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Regulated Cleavages at the West Nile Virus NS4A-2K-NS4B Junctions Play a Major Role in Rearranging Cytoplasmic Membranes and Golgi Trafficking of the NS4A Protein

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A common feature associated with the replication of most RNA viruses is the formation of a unique membrane environment encapsulating the viral replication complex. For their part, flaviviruses are no exception, whereupon infection causes a dramatic rearrangement and induction of unique membrane structures within the cytoplasm of infected cells. These virus-induced membranes, termed paracrystalline arrays, convoluted membranes, and vesicle packets, all appear to have specific functions during replication and are derived from different organelles within the host cell. The aim of this study was to identify which protein(s) specified by the Australian strain of West Nile virus, Kunjin virus (KUNV), are responsible for the dramatic membrane alterations observed during infection. Thus, we have shown using immunolabeling of ultrathin cryosections of transfected cells that expression of the KUNV polyprotein intermediates NS4A-4B and NS2B-3-4A, as well as that of individual NS4A proteins with and without the C-terminal transmembrane domain 2K, resulted in different degrees of rearrangement of cytoplasmic membranes. The formation of the membrane structures characteristic for virus infection required coexpression of an NS4A-NS4B cassette with the viral protease NS2B-3pro which was shown to be essential for the release of the individual NS4A and NS4B proteins. Individual expression of NS4A protein retaining the C-terminal transmembrane domain 2K resulted in the induction of membrane rearrangements most resembling virus-induced structures, while removal of the 2K domain led to a less profound membrane rearrangement but resulted in the redistribution of the NS4A protein to the Golgi apparatus. The results show that cleavage of the KUNV polyprotein NS4A-4B by the viral protease is the key initiation event in the induction of membrane rearrangement and that the NS4A protein intermediate containing the uncleaved C-terminal transmembrane domain plays an essential role in these membrane rearrangements.

A common feature of plus-strand RNA viruses is their ability to induce cytoplasmic membrane rearrangements that apparently facilitate efficient RNA replication (2, 8–10, 21–23, 28, 32, 33, 36, 38). In most cases, these membrane structures share common features, such as small 70- to 100-nm vesicles containing thread-like inclusions (dependent on fixation protocols), or double membrane vesicles enclosing the replicating viral RNA. Flaviviruses appear unique in that they induce at least two or three characteristic structures including convoluted membranes (CM), paracrystalline (PC) arrays, and vesicle packets/smooth membrane structures (30). Our detailed ultrastructural analyses, using an Australian strain of West Nile virus, Kunjin virus (KUNV), as a model flavivirus, have revealed that each of these membrane structures can be immunostained with distinct antibodies to different constellations of both virus-specific and host-specific proteins, strongly suggesting that each membrane structure performs a precise function during replication (21, 22, 25, 38).

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Early seminal studies established a relationship of the KUNV nonstructural (ns) proteins and the viral RNA within membrane fractions prepared from cytoplasmic extracts and sedimentation through sucrose density gradients (4, 5). The fast-sedimenting or "heavy" membranes contained the virus-specified RNA and ns proteins (NS1, NS2A, NS3, NS4A and NS5) and virtually all of the assessed RNA-dependent RNA polymerase activity. The apparent membrane association of these ns proteins was also confirmed later by immunofluorescence (IF) and cryoimmunoelectron microscopy (cryo-IEM), whereby each of these viral proteins was observed to specifically label one or more of these KUNV-induced membrane structures (25, 38). The specific role of the KUNV ns proteins in induction of the characteristic membranes was highlighted in Vero cells stably expressing KUN replicon RNA (lacking structural proteins) where all the virus-induced membranes were clearly observed (26). These studies also identified that a certain threshold level of RNA replication was required before membrane induction could occur, indicating a close relationship between viral RNA and protein synthesis and cellular membrane biogenesis (26).

