รายงานวิจัยฉบับสมบูรณ์

โครงการ การเข้าเซลล์ของไวรัสไข้เลือดออกโดยอาศัยและไม่อาศัยตัวตอบรับบนผิวเซลล์

โดย Visiting Prof. Duncan R. Smith และคณะ

พฤษภาคม 2547

สัญญาเลขที่ BGJ4680018

รายงานวิจัยฉบับสมบูรณ์

โครงการ การเข้าเซลล์ของไวรัสไข้เลือดออกโดยอาศัยและไม่อาศัยตัวตอบรับบนผิวเซลล์

คณะผู้วิจัย	สังกัด
1. Visiting Prof. Duncan R. Smith	สถาบันอณูชีววิทยาและพันธุศาสตร์ มหาวิทยาลัยมหิดล
2. นางสาวสุมาลี จินดาดำรงเวช	สถาบันอณูชีววิทยาและพันธุศาสตร์ มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

สัญญาเลขที่ BGJ4680018

โครงการ "การเข้าเซลล์ของไวรัสไข้เลือดออกโดยอาศัยและไม่อาศัยตัวตอบรับบนผิวเซลล์"

รายประจำปี

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_2 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_2 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^2 tissue culture flasks containing 20ml of growth medium. Prior to confluence, when the cell number reached 1.0 x 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% $NaHCO_3$, 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 $MgCl_2$, 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 g) 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 10^7 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 x 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 witrh 5% H_2O_2 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.

Immunoflourescence 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 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 1×10^7 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 1×10^5) 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 g/per reaction of the two anti-GRP 78 polyclonal antibodies as well as 10 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. Thepparit, 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

Arch Virol (2004) 149: 915–927
DOI 10.1007/s00705-003-0263-x

Archives of Virology
Printed in Austria

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

S. Jindadamrongwech, C. Thepparit, and D. R. Smith

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

Received June 18, 2003; accepted October 17, 2003
Published online January 5, 2004

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_2 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_2 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^2 tissue culture flasks containing 20 ml of growth medium. Prior to confluence, when the cell number reached 1.0×10^7 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% $NaHCO_3$, 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 $MgCl_2$, 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 × g to remove membranous organelles and then at 20,000 × 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, 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 1×10^7 pfu dengue serotype 2 virus in 1% skim milk in TBS. Hybridization was undertaken at 4 °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 (1×10^6) 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 °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 °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% H_2O_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.

Immunoflourescence 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 1×10^7 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 1×10^5) 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.

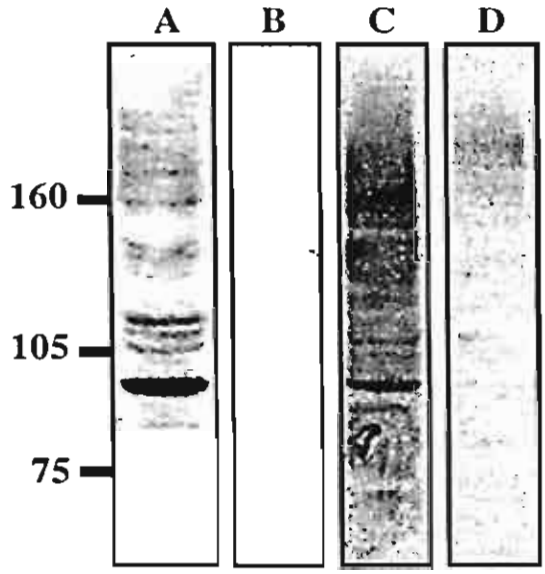

Fig. 1. VOPBA analysis of dengue virus serotype 2 on HepG2 cell membranes. Cell membranes were prepared from HepG2 and separated by SDS-PAGE. Proteins were transferred to solid matrix support and incubated with dengue virus serotype 2. Position of binding of dengue serotype 2 was detected by using a 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

