antibody (B) or no antibody (A). Binding of the dengue virus was visualized using a pan-specific monoclonal antibody directed against the dengue virus E protein and a FITC-conjugated secondary antibody. Samples were viewed under a fluorescent microscope. Original magnification  $\times 400$

of infection was carried out using  $10\ \mu\text{g}$  per reaction of the two anti-GRP 78 polyclonal antibodies as well as  $10\ \mu\text{g}$  of an irrelevant antibody (directed against human cytochrome C). Experiment was undertaken twice, once in triplicate with three assays of each virus titer, and once in triplicate with six assays of each virus titer. Results (Fig. 7) show an inhibition to about 60% of control levels with pre-incubation of the cells with the antibody directed against the N-terminus of GRP 78 (BiP) while pre-incubation of HepG2 cells with an antibody directed against the C-terminus of GRP 78 (BiP) shows an enhancement to approximately 180% of control levels. The enhancement of infection with the antibody directed against the C-terminus of GRP 78 (BiP) was shown to be concentration dependent (Fig. 7).

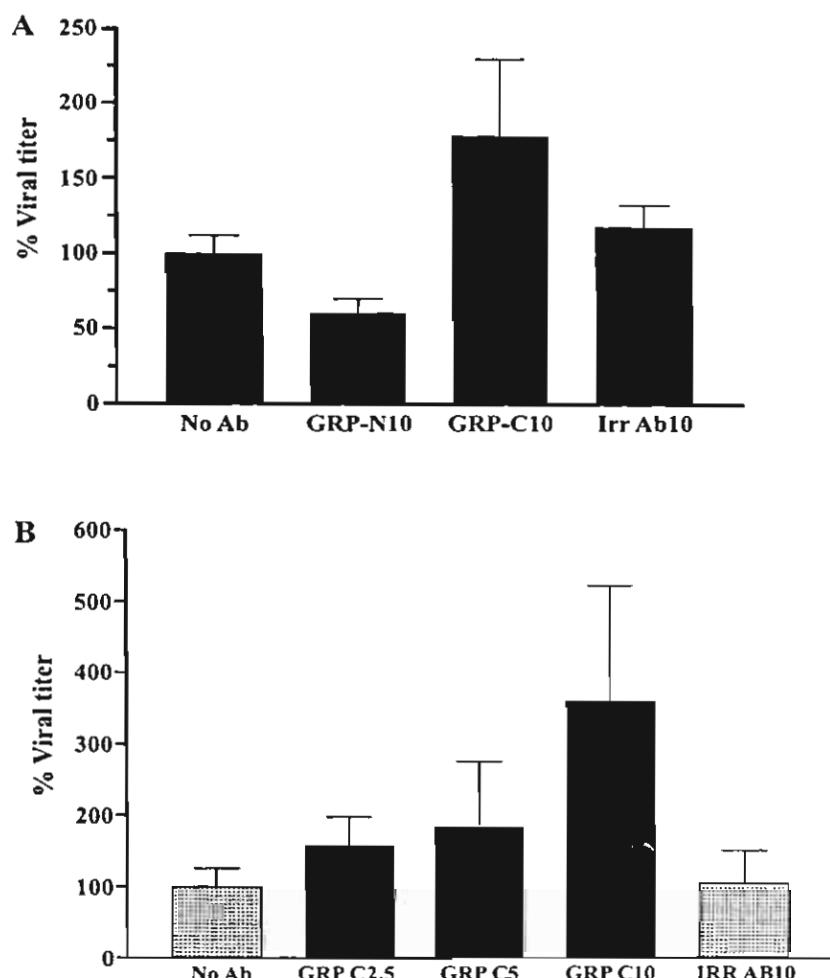


**Fig. 6.** Virus production curve of dengue serotype 2 in HepG2 cells. HepG2 cells were infected with dengue serotype 2 at a multiplicity of infection of 1 and un-internalized viruses inactivated with acid glycine at pH 3.0. Virus titer of the growth medium was determined hourly by plaque assay between 10 h and 24 h post infection

### Discussion

Glucose regulated protein 78 is known to be primarily resident in the endoplasmic reticulum [16] where it is believed to function as a chaperonin involved in the folding and assembly of MHC class 1 antigens [21]. GRP 78 was first identified as an immunoglobulin heavy chain binding protein termed BiP [17] and it was shown to bind to immunoglobulin heavy chains in the absence of light chains in pre-B lymphocytes. Recently, several studies have shown GRP78 (BiP) to be expressed on the cell surface [13, 26, 28, 30] a finding confirmed by this study. In the most recent of these studies [26] global profiling on five different cell lines showed an abundance of ER chaperone proteins (including GRP78, GRP75, HSP70, HSP60, HSP54 and HSP27) on the cell surface, confirming several studies that had previously identified HSP70 on the surface of a number of cell types [1, 3–5, 14, 20]. While the functionality of these proteins on the cell surface remains to be elucidated, it is interesting to note that GRP78 (BiP) has previously been identified as a co-receptor protein for the non-enveloped Coxsackievirus A9 [29]. In this system, GRP 78 (BiP) acts as a binding protein for the Coxsackievirus A9, but internalization of the virus requires the expression of major histocompatibility complex class I molecules [28].