Our complementary studies have indicated that the CM/PC structures appear to be derived from membranes of the rough endoplasmic reticulum and the intermediate compartment (22). In contrast, the vesicle packets (the site of viral RNA synthesis) contained protein markers for the *trans*-Golgi region

(22). This collection of cellular markers prompted us to investigate the biogenesis of the virus-induced membrane structures and the roles for both the viral and the host cell proteins in this process. Currently, only limited data are available indicating a role for any of the flavivirus proteins in membrane rearrangements. Studies with the *Hepacivirus* hepatitis C virus (HCV) proteins revealed the presence of structures similar to the induced CM/PC in cells transfected with NS4B-expressing plasmid (8). However, a role for HCV NS4B in virus replication is not currently known, and it appears to localize differently than NS4B of other flaviviruses (12, 37). A recent study by Chua et al. (6) has suggested a role for dengue virus NS3 in some membrane alterations; however, no electron microscopy studies were performed to confirm the observations. Here we describe the first report on the essential role of flavivirus ns protein NS4A in the induction of membrane structures CM/PC, characteristic of flavivirus infection, and define the cleavage of NS4A-4B polyprotein intermediate by the viral NS2B-3 protease as the key event for the initiation of the membrane induction. We also provide some compelling evidence to suggest that the removal of the 2K domain from NS4A allows targeting of the mature NS4A protein to Golgi membranes implicated in the formation of the viral replication complex (22).

(This work was conducted by J. Roosendaal in partial fulfillment of the requirements for a Masters by research degree from the University of Wageningen, Wageningen, The Netherlands.)

MATERIALS AND METHODS

Plasmid construction. The Semliki Forest virus (SFV) replicon expression vector pSFV1 (Invitrogen) was used to engineer constructs expressing different KUN genes and gene cassettes. All inserted genes were generated by PCR with appropriate primer pairs containing incorporated restriction digest sites and were named according to the gene(s) inserted within. The primer sequences were derived from the sequence of KUNV RNA (7, 14), and genes were amplified from constructs previously described (14). Restriction digest and insertion into the BamHI- or SmaI-linearized pSFV1 was used to generate the expression constructs. All inserts contained a Kozak sequence and an individual ATG start codon at the N terminus with a corresponding stop codon at the C terminus. The pSFV-NS2B-3pro construct encoding functional KUNV protease contained the complete KUNV NS2B gene and first 187 codons of KUNV NS3 gene.

Cells and transfection conditions. Baby hamster kidney (BHK) and Vero cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and penicillin-streptomycin. Cells were passaged the day preceding electroporation to ensure optimal growth conditions. In vitro-transcribed RNAs of the individual pSFV1-derived constructs encoding KUNV gene/gene cassettes were generated according to the manufacturer's protocol using SP6 RNA polymerase (Boehringer Mannheim) and SpeI-linearized plasmid DNAs as templates. In the case of pSFV1-NS4B, the DNA was linearized with SapI located further downstream of the SpeI site because an SpeI site is located within the NS4B sequence. Electroporation of BHK was performed with the Bio-Rad Gene Pulser II electroporator using conditions specified previously (13, 14). Electroporation of Vero cells was performed as described previously (14, 15), except that 6×10^6 cells were used for each electroporation and conditions were 290 V and 25 mF involving 4 pulses at 4-s intervals.

Radiolabeling and immunoprecipitation. pSFV1-transfected BHK cells were grown on 60-mm^2 culture dishes, and at 11 h posttransfection, the cells were starved in methionine-cysteine-deficient medium for 60 min and subsequently radiolabeled with 50μCi/ml [^{35}S]methionine-cysteine (Trans-label; ICN) for a further 120 min. The radiolabeled cells were incubated on ice with lysis buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% Na deoxycholate, 0.1% sodium dodecyl sulfate (SDS) containing 4-(2-aminoethyl) benzenesulfonyl fluoride (0.1 M), aprotinin (2 mg/ml), and leupeptin (1 mg/ml)] for 10 min and harvested by scraping into microcentrifuge tubes and kept on ice for an additional 10 min. The lysate was centrifuged for 10 min at 12,000 × *g* at 4°C, and the resulting supernatant was collected for analysis either directly or after immunoprecipitation with rabbit anti-NS2B, anti-NS3, anti-NS4A, or anti-NS4B antibodies as previously described (25, 37, 38). Isolated proteins were resolved on 12.5% polyacrylamide–SDS gels that were fixed and incubated in Amplify (Amersham) before visualization by autoradiography.

