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Identification of human hnRNP C1/C2 as a dengue virus NS1-interacting protein

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ABSTRACT

Dengue virus nonstructural protein 1 (NS1) is a key glycoprotein involved in the production of infectious virus and the pathogenesis of dengue diseases. Very little is known how NS1 interacts with host cellular proteins and functions in dengue virus-infected cells. This study aimed at identifying NS1-interacting host cellular proteins in dengue virus-infected cells by employing co-immunoprecipitation, two-dimensional gel electrophoresis, and mass spectrometry. Using lysates of dengue virus-infected human embryonic kidney cells (HEK 293T), immunoprecipitation with an anti-NS1 monoclonal antibody revealed eight isoforms of dengue virus NS1 and a 40-kDa protein, which was subsequently identified by quadrupole time-of-flight tandem mass spectrometry (Q-TOF MS/MS) as human heterogeneous nuclear ribonucleoprotein (hnRNP) C1/C2. Further investigation by co-immunoprecipitation and co-localization confirmed the association of hnRNP C1/C2 and dengue virus NS1 proteins in dengue virus-infected cells. Their interaction may have implications in virus replication and/or cellular responses favorable to survival of the virus in host cells.

Introduction

Dengue virus is a mosquito-borne human pathogen which causes a serious public health concern around the world with approximately 100 million cases of dengue infection and 500,000 cases of hospitalizations per annum [1]. The fatality rate of the affected individuals is about 1–5% and occurs mostly in children [1]. However, the mechanisms involved in the pathogenesis of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) remain unraveled.

Dengue virus is a positive, single-stranded RNA virus in the genus *Flavivirus* of the family *Flaviviridae* and contains a 11-kb genome encoding three-structural proteins (capsid, C; premembrane, prM; and envelope, E) and seven-nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [2]. In virus-infected cells, newly synthesized NS1 appears as a monomer in the lumen of the endoplasmic reticulum (ER) and subsequently undergoes glycosylation and dimerization as the protein is transported along the host secretory pathway to the cell surface and eventually to the extracellular milieu [3–5]. The exact roles of NS1 in each compartment are not clearly understood.

Secreted NS1 is found to activate complements in the presence or absence of specific antibodies and interact with human complement regulatory protein clusterin, potentially leading to viral and host immune complex formation and subsequent plasma leakage [6,7]. The correlation between levels of secreted NS1 and disease severity has also been observed [7,8]. Unlike the secreted form, cell surface-associated NS1 requires cross-linking of specific antibodies to induce efficient complement activation and intracellular signal transduction in response to dengue virus infection [3,7].

How the NS1 molecule functions inside virus-infected cells is still elusive. A number of previous studies propose the role of intracellular NS1 in the maturation process of dengue virus [9–11]. The NS1 molecule co-localizes with double-stranded dengue viral RNA (dsRNA), and associates with intracellular membrane structures,

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which are presumed sites of virus replication, and possibly with other viral nonstructural proteins, including NS2A, NS3, NS4A, and NS5 to form viral replication complexes in virus-infected cells [9–12]. Very little is known about the interplay between dengue virus NS1, host proteins, and cellular responses during dengue virus infection. We therefore hypothesized that the intracellular NS1 may interact with host cellular proteins to facilitate its proper folding, trafficking and/or to promote favorable environment for virus production in the host cell. Biochemical and proteomic approaches were utilized in this study to identify NS1-interacting proteins and subsequently confirm the protein–protein interaction.

Materials and methods

Cell line, virus, and antibodies. A human embryonic kidney epithelial cell line, 293T, dengue virus serotype 2 strain 16681, and mouse monoclonal antibodies recognizing linear epitopes (1B2, NS1-1F, NS1-3F, and NS1-4F) or conformational epitopes (NS1-8.2 and 1A4) on dengue virus NS1 were obtained as described previously [13–15]. A mouse anti-human hnRNP C1/C2 monoclonal antibody (clone 4F4) was purchased from Santa Cruz Biotechnology, Inc., CA, USA.

