Class II ADP-ribosylation Factors Are Required for Efficient Secretion of Dengue Viruses

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Background: To date, very few cellular factors required for secretion of flaviviruses have been described.

Results: Simultaneous depletion of class II Arf (Arf4 and Arf5) blocks dengue flavivirus secretion, without altering the constitutive secretory pathway. Dengue glycoprotein prM interacts with Arf4 and Arf5.

Conclusion: Arf4 and Arf5 play a crucial role in dengue flavivirus secretion.

Significance: Our findings reveal a molecular mechanism of dengue flavivirus secretion.

Identification and characterization of virus-host interactions are very important steps toward a better understanding of the molecular mechanisms responsible for disease progression and pathogenesis. To date, very few cellular factors involved in the life cycle of flaviviruses, which are important human pathogens, have been described. In this study, we demonstrate a crucial role for class II Arf proteins (Arf4 and Arf5) in the dengue flavivirus life cycle. We show that simultaneous depletion of Arf4 and Arf5 blocks recombinant subviral particle secretion for all four dengue serotypes. Immunostaining analysis suggests that class II Arf proteins are required at an early pre-Golgi step for dengue virus secretion. Using a horseradish peroxidase protein fused to a signal peptide, we show that class II Arfs act specifically on dengue virus secretion without altering the secretion of proteins through the constitutive secretory pathway. Co-immunoprecipitation data demonstrate that the dengue prM glycoprotein interacts with class II Arf proteins but not through its C-terminal VXPV motif. Finally, experiments performed with replication-competent dengue and yellow fever viruses demonstrate that the depletion of class II Arfs inhibits virus secretion, thus confirming their implication in the virus life cycle, although data obtained with West Nile virus pointed out the differences in virus-host interactions among flaviviruses. Our findings shed new light on a molecular mechanism used by dengue viruses during the late stages of the life cycle and demonstrate a novel function for class II Arf proteins.

The four serotypes of dengue virus (DENV), which are members of the Flavivirus genus in the Flaviridae family, are the most important vector-borne viruses, and they cause 50–100 million cases of infection per year, including 500,000 severe cases (1–3).

Assembly of DENV, like other flaviviruses, occurs at membranes of the endoplasmic reticulum (ER) (4). Virions bud into the lumen of this organelle and, before being released, traffic through the host cell secretory pathway where the cellular protein furin cleaves pre-membrane (prM) protein, resulting in the release of the pr peptide and formation of mature virions (5–7). During flavivirus infection, in addition to infectious mature virions, noninfectious subviral particles are produced and traffic along the same secretory pathway as infectious particles before being released by the host cell (8). Similar recombinant subviral particles (RSPs) can form in the absence of cap-sid in cells transfected solely with prM and envelope (E) glycoproteins (9–12). In a previous work, we have developed RSPs for the four dengue serotypes and have shown that they mimic budding, secretion, and maturation of DENV (12). Therefore, dengue RSP represents a safe and convenient tool for the study of virus-host interactions during DENV secretion in host cells.

Currently, the viral-host interactions during the DENV life cycle are still poorly characterized. Moreover, most studies in this field focus on the maturation process, and less research has been done to investigate the molecular mechanisms supporting secretion (13, 14). We have previously reported the development of human stable cell lines that constitutively secrete RSPs of all four dengue serotypes and their use for screening a human siRNA library targeting specifically 122 genes involved in cellular membrane trafficking (12). We noticed that two members of the ADP-ribosylation factor (Arf) family, Arf1 and Arf6, which represent the most studied Arf proteins (15), as well as an Arf-related gene, the ADP-ribosylation factor interacting protein 2 (Arfaptin 2), showed either significant reduction or enhance-

DPS, dengue patient serum; ER, endoplasmic reticulum; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; E, envelope; TRITC, tetramethylrhodamine isothiocyanate; BFA, brefeldin A.
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ment of RSP release (12), strongly suggesting a role for members of the Arf family in late stages of the DENV life cycle.