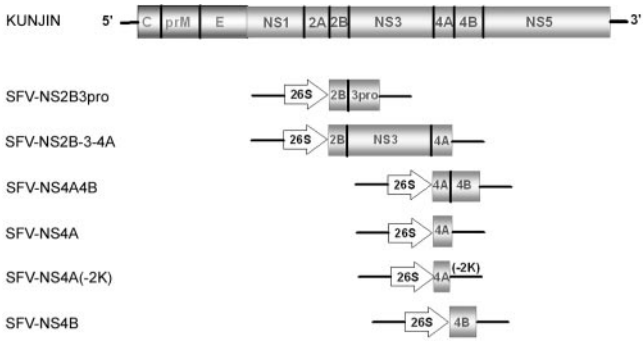

FIG. 1. Schematic representation of KUNV genes/gene cassettes encoded in the Semliki Forest virus replicon vectors. All constructs were generated via PCR and appropriate restriction digest, and where required, signal sequences were included to ensure correct translocation of the expressed polyproteins into the lumen of the rough endoplasmic reticulum. The NS4A(−2K) C terminus was determined by previous studies (17), and the NS2B-3pro construct contained the first 187 amino acids of NS3 required for efficient in *trans* proteolytic processing at cytoplasmic dibasic sites within the KUN polyproteins. The 26S promoter represents the subgenomic promoter of pSFV used for expression of foreign genes.

Immunofluorescence analysis. At 11 h posttransfection, cells were washed with phosphate-buffered saline and fixed with 4% paraformaldehyde in phosphate-buffered saline for 10 min at room temperature, followed by an additional 10 min with 4% paraformaldehyde containing 0.1% Triton X-100. The fixed cells were subsequently incubated with monospecific rabbit antisera to KUNV NS3, NS4A, or NS4B or to a monoclonal antibody raised against the Golgi marker protein Giantin (18). Bound antibody was subsequently visualized by IF with goat anti-rabbit immunoglobulin G (IgG) conjugated to fluorescein isothiocyanate (FITC; Edward Keller, Australia) or to Oregon green (Molecular Probes, Leiden, The Netherlands) or with anti-mouse IgG conjugated to Texas Red (Molecular Probes) and viewed with a Nikon E600 UV microscope. Images were captured on a Leica DC100 charge-coupled device cooled digital camera before presentation using Adobe Photoshop software.

Cryofixation, sectioning, and electron microscopy. All procedures have been described previously (23, 24, 38). Ultrathin cryosections (~50 nm) were cut, collected in a mix of 19:1 sucrose-methylcellulose, and immunolabeled with rabbit antisera raised against KUN NS4A or NS3. Bound antibodies were visualized with 10 nm protein A gold (Utrecht University, Utrecht, The Netherlands) and viewed on a Joel 1010 transmission electron microscope. Images were collected and composed into figures using Adobe Photoshop software.

RESULTS

Expression of the KUNV genes/gene cassettes by the SFV replicon vector. As depicted in Fig. 1, several SFV replicon constructs that encompassed the KUNV genes from NS2B to NS4B were prepared. This region was initially chosen as the specified individual proteins were observed to localize to induced membranes in infected cells (25, 37, 38). In addition, we have not observed membrane rearrangements when either NS1 or NS5 was expressed alone (J. M. Mackenzie, A. Khromykh, and E. G. Westaway, unpublished observations). In some of the experiments, the vector encoding the KUNV viral protease NS2B-3pro was included in the transfections to ensure correct processing of the polyprotein intermediates. The SFV replicon system was employed because expression from the SFV replicon vector yields high levels of proteins and