In this report we have demonstrated that GRP78 (BiP) functions as a receptor element on liver cells for dengue virus serotype 2. Interestingly, antibodies directed against the N terminus of GRP 78 (BiP) inhibit both binding and infection, while those directed against the C-terminus of the protein enhance infection (but not no-



**Fig. 7.** **A** Antibody inhibition test. HepG2 cells were pre-incubated with either no antibody (No Ab), 10 µg of a polyclonal antibody directed against the N-terminus (GRP-N10) or C-terminus (GRP-C10) of GRP 78 (BiP) or 10 µg of an irrelevant monoclonal antibody (Irr Ab10) and subsequently incubated with dengue serotype 2 at a multiplicity of infection of 0.1. After 24 h the level of virus in the medium was assayed by plaque titer on Vero cells. Results are normalized against level of virus produced with no antibody pre-incubation and are the sum of two independent experiments carried out in triplicate with treble or six fold assay of titer. Error bars represent SEM. **B** Antibody inhibition test. HepG2 cells were pre-incubated with either no antibody (No Ab), 2.5 µg (GRP C2.5), 5 µg (GRP C5) or 10 µg (GRP C10) of a polyclonal antibody directed against the C-terminus of GRP 78 (BiP) or 10 µg of an irrelevant monoclonal antibody (IRR AB10) and subsequently incubated with dengue virus serotype 2 at a multiplicity of 0.1. After 24 h the level of virus in the medium was assayed by plaque titer on Vero cells. Results are normalized against level of virus produced with no antibody pre-incubation and are the sum of one experiment undertaken in triplicate with treble assay of titer. Error bars represent SEM

ticeably binding of the virus), albeit by approximately the same magnitude. While binding of proteins to GRP 78 (BiP) is believed to occur at the C-terminus of the protein, regulatory domains governing binding and acting through conformational changes in GRP 78 (BiP) are located at the N-terminus [10 and references therein], and as such it is likely that conformational changes induced through the binding of C-terminal antibodies may serve to enhance the ability of the virus to bind to the receptor, although it is possible that binding of the C-terminal antibody to GRP 78 (BiP) serves to activate the cell leading to enhanced viral replication.

Evidence from the western blot would suggest that it is actually a variant form of GRP 78 (BiP) that predominantly binds the dengue virus, although whether this is a translational or post-translational modification remains to be established, although post-translationally modified forms of GRP 78 (BiP) have been reported [15]. The immunoglobulin heavy chain binding function of GRP 78 (BiP) may be particularly significant in its interaction with the dengue virus as evidence has suggested that the functional binding motif of the dengue virus E protein resides in domain III and is characterized by an immunoglobulin like structure [11]. While this study has provided functional evidence that GRP78 (BiP) acts as a receptor element for dengue serotype 2 on liver cells by a virus reduction assay, further validation will require the transfection of a GRP78 (BiP) clone into a non-susceptible cell line to demonstrate the conversion of the cell line to a susceptible one. Given the dearth of non-susceptible cell lines, this may prove somewhat problematic. Finally, the presence of additional (albeit weaker) bands on the VOPBA analysis, coupled with only partial inhibition seen with the antibody directed against the N-terminus of GRP 78 (BiP) suggests that additional protein elements play a role in facilitating the entry of the virus into liver cells.

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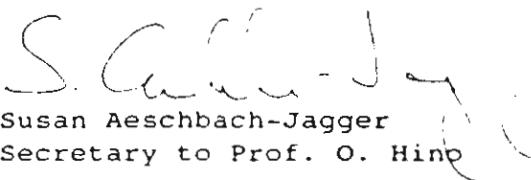
## VERSION 1

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specific heterogeneity of dengue virus binding proteins on  
hepg2 human liver cells

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**VIRUS OVERLAY PROTEIN BINDING ASSAY (VOPBA) REVEALS SEROTYPE SPECIFIC HETEROGENEITY OF DENGUE VIRUS BINDING PROTEINS ON HEPG2 HUMAN LIVER CELLS.**

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**Key Words:** dengue, flavivirus, HepG2, liver, receptor

**Running title:** Dengue virus binding proteins

**ABSTRACT**

**Objective:** This study sought to investigate the presence of dengue virus binding proteins expressed on the surface of HepG2 cells and to determine if there were serotype specific differences in binding.