Arf proteins belong to the Ras superfamily of small GTPases. They cycle between cellular membranes and the cytosol, playing critical roles in membrane trafficking via the recruitment of various coat proteins (16, 17), initiating membrane curvature (18), and by modulating the activity of several lipid-modifying enzymes (19, 20). Six Arf proteins have been identified so far, with only five expressed in humans; Arf2 has been lost (20). Based on amino acid sequence identity, the six Arfs were grouped into three classes (21) as follows: class I (Arf1–3), class II (Arf4 and -5), and class III (Arf6). Functionally, class I members are known to be involved in the assembly of different types of coat complexes onto budding vesicles along the secretory pathway (22), and class III (viz. Arf6) regulates endosome-membrane traffic and structural organization at the cell surface (23). Less is known about the function of class II proteins, although Arf4 involvement in the trafficking of rhodopsin has recently been documented (24, 25).

In this study, we investigated the potential role of all Arf family members during DENV secretion using dengue RSPs as a model system. We have identified Arf4 and Arf5 as two novel cellular factors involved in dengue virus secretion.

EXPERIMENTAL PROCEDURES

Cells, Viruses, and Antibodies—HeLa cells, human embryonic kidney cells (293T), and human hepatic cells (HepG2) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C with 5% CO₂. Dengue RSP-producing cell lines (HeLa-prME-DENV1, -DENV2, -DENV3, and -DENV4), which were established using the codon optimized DENV prME gene as described previously (12), were cultured in the same medium containing 500 µg/ml hygromycin. Mosquito Aedes pseudoscutellaris (AP61) cells were grown in L-15 medium containing 10% FBS and 1% tryptophan at 28 °C.

All work with infectious flavivirus, including Israeli WNV strain IS-98-ST1, DENV1 strain d1d FGA/NA, DENV4 strain 63632/76 (Burma), and YFV strain (Asibi), was performed in a biosafety level 3 laboratory (Institut Pasteur, Paris, France). HepG2 cells were used to study the effect of depletion of class II Arfs by siRNAs on flavivirus replication. Virus titration of DENV1 and DENV4 was performed on AP61 cells, whereas titration of YFV and WNV was performed using VeroE6 and BHK21, respectively.

The mouse anti-E monoclonal antibodies (mAb) 4E11 and 4G2 were provided by Dr. A. Amara (Institut Pasteur, Paris, France). The mouse anti-prME antibody and sera from a patient with dengue virus infection were kindly provided by Dr. Philippe Buchy (Institut Pasteur, Cambodia). The mouse anti-prM monoclonal antibody prM-6.1 (26) was kindly provided by Dr. Sittisombut Nopponr (Chiang Mai University, Chiang Mai, Thailand). Other antibodies used were anti-GAPDH mAb from Abcam (Cambridge, MA), anti-Arf4 from ProteinTech Group Inc. (Chicago, IL), and anti-Arf5 mAb from Abnova Corp. (Walnut, CA).

Constructs—For the construction of GFP fusion proteins, Arf4 (NM_001660) and Arf5 (NM_001662) cDNAs were purchased from OriGene Technologies (Rockville, MD). A GFP cDNA derived from the pGFP-N1 plasmid (Clontech) was fused to the C-terminal end of Arf4 and Arf5 and subcloned into pcDNA3.1 (Invitrogen).

For rescue experiments, mutations were introduced into Arf5 or DENV prME by PCR-mediated mutagenesis at the site targeted by the Arf5 siRNA (L-011584), so that the original sequence (5-ACCACAATCTGTACAAA-3) was changed to (5-AC-GAGATACTCTATAAG-3), thus creating a siRNA-resistant Arf5 sequence (Arf5SR). Both wild-type Arf5 and Arf5SR were then subcloned into pcDNA3.1 using BamHI and XhoI restriction enzymes.

To obtain separate prM and E constructs, prM or E was amplified by PCR using codon-optimized DENV1 prME as template and subcloned into pcDNA3.1 vector using BamHI and XhoI restriction enzymes. A signal sequence of vesicular stomatitis virus G envelope glycoprotein was inserted in-frame upstream of either prM or E cDNA.