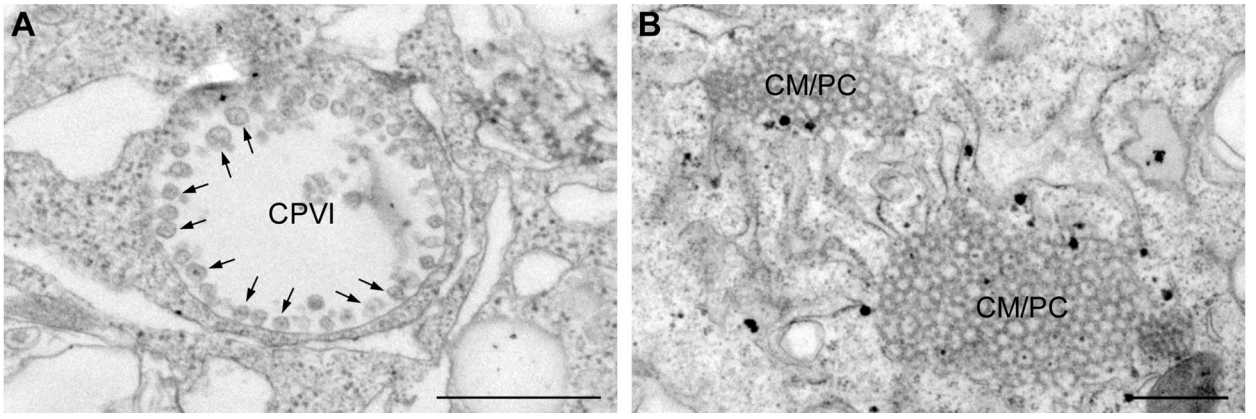

FIG. 2. Distinct membrane rearrangements induced upon cytoplasmic replication of either the Semliki Forest virus replicon (A) or the Kunjin virus replicon (B). Abbreviations: CPVI, cytopathic vacuoles characteristic of alphavirus replication; CM/PC, convoluted membranes/paracrystalline arrays characteristic for flavivirus replication. Bars, 500 nm.

because cytoplasmic membranes utilized by SFV (i.e., endosome/lysosomes) (9) are quite distinct from those involved in *Flavivirus* replication (i.e., endoplasmic reticulum [ER]/intermediate compartment and *trans*-Golgi) (22), as depicted in Fig. 2. Therefore, we envisaged no interference in membrane induction by the SFV replicon vector. In addition, no interference of replication was observed when the SFV replicon was used as a helper system for the packaging of the KUNV replicon (13).

To ascertain the level of expression of the KUNV ns polyproteins and their ability to be cleaved when cotransfected with the KUNV protease (NS2B-3pro), BHK cells were transfected with the SFV replicon constructs, and newly synthesized proteins were metabolically radiolabeled with [^{35}S]methionine-