**Methods:** HepG2 cell membrane proteins were extracted and separated by SDS-PAGE, transferred to nitrocellulose membranes and incubated with dengue virus serotypes 2, 3 and 4 under varying hybridization conditions. The positions of dengue virus binding proteins were established with a pan specific anti-dengue virus monoclonal antibody.

**Results:** Dengue virus binding proteins were seen at approximately 78-80, 90, 98, and 102 kDa for dengue serotype 2, 90, 130 and 182 kDa for dengue serotype 3 and 90 and 130 kDa for dengue serotype 4. Binding of the serotypes 3 and 4 was significantly altered by the hybridization conditions, while serotype 2 was affected to a lesser extent.

**Conclusions:** The virus overlay assay used here provides further evidence that there is a serotype specific component regulating the entry of the dengue virus into cells. Given that several virus binding proteins are seen for each serotype, multiple proteins may be required to facilitate the entry of the virus into some cell types.

## INTRODUCTION

The tissue tropism of a virus is a key determinant of viral pathogenicity, and this is often modulated by the presence or absence of appropriate molecules on the surface of a cell that can be used by the virus to gain entry into that cell. The dengue virus consists of four closely related but antigenically distinct serotypes termed dengue 1 to dengue 4 and is known to infect and replicate in several cell types, however the nature of the viral receptors is still unclear, although evidence suggests that the entry of the virus may be modulated by both cell and serotype specific factors [1].

While immune cells such as monocytes/macrophages and T lymphocytes have been proposed to be the main targets of the dengue virus [2], non-immune cells such as hepatocytes, endothelial cells, and brain cells have also been reported as potential targets [3 – 6]. Liver specimens from fatal cases of dengue fever have been shown to contain dengue virus antigens [7], and the virus itself has been isolated from the liver of fatal cases of dengue [8]. Additionally, changes in liver function have been associated with severe cases of dengue infection [9] suggesting that hepatocytes may be a target for the virus especially in the severe, fatal cases [10]. In a recent report we used the virus overlay protein binding assay to identify and characterize a dengue virus serotype 2 binding molecule expressed on the surface of HepG2 cells (a human liver cell line) and showed that antibodies to GRP78 inhibited infection, although only by approximately 2 fold [11]. Given that the dengue virus comprises of four closely related, but antigenically distinct serotypes we sought to further evaluate the binding of two additional serotypes of the dengue virus to HepG2 membrane proteins to determine the similarity of binding of these additional serotypes.

## MATERIALS AND METHODS

### Virus and cells

Dengue virus serotype 2 (strain 16681), serotype 3 (strain 16562), and serotype 4 (strain 1036) were kindly provided by Dr. Siritorn Butrapet (Center for Vaccine Development, Mahidol University, Thailand). Each serotype was propagated in Vero cells at 37 °C, 5% CO<sub>2</sub> in Dulbecco's Modified Eagle Medium (DMEM; HyClone, Logan, Utah, USA) supplemented with 5% heat inactivated fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, USA) and 100 units of penicillin and 100µg streptomycin/ml. Virus was purified by sucrose gradient ultracentrifugation at 4 °C and resuspended in TNF buffer (10 mM Tris-HCl, pH 7.5, 140mM NaCl, 1mM EDTA) containing 1% BSA. The human hepatoma cell line (HepG2) was cultured at 37°C, 10% CO<sub>2</sub> in 10% FBS-DMEM supplemented with 100 units of penicillin and 100µg streptomycin/ml.

### Cell membrane Preparation

HepG2 cells were scraped from culture plates and washed in TBS (50mM Tris HCl, pH 7.6, 150mM NaCl). Cells were lysed in ice cold buffer M (100mM Tris-HCl, pH 8.0, 2 mM MgCl<sub>2</sub>, 1mM EDTA, 0.2% Triton X-100, 1 mM PMSF) by vortexing and centrifuged at 600g for 3 minutes to remove nuclei and cell debris. The supernatant was collected and centrifuged at 6,000g for 5 minutes to remove membranous organelles and at 20,000g to pellet membrane proteins. The pellet was resuspended in buffer M and keep at -80°C until required. Protein concentrations were determined by the Bradford method [12].