For site-directed mutagenesis, the VXPT motif in the C-terminal portion of prM was either deleted (prME-ΔVXPT) or mutated to AXAX (prME-AXAX) by overlapping PCR using codon-optimized DENV1 prME as template and then subcloned into pcDNA3.1 vector using BamHI and XhoI restriction enzymes.

siRNA Experiments—All siRNAs, including nontargeting (NT) siRNA (D-001206) and transfection reagents DharmaFECT 1 (T-2001), were purchased from Dharmacon (Research Inc., Lafayette, CO). Arf1 siRNA (L-011580), Arf3 siRNA (L-011581), Arf4 siRNA (L-011582), Arf5 siRNA (L-011584), Arf6 siRNA (L-004008), and COP β (β-COP) siRNA (L-017940) were provided as SMARTpool ON-TARGET plus siRNA, which are pools of four siRNAs targeting various sites in a single gene. DENV1 E siRNA (AGATCCAGCTGAC- CGATT), Arf4i siRNA (AGACAACCAUUUGUAAUA), and Arf5i siRNA (CCCAAAUCCUGUAACACCU) were provided as individual siRNAs.

For siRNA experiments, reverse transfection was performed using DharmaFECT 1 reagents. Briefly, siRNAs were added to 24-well plates in DMEM without FBS and antibiotics. Twenty minutes later 0.8 ml of cells (120,000 cells/ml in DMEM supplemented with 10% FBS) were added to each well so that the final siRNA concentration was 100 nM. Cells were incubated at 37 °C for 48 h. Medium was then replaced with 0.3 ml of DMEM containing 2% FBS (without antibiotics), and 14 h later, supernatant containing secreted RSP was collected and cleared by centrifugation at 4000 rpm for 15 min. Cells were lysed in buffer containing 1% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), and 1 mM EDTA for 15 min on ice, with frequent vortexing.

In rescue experiments, HeLa-prME-DENV1 cells were transfected with either Arf5SR or with an empty vector to derive three stable cell lines, following culture in medium containing 400 µg/ml G418 for 3 weeks. These cell lines were then subjected to siRNA experiments as described above.

Gel Electrophoresis, Western Blot, and RSP Quantification—4–12% NuPAGE Novex BisTris gels (Invitrogen) were used for SDS-PAGE. For Western blotting, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. The mem-
branes were blocked overnight in 5% milk in PBS containing 0.1% Tween 20 solution and incubated with primary antibody for 1 h. After repeated washes, membranes were incubated for 1 h at room temperature with a horseradish peroxidase-labeled secondary antibody (Zymed Laboratories Inc.), and proteins were visualized using ECL Western blot detection reagent (Invitrogen) and exposure of blots to x-ray films. To quantify RSP secretion, the mean luminescence and area of supernatant and cell lysate E signals on Western blot results were measured by densitometry using the Adobe Photoshop software (Adobe Systems Incorporated, San Jose, CA). For each condition, the relative amount of RSP in supernatant was estimated by calculating the ratio E supernatant/E cell lysate. Ratios were then normalized to that of NT siRNA and expressed in percentage of reduction of secretion.

Flow Cytometry Analysis—HeLa-prME-DENV1 cells were simultaneously transfected with either Arf4GFP or Arf5GFP together with siRNAs targeting Arf4 or Arf5. Medium was replaced 4 h post-transfection with DMEM containing 10% FBS. Cells were fixed in 3.2% paraformaldehyde 48 h later and analyzed using a FACSCalibur flow cytometer (BD Biosciences).

Virus Titration—DENV and WNV were titrated by focus immunodetection assay, essentially as described previously (27). Briefly, 10^6 cells were seeded in 24-well plates. After 1 day, cells were washed three times, and 10-fold serial dilutions of infectious supernatants (10^-2 to 10^-5 for DENV and 10^-3 to 10^-6 for WNV) were added to cells for 90 min at 28 °C. Cells were subsequently incubated in a final volume of 800 μl of L-15 medium supplemented with 10% FBS.