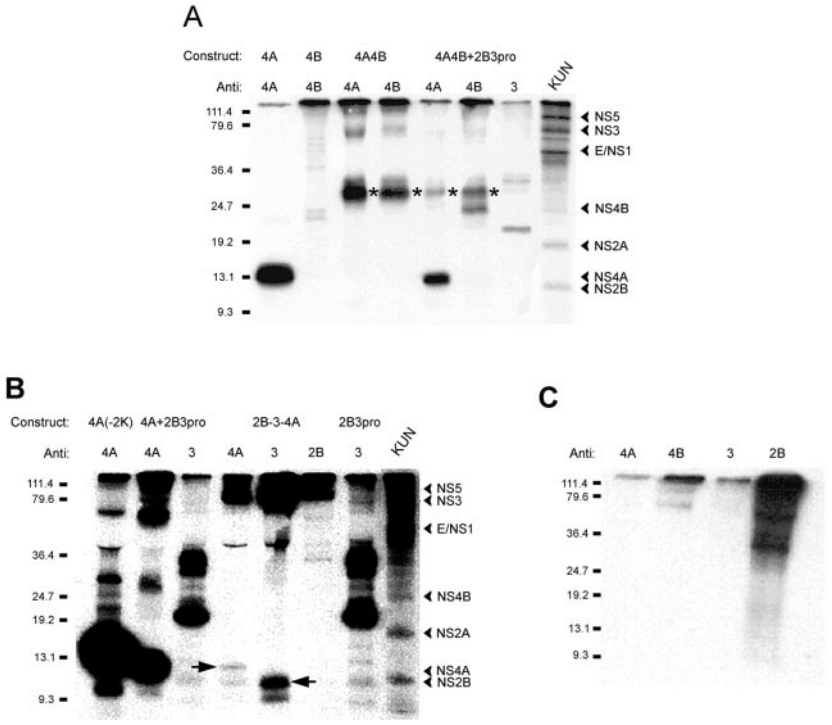

FIG. 3. Expression of KUNV genes/gene cassettes by the SFV replicon vector results in correct processing of the individual proteins and reveals a requirement of the viral protease for cleavage of NS4A and NS4B. BHK cells were electroporated and radiolabeled at 11 h posttransfection with [^{35}S]methionine-cysteine. Individual KUNV proteins were then isolated from the collected lysates by RIP using monospecific rabbit antisera (A and B). Protein migration was assessed against a [^{35}S]methionine-cysteine-radiolabeled lysate generated from KUN-infected BHK cells and by the predicted molecular mass. Note that the expression of the NS2B-3pro construct yields an NS3 species of ~20 kDa corresponding to the first 187 amino acids of the NS3 protein that is isolated after RIP with anti-NS3 antibodies. Uncleaved NS4A-NS4B polyproteins are indicated by asterisks in panel A. Mock lysates are shown in panel C.

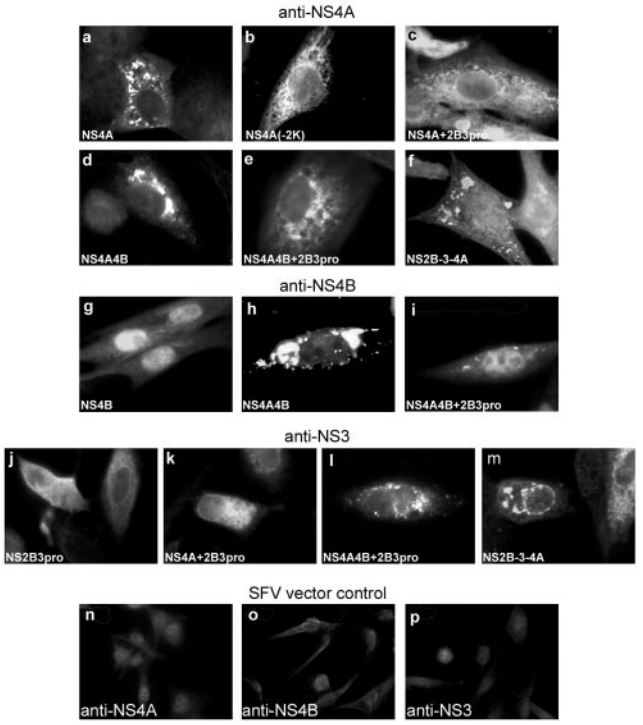

FIG. 4. Subcellular localization of the expressed KUN proteins as observed by IF. BHK cells transfected as indicated were fixed with 4% paraformaldehyde–0.05% Triton X-100, and distribution of the individual expressed proteins was observed using monospecific rabbit antisera and anti-rabbit FITC-conjugated IgG. Some smaller foci were also observed in cells expressing NS4A (a) and NS4A(−2K) (b). Large perinuclear foci were only observed in cells transfected with NS4A plus NS2B-3pro (c and k), NS4A-4B (d and h), NS4A-4B plus NS2B-3pro (e, i, and l), and NS2B-3-4A (f and m). Also note nuclear translocation of NS4B when expressed alone (g) or when cleaved from the polyproteins (i) but not when the viral protease was absent (h). SFV-vector-only-transfected BHK cells immunostained with the indicated anti-KUNV antibodies served as controls.

