ARTICLE
Journal of Cellular Biochemistry 113:1681–1691 (2012)

Journal of Cellular Biochemistry

Endothelial Cell Surface Expression of Protein Disulfide Isomerase Activates β1 and β3 Integrins and Facilitates Dengue Virus Infection

Shu-Wen Wan,[1] Chiou-Feng Lin,[1,2,3,4] Yi-Tien Lu,[3] Huan-Yao Lei,[1,3,4] Robert Anderson,[5] and Yee-Shin Lin[1,3,4]*

[1]*Institute of Basic Medical Sciences, National Cheng Kung University Medical College, Tainan, Taiwan*
[2]*Institute of Clinical Medicine, National Cheng Kung University Medical College, Tainan, Taiwan*
[3]*Department of Microbiology and Immunology, National Cheng Kung University Medical College, Tainan, Taiwan*
[4]*Center of Infectious Disease and Signaling Research, National Cheng Kung University, Tainan, Taiwan*
[5]*Departments of Microbiology & Immunology and Pediatrics, Dalhousie University, Halifax, Nova Scotia, Canada*

ABSTRACT

Infection with dengue virus (DENV) causes diseases ranging from mild dengue fever to severe hemorrhage or shock syndrome. DENV infection of endothelial cells may cause cell apoptosis or vascular leakage and result in clinical illness of hemorrhage. However, the endothelial cell molecules involved in DENV infection and the mechanisms governing virus–cell interactions are still uncertain. Since protein disulfide isomerase (PDI) reducing function at the cell surface was shown to be required for entry of certain viruses and bacteria, we explored the role of PDI expressed on endothelial cell surface in DENV infection. Using siRNA to knock down PDI, DENV infection was reduced which could be reversed by treating cells with a reducing agent Tris(2-carboxyethyl)phosphine hydrochloride (TCEP). DENV-induced PDI surface expression was mediated through the Lys-Asp-Glu-Leu (KDEL) receptor-Src family kinase signal pathway. Furthermore, cell surface PDI colocalized with β1 and β3 integrins after DENV infection, and the activation of integrins was blocked by PDI inhibition. Finally, blockade of PDI inhibited DENV entry into endothelial cells. Our findings suggest a novel mechanism whereby surface PDI which causes integrin activation is involved in DENV entry, and DENV infection further increases PDI surface expression at later time points. These findings may have implications for anti-DENV drug design. J. Cell. Biochem. 113: 1681–1691, 2012.

KEY WORDS: VIRAL INFECTION; PROTEIN DISULFIDE ISOMERASE; INTEGRINS

Dengue virus (DENV), a Flavivirus of global public health importance, causes diseases ranging from mild dengue fever to severe life-threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Thrombocytopenia, vascular leakage, and liver damage are major clinical manifestations of DHF/DSS [Tan and Alonso, 2009]. A number of mechanisms including antibody-dependent enhancement [Halstead, 2003; Huang et al., 2006], viral serotype variation [Bielefeldt-Ohmann, 1997], abnormal immune activation [Clyde et al., 2006], and autoimmunity [Lin et al., 2011] have been implicated in the pathogenesis of DHF/DSS.

Increased capillary permeability or endothelial damage may cause vascular leakage and result in clinical illness in DHF/DSS. Although it remains controversial whether endothelial cells are a target for DENV infection, studies on dengue patient tissue specimens showed that dengue viral antigens can be detected in the liver and lung endothelium [Jessie et al., 2004]. Microvascular endothelial cell apoptosis was demonstrated in pulmonary and intestinal tissues from fatal cases of DHF/DSS [Limonta et al., 2007]. Furthermore, DENV can infect endothelial cells in vitro and lead to chemokine production, complement activation, and apoptosis

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: National Science Council; Grant numbers: NSC99-2321-B006-008, NSC100-2325-B006-007; Grant sponsor: National Health Research Institutes; Grant number: NHRI-100A1-PDCO-0209115; Grant sponsor: Multidisciplinary Center of Excellence for Clinical Trial and Research, Department of Health; Grant number: DOH100-TD-B-111-002.

*Correspondence to: Dr. Yee-Shin Lin, Department of Microbiology and Immunology, National Cheng Kung University Medical College, 1 University Road, Tainan 701, Taiwan. E-mail: yslin1@mail.ncku.edu.tw

Received 13 December 2011; Accepted 14 December 2011 • DOI 10.1002/jcb.24037 • © 2011 Wiley Periodicals, Inc.
Published online 22 December 2011 in Wiley Online Library (wileyonlinelibrary.com).

[Avirutnan et al., 1998]. Finally, studies using a mouse model of DENV-induced hemorrhage showed that endothelial cells can be targets of DENV infection and become apoptotic [Chen et al., 2007; Yen et al., 2008]. However, the endothelial cell molecules involved in DENV infection and the mechanisms governing the virus–cell interactions are still uncertain.

A role for protein disulfide isomerase (PDI) at the cell surface has been demonstrated in the entry of several viruses, including HIV [Ryser and Fluckiger, 2005; Ou and Silver, 2006], Newcastle disease virus [Jain et al., 2009], and Sindbis virus [Abell and Brown, 1993]. A recent study also showed that Chlamydia attachment and entry required PDI and that the thiol-mediated oxidoreductive function of PDI was essential for entry, although the precise target of PDI activity remains unclear [Abromaitis and Stephens, 2009]. PDI is mainly localized in the endoplasmic reticulum (ER) and acts as a chaperone. However, it is also detected in the nuclear envelope, cytosol, and other endomembranes, such as the Golgi, secretory vesicles, and plasma membrane [Turano et al., 2002]. In addition, PDI is exported from the ER despite possessing the Lys-Asp-Glu-Leu (KDEL) retention sequence, and is deposited on the cell surface [Yoshimori et al., 1990; Terada et al., 1995]. Cell surface PDI has reducing activity for exterior thiols [Turano et al., 2002].

PDI mediates platelet aggregation and secretion by activating αIIbβ3 integrin [Manickam et al., 2008]. PDI is involved in β1 and β7 integrin-mediated lymphocyte adhesion [Mou et al., 1998] and enhances αvβ3 integrin adherence to its ligand vitronectin on Mn^{2+}-stimulated endothelial cells [Swiatkowska et al., 2008]. A variety of viruses infect cells by using integrins as receptors or co-receptors. For example, Foot-and-mouth disease virus binds to Arg-Gly-Asp (RGD)-associated integrins αvβ1, αvβ3, and αvβ6 [Jackson et al., 2002]. In addition, integrin αvβ3 acts as a co-receptor for human cytomegalovirus [Wang et al., 2005]. Notably, a recent report showed that DENV induced upregulated expression of β3 integrin which was associated with DENV entry into human endothelial cells [Zhang et al., 2007]. In the present study, we investigated the role of PDI on endothelial cell surface in DENV infection and its correlation with integrins. We found that cell surface expression of PDI and subsequent activation of β1 and β3 integrins facilitates DENV infection in endothelial cells.

MATERIALS AND METHODS

CELL AND VIRUS CULTURES

Human microvascular endothelial cell line-1 (HMEC-1) was obtained from the Centers for Disease Control and Prevention (Atlanta, GA), and passaged in culture plates using endothelial cell growth medium M200 (Cascade Biologics, OR) containing 2% fetal bovine serum, 1 μg/ml hydrocortisone, 10 ng/ml epidermal growth factor, 3 ng/ml basic fibroblast growth factor, 10 μg/ml heparin, and antibiotics.

DENV2 (PL046) was propagated in C6/36 cells. Briefly, C6/36 monolayers were incubated with DENV at multiplicity of infection (MOI) of 0.01 at 28°C in 5% CO_2 for 5 days. The cultured medium was harvested, and cell debris was removed by centrifugation at 900*g* for 10 min. After further centrifugation at 16,000*g* for 10 min, the virus supernatant was collected and stored at −70°C until experiments. Virus titer was determined by plaque assay using BHK-21 cells.

INFECTION OF ENDOTHELIAL CELLS WITH DENV

HMEC-1 cells were inoculated with DENV for 1 h at 37°C. For Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) pretreatment, cells were cultured with 0.01 mM of TCEP (Sigma–Aldrich, MO) for 10 min at room temperature and then washed twice with non-TCEP medium to remove TCEP before addition of DENV. After centrifugation to remove virus particles in the culture supernatant, 2×10^5 cells were seeded into each well of six-well tissue-culture plates or 5×10^4 cells were seeded into each well of 24-well tissue-culture plates. TCEP pretreatment had no effect on cell attachment. To clarify the pathway of PDI surface expression, the infected cells were treated with ER–Golgi inhibitor brefeldin A (BFA; Sigma–Aldrich) or Src family kinase (SFK) inhibitors, PP1 and PP2 (TOCRIS Bioscience, MO) at 6 h before cell harvesting. Culture media and infected cells were harvested at various time points after DENV infection for further experiments.