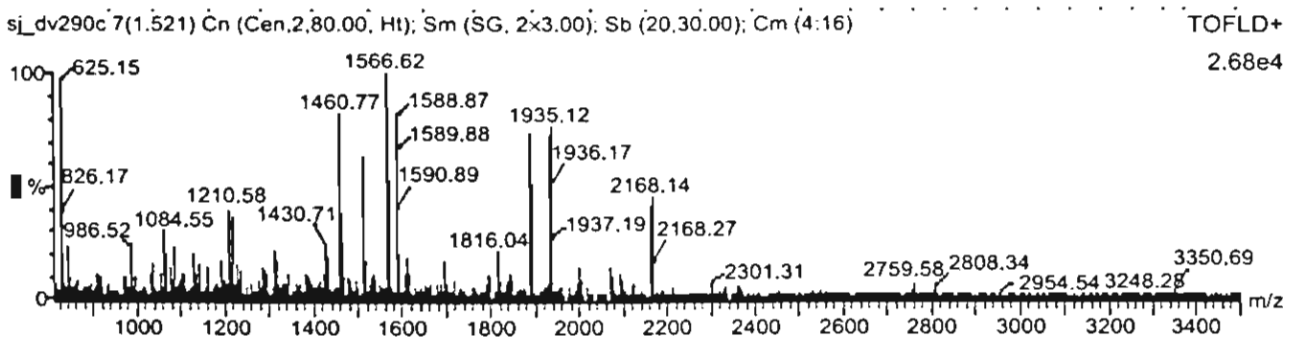

Fig. 2. Spectra received from APAF (see Materials and methods) of MALDI-TOF analysis of dengue virus binding band from VOPBA experiment