cysteine and analyzed by SDS-polyacrylamide gel electrophoresis after precipitation with antibodies specific to KUNV proteins (Fig. 3). A lysate of radiolabeled KUNV-infected Vero cells was used as a marker for the identification of each KUNV protein. All the constructs showed high levels of expression of the KUNV genes, except for the NS4B protein, where its cytoplasmic expression appeared at a lower level, presumably due to its' translocation to the nucleus (Fig. 4). In all cases, the produced KUNV proteins were specifically detected with the appropriate antibodies. Interestingly, radioimmunoprecipitation (RIP) analysis of the lysate from pSFV-NS4A-4B-transfected cells using anti-NS4A and anti-NS4B antibodies showed a single protein species of about 30 kDa and no individual proteins that migrated to the predicted sizes for NS4A or NS4B (Fig. 3A). The results suggest that the NS4A-4B polyprotein could not be cleaved by the host signal peptidase alone under these conditions. However, when pSFV-NS4A-4B was cotransfected with pSFV-NS2B-3pro, NS4A-4B was cleaved at the previously proposed "2K" site (17), liberating the NS4A and NS4B proteins (Fig. 3A). Expression of pSFV-NS2B-3-4A also revealed predominately correct processing to release the mature proteins (Fig. 3B), albeit the efficiency of cleavage was significantly lower than when NS2B-3pro was supplied in *trans*. This less-efficient cleavage profile may reflect the inefficient cleavage at the NS3-NS4A junction by NS2B-3pro mediated in *cis*, as described for Murray Valley encephalitis virus (MVEV) (19). Note that longer exposure of the film clearly shows NS4A and NS2B bands in the anti-NS4A and anti-NS2B immunoprecipitates of the products expressed from the constructs pSFV-NS2B-3-4A and pSFV-NS2B-3pro, respectively (data not shown). However, it is noted that some aberrant migration patterns of the NS4A protein species were observed that cannot be explained. Most notable was the apparent slower migration of NS4A(−2K) protein when expressed alone in the absence of NS2B-3pro (Fig. 3B, lane 1). In all other cases, the migration pattern of NS4A appeared consistent with processing at the 2K site, such that the NS4A released from the NS4A-NS4B polyprotein in the presence of NS2B-3pro migrates faster than NS4A expressed alone with 2K still attached (compare lanes 1 and 5 in Fig. 3A). Similar NS4A protein species were also observed from the NS4A plus NS2B-3pro and NS2B-3-4A transfected cells (Fig. 3B lanes 2 and 5). Thus, we can only suggest that the migration of NS4A in the absence of the viral protease is somehow influenced by its decreased hydrophobicity. It is noteworthy that a similar anomalous migration profile was also described during the first positive identification of KUNV NS4A (34), whereby inconsistent migration profiles were shown depending on the gel conditions utilized.

These results show that all of the KUNV proteins were expressed from the SFV replicons and that correct processing of the expressing polyproteins occurred when the required factors (KUNV protease) were present. As was observed for transient expression of yellow fever virus (YFV) NS4A-4B from recombinant vaccinia virus (17), there appears to be a requirement for cleavage by the KUNV NS2B-3 protease at the proposed "4A/2K" site as a prerequisite for the cleavage by cellular signalase to release the NS4A and NS4B proteins.

Subcellular distribution of the KUNV polyproteins as observed by IF. To assess the general subcellular distribution of the expressed KUNV polyproteins, BHK cells were transfected with the SFV replicon constructs and were fixed at 11 h post-transfection for IF analysis (Fig. 4). Fixed cells were immunostained with monospecific rabbit antisera to KUNV NS4A, NS4B, and NS3 and visualized with FITC-conjugated goat anti-rabbit IgG.

In all transfections, the expressed proteins were readily visualized. Staining with anti-NS4A antibodies of cells expressing NS4A (Fig. 4a) and NS4A(−2K) (Fig. 4b) revealed a thickened reticulum-like localization pattern with small isolated puncta dispersed within the cytoplasm. Cells expressing NS4A plus NS2B-3pro, NS4A-4B, NS4A-4B plus NS2B-3pro, and NS2B-3-4A and immunostained with anti-NS4A antibodies displayed a thickened reticular network with apparent large foci throughout the cytoplasm (Fig. 4c to f). These large foci were also readily immunostained with anti-NS4B and anti-NS3 antibodies in the corresponding cells (Fig. 4 h, i, k to m). Noticeably, NS4B translocated to the nucleus when expressed alone (Fig. 4g), as we had observed previously (37). However, this did not occur when the NS4B was expressed within the NS4A-4B cassette (Fig. 4h). In contrast, coexpression of the