Following the appropriate incubation time at 28 °C (5 days for DENV and 3 days for WNV), cells were fixed in 3% formaldehyde for 20 min, permeabilized with 0.1% Triton X-100 for 4 min at room temperature, and incubated for 30 min at 37 °C with the mouse monoclonal 4G2 antibody that can detect E protein from different flaviviruses (28). Cells were incubated with secondary anti-mouse HRP-conjugated antibodies for 1 h at 37 °C, and nuclei were revealed using dianinobenzidine (Sigma).

YFV was titrated by plaque assay using Vero cells seeded in 24-well plates. One day after seeding, 10-fold serial dilutions (10^-3 to 10^-6) of infectious supernatants were added, and cells were incubated for 90 min at 37 °C. Cells were subsequently incubated in a final volume of 800 μl of L-15 medium supplemented with 10% FBS and incubated at 37 °C for 5 days. After repeated washings, cells were stained with 3% formaldehyde crystal violet for 20 min at room temperature, and plaques were manually counted.

Fluorescence Microscopy—For fluorescence microscopy, HeLa-prME-DENV1 cells grown on glass coverslips were fixed, permeabilized, and incubated with the following antibodies: anti-E (1:1000), anti-calretucin (1:200), anti-ERGIC53 mAb (1:1000), and anti-Golgin-97 mAb (1:50) followed by incubation with appropriate secondary antibodies conjugated with fluorescein isothiocyanate (FITC) or TRITC. Nuclei were stained with DAPI, and coverslips were mounted on glass slides for analysis. Fixed cells were visualized under an AxioObserver inverted motorized fluorescent microscope, using the Apo-Tome module piloted through the Axiovision 4.6 software, and images were acquired through a high resolution MRm AxioCam CCD camera (Carl Zeiss, Jena, Germany).

RESULTS
Silencing of Class II Arf Expression Inhibits Secretion of Dengue Recombinant Subviral Particles—To investigate the effect of all Arf family members on the secretion of DENV1 RSP, we transfected the HeLa-prME-DENV1 stable cell line (which constitutively secretes RSPs for DENV1) with siRNAs targeting each Arf gene. Cells treated with either NT siRNA or siRNA targeting the E glycoprotein of DENV1 were used as negative and positive controls, respectively. RSP secretion was assessed by visualizing dengue E protein in the supernatant by Western blotting. As expected, E protein expression was abolished and could not be detected in both supernatant and cell lysate after specific DENV1 E siRNA treatment (Fig. 1A, lane 9). Targeting of single Arfs did not induce any significant variation in the secretion of RSPs (Fig. 1A, upper panel, lanes 4–8), with the exception of Arf1, whose depletion partially reduced RSP secretion (Fig. 1A, upper panel, lane 4). This result is consistent with our previous data generated by screening a library of 122 siRNAs targeting genes involved in membrane traffic (12). In contrast, no significant change in intracellular expression levels of E was observed after knockdown of any of the Arfs (Fig. 1A, middle panel, lanes 4–8). It has been reported previously that Arfs could play redundant roles and that cooperation of two Arfs at the same site might be a general mechanism of Arf activity (29). Therefore, we decided to investigate the effect of all possible combinations of double Arf knockdowns on the secretion of DENV1 RSPs (Fig. 1B). For all conditions, no significant...
variations in expression of E protein and GAPDH were observed in cell lysates (Fig. 1B, middle and lower panels). In contrast, combinations of siRNAs targeting Arf1/H110014, Arf1/H110015, and Arf4/H110015 induced a clear decrease of secretion of RSPs (Fig. 1B, lanes 3, 4, and 9, respectively). Interestingly, the most dramatic reduction of DENV1 RSP secretion was observed after down-regulation of both members of class II Arfs (Arf4 and Arf5), suggesting a crucial role for these proteins in the release of RSPs (Fig. 1B, lane 9). Moreover, as the depletion of Arf4 or Arf5 alone did not decrease RSP secretion (Fig. 1A, lanes 6 and 7), our results also suggest that class II Arf proteins could compensate for each other during RSP secretion.