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

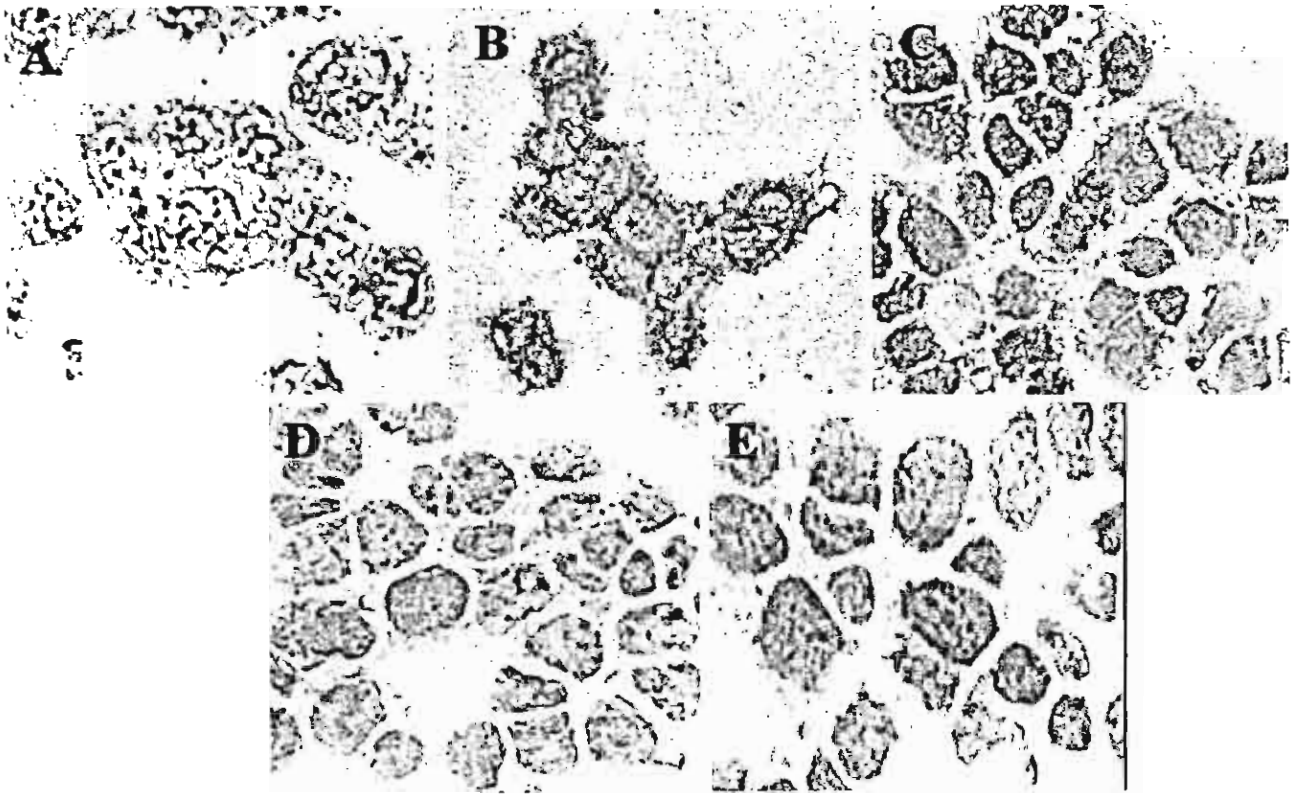

Fig. 3. Expression of GRP78 on the cell surface of HepG2 cells. HepG2 cells were grown on glass slides and reacted with polyclonal antibodies against the N-terminus (**A** and no primary antibody control, **D**) or C-terminus (**B** and no primary antibody control, **E**) of GRP 78 (BiP) as well as an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

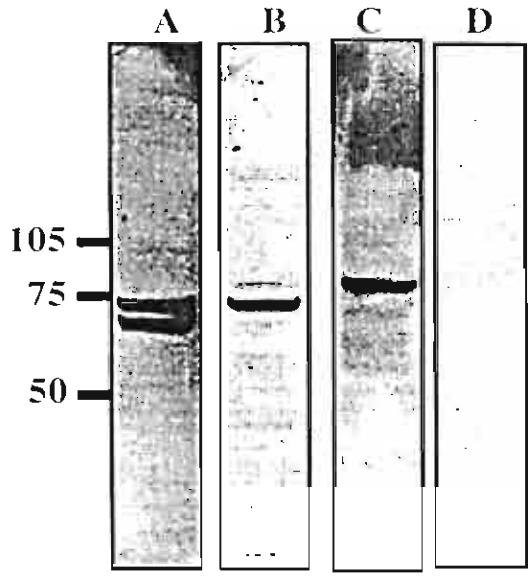

Fig. 4. Western blot and VOPBA analysis. HepG2 cell membrane proteins were extracted, separated by SDS-PAGE and transferred to solid matrix support. Filters were probed with polyclonal antibodies directed against either the N-terminus (**A**) or C-terminus (**B**) of GRP 78 (BiP), or incubated with dengue 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