Dengue virus infection and immunoprecipitation. 293T cells were infected with dengue virus at a multiplicity of infection (MOI) of 1 and harvested at indicated time points post-infection to verify the percentage of dengue virus infection by immunofluorescence staining for viral E and NS1 antigen expression and flow cytometry [15]. Mock-infected cells served as negative controls. Clear lysates were prepared from mock and dengue virus-infected cells and then subjected to immunoprecipitation using a mouse isotype-matched control IgG1 or IgG2a antibody (MOPC 21 or UPC 10, Sigma, St. Loius, MO, USA), a mouse anti-NS1 monoclonal antibody (1A4, IgG2a), or a mouse anti-human hnRNP C1/C2 (4F4, IgG1) according to a previously described method with minor modifications [15].

Two-dimensional (2-D) gel electrophoresis and mass spectrometry. Immunoprecipitated proteins (200 μg) were subjected to the first dimensional separation by isoelectric focusing (IEF) on Immobiline DryStrip (nonlinear pH 3–10, 7-cm long; Amersham Bioscience) and the second dimensional separation in 12% SDS–polyacrylamide gel [16]. Separated protein spots were visualized by staining with Coomassie brilliant blue R-250 and the protein spot of interest was excised and in-gel digested with trypsin. Peptides were extracted from the gel pieces, purified, and analyzed by Q-TOF Ultima mass spectrometer (Micromass, Manchester, UK) according to the methods described previously [16].

Immunoblot analysis. Immunoprecipitated proteins, which had been heated or left unheated at 95 °C for 5 min in the presence or absence of 5% mercaptoethanol, were separated by electrophoresis in 10% SDS–polyacrylamide gel and transferred to a PVDF membrane (Millipore Corporation, Billerica, USA) using a SemiPhor semi-dry transphor unit (Amersham Bioscience). The membrane was processed as described previously [15], except that a mixture of mouse anti-NS1 monoclonal antibodies described above or a mouse anti-human hnRNP C1/C2 monoclonal antibody (4F4) was utilized in this study. The immunoreactive proteins were visualized by Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer Applied Biosystems, Foster City, CA, USA).

Double immunofluorescence staining. Mock and dengue virus-infected cells on glass coverslips were fixed with 3.7% formaldehyde in PBS for 7 min followed by absolute methanol for 10 min at room temperature (RT). The cells were incubated with a mouse isotype-matched control IgG1 antibody (MOPC 21) or a mouse anti-human hnRNP C1/C2 monoclonal antibody (4F4) at the dilution of 1:500 for 1 h at RT. Successive incubations of the cells for 30 min at RT in the dark were then performed using a Cy3-conjugated rabbit anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at the dilution of 1:4000, 10% normal mouse serum in PBS, and 20 μg/ml of a FITC-conjugated mouse anti-NS1 monoclonal antibody (1A4). The stained cells were visualized under a laser-scanning confocal microscope (LSM 510 Meta, Carl Zeiss, Jena, Germany).

Results and discussion

Determination of a suitable condition for preparation of dengue virus-infected cell lysates

Initially, HEK 293T cell line that had been infected with dengue virus at an MOI of 1 were collected daily for 3 days and assessed for