The use of pooled siRNAs, targeting four different sites in the same gene, ensured an efficient knockdown of targeted mRNA but could have resulted in an enhancement of off-targeting effect. To verify that the inhibition of DENV1 RSP secretion was observed after down-regulation of both members of class II Arfs (Arf4 and Arf5), suggesting a crucial role for these proteins in the release of RSPs (Fig. 1B, lane 9). Moreover, as the depletion of Arf4 or Arf5 alone did not decrease RSP secretion (Fig. 1A, lanes 6 and 7), our results also suggest that class II Arf proteins could compensate for each other during RSP secretion.

The use of pooled siRNAs, targeting four different sites in the same gene, ensured an efficient knockdown of targeted mRNA but could have resulted in an enhancement of off-targeting effect. To verify that the inhibition of DENV1 RSP secretion specifically resulted from class II Arf depletion, the effect of individual siRNAs targeting Arf4 and/or Arf5 genes (Arf4i and Arf5i), respectively, was investigated and compared with that of the pooled siRNAs (Fig. 1C, lanes 6–8 and 2–4, respectively). Similar expression for GAPDH was observed for all conditions tested (Fig. 1C, lower panel). Combinations of either individual or pooled siRNAs targeting Arf4+/5 led to a severe reduction of secreted RSPs (Fig. 1C, lanes 4 and 8, upper panel). Similar results were obtained with another set of individual siRNAs (data not shown). Altogether, our data demonstrate that blocking of dengue RSP secretion did not result from an off-targeting effect of siRNAs and, therefore, was specifically due to class II Arf depletion.

To verify the efficacy and specificity of siRNA knockdown, Arf4 and Arf5 expression was monitored by Western blotting using either anti-Arf4 or anti-Arf5 antibodies. A band with an apparent molecular mass of 21 kDa, corresponding to the predicted electrophoresis mobility of Arf4 or Arf5 protein, was readily observed in cells treated with NT siRNA but not in cells transfected with their specific siRNAs (Fig. 1C, 3rd and 4th panels). To further measure siRNA knockdown efficiency, we used a recombinant Arf protein fused at its C terminus to a GFP tag, so that siRNA targeting to Arf would also reduce expression of GFP. HeLa-prME-DENV1 cells were transiently transfected with Arf4GFP or Arf5GFP in combination with Arf4 or Arf5 siRNAs as in C. The efficiency of knockdown was assessed by calculating the percentage of GFP-positive cells by flow cytometry.
in the percentage of Arf4-GFP cells was observed after Arf5 siRNA treatment (Fig. 1D, upper panel, 31.2%), confirming the specificity of Arf4 siRNAs treatment. Similar results were obtained with Arf5-GFP cells and Arf5 siRNA. Arf5 siRNA treatment could reduce the percentage of Arf5-GFP-positive cells by over 90% (Fig. 1D, lower panel) but not that of Arf4-GFP. These results demonstrate that treatment with Arf4 and Arf5 siRNAs could efficiently knockdown expression of corresponding proteins.

Expression of siRNA-resistant Arf5 Rescues Secretion of Dengue Recombinant Subviral Particles—To further confirm the specific role of class II Arfs in secretion of DENV RSPs, we performed a rescue experiment. We engineered cells that express an siRNA-resistant form of Arf5 (Arf5SR) in which six nucleotides in the targeting site of Arf5i siRNA were substituted by introducing silent mutations that did not modify the G/C content (Fig. 2A). Under these conditions, treatment with siRNA on these cells would only target endogenous Arf5 with-
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The release of HRP into the medium is quantified by its enzymatic activity as described previously (34). HeLa cells were transiently transfected with ss-HRP in combination with various treatments, including siRNA targeting the β-COP subunit of the COP I coatmer, which interferes with the constitutive secretory pathway (34–36). HeLa-prME-DENV1 cells were also treated with the same siRNAs. BFA treatment was used as control. Two days post-transfection, peroxidase activity or RSPs in cell supernatants were measured by chemiluminescence or Western blot, respectively, and then normalized to those of control cells transfected with NT siRNA. No difference was observed in ss-HRP secretion after Arf4 + 5 down-regulation when compared with NT controls, whereas Arf4 + 5 siRNA reduced RSP secretion by 80% (Fig. 4C). BFA treatment induced a clear reduction of both ss-HRP and RSP secretion. Treatment with β-COP siRNA led to a drastic 60% reduction of secreted ss-HRP into the supernatant, whereas it only slightly affected RSP secretion by 20% (Fig. 4C). These results demonstrate that class II Arf proteins are not required for the generic constitutive secretory pathway and that they play a specific role in the secretion of dengue RSPs.