FLOW CYTOMETRY

For surface staining, cells were stained with anti-PDI (Stressgen Biotechnologies, BC, Canada) or rabbit control IgG (Santa Cruz Biotechnology, CA), anti-β1 integrin (clone TDM29, Millipore, MA), anti-β3 integrin (clone VI-PL2), or mouse isotype control IgG_1 (BD Biosciences, San Jose, CA) at 4°C for 1 h. After washing twice with PBS, the samples were incubated with 1 μl of 1 mg/ml Alexa-488-conjugated donkey anti-rabbit IgG or goat anti-mouse IgG (Invitrogen, CA) at 4°C for 30 min. The cells were then fixed with 1% formaldehyde in PBS at room temperature for 10 min and washed twice with PBS. For intracellular staining, cells were fixed with 1% formaldehyde in PBS at room temperature for 10 min. After washing, the cells were stained with anti-DENV envelope (E) monoclonal antibody (clone 137-22, obtained from H.Y. Lei) and isotype control IgG_1 (BD Biosciences) in permeabilization buffer (2% bovine serum albumin, 0.1% saponin, and 0.1% sodium azide) at 4°C for 30 min. After washing twice with PBS, samples were incubated with 1 μl of 1 mg/ml Alexa-488-conjugated goat anti-mouse IgG (Invitrogen) at 4°C for 30 min. Cells were fixed with 1% formaldehyde in PBS at room temperature for 10 min. After washing, the cells were stained with FITC-anti-DENV E monoclonal antibody (clone 137-22) and FITC-isotype control IgG_1 (BD Biosciences) in permeabilization buffer at 4°C for 1 h. For confirmation of knockdown efficiency of KDEL receptor, cells were fixed with 1% formaldehyde in PBS at room temperature for 10 min. After washing, cells were stained with anti-KDEL receptor antibody (Stressgen) or isotype control IgG_1 (BD Biosciences) in PBS at 4°C for 1 h. After washing twice with PBS, samples were incubated with 1 μl of 1 mg/ml Alexa-488-conjugated goat anti-mouse IgG (Invitrogen) at 4°C for 30 min. The samples were analyzed by flow cytometry (FACSCalibur; BD Biosciences) with excitation set at 488 nm. The percentage of positive cells was determined by comparison with the isotype control and the percentage of cells with higher PDI or integrin expression was compared with mock (uninfected) cells for statistical analysis.

WESTERN BLOTTING

For whole cell extracts, the cells were lysed with a buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris (pH 7.5), 5 mM EDTA, 5 mM NaN_3, 10 mM NaF, 10 mM sodium prophosphate and protease inhibitor mix cocktail I (Sigma). After being freeze-thawed once, cell lysates were centrifuged at 14,000*g* at 4°C for 20 min. The surpernatant was collected and boiled in sample buffer for 15 min. The biotinylated surface proteins were prepared using the Cell Surface Protein Isolation Kit (Pierce, IL). The whole cell extracts and cell fractions were resolved on SDS–PAGE and electrotransferred onto polyvinylidene difluoride membranes (Millipore, MA) using a semi-dry cell transfer blot (Bio-Rad). After blocking, the blots were incubated with rabbit antibody against PDI (Stressgen) and antibody against β3 integrin (Cell Signaling Technology), or mouse antibody against β1 integrin (clone 18/CD29, BD Biosciences). Mouse antibodies specific for β-actin (Sigma–Aldrich) and GAPDH (Santa Cruz Biotechnology) were used for internal controls. Finally, blots were hybridized with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Cell Signaling Technology) and developed using an ECL substrate kit (Millipore).

CONFOCAL MICROSCOPY

For immunofluorescence staining, HMEC-1 cells were grown on glass slides and infected with DENV as described above. For surface staining, cells were stained with Alexa-594-conjugated Cholera Toxin Subunit B (CT-B; Invitrogen) and anti-PDI (Stressgen) at 37°C for 30 min. After washing, the cells were fixed with 1% formaldehyde in PBS at room temperature for 10 min, followed by Alexa-633-conjugated goat anti-rabbit IgG (Invitrogen) at room temperature for 2 h. After washing, cells were stained with antibodies against DENV E protein in permeabilization buffer at 4°C for 1 h and Alexa-488-conjugated goat anti-mouse IgG (Invitrogen) at room temperature for 2 h. For colocalization assay, cells were fixed with 1% formaldehyde in PBS at room temperature for 10 min. After washing, cells were stained with antibodies against PDI (Stressgen) or phosphorylated SFKs (Cell Signaling Technology) at 4°C for 1 h followed by Alexa-594-conjugated donkey anti-rabbit IgG (Invitrogen) or Dylight-546-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, PA) at 4°C for 30 min. Washed cells were next stained with antibodies against active form β1 integrin (clone HUTS-4), αvβ3 integrin (clone LM609) (Millipore), KDEL receptor (Stressgen), or GM130 (BD Transduction Laboratories) at 4°C for 1 h and then Alexa-488-conjugated goat anti-mouse IgG or Alexa-633-conjugated goat anti-mouse IgG (Invitrogen) at 4°C for 30 min. After washing, cells were stained with FITC-conjugated antibodies against DENV E protein in permeabilization buffer at 4°C for 1 h. The nuclei of cells were stained with DAPI (Calbiochem). Fluorescence image studies were performed using a laser confocal microscope (Olympus FV-1000).

ADHESION ASSAY

The 96-well plates were coated with fibrinogen (100 μg/ml), vitronectin (5 μg/ml; Calbiochem), fibronectin (1 μg/ml) or collagen I (1 μg/ml; Sigma–Aldrich) for 1 h at 37°C. After blocking with 1% bovine serum albumin in PBS, 5×10^4 cells in 100 μl serum-free medium were added to the plate and incubated for 1 h at 37°C. After washing to remove the non-adherent cells, the cells were fixed and stained with 0.1% crystal violet aqueous solution in 20% methanol for 15 min. After washing with water, the stained cells were dissolved with 33% acetic acid. The optical density at 590 nm was measured using a microplate reader.

TRANSFECTION WITH siRNA

4×10^5 HMEC-1 cells were seeded into each well of six-well plates and transfected with 20 nM of β1 integrin (HSS105560) and β3 integrin (HSS179982; Invitrogen), 100 nM of PDI siRNA (5′-GGACCATGAGAACATCGTC-3′) [Park et al., 2006], KDEL receptor siRNA (5′-GGUGUUCACUGCCCGAUAU-3′) [Pulvirenti et al., 2008], or control siRNA (5′-ACTACCGTTGTTATAGGTG-3′) [Ou and Silver, 2006] by lipofectamin™ 2000 (Invitrogen). After 72 h, the transfection efficiency was confirmed using Western blot analysis for PDI, β1, and β3 integrin, or using flow cytometry for KDEL receptor.

VIRAL ENTRY ASSAY

Cells were pretreated with 0.01 mM of TCEP for 10 min at room temperature or incubated with 50 μg/ml of function-blocking antibodies against PDI (Alexis Biochemicals, Switzerland), β1 integrin (clone 6S6) and β3 integrin (clone B3A; Millipore) for 30 min at 4°C and then washed twice with medium. Cells were then inoculated with DENV for 2 h at 37°C, and the supernatant containing unbound virus was removed by centrifugation. Residual virus was inactivated with acid citrate buffer (pH 3.0) and removed by extensive washing with medium. The cells were then freeze-thawed three times, and the titers of intracellular viruses were detected by plaque assay.

STATISTICS

Comparisons between various treatments were performed by unpaired *t*-test with SigmaPlot version 10.0. Statistical significance was set at $P < 0.05$.