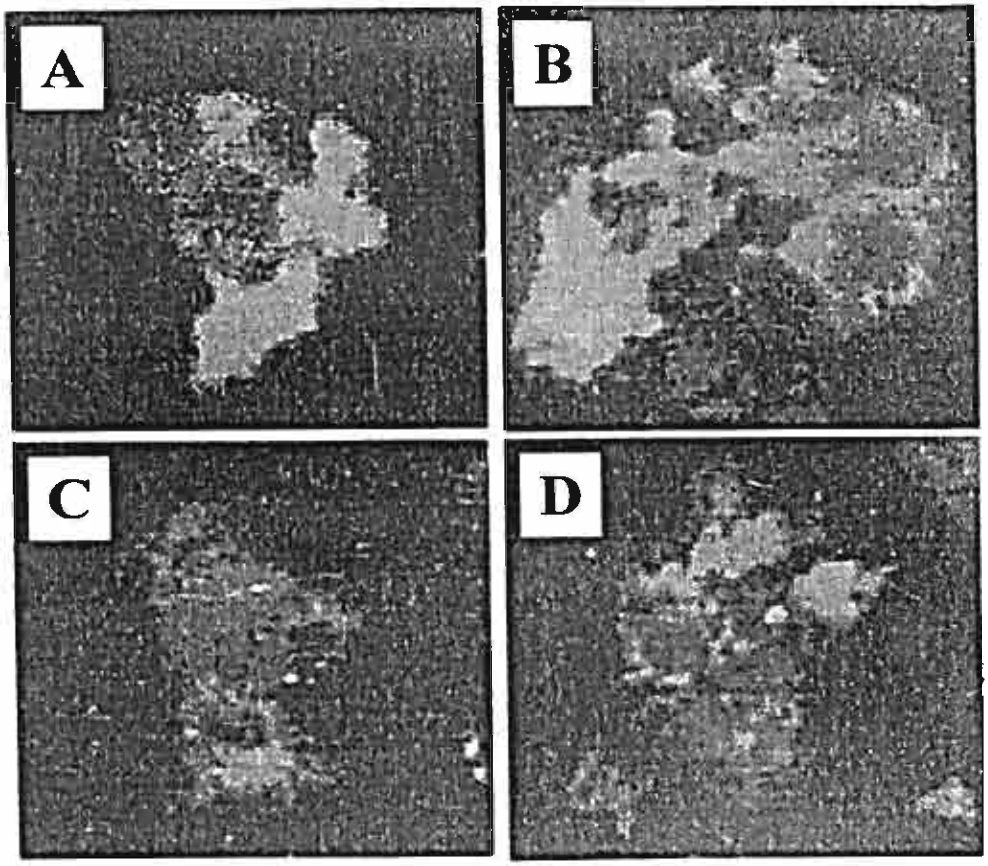

Fig. 5. Fluorescence microscopy of dengue virus serotype 2 binding to HepG2 cells. HepG2 cells were grown on glass slides and incubated with the dengue virus after pre-incubation with antibodies directed against the N-terminus (**C**) or C-terminus (**D**) of GRP78 (BiP) or an irrelevant antibody (**B**) 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 ×400

of infection was carried out using 10 μg/per reaction of the two anti-GRP 78 polyclonal antibodies as well as 10 μg 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).

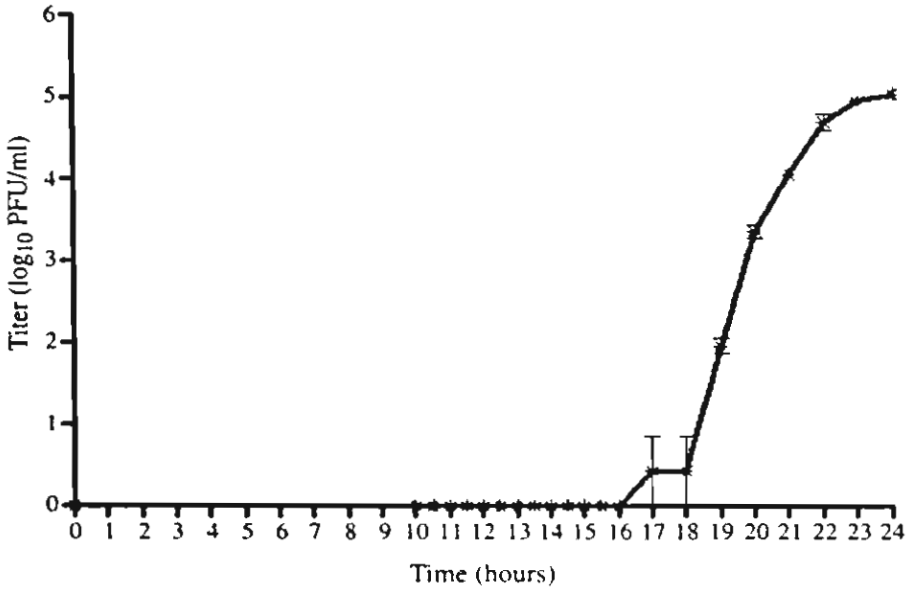

Fig. 6. Virus production curve of dengue serotype 2 in HepG2 cells. HepG2 cells were infected with dengue serotype 2 at a multiplicity of infection of 1 and un-internalized viruses inactivated with acid glycine at pH 3.0. Virus titer of the growth medium was determined hourly by plaque assay between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