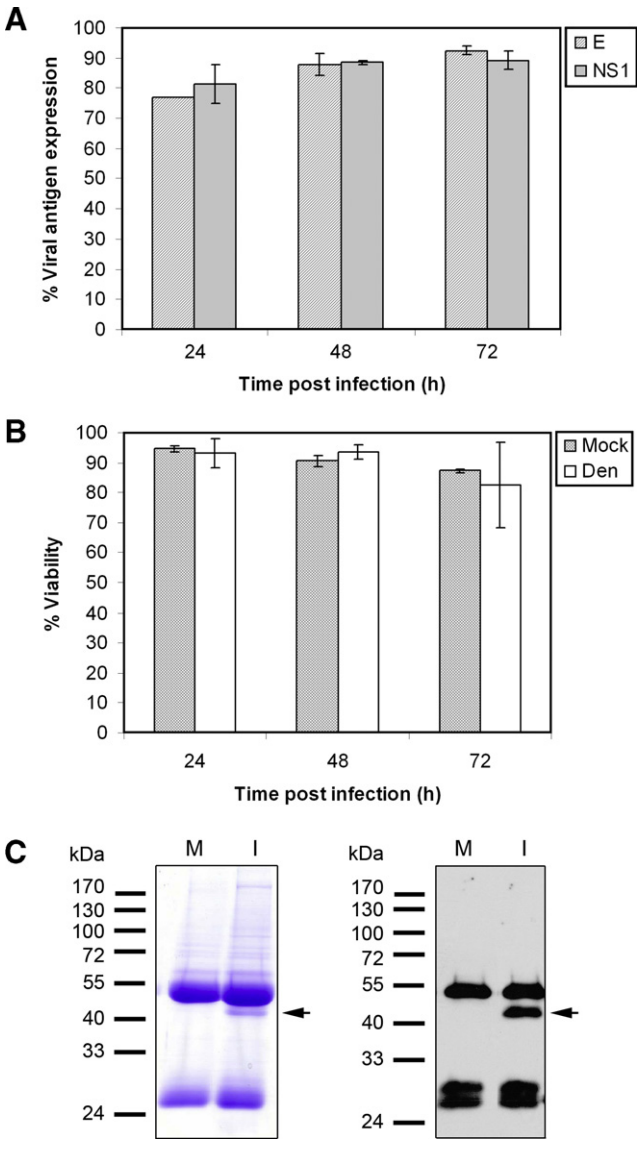

Fig. 1. Dengue virus infection of HEK 293T cells. Mock and virus-infected cells were assessed for (A) the expression of viral E and NS1 by immunofluorescence staining and flow cytometry and (B) the percentage of cell viability by trypan blue exclusion. Mock (M) and dengue virus-infected (I) cell lysates were subjected to immunoprecipitation using an anti-NS1 monoclonal antibody. Precipitated proteins were electrophoresed in a 10% SDS–polyacrylamide gel under reducing and heated conditions and visualized by Coomassie blue staining (C, left panel) or immunoblot analysis probed with an anti-NS1 specific antibody (C, right panel). Arrows indicate the dengue virus NS1 protein.

the percentage of dengue virus infection and cell viability. Mock-infected cells served as negative controls. Infection of HEK 293T cells with dengue virus for 48 h resulted in high levels of viral antigen expression (Fig. 1A) but low percentage of cell death (Fig. 1B). As a result, we employed this condition for preparing cell lysates and tested whether dengue virus NS1 could be immunoprecipitated from these samples using an anti-NS1 monoclonal antibody.

Analysis by SDS–PAGE and Coomassie blue staining revealed that, under reducing and heated conditions, a unique 46-kDa protein band was detectable in the precipitated sample from virus-infected cell lysate (Fig. 1C, left panel, lane I), but not in that from mock control (Fig. 1C, left panel, lane M). The reactivity with the anti-NS1 antibody in immunoblot analysis (Fig. 1C, right panel, lane I) strongly indicated that the specific 46-kDa protein was monomeric NS1 of dengue virus. Two additional protein bands of about 25 and 50 kDa were also observed in the precipitated samples from mock and dengue virus-infected cell lysates (Fig. 1C, left panel, lanes M and I), corresponding to light and heavy chains of IgG used for the immunoprecipitation (Fig. 1C, right panel, lanes M and I). These results therefore demonstrated that the infecting condition used in this study was adequate for establishment of dengue virus-infected samples for immunoprecipitation.

Identification of dengue virus NS1-interacting proteins by 2-D gel electrophoresis and mass spectrometry

Our results of SDS–PAGE could not show clearly any other proteins that were co-precipitated with dengue virus NS1 following immunoprecipitation with the specific antibody. This may have been due to insufficient amounts of the starting materials used for immunoprecipitation and/or the limited ability of SDS–PAGE to separate proteins with similar molecular sizes. We therefore prepared large amounts of mock and dengue virus-infected cell lysates, utilized them for immunoprecipitation using an isotype-matched control antibody or an anti-NS1 monoclonal antibody, and analyzed precipitated proteins by 2-D gel electrophoresis.

Immunoprecipitation of dengue virus-infected cell lysate with the isotype-matched control antibody resulted in the appearance of two major sets of protein spots of about 25 and 50 kDa and other minor protein spots on the 2-D SDS–polyacrylamide gel with similar patterns observed in the mock-infected cell lysate precipitated with the anti-NS1 antibody [Fig. 2A, Mock (anti-NS1) and DENV-2 (control IgG)]. Immunoblot analysis of the 25- and 50-kDa protein spots using rabbit anti-mouse immunoglobulin antibody suggested that these proteins may correspond to different isoforms