RESULTS

TREATMENT WITH DISULFIDE REDUCING AGENT ENHANCES DENV INFECTION INTO ENDOTHELIAL CELLS

Because of the role of PDI-mediated disulfide bond reduction in viral and bacterial entry [Abell and Brown, 1993; Ryser and Fluckiger, 2005; Ou and Silver, 2006; Abromaitis and Stephens, 2009; Jain et al., 2009], we first tested whether it was the reducing function which had a role in DENV entry into cells. HMEC-1 cells were infected with DENV2 (PL046) for various time periods in the presence or absence of membrane-impermeable disulfide reducing agent TCEP. Figure 1A shows that TCEP enhanced DENV infection into HMEC-1 cells up to 48 h as determined by DENV E protein expression. We next used siRNA to knock down PDI and found that DENV infection was reduced, and this inhibitory effect was rescued by addition of TCEP (Fig. 1B). Our results, therefore, show that cell surface disulfide bond reduction can enhance DENV infection in the endothelial cells. To exclude the possibility that the increased DENV infection was due to the cytotoxicity of TCEP, the release of lactate dehydrogenase (LDH) into the supernatant was measured and cell

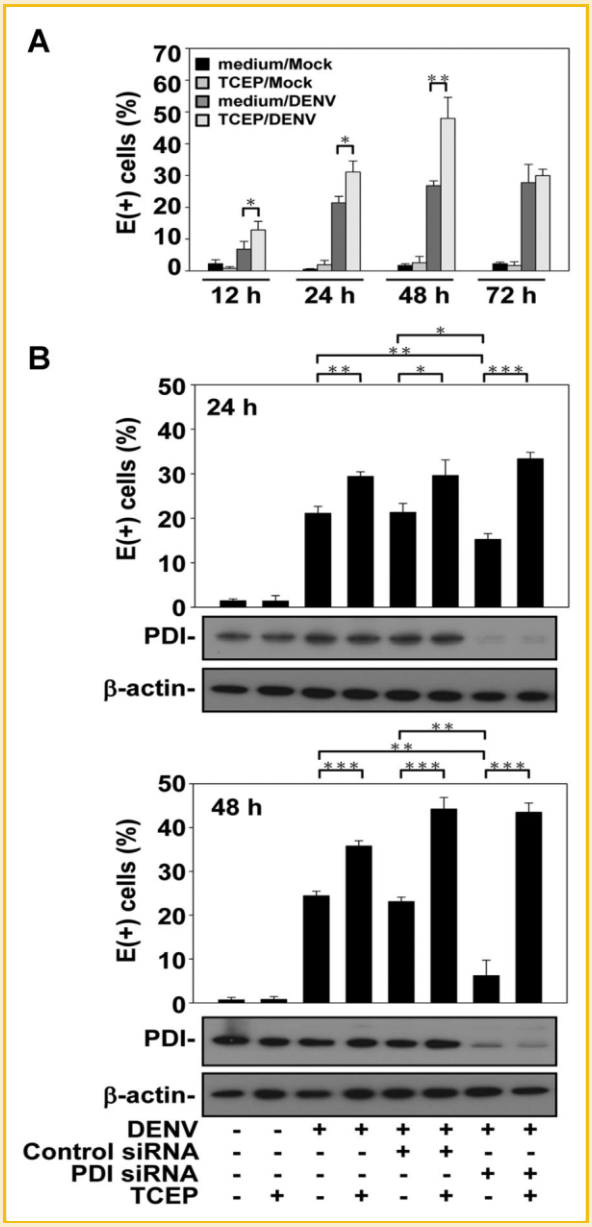

Fig. 1. Treatment with the reducing agent TCEP increases DENV infection in endothelial cells. A: HMEC-1 cells were pretreated with or without 0.01 mM TCEP at room temperature for 10 min and then infected with DENV (MOI = 100) at 37°C for 1 h. After various time periods, cells were stained with anti-DENV E protein antibodies at 4°C for 1 h, followed by Alexa-488-conjugated goat anti-mouse IgG at 4°C for 30 min. The percentages of E (+) cells were analyzed using flow cytometry. Results shown are means ± SD for triplicate cultures. B: Non-transfected, control siRNA-transfected, or PDI siRNA-transfected cells were infected with DENV with or without pretreatment with 0.01 mM TCEP at room temperature for 10 min. At 24 or 48 h post-infection, cells were stained with anti-DENV E protein antibodies at 4°C for 1 h, followed by Alexa-488-conjugated goat anti-mouse IgG at 4°C for 30 min. The percentages of E (+) cells were analyzed using flow cytometry. Results shown are means ± SD for triplicate cultures. The expression of PDI in the cells of each group is shown using Western blotting. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

viability was evaluated by WST-8 assay. TCEP had no marked influence on the extracellular LDH leakage and cell viability (data not shown). A recent study noted that DENV replication and assembly took place close to the ER. These authors found that viral proteins colocalized with PDI in infected cells [Welsch et al., 2009]. To investigate the possibility that PDI knockdown could influence viral replication at a post-entry step, cells were first infected with DENV followed by PDI knockdown. Results showed that both E and NS3 protein levels in DENV-infected, PDI siRNA post-transfected cells were not significantly different from those transfected with control siRNA or infected with DENV alone at 24 h post-infection (Fig. S1). The group with PDI siRNA pretreatment before DENV infection was a control showing an inhibition similar to that in Figure 1B.

DENV INDUCES CELL SURFACE EXPRESSION OF PDI THROUGH THE ER–GOLGI SECRETION PATHWAY

In order to further elucidate the role of PDI in DENV infection, we determined cell surface PDI expression following DENV infection at various time periods. By immunostaining with PDI-specific antibodies followed by flow cytometric analysis, the percentages of cells expressing surface PDI increased time-dependently with DENV infection (Fig. 2A). The surface expression of PDI increased dramatically at an MOI of 100; however, the increase of cell surface PDI could also be observed at an MOI of 1 and 10 at later time points (72 and 96 h; Fig. S2). To further confirm the surface expression of PDI, cell surface proteins were labeled with a cell-impermeable biotinylating reagent and precipitated with immobilized streptavidin. Western blotting showed that DENV infection increased the cell surface PDI level in an MOI-dependent manner (Fig. 2B, upper panel). There was no significant change in total PDI levels when cells were infected with different MOI of DENV (Fig. 2B, lower panel). The localization of PDI on DENV-infected endothelial cell surface was further confirmed by staining with PDI-specific antibodies and CT-B in non-fixed cells and then DENV E protein-specific antibodies after fixation. CT-B binds to specific gangliosides and is therefore a marker for lipid rafts on the plasma membrane enriched in cholesterol and sphingolipids. The confocal images showed that PDI colocalized with CT-B on plasma membrane of DENV-infected E-positive cells (Fig. 2C).

Some chaperones traffic from ER to Golgi complex and bind to the KDEL receptor, and subsequently activate the SFKs in the Golgi complex and regulate Golgi-to-plasma membrane transport [Pulvirenti et al., 2008; Capitani and Sallese, 2009]. PDI is an ER chaperone with a KDEL sequence [Yoshimori et al., 1990]. Treatment with BFA, a blocker of the ER–Golgi secretion pathway, showed a decrease in PDI translocation to the surface of DENV-infected cells in a dose-dependent manner (Fig. 3A). There was no toxic effect of BFA on HMEC-1 cells (data not shown). In DENV infection, the colocalization of PDI and KDEL receptor spread from the perinuclear site to cell periphery, but BFA blocked this trafficking (Fig. 3B). Using KDEL receptor siRNA to knock down KDEL receptor expression as demonstrated by flow cytometric analysis (Fig. 3C), there was reduced surface expression of PDI (Fig. 3D). In addition, DENV infection of endothelial cells as determined by viral E protein expression was also decreased when cells were transfected with