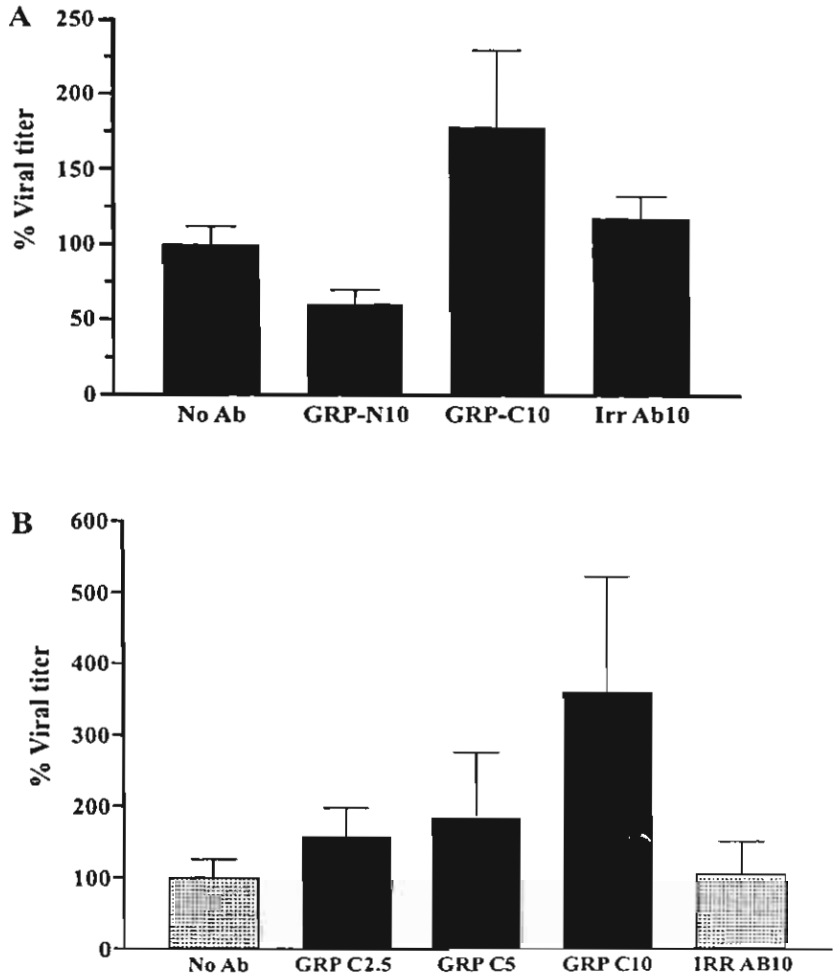

Fig. 7. **A** Antibody inhibition test. HepG2 cells were pre-incubated with either no antibody (No Ab), 10 μg of a polyclonal antibody directed against the N-terminus (GRP-N10) or C-terminus (GRP-C10) of GRP 78 (BiP) or 10 μg of an irrelevant monoclonal antibody (Irr Ab10) and subsequently incubated 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.

Acknowledgements

This work was supported by grant number BRG/04/2544 from the Thailand Research Fund. SJ and CT are Royal Golden Jubilee Fund Scholars. The authors thank Siritorn Butrapet for kindly providing some materials used in this study. Mass spectrometry analysis was undertaken commercially and we are required to state that "This research was facilitated by access to the Australian Proteome Analysis Facility under the Australian Government's Major National Research Facilities Program".

References

1. Asea A, Kraeft SK, Kurt-Jones EA, Stevenson MA, Chen LB, Finberg RW, Koo GC, Calderwood SK (2000) HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. Nat Med 6: 435–442
2. Bielefeldt-Ohmann H, Meyer M, Fitzpatrick DR, Mackenzie JS (2001) Dengue virus binding to human leukocyte cell lines: receptor usage differs between cell types and virus strains. Virus Res 73: 81–89
3. Botzler C, Issels R, Multhoff G (1996) Heat-shock protein 72 cell-surface expression on human lung carcinoma cells in associated with an increased sensitivity to lysis mediated by adherent natural killer cells. Cancer Immunol Immunother 43: 226–230