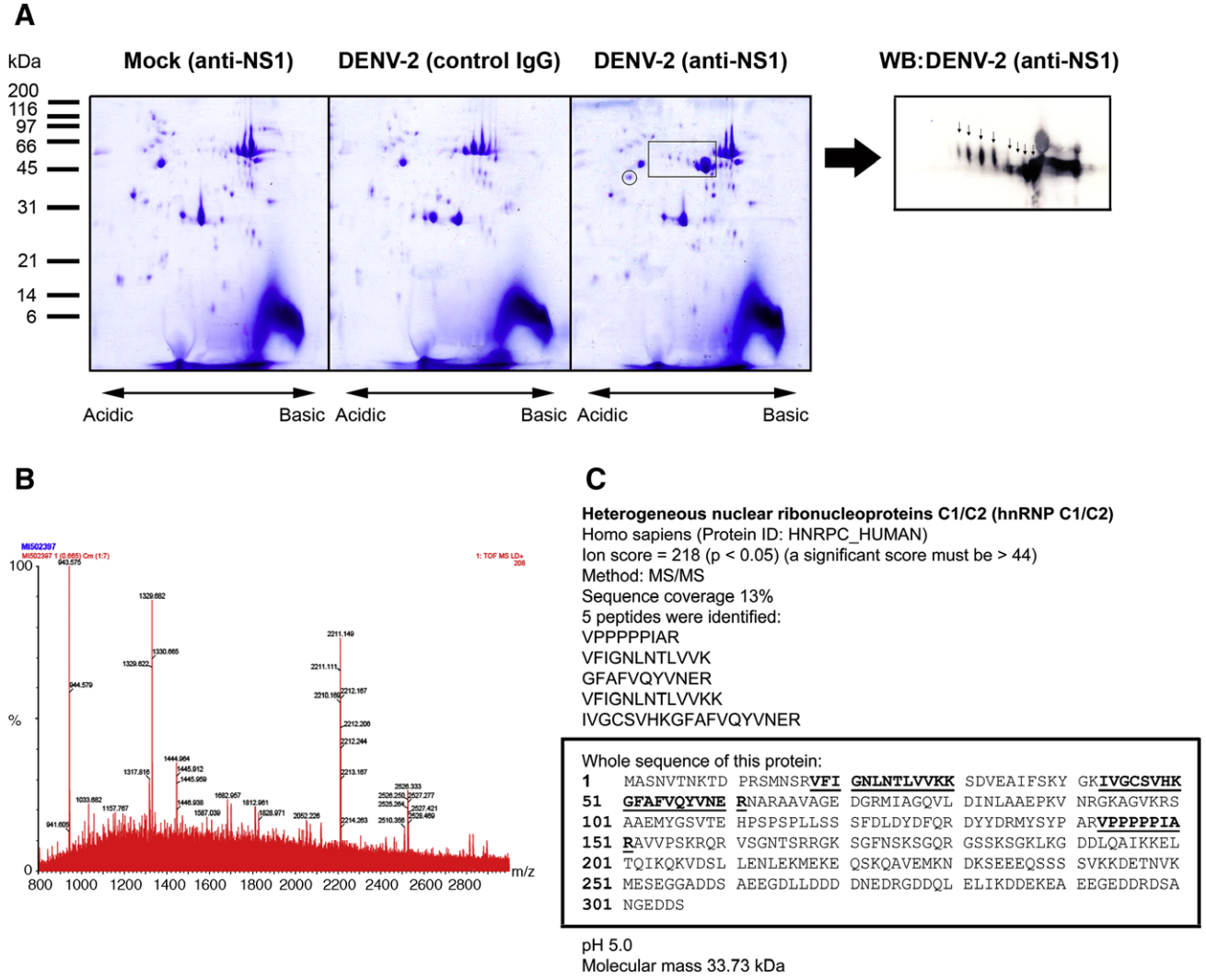

Fig. 2. Identification of hnRNP C1/C2 by 2-D gel electrophoresis and mass spectrometry. Mock and dengue virus-infected cell lysates were subjected to immunoprecipitation using either an anti-NS1 monoclonal antibody or an isotype-matched control antibody. (A) Precipitated proteins were separated by 2-D gel electrophoresis and visualized by Coomassie blue staining. A 40-kDa protein spot of interest (circled) and eight isoforms of dengue virus NS1 (indicated with a rectangle) are shown in DENV-2 (anti-NS1). The NS1 isoforms were verified by immunoblot analysis [WB: DENV-2 (anti-NS1)]. (B) The 40-kDa protein spot was processed for Q-TOF MS/MS analysis. The profile of mass/charge ratio of peptides is shown. (C) Five peptides resulting from the MS/MS analysis are shown to match the human heterogeneous nuclear ribonucleoproteins C1/C2.