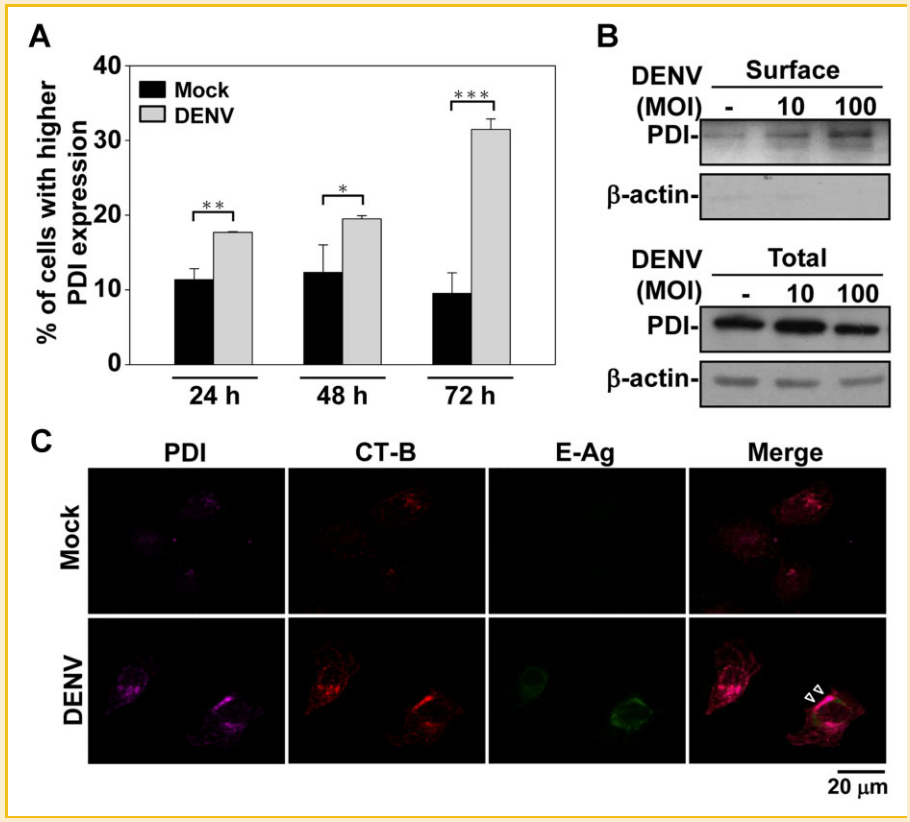

Fig. 2. DENV infection increases cell surface expression of PDI. A: HMEC-1 cells were infected with DENV (MOI = 100) for different time intervals, the surface level of PDI was detected using flow cytometry. Results shown are means ± SD for triplicate cultures. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. B: At 72 h post-infection (MOI = 10 or 100), the cells were surface-labeled with biotin and the biotinylated surface proteins were precipitated with streptavidin-conjugated agarose beads. PDI present in cell surface fraction and total PDI were determined by Western blotting. C: At 72 h post-infection, the cell surface PDI (purple), plasma membrane marker Cholera Toxin Subunit B (CT-B, red), and total viral E protein (green) were detected using confocal microscopy. Open arrowheads indicate the expression of PDI is on the surface of endothelial cells. Mock-infected cells served as controls.

KDEL receptor siRNA compared with cells treated with control siRNA or with DENV alone (Fig. 3E). Our results therefore showed that the increased surface expression of PDI in DENV infection was dependent on the KDEL receptor on the ER–Golgi complex. In addition, signaling downstream of the KDEL receptor induced the activation of a Golgi pool of Src kinases and a phosphorylation cascade that subsequently activated intra-Golgi trafficking. We further found that DENV induced SFK phosphorylation which localized both on the plasma membrane and the Golgi complex (as demonstrated by GM130 staining). BFA inhibited phosphorylation of SFKs on the Golgi complex, which also led to the reduced expression of phosphorylated SFKs on the plasma membrane (Fig. 4A). There was only slight phosphorylation of SFKs in both mock and BFA-treated cells (data not shown). We next showed that SFK inhibitors PP1 and PP2 decreased the DENV-induced surface expression of PDI (Fig. 4B). In addition, DENV infection of endothelial cells as determined by viral E protein expression was also decreased when cells were treated with PP1 or PP2 (Fig. 4C). A previous report showed that inhibition of SFK activity prevented maturation of the E protein of another flavivirus, West Nile Virus [Hirsch et al., 2005]. This might be a possible explanation for the reduced E protein expression after PP1 or PP2 treatment. These results indicate that DENV-induced PDI surface expression is mediated through the KDEL receptor-SFK signal pathway on the ER–Golgi complex.

EFFECTS OF PDI ON β1 AND β3 INTEGRIN ACTIVATION AND DENV INFECTION

PDI can affect the function of αvβ3 integrin at the surface on endothelial cells [Swiatkowska et al., 2008]. In addition, the expression of β3 integrin is increased in DENV-infected endothelial cells, which is associated with viral entry into the cells [Zhang et al., 2007]. Since β1 and β3 integrins are abundantly expressed in endothelial cells [Silva et al., 2008], we next determined whether PDI caused an effect on β1 and β3 integrins after DENV infection. Immunostaining and flow cytometic analysis showed that cell surface expression of β1 and β3 integrins was increased by DENV infection, but not by UV-inactivated DENV (Fig. 5A). To further detect the colocalization of PDI with β1 and β3 integrins on the cell surface, DENV-infected endothelial cells were stained with antibodies against DENV E protein, PDI, and active-form β1 or αvβ3 integrin. The confocal overlay images showed that PDI colocalized with β1 and β3 integrins on the surface of DENV-infected cells (Fig. 5B). In addition, the interaction of αvβ3 integrin with PDI was increased after DENV infection by co-immunoprecipitation assay (Fig. S3).