Dengue prM Interacts with Class II Arfs in HeLa-prME Cells—
To establish whether DENV glycoproteins could interact with class II Arfs in mammalian cells, co-immunoprecipitation experiments were performed. Lysates from HeLa and HeLa-prME-DENV1 cells were incubated with either normal human serum or dengue patient serum (DPS), obtained from a patient infected by DENV1, and subjected to immunoprecipitation. The presence of DENV E, prM, Arf4, and Arf5 in immunoprecipitates was analyzed by Western blotting. A clear band corresponding to Arf4 was detected in cell lysates from HeLa-prME-DENV1 but not parental HeLa cells. Our results indicate an interaction between class II Arfs and DENV glycoproteins.

To test which glycoprotein, prM or E, could be involved in this interaction with class II Arfs, we first subcloned prM and E into two separate constructs and transfected them into 293T cells individually or in combination. Cell lysates were subjected to immunoprecipitation using the DPS anti-DENV1 serum, and co-immunoprecipitation of Arf4 was analyzed by Western blotting. A clear band corresponding to Arf4 was detected in cells transfected with prM and E in combination (Fig. 5B, lane 12, lower panel), and a weak band was also observed in cells expressing prM (Fig. 5B, lane 10, lower panel). We noted that E and prM were expressed at much lower levels when these proteins were expressed individually than in combination (Fig. 5B, lanes 2–4, upper and middle panels). To exclude the possibility that the failure to precipitate Arf4 in cells expressing E individually may have resulted from the low expression level of E, we used two monoclonal antibodies, prM-6.1 and 4G2, which recognize prM and E, respectively (26, 28), for immunoprecipitation from HeLa-prME-DENV1 cell lysate. These two monoclonal antibodies efficiently pulled down their specific glycoproteins and a trace amount of others (Fig. 5C, upper two panels). We found that both class II Arfs were immunoprecipitated with anti-prM antibody but not with 4G2 (Fig. 5C, lower two panels), indicating that prM but not E protein interacts with class II Arfs.
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To gain insight into the molecular mechanism underlying this interaction, we inspected prM sequence. It has been reported that a VXPX motif in the C-terminal portion of rhodopsin was recognized by Arf4 (24, 25). We found a similar motif at the C-terminal end of prM, viz. V161XP163X, which was conserved in all four serotypes used in our experiments (Fig. 5D). To test whether this sequence was important for interaction of DENV prM with class II Arfs, we produced mutated forms of prME with either a deletion of the VXPX motif (prME-ΔVXPX) or a substitution of valine 161 and proline 163 with alanines (prME-AXAX) and transfected them into 293T cells. No RSPs could be detected in supernatants of cells transfected with prME-ΔVXPX, whereas RSPs were detected after transfection with prME-AXAX (Fig. 5E, upper panel). The absence of RSPs in the supernatant of prME-ΔVXPX-expressing cells could be explained by the significant reduction of expression levels of prM and E in corresponding cell lysates (Fig. 5E, lane 2, middle two panels). This result indicates that the VXPX motif has an important role for expression and/or stability of the prM protein, but the Val-161 and Pro-163 were not critical residues. Cell lysates of cells transfected with prME-ΔVXPX and prME-AXAX were then subjected to immunoprecipitation with anti-DENV1 sera. We found that Arf4 could be pulled down from cells transfected with either prME of prME-AXAX (Fig. 5F, lower panel, lanes 10 and 12) but not from those expressing the deletion mutant (Fig. 5F, lower panel, lane 11), which also resulted in a much reduced amount of prM protein in the cell lysate (Fig. 5F, lane 3, upper panel). These results show that although the VXPX in the C-terminal portion of prM is important for the expression of prM, it is not the motif recognized by Arf4.