of IgG light and heavy chains, respectively (data not shown). Apart from the detection of these protein spots, immunoprecipitation of dengue virus-infected cell lysate with the anti-NS1 specific antibody yielded a unique profile of eight protein spots of approximately 46 kDa as well as a single 40-kDa protein spot [Fig. 2, DENV-2 (anti-NS1)] which were not detectable in other precipitated control samples [Fig. 2, Mock (anti-NS1) and DENV-2 (control IgG)]. The former set of protein spots likely represented various isoforms of dengue virus NS1 owing to their reactivity with an anti-NS1 antibody in the immunoblot analysis [Fig. 2A—the rightmost inset, WB: DENV-2 (anti-NS1)]. The unknown 40-kDa protein spot, which was co-precipitated with dengue virus NS1, was subsequently excised, in-gel digested with trypsin and processed for mass spectrometry. Spectra of the eluting peptides obtained from mass spectrometric analysis were shown in Fig. 2B. Five peptides were identified and, based on the SwissProt database, were deduced to be from human heterogeneous nuclear ribonucleoproteins (hnRNP) C1/C2 (SwissProt accession number **P07910**) (Fig. 2C). The identified peptides were common to both isoforms of hnRNP C and corresponded to sequence coverage of about 13% of the protein (Fig. 2C).

Co-immunoprecipitation of hnRNP C1/C2 and dengue virus NS1

Human hnRNP C1/C2 are members of the heterogeneous nuclear ribonucleoprotein family which consists of 20 major hnRNP proteins (designated hnRNP A1 through U) with molecular sizes of approximately 36–120 kDa [17,18]. Alternative mRNA splicing of hnRNP C proteins with a 13-amino acid deletion occurring after glycine 106 or serine 107 generates two isoforms of proteins, hnRNP C1 (290 amino acids) and hnRNP C2 (303 amino acids) [19,20]. The hnRNP C1 and C2 proteins (41 and 43 kDa) are involved in mRNA biogenesis and contain important conserved motifs essential for RNA binding, protein–protein interaction and nuclear localization [17,18,21–23]. In order to determine whether the hnRNP C1/C2 interacts with dengue virus NS1 in virus-infected cells, immunoprecipitation of mock and dengue virus-infected cell lysates was performed using isotype-matched control antibodies or monoclonal antibodies specific against human hnRNP C1/C2 or dengue virus NS1. The presence of the two proteins in each precipitated sample was then analyzed by the immunoblot assay under nonreducing and unheated conditions. The lysates prior to immunoprecipitation were also included in this assay as controls for detection of hnRNP C1/C2 and dengue virus NS1.

As expected, hnRNP C1/C2 was observed in mock and dengue virus-infected cell lysate while dengue virus NS1 was detected only in the latter sample (Fig. 3, input). Using the anti-NS1 monoclonal antibody for immunoprecipitation, dengue virus NS1 and hnRNP C1/C2 were pulled down from virus-infected lysate, but not from mock-infected control (Fig. 3, IP: αNS1). On the contrary, immunoprecipitation with the anti-hnRNP C1/C2 monoclonal antibody yielded only the hnRNP C1/C2 in mock and dengue virus-infected samples, but no NS1 dimer was observed (Fig. 3, IP: αhnRNP). As negative controls, immunoprecipitation with either isotype-matched control antibody did not give any specific band of NS1 dimer; however, relatively low levels of hnRNP C1/C2 could be detected probably as a result of certain extents of nonspecific binding to protein G–Sepharose beads (Fig. 3, IP: IgG1 and IgG2a). Taken together, these findings suggested that the interaction between the dengue NS1 and hnRNP C1/C2 occurred in virus-infected cells. Inability to co-immunoprecipitate dengue virus NS1 using the anti-hnRNP C1/C2 antibody could be due to either masking of the hnRNP C1/C2 epitope by the interacting protein as suggested by a previous work on the Epstein-Barr virus SM protein [24], or only a small portion of the total hnRNP C1/C2 population involved in the dengue virus NS1 interaction.