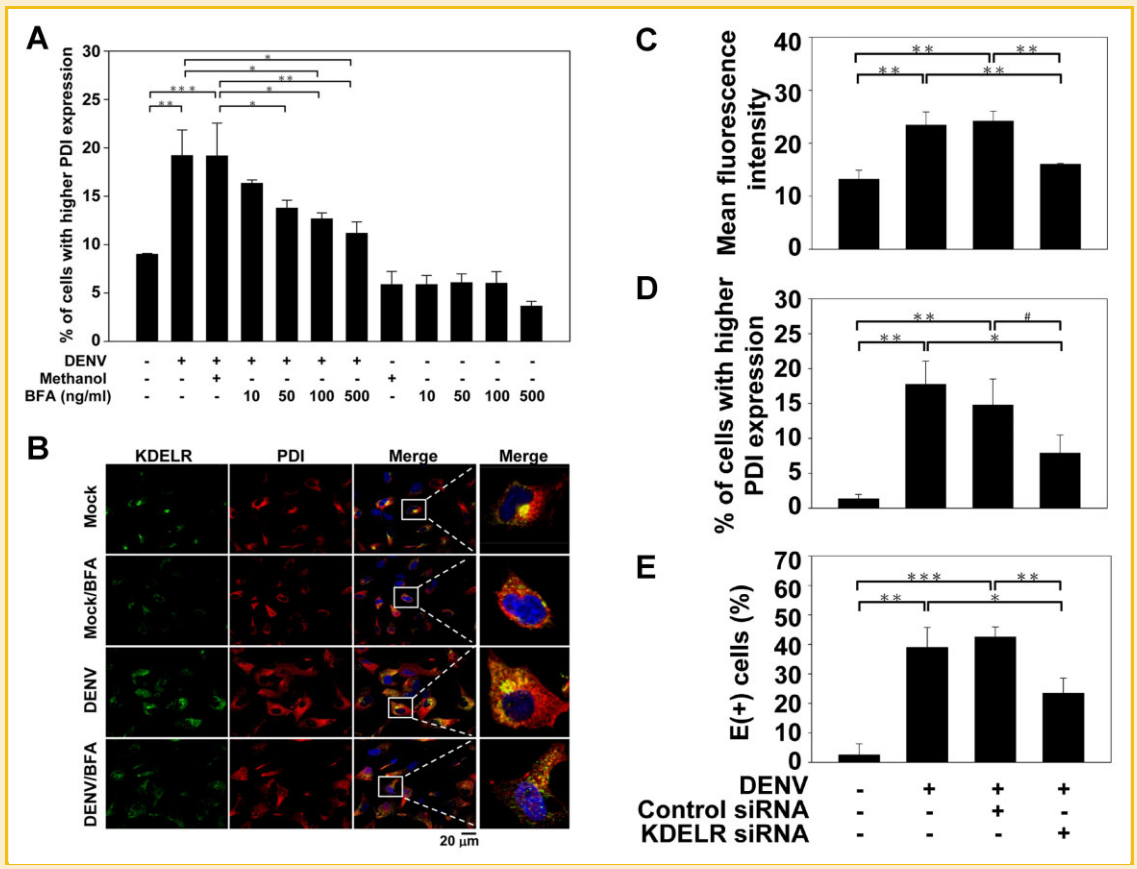

Fig. 3. DENV-induced PDI surface expression is mediated through the KDEL receptor-mediated pathway on the ER–Golgi complex. A: HMEC-1 cells were infected with DENV (MOI = 100). Brefeldin A (BFA) or methanol control was added 6 h before cell harvesting at 72 h post-infection. The surface level of PDI was detected using flow cytometry. B: HMEC-1 cells were infected with DENV and then double-stained for PDI (red) and KDEL receptor (KDELR, green) at 72 h post-infection. The colocalization of KDELR with PDI (yellow) was analyzed by confocal microscopy. Mock-infected cells served as controls. BFA was added 6 h before cell harvesting. C: Non-transfected, control siRNA-transfected, or KDELR siRNA-transfected cells were infected with DENV. At 72 h post-infection, the KDELR expression was determined using flow cytometry to confirm the siRNA knockdown efficiency. Results shown are means ± SD for triplicate cultures. D: The surface level of PDI was detected using flow cytometry. Results shown are means ± SD for triplicate cultures. #$P = 0.059$. E: Cells were stained with anti-DENV2 E protein antibodies. The percentages of E (+) cells were analyzed using flow cytometry. Results shown are means ± SD for triplicate cultures. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Several reports demonstrated PDI-dependent disulfide exchange on β1 and β3 integrin activation on the surface of platelets or endothelial cells [Manickam et al., 2008; Swiatkowska et al., 2008]. In addition to the increased surface expression of integrins, we further demonstrated DENV-induced integrin activation by detecting the expression of active-form β1 integrin on the cell surface and showing that this activation effect was inhibited by bacitracin (Fig. S4A), which is a membrane-impermeable agent capable of interfering with PDI function [Mandel et al., 1993]. Fibronectin and collagen are the ligands for β1 integrin, whereas fibrinogen, vitronectin, and fibronectin are the ligands for β3 integrin [Silva et al., 2008]. Our results showed that the affinity of β1 and β3 integrins to their ligands was elevated by DENV infection. Treatment with bacitracin resulted in an inhibition of DENV-induced adhesion to the ligands of β1 and β3 integrins (Fig. S4B). When cells were transfected with PDI siRNA, the affinity of β1 and β3 integrins to their ligands was decreased in PDI siRNA-transfected cells compared with that of control siRNA-transfected cells (Fig. 5C). Moreover, the surface expression of active-form β1 integrin was decreased after PDI knockdown (Fig. S5). These results indicate that PDI plays an important role in β1 and β3 integrin activation. To further confirm the role of surface β1 and β3 integrins, treatment of cells with function-blocking β1 or β3 integrin antibodies caused inhibition of DENV infection as demonstrated by reduced E protein expression in the endothelial cells. Treatment with anti-PDI antibodies or in combination with anti-β1 and anti-β3 integrin antibodies showed a similar inhibitory effect (Fig. 5D). While treatment with anti-β1 integrin blocking antibodies caused inhibition of DENV infection, treatment of cells with β1 integrin-activating antibodies caused enhancement of DENV infection. Furthermore, while treatment with blocking anti-β3 integrin antibodies caused inhibition of DENV infection, treatment with non-blocking β3 integrin antibodies had no effect on DENV infection (Fig. S6). As a negative control, treatment of cells with anti-αvβ5 integrin antibodies only caused slight inhibition (data not shown). These results indicate that PDI is required not only for the activation of β1 and β3 integrins but also DENV infection.

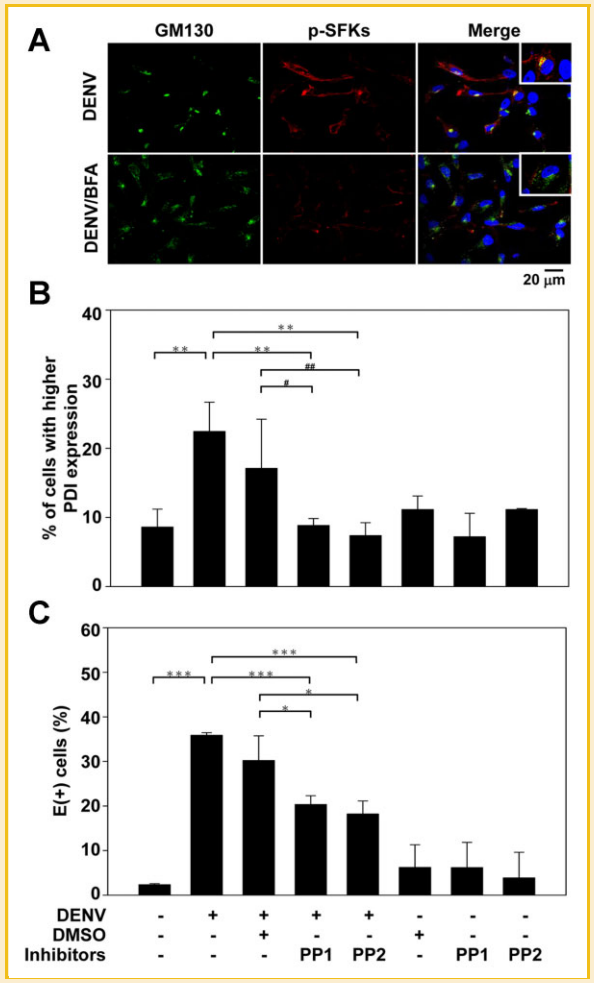

Fig. 4. DENV-induced PDI surface expression is mediated through the SFK signaling pathway on the ER–Golgi complex. A: HMEC-1 cells were infected with DENV (MOI = 100) and then double-stained for phosphorylated SFKs (p-SFKs, red) and GM130 (green) at 72 h post-infection. The colocalization of GM130 with p-SFKs (yellow) was analyzed by confocal microscopy. Nuclei were stained with DAPI (blue). Brefeldin A (BFA) was added 6 h before cell harvesting. B: Cells were infected with DENV and the surface level of PDI was detected using flow cytometry at 72 h post-infection. The p-SFKs inhibitors PP1 and PP2 (10 μM) were added 6 h before cell harvesting. Results shown are means ± SD for triplicate cultures. #$P = 0.116$; ##$P = 0.083$. C: Cells were stained with anti-DENV2 E protein antibodies. The percentages of E (+) cells were analyzed using flow cytometry. PP1 and PP2 (10 μM) were added 6 h before cell harvesting. Results shown are means ± SD for triplicate cultures. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

PDI-MEDIATED INTEGRIN ACTIVATION ENHANCES DENV ENTRY

A previous study indicated that β3 integrin was associated with DENV entry into human endothelial cells [Zhang et al., 2007]. To determine the role of integrin in DENV entry, we determined the viral titers after DENV entry for 2 h. We used siRNA to knock down β1 and β3 integrins and found that DENV entry was reduced and the inhibitory effect was slightly enhanced in double knockdown cells (Fig. 6A). To further investigated the role of PDI on DENV entry, we next used siRNA to knock down PDI and found that DENV entry was reduced, and this inhibitory effect was rescued by addition of TCEP (Fig. 6B). Treatment of cells with anti-PDI, anti-β1, or anti-β3 integrin antibodies also caused inhibition of DENV entry (Fig. 6C). Together, our results suggest the involvement of PDI in DENV entry, which is further enhanced by DENV-induced PDI expression on the endothelial cell surface.

DISCUSSION

A requirement for cell surface PDI activity has been shown in the infection of a number of viruses, including HIV [Ryser and Fluckiger, 2005; Ou and Silver, 2006], Newcastle disease virus [Jain et al., 2009], and Sindbis virus [Abromaitis and Stephens, 2009]. PDI may directly interact with and modify viral proteins for viral entry. For example, cell surface PDI activity is required for the fusion of Newcastle disease virus and cell membranes, which is mediated by the reduction of disulfide bonds in the virus fusion protein [Jain et al., 2009]. For HIV, PDI attaches via the CD4-gp120 complex and modulates the conformation of gp120 by thiol reduction, and this leads to HIV entry into cells [Ryser and Fluckiger, 2005]. In addition to the documented roles of PDI in viral entry, PDI has also been shown to be involved in bacterial entry as in the case of Chlamydia, in which the thiol-mediated oxidoreductive function of PDI is required for bacterial entry into cells but not for attachment [Abromaitis and Stephens, 2009]. It was speculated that Chlamydia binds to cell surface protein(s) that is associated with PDI. However, the target of PDI enzymatic activity that facilitates Chlamydia entry remains unclear. In this study, we show that PDI modifies host proteins for viral entry. Cell surface expression of PDI activates β1 and β3 integrins and further enhances DENV infection. Nevertheless, the possibility of a direct PDI effect on DENV proteins as shown in other viruses [Abell and Brown, 1993] cannot be excluded.