Dengue Viruses Are Sensitive to Class II Arf Depletion—RSPs are not fully representative of the DENV viral life cycle as only the structural viral genes are expressed and not the viral replication machinery. To determine whether class II Arf proteins would affect the life cycle of fully replicative DENV, we infected human hepatic HepG2 cells with DENV1 (d1d FGA/NA strain) or DENV4 (63632/76 strain) viruses, which are the parent viral strains used to design DENV1 and DENV4 RSPs, respectively. HepG2 cells were used in these experiments because they are highly susceptible to infection with the 63632/76 DENV4 viral strain, as this virus was isolated from a patient who died from liver failure due to dengue infection. Cells were first transfected with the specified siRNAs, which did not cause any detectable cytotoxicity (data not shown). Two days post-transfection, HepG2 cells were infected with DENV1 or DENV4. An input of 20 pfu/cell, i.e. multiplicity of infection of 20 for DENV1 or of 1 for DENV4, was required to infect HepG2 cells at a significant level. Production of progeny viruses was evaluated by titration of culture supernatants collected on day 2 post-infection on AP61 cells. A significant albeit moderate reduction in virus progeny production was observed in infected HepG2 cells treated concomitantly with Arf4 and Arf5 siRNAs, when compared with control (≈2.5-fold for DENV1, Fig. 6A; ≈5-fold for DENV4, Fig. 6B). As expected, Arf5 was efficiently silenced in AP61 cells. A significant albeit moderate reduction in virus progeny production was observed in infected HepG2 cells treated concomitantly with Arf4 and Arf5 siRNAs, when compared with control (≈2.5-fold for DENV1, Fig. 6A; ≈5-fold for DENV4, Fig. 6B). As expected, Arf5 was efficiently silenced in AP61 cells.
As HepG2 cells are permissive to hepatotropic YFV and neurotropic WNV, these cells were exposed to YFV strain Asibi and WNV strain IS-98-ST1 at an multiplicity of infection of 1. To address the role of class II Arfs in virus progeny production, HepG2 cells were treated during 48 h with specific siRNAs prior to YFV and WNV infection. Interestingly, siRNA treatment reduced YFV release by 10-fold (Fig. 6C), whereas inhibition of class II Arf proteins was ineffective in reducing WNV progeny production (Fig. 6D). These results suggest that Arf4 and Arf5 may play a role in YFV but not in WNV release from HepG2 cells. Altogether our data indicate that class II Arf proteins exhibit a differential involvement in the secretion of flaviviruses from infected cells.

**DISCUSSION**

Virus-host interactions during flavivirus secretion are poorly understood, and very few cellular factors involved in this process have been described so far (13, 14). We have used an original approach, based on the development of dengue RSPs, to identify two novel host cellular factors, Arf4 and Arf5, involved in DENV secretion. Our results demonstrate that simultaneous depletion of Arf4 and Arf5 blocks RSP secretion for all four dengue serotypes. Experiments with parental viruses used to construct the RSPs show that the life cycle of DENV is significantly affected by targeting class II Arfs. Interestingly, we also tried Arf4 and Arf5 depletion on cells infected with yellow fever flavivirus and found it partially blocks virus production as with DENV1 and -4 viruses, suggesting a role for Arf4 and Arf5 in the life cycle of hepatotropic flaviviruses. Moreover, our results show that class II Arfs are functionally redundant and can complement each other, as previously reported for some other members of the family (29). By using antibodies specific for prM and E, or by expressing prM and E individually in mammalian cells, we have found that it is the prM protein that interacts with human class II Arf.