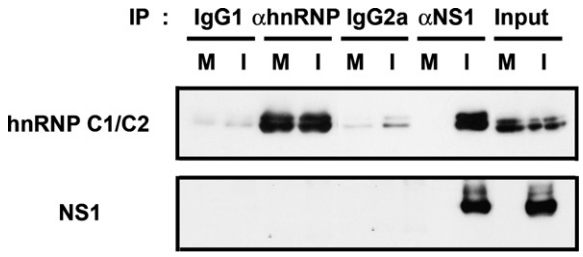

Fig. 3. Co-immunoprecipitation of hnRNP C1/C2 and dengue virus NS1. Mock (M) and dengue virus-infected (I) cell lysates were processed for immunoprecipitation (IP) using isotype-matched control antibodies (IgG1 or IgG2a), an anti-hnRNP C1/C2 monoclonal antibody (clone 4F4, IgG1), or an anti-NS1 monoclonal antibody (clone 1A4, IgG2a). Each precipitated sample was detected for the presence of hnRNP C1/C2 and dengue virus NS1 by immunoblot analysis using specific antibodies. Mock and virus-infected cell lysates prior to the immunoprecipitation (input) served as controls for the protein detection.

Co-localization of hnRNP C1/C2 and dengue virus NS1

Further investigation was carried out to determine sub-cellular distribution of hnRNP C1/C2 and dengue virus NS1 by double immunofluorescence staining and confocal microscopy. hnRNP C1/C2 was found predominantly in the nucleus and, to a lesser extent, in the cytoplasm of both mock (data not shown) and dengue virus-infected cells with an evenly distributed pattern (Fig. 4A and B, hnRNP C1/C2). Although hnRNP C1/C2 is primarily a nuclear protein in the cells, it can be translocated to the cytoplasm following the induction of apoptosis via Rho-activated kinase activation [25] or during poliovirus and human rhinovirus infection through an apoptosis-independent mechanism [26,27]. Nevertheless, an increase in the nuclear efflux of the hnRNP C1/C2 was not observed upon dengue virus infection in our study (data not shown).

Unlike the hnRNP C1/C2, dengue virus NS1 was detected as large foci mainly in the cytoplasm of dengue virus-infected cells (Fig. 4A and B, NS1), consistent with the staining pattern observed in previous studies [7,10,15]. A partial co-localization of the hnRNP C1/C2 and NS1 was detectable in some dengue virus-infected cells particularly at the perinuclear regions (Fig. 4A and B, Merge). This observation may help to explain the incapability to detect reciprocal co-immunoprecipitation with the anti-hnRNP C1/C2 antibody (Fig. 3, IP: αhnRNP). Consistent findings on the co-localization and the co-immunoprecipitation of hnRNP C1/C2 with dengue virus NS1 was observed not only in HEK 293T cell line (Fig. 3 and 4) but also in other three human cell lines (including liver HepG2, fibroblast HF and endothelial EAhy926) and primary human umbilical vein endothelial cells, HUVEC, (data not shown), confirming that this interaction is genuine, but probably transient and involved in only a part of total host cellular hnRNP C1/C2.

A previous study using a yeast two hybrid system identified human signal transducer and activator of transcription 3β (STAT3β) as a dengue virus NS1-interacting protein which may be implicated in the induction of IL-6 levels and subsequent plasma leakage [28]. By utilizing different approach, we demonstrated herein that hnRNP C1/C2 is additional host cellular protein that associates with dengue virus NS1 in virus-infected cells, but mechanisms of this protein–protein interaction are not known. The common structural motifs on hnRNP C1/C2 [17,22,23] may interact with dengue virus NS1 directly or indirectly through a link of other host proteins, viral proteins, and/or viral RNA resulting in the formation of viral replication complexes. This notion was supported by a previous study on the role of hnRNP C1 in the initiation of positive-strand viral RNA synthesis in poliovirus-infected cells through its interaction with the 3′-end of negative strand RNA of poliovirus and other three viral protein precursors, polypeptide 3CD, P2, and P3, crucial for poliovirus replication [29]. In addition, detection of various