Flavivirus replication and assembly occur close to the ER in that viral proteins colocalize with PDI in infected cells [Mackenzie and Westaway, 2001; Welsch et al., 2009]. However, the role of PDI in virus replication has not been explored. In our study, we found that knockdown of PDI after DENV entry has no effect on DENV replication in endothelial cells at least up to 24 h (Fig. S1). Nevertheless, by 48 and 72 h DENV replication as indicated by E (+) cells was significantly slower in the PDI knockdown group than in the control siRNA and DENV alone groups (data not shown). Therefore, besides a role of DENV entry, PDI may also be involved at other stages in the DENV replication cycle. The role that PDI might play in post-entry steps needs to be further evaluated. PDI may be particularly important in the amplification of DENV infection in cells which are less susceptible to DENV, like endothelial cells. In addition, PDI was found to colocalize with DENV E protein and integrin (Fig. 5B, solid arrowheads) in DENV-infected cells. Of note, PDI colocalized with integrin only on the cell surface (Fig. 5B, open arrowheads). Therefore, surface PDI may play a role in integrin activation (Figs. 5C, S4, and S5), and further enhance viral entry.

A recent study demonstrated a role for the actin cytoskeleton in the initial stage of DENV infection in endothelial cells. The binding of DENV to HMEC-1 cells induced the formation of actin-containing filopodia which is required for viral entry [Zamudio-Meza et al.,

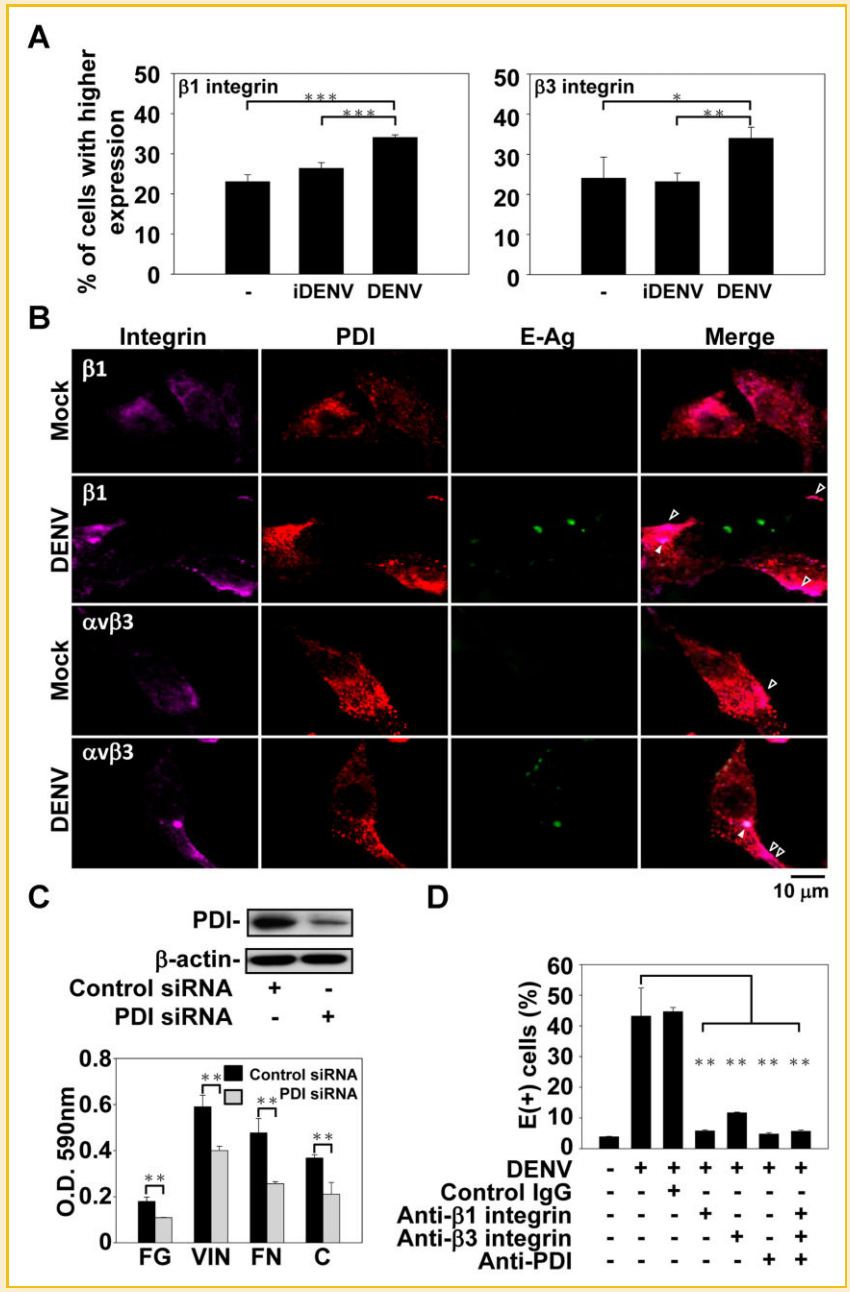

Fig. 5. Blockade of PDI reduces β1 and β3 integrin activation and DENV infection in endothelial cells. A: HMEC-1 cells were infected with DENV or UV-inactivated DENV (iDENV) (MOI = 100) for 72 h and surface stained with anti-β1 and β3 integrin antibodies. The percentages of cells with surface β1 and β3 integrins were analyzed using flow cytometry. Results shown are means ± SD for triplicate cultures. B: DENV-infected cells were triple-stained for PDI (red), β1 and β3 integrins (purple), and viral E protein (green). Solid arrowheads indicate the colocalization of PDI with β1 or β3 integrins and viral E protein. Open arrowheads indicate the colocalization of surface β1 or β3 integrins with PDI. Mock-infected cells served as negative controls. C: After knockdown of PDI, cell adhesion to fibrinogen (FG, 100 μg/ml), vitronectin (VIN, 5 μg/ml), fibronectin (FN, 1 μg/ml), and collagen I (C, 1 μg/ml) was measured at optical density of 590 nm. Results shown are means ± SD for triplicate cultures. The expression of PDI in the cells of each group is shown using Western blotting. D: Cells were infected with DENV in the presence or absence of anti-β1 and β3 integrins and anti-PDI antibodies, then stained with anti-DENV2 E protein antibodies. The percentages of E (+) cells were analyzed using flow cytometry. Results shown are means ± SD for triplicate cultures. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

2009]. In addition, small GTPase Rac1 plays a role in the regulation of the actin cytoskeleton during DENV infection. Studies showed that actin is essential for DENV entry into and release from endothelial-like ECV304 cells [Wang et al., 2010]. We found that DENV infection of endothelial cells as determined by viral E protein expression was also decreased when cells were treated with the actin cytoskeleton-disrupting agent, cytochalasin D (Fig. S7A). Disorganization of actin not only prevented infection, but also inhibited DENV-induced surface expression of PDI (Fig. S7B). In addition, UV-inactivated DENV did not induce the surface expression of PDI

(data not shown). Therefore, only actively replicating DENV could upregulate PDI expression.

The ER chaperone proteins containing a KDEL sequence traffic from ER to Golgi complex, bind to the KDEL receptor and subsequently activate the SFKs in the Golgi complex thereby regulating Golgi-to-plasma membrane transport [Pulvirenti et al., 2008; Capitani and Sallese, 2009]. We found that DENV-induced PDI surface expression is mediated through the KDEL receptor-SFK signal pathway on the ER–Golgi complex. Treatment with BFA, a blocker of the ER–Golgi secretion pathway, showed a decrease in PDI translocation to the surface of DENV-infected cells. However, the mechanism by which DENV upregulates expression of PDI on the cell surface still remains to be elucidated.

PDI may act as a target for autoimmune responses. Previous studies showed the occurrence of autoantibodies against PDI in a spontaneous hereditary hepatitis rat model and in patients with hepatic disorders [Yokoi et al., 1993; Nagayama et al., 1994]. Also, PDI is a major target of post-streptococcal autoantibodies by sharing similar epitopes with streptolysin O [Aran et al., 2010]. We previously demonstrated that antibodies against DENV non-structural protein 1 (NS1) cross-reacted with endothelial cells and caused their activation and apoptosis [Lin et al., 2002, 2005]. We have previously shown that PDI is a candidate autoantigen recognized by anti-DENV NS1 antibodies [Cheng et al., 2009].