**FIGURE 5. Interaction of dengue prM with human class II Arf.** A, lysates of HeLa and HeLa-prME-DENV1 cells were immunoprecipitated (IP) with normal human sera (NHS, lanes 3 and 4) or DPS (lanes 5 and 6). E, prM, Arf4, and Arf5 expression (lanes 1 and 2) was tested in all cell lysates (CL) as input control. Immune complexes were detected by Western blot (WB) using anti-prME, anti-Arf4, or anti-Arf5 antibody. The bands on the top of the Western blot results using Arf4 or Arf5 antibodies are light chains of IgG used for immunoprecipitation and can also be observed in B, C, and F. B, 293T cells were transfected with prM and E, either individually or in combination, and cell lysates were immunoprecipitated with normal sera (lanes 5–8) or DPS (lanes 9–12). Empty vector (ev) served as the control. Arf4 expression was tested in all cell lysates as input control (lanes 1–4). Immune complexes were detected by Western blot using anti-prME or anti-Arf4 antibody. C, lysates of HeLa-prME-DENV1 cells were immunoprecipitated with anti-E monoclonal antibody 4G2 (lane 1) or anti-prM monoclonal antibody prM-6.1 (lane 2). Immune complexes were detected by Western blot using 4E11 conjugated with HRP, anti-prME, anti-Arf4, or anti-Arf5 antibody. D, alignment of prM of four DENV serotypes, with the consensus sequence below, revealed a VXP motif in the C terminus, shown in boldface and highlighted. E, 293T cells were transfected with prME, prME-ΔVXP, or prME-AXAX. Glycoprotein prM or E protein in both supernatant and cell lysates were analyzed by Western blot using mouse anti-prME IgG. GAPDH was used as control. F, 293T cells were transfected with prME, prME-ΔVXP, or prME-AXAX, or empty vector, and cell lysates were immunoprecipitated with normal human sera (lanes 5–8) or DPS (lanes 9–12). Arf4 expression was tested in all cell lysates as input control (lanes 1–4). Immune complexes were detected by Western blot analysis using anti-prME or anti-Arf4 antibody.
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The effect of Arf4 and Arf5 down-regulation was evaluated on replication-competent viruses by measuring viral titers in the supernatants of infected cells 2 days post-infection using either focus immunodetection assay for DENV1 (A), DENV4 (B), and WNV (D) or plaque assay for yellow fever virus (C). Results are shown as mean ± S.D. of triplicate measurements from one experiment. Similar results were obtained in two other infections.

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Arf4 and Arf5 play a crucial role in dengue virus secretion. The effect of Arf4 and Arf5 down-regulation was evaluated on replication-competent viruses by measuring viral titers in the supernatants of infected cells 2 days post-infection using either focus immunodetection assay for DENV1 (A), DENV4 (B), and WNV (D) or plaque assay for yellow fever virus (C). Results are shown as mean ± S.D. of triplicate measurements from one experiment. Similar results were obtained in two other infections.
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Arf4 and Arf5 are not as efficient in the virus infection experiments as opposed to the RSPs. One possible reason may be the lower expression level of prME in DENV-infected cells than that in HeLa-prME cells, in which a codon-optimized prME gene is continually expressed. Codon optimization enhances the expression of the prME gene in mammalian cells without changing the amino acid of the prME protein (12). As more particles form in HeLa-prME cells, their secretion may need more Arf4/5 proteins, which makes HeLa-prME cells a sensitive tool to identify cellular factors required for DENV secretion. In our experiment, although the siRNA reduced almost 90% Arf4/5 protein, there were detectable levels of Arf4/5 proteins in host cells. The remaining Arf4/5 proteins were not sufficient to support the secretion of RSPs but might still be able to partially support the secretion of replicative dengue virus because their prME level is lower and does not require high levels of Arf4/5 proteins. In the future, we will design some approaches other than siRNA to test this hypothesis.

The function of class II Arfs has so far remained elusive. Our results reveal a novel function for these proteins, the less studied and known members of the Arf family. As there are no antiviral therapies approved for use against flaviviruses, a possible strategy is to consider the new characterized host cell components as potential therapeutic targets for drug development (47).

REFERENCES

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