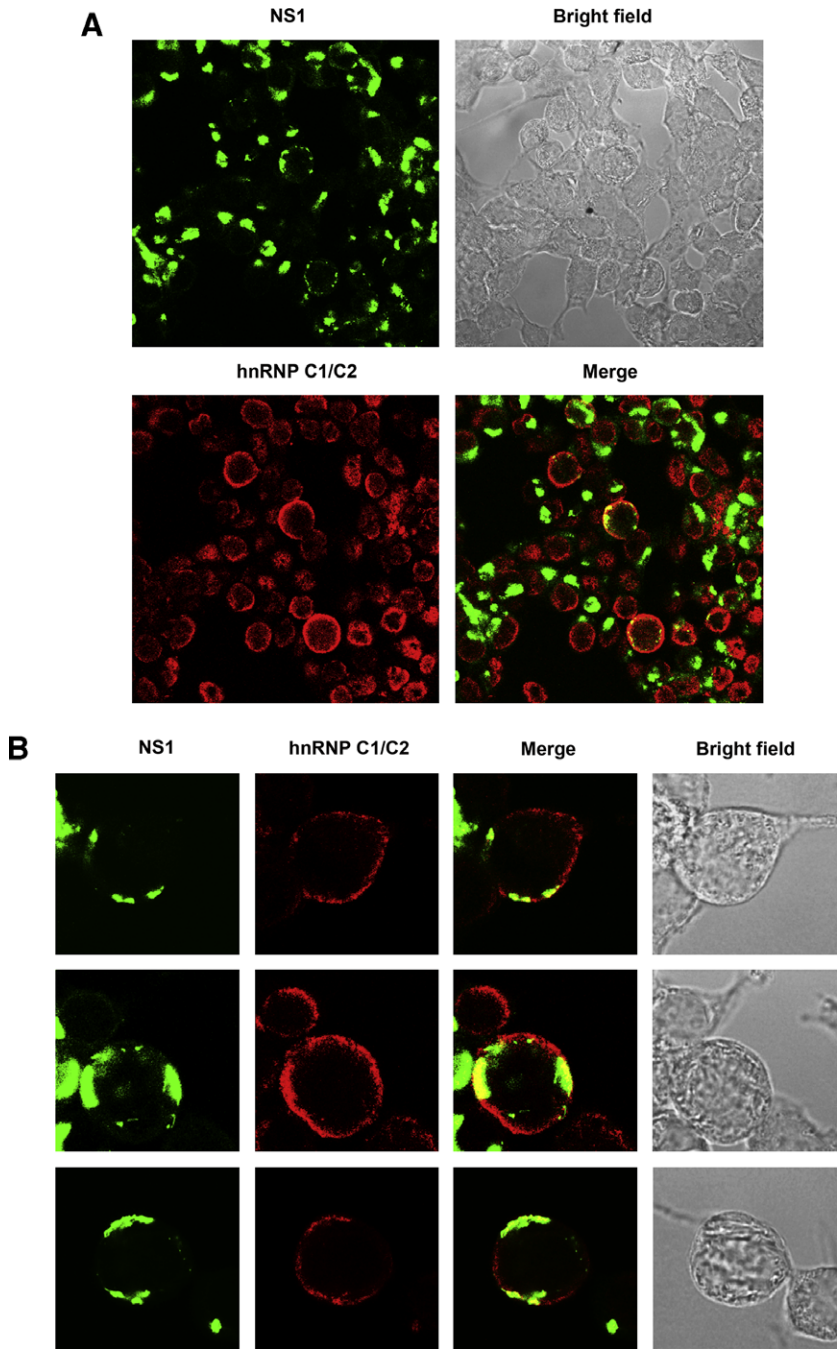

Fig. 4. Co-localization of hnRNP C1/C2 and dengue virus NS1. Dengue virus-infected cells were subjected to double immunofluorescence staining and observed for localization of hnRNP C1/C2 (red) and dengue virus NS1 (green) by using a laser-scanning confocal microscope with a 63× objective lens (A,B). Individual images that had been captured in the same fields are merged and areas where co-localization of both the proteins occurs are shown in yellow. Three representative fields of the stained cells with a 5× magnification are shown in (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

isoforms of dengue virus NS1 with distinct isoelectric points (p*I*) by 2-D gel electrophoresis in our study implied that differential post-translational modifications of NS1 might occur in virus-infected cells and give rise to generation of different isoforms of the protein, each of which potentially exerts different functions. Which particular isoform of dengue virus NS1 interacts with hnRNP C1/C2 and whether this association is involved in virus replication and host cellular responses during dengue virus infection require further investigations.

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