In the present study, we reveal a novel mechanism of PDI that is to facilitate DENV entry into cells, which is at least in part mediated via activation of β1 and β3 integrins. A number of DENV receptor candidate molecules have been identified in different cell types, including heparin sulfate [Chen et al., 1997], DC-SIGN [Navarro-Sanchez et al., 2003], heat shock proteins [Reyes-Del Valle et al., 2005], and GRP78 (BiP) [Jindadamrongwech et al., 2004]. In addition, CLEC5A interacts with DENV in a manner which does not result in viral entry but stimulates the release of proinflammatory cytokines [Chen et al., 2008]. Furthermore, clathrin-mediated endocytosis plays a pivotal role in DENV entry [van der Schaar et al., 2008], although in some DENV serotypes or different cell types it may be clathrin-independent [Acosta et al., 2009]. A recent report showed that β3 integrin is associated with DENV entry into endothelial cells [Zhang et al., 2007]. Another report also described the clathrin-mediated endocytosis of αvβ3 integrin as well as virus–αvβ3 integrin complex [Joki-Korpela et al., 2001]. The role of β1 or β3 integrins in the clathrin-mediated endocytosis in DENV infection needs to be further clarified. In the present study, we found that DENV can upregulate surface PDI which in turn regulates β1 and β3 integrins. Blockade of PDI inhibited DENV-induced β1 and β3 integrin activation as well as DENV infection in endothelial cells. In addition to DENV2 which is used in this study, the role of PDI in infection with other DENV serotypes and in other cell types remains

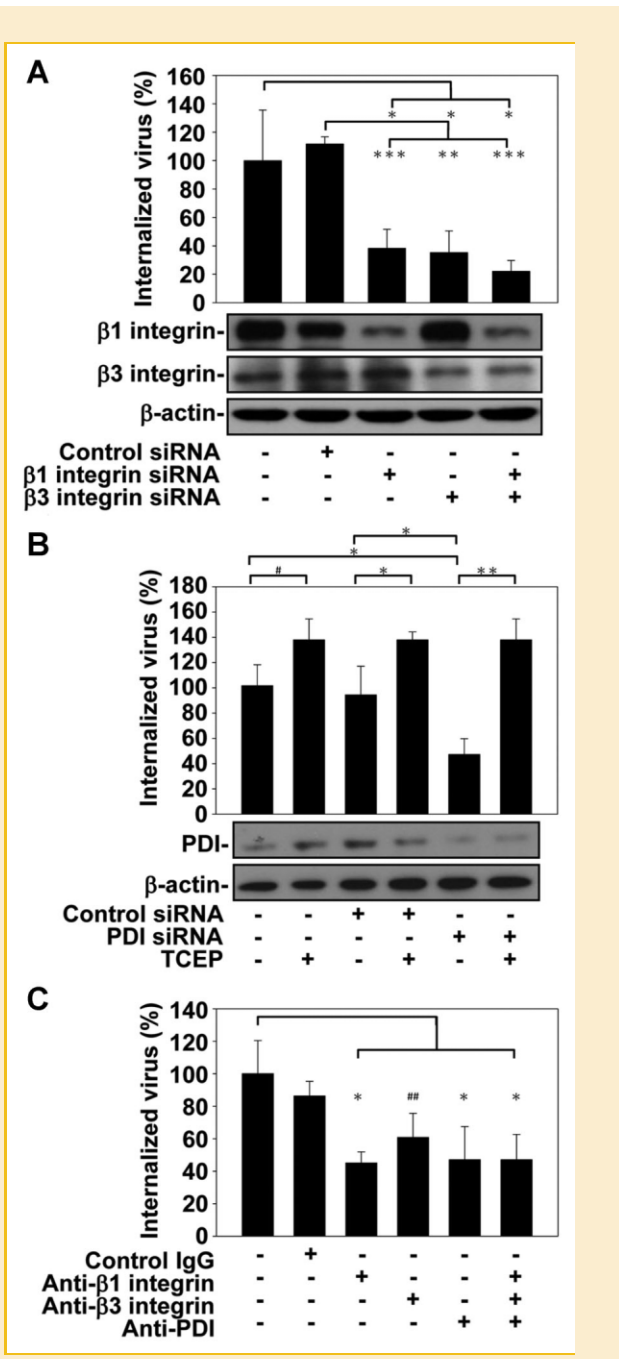

Fig. 6. PDI and β1 and β3 integrins are required for DENV entry. A: Non-transfected, control siRNA-transfected, β1 or β3 integrins siRNA-transfected cells were inoculated with DENV (MOI = 100) for 2 h. After thorough removal of unbound virus, the cells were lysed by freeze-thaw cycles and internalized virus was quantified by plaque assay. The internalized virus titers from non-transfected cells were normalized to 100%. The expression of β1 and β3 integrins in the cells of each group is shown using Western blotting. B: Non-transfected, control siRNA-transfected, or PDI siRNA-transfected cells were pretreated with or without 0.01 mM TCEP and then inoculated with DENV (MOI = 100) for 2 h. After thorough removal of unbound virus, the cells were lysed by freeze-thaw cycles and internalized virus was quantified by plaque assay. The internalized virus titers from non-transfected cells were normalized to 100%. The expression of PDI in the cells of each group is shown using Western blotting. C: Cells were pretreated with functional blocking antibodies against β1 and β3 integrins and PDI for 30 min at 4°C and then infected with DENV (MOI = 100) for 2 h. The cells were lysed by freeze-thaw cycles and the internalized virus was quantified by plaque assay. The internalized virus titers of non-treated cells were normalized to 100%. Results shown are means ± SD for triplicate cultures. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $\#P = 0.055$; $\#\#P = 0.054$.

to be investigated. The possible implications of the findings in this study for anti-DENV drug design are potentially exciting topics for future investigation.

ACKNOWLEDGMENTS

This work was supported by Grants NSC99-2321-B006-008 and NSC100-2325-B006-007 from the National Science Council, Taiwan, NHRI-100A1-PDCO-0209115 from National Health Research Institutes, Taiwan, and DOH100-TD-B-111-002 from Multidisciplinary Center of Excellence for Clinical Trial and Research, Department of Health, Taiwan. We thank Dr. Tamotsu Yoshimori (Osaka University) and Dr. Billy Tsai (University of Michigan Medical School) for helpful discussions and critical reading of the manuscript. We also thank Y.W. Wu and A.Y.H. Hsu for technical assistance with FITC-labeling anti-DENV E antibodies.

REFERENCES

Abell BA, Brown DT. 1993. Sindbis virus membrane fusion is mediated by reduction of glycoprotein disulfide bridges at the cell surface. J Virol 67:5496–5501.

Abromaitis S, Stephens RS. 2009. Attachment and entry of Chlamydia have distinct requirements for host protein disulfide isomerase. PLoS Pathog 5: e1000357. DOI: 10.1371/jounal.ppat.1000357.

Acosta EG, Castilla V, Damonte EB. 2009. Alternative infectious entry pathways for dengue virus serotypes into mammalian cells. Cell Microbiol 11:1533–1549.

Aran A, Weiner K, Lin L, Finn LA, Greco MA, Peppard P, Young T, Ofran Y, Mignot E. 2010. Post-streptococcal auto-antibodies inhibit protein disulfide isomerase and are associated with insulin resistance. PLoS ONE 5:e12875. DOI: 10.1371/journal.pone.0012875.

Avirutnan P, Malasit P, Seliger B, Bhakdi S, Husmann M. 1998. Dengue virus infection of human endothelial cells leads to chemokine production, complement activation, and apoptosis. J Immunol 161:6338–6346.

Bielefeldt-Ohmann H. 1997. Pathogenesis of dengue virus diseases: Missing pieces in the jigsaw. Trends Microbiol 5:409–413.

Capitani M, Sallese M. 2009. The KDEL receptor: New functions for an old protein. FEBS Lett 583:3863–3871.

Chen Y, Maguire T, Hileman RE, Fromm JR, Esko JD, Linhardt RJ, Marks RM. 1997. Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. Nat Med 3:866–871.

Chen HC, Hofman FM, Kung JT, Lin YD, Wu-Hsieh BA. 2007. Both virus and tumor necrosis factor alpha are critical for endothelium damage in a mouse model of dengue virus-induced hemorrhage. J Virol 81:5518–5526.

Chen ST, Lin YL, Huang MT, Wu MF, Cheng SC, Lei HY, Lee CK, Chiou TW, Wong CH, Hsieh SL. 2008. CLEC5A is critical for dengue-virus-induced lethal disease. Nature 453:672–676.

Cheng HJ, Lin CF, Lei HY, Liu HS, Yeh TM, Luo YH, Lin YS. 2009. Proteomic analysis of endothelial cell autoantigens recognized by anti-dengue virus nonstructural protein 1 antibodies. Exp Biol Med 234:63–73.

Clyde K, Kyle JL, Harris E. 2006. Recent advances in deciphering viral and host determinants of dengue virus replication and pathogenesis. J Virol 80:11418–11431.

Halstead SB. 2003. Neutralization and antibody-dependent enhancement of dengue viruses. Adv Virus Res 60:421–467.