### **Viral overlay protein binding assay (VOPBA)**

A total of 80 $\mu$ g HepG2 membrane proteins per lane were separated on an 8% SDS-PAGE gel and transferred overnight to nitrocellulose membranes by the WetBlot technique (Bio-Rad, Richmond, CA, USA). The membranes were blocked with 5% skim milk in TBS at room temperature (RT) for 1 hour with constant agitation. A total of 1x10<sup>7</sup> pfu purified dengue virus in 1% skim milk in TBS was incubated with the membranes for 2 hours at 4°C, RT, or 37 °C as indicated in the results. After three washes with TBS, the membranes were incubated with a pan specific anti-dengue virus monoclonal antibody (HB114), a gift from Dr. Siritorn Butrapet, at a dilution of 1:100 in 5% skim milk in TBS for 2 hours at RT. The membranes were washed and then incubated with HRP-conjugated rabbit anti-mouse IgG (Sigma Chemical Co., St Louis, MO, USA) at a dilution of 1:3000 in 5% skim milk in TBS for 1 hour at RT. The signal was generated by the ECL Plus Western Blotting Analysis kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). A reaction with no virus was used as the negative control in each experiment.

## RESULTS

To characterize the binding of the dengue virus serotypes 2, 3 and 4 to extracellular membrane proteins from HepG2 cells, membrane proteins were extracted and separated by electrophoresis through 8% SDS-PAGE gels and proteins transferred to nitrocellulose membranes by Wetblot electroblotting. Membranes were incubated with purified virus and the position of virus binding detected using a pan-specific anti-dengue monoclonal antibody. The initial virus overlay was undertaken at 4°C for 2 hours (Figure 1). The pattern of virus binding for serotype 2 was as reported previously [11], with a major virus binding band of 78-80 kDa, and three weaker bands of 90, 95 and 102 kDa, several bands between 130 and 150 kDa and 180 and 250 kDa. At an overlay temperature of 4°C, neither dengue serotype 3 nor serotype 4 bound efficiently to the membrane proteins. No signal was seen in the negative control lane (no virus).

Increasing the temperature of overlay to R/T (25°C) resulted in the loss of the 95kDa band for dengue serotype 2 as well as loss of some of the higher molecular weight weaker bands (Figure 1). Dengue virus serotype 3 produced a significant banding pattern at R/T with predominant bands of approximately 90 kDa, 130 kDa and 180 kDa and weaker bands of approximately 80 kDa and 100 kDa. Weak virus binding was observed for dengue serotype 4 at 78-80, 90 and approximately 130 kDa. Increasing the temperature further to 37°C for dengue serotypes 3 and 4 resulted in a further enhancement of the dengue binding signal. For serotype 3, predominant bands were noted at 90, 130 and 180 kDa, while for dengue serotype 4, predominant bands were observed at 90 and 130 kDa.

## DISCUSSION

Previous studies on dengue virus binding proteins using the virus overlay protein binding assay (VOPBA) have revealed variable sizes of protein bands [13-17]. However, these studies have used a range of different cells and virus serotypes, and as such comparisons between them are difficult. This is compounded by the fact that both dengue serotype and cell type may influence which protein receptors are used to effect cell entry [1, 18]. Additionally it has been reported that the dengue virus may have differences in cell tropism as determined by previous cell passage history [19-20]. Hence, it is difficult to compare between different studies, making identification of the dengue virus receptor complex. The variations due to cell type were excluded in this study by using the human hepatoma cell line, HepG2, as a target cell for dengue virus binding. We found that DEN-2 (strain 16681) predominantly bound a protein band of approximately 78-80 kDa from HepG2 membrane, while DEN-3 (strain 16562) and DEN-4 (strain 1036) bound the same two protein bands of approximately 90 and 130 kDa, as well as an additional 182 kDa in DEN-3. The absence of the 78-80 kDa band found in dengue 2 and previously characterized as GRP78 [11] from VOPBA of serotypes 3 and 4 would suggest that GRP78 may well be a serotype specific receptor element. Interestingly, while the hybridization of dengue serotype 2 to HepG2 membrane proteins is relatively unaffected by the hybridization temperature, serotypes 3 and 4 are sensitive to the temperature used. Previous VOPBA studies using C6/36 cells (derived from *Aedes albopictus*) and dengue serotype 4 have also noted different binding patterns at different temperatures with enhanced binding at higher hybridization temperatures [14, 17] suggesting that protein labiality may play a role in binding of the dengue virus to its cognate receptor proteins.

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**FIGURE LEGEND**

**Figure 1** Dengue virus binding to HepG2 cell membrane proteins at different temperatures by virus overlay protein binding assay (VOPBA). M represents the protein markers in kilodaltons