Hirsch AJ, Medigeshi GR, Meyers HL, DeFilippis V, Fruh K, Briese T, Lipkin WI, Nelson JA. 2005. The Src family kinase c-Yes is required for maturation of West Nile virus particles. J Virol 79:11943–11951.

Huang KJ, Yang YC, Lin YS, Huang JH, Liu HS, Yeh TM, Chen SH, Liu CC, Lei HY. 2006. The dual-specific binding of dengue virus and target cells for the antibody-dependent enhancement of dengue virus infection. J Immunol 176:2825–2832.

Jackson T, Mould AP, Sheppard D, King AM. 2002. Integrin αvβ1 is a receptor for foot-and-mouth disease virus. J Virol 76:935–941.

Jain S, McGinnes LW, Morrison TG. 2009. Role of thiol/disulfide exchange in Newcastle disease virus entry. J Virol 83:241–249.

Jessie K, Fong MY, Devi S, Lam SK, Wong KT. 2004. Localization of dengue virus in naturally infected human tissues, by immunohistochemistry and in situ hybridization. J Infect Dis 189:1411–1418.

Jindadamrongwech S, Thepparit C, Smith DR. 2004. Identification of GRP 78 (BiP) as a liver cell expressed receptor element for dengue virus serotype 2. Arch Virol 149:915–927.

Joki-Korpela P, Marjomaki V, Krogerus C, Heino J, Hyypia T. 2001. Entry of human parechovirus 1. J Virol 75:1958–1967.

Limonta D, Capo V, Torres G, Perez AB, Guzman MG. 2007. Apoptosis in tissues from fatal dengue shock syndrome. J Clin Virol 40:50–54.

Lin CF, Lei HY, Shiau AL, Liu HS, Yeh TM, Chen SH, Liu CC, Chiu SC, Lin YS. 2002. Endothelial cell apoptosis induced by antibodies against dengue virus nonstructural protein 1 via production of nitric oxide. J Immunol 169:657–664.

Lin CF, Chiu SC, Hsiao YL, Wan SW, Lei HY, Shiau AL, Liu HS, Yeh TM, Chen SH, Liu CC, Lin YS. 2005. Expression of cytokine, chemokine, and adhesion molecules during endothelial cell activation induced by antibodies against dengue virus nonstructural protein 1. J Immunol 174:395–403.

Lin YS, Yeh TM, Lin CF, Wan SW, Chuang YC, Hsu TK, Liu HS, Liu CC, Anderson R, Lei HY. 2011. Molecular mimicry between virus and host and its implications for dengue disease pathogenesis. Exp Biol Med 236:515–523.

Mackenzie JM, Westaway EG. 2001. Assembly and maturation of the flavivirus Kunjin virus appear to occur in the rough endoplasmic reticulum and along the secretory pathway, respectively. J Virol 75:10787–10799.

Mandel R, Ryser HJ, Ghani F, Wu M, Peak D. 1993. Inhibition of a reductive function of the plasma membrane by bacitracin and antibodies against protein disulfide-isomerase. Proc Natl Acad Sci USA 90:4112–4116.

Manickam N, Sun X, Li M, Gazitt Y, Essex DW. 2008. Protein disulphide isomerase in platelet function. Br J Haematol 140:223–229.

Mou Y, Ni H, Wilkins JA. 1998. The selective inhibition of β1 and β7 integrin-mediated lymphocyte adhesion by bacitracin. J Immunol 161:6323–6329.

Nagayama S, Yokoi T, Tanaka H, Kawaguchi Y, Shirasaka T, Kamataki T. 1994. Occurrence of autoantibody to protein disulfide isomerase in patients with hepatic disorder. J Toxicol Sci 19:163–169.

Navarro-Sanchez E, Altmeyer R, Amara A, Schwartz O, Fieschi F, Virelizier JL, Arenzana-Seisdedos F, Despres P. 2003. Dendritic-cell-specific ICAM3-grabbing non-integrin is essential for the productive infection of human dendritic cells by mosquito-cell-derived dengue viruses. EMBO Rep 4:723–728.

Ou W, Silver J. 2006. Role of protein disulfide isomerase and other thiol-reactive proteins in HIV-1 envelope protein-mediated fusion. Virology 350:406–417.

Park B, Lee S, Kim E, Cho K, Riddell SR, Cho S, Ahn K. 2006. Redox regulation facilitates optimal peptide selection by MHC class I during antigen processing. Cell 127:369–382.

Pulvirenti T, Giannotta M, Capestrano M, Capitani M, Pisanu A, Polishchuk RS, San Pietro E, Beznoussenko GV, Mironov AA, Turacchio G, Hsu VW, Sallese M, Luini A. 2008. A traffic-activated Golgi-based signalling circuit coordinates the secretory pathway. Nat Cell Biol 10:912–922.

Reyes-Del Valle J, Chavez-Salinas S, Medina F, Del Angel RM. 2005. Heat shock protein 90 and heat shock protein 70 are components of dengue virus receptor complex in human cells. J Virol 79:4557–4567.

Ryser HJ, Fluckiger R. 2005. Progress in targeting HIV-1 entry. Drug Discov Today 10:1085–1094.

Silva R, D'Amico G, Hodivala-Dilke KM, Reynolds LE. 2008. Integrins: The keys to unlocking angiogenesis. Arterioscler Thromb Vasc Biol 28:1703–1713.

Swiatkowska M, Szymanski J, Padula G, Cierniewski CS. 2008. Interaction and functional association of protein disulfide isomerase with αvβ3 integrin on endothelial cells. FEBS J 275:1813–1823.

Tan GK, Alonso S. 2009. Pathogenesis and prevention of dengue virus infection: State-of-the-art. Curr Opin Infect Dis 22:302–308.

Terada K, Manchikalapudi P, Noiva R, Jauregui HO, Stockert RJ, Schilsky ML. 1995. Secretion, surface localization, turnover, and steady state expression of protein disulfide isomerase in rat hepatocytes. J Biol Chem 270:20410–20416.

Turano C, Coppari S, Altieri F, Ferraro A. 2002. Proteins of the PDI family: Unpredicted non-ER locations and functions. J Cell Physiol 193:154–163.

van der Schaar HM, Rust MJ, Chen C, van der Ende-Metselaar H, Wilschut J, Zhuang X, Smit JM. 2008. Dissecting the cell entry pathway of dengue virus by single-particle tracking in living cells. PLoS Pathog 4: e1000244. DOI: 10.1371/journal.ppat.1000244.

Wang X, Huang DY, Huong SM, Huang ES. 2005. Integrin αvβ3 is a coreceptor for human cytomegalovirus. Nat Med 11:515–521.

Wang JL, Zhang JL, Chen W, Xu XF, Gao N, Fan DY, An J. 2010. Roles of small GTPase Rac1 in the regulation of actin cytoskeleton during dengue virus infection. PLoS Negl Trop Dis 4: e809. DOI: 10.1371/journal.pntd.0000809.

Welsch S, Miller S, Romero-Brey I, Merz A, Bleck CK, Walther P, Fuller SD, Antony C, Krijnse-Locker J, Bartenschlager R. 2009. Composition and three-dimensional architecture of the dengue virus replication and assembly sites. Cell Host Microbe e5:365–375.

Yen YT, Chen HC, Lin YD, Shieh CC, Wu-Hsieh BA. 2008. Enhancement by tumor necrosis factor alpha of dengue virus-induced endothelial cell production of reactive nitrogen and oxygen species is key to hemorrhage development. J Virol 82:12312–12324.

Yokoi T, Nagayama S, Kajiwara R, Kawaguchi Y, Horiuchi R, Kamataki T. 1993. Identification of protein disulfide isomerase and calreticulin as autoimmune antigens in LEC strain of rats. Biochim Biophys Acta 1158:339–344.

Yoshimori T, Semba T, Takemoto H, Akagi S, Yamamoto A, Tashiro Y. 1990. Protein disulfide-isomerase in rat exocrine pancreatic cells is exported from the endoplasmic reticulum despite possessing the retention signal. J Biol Chem 265:15984–15990.

Zamudio-Meza H, Castillo-Alvarez A, Gonzalez-Bonilla C, Meza I. 2009. Cross-talk between Rac1 and Cdc42 GTPases regulates formation of filopodia required for dengue virus type-2 entry into HMEC-1 cells. J Gen Virol 90:2902–2911.

Zhang JL, Wang JL, Gao N, Chen ZT, Tian YP, An J. 2007. Up-regulated expression of β3 integrin induced by dengue virus serotype 2 infection associated with virus entry into human dermal microvascular endothelial cells. Biochem Biophys Res Commun 356:763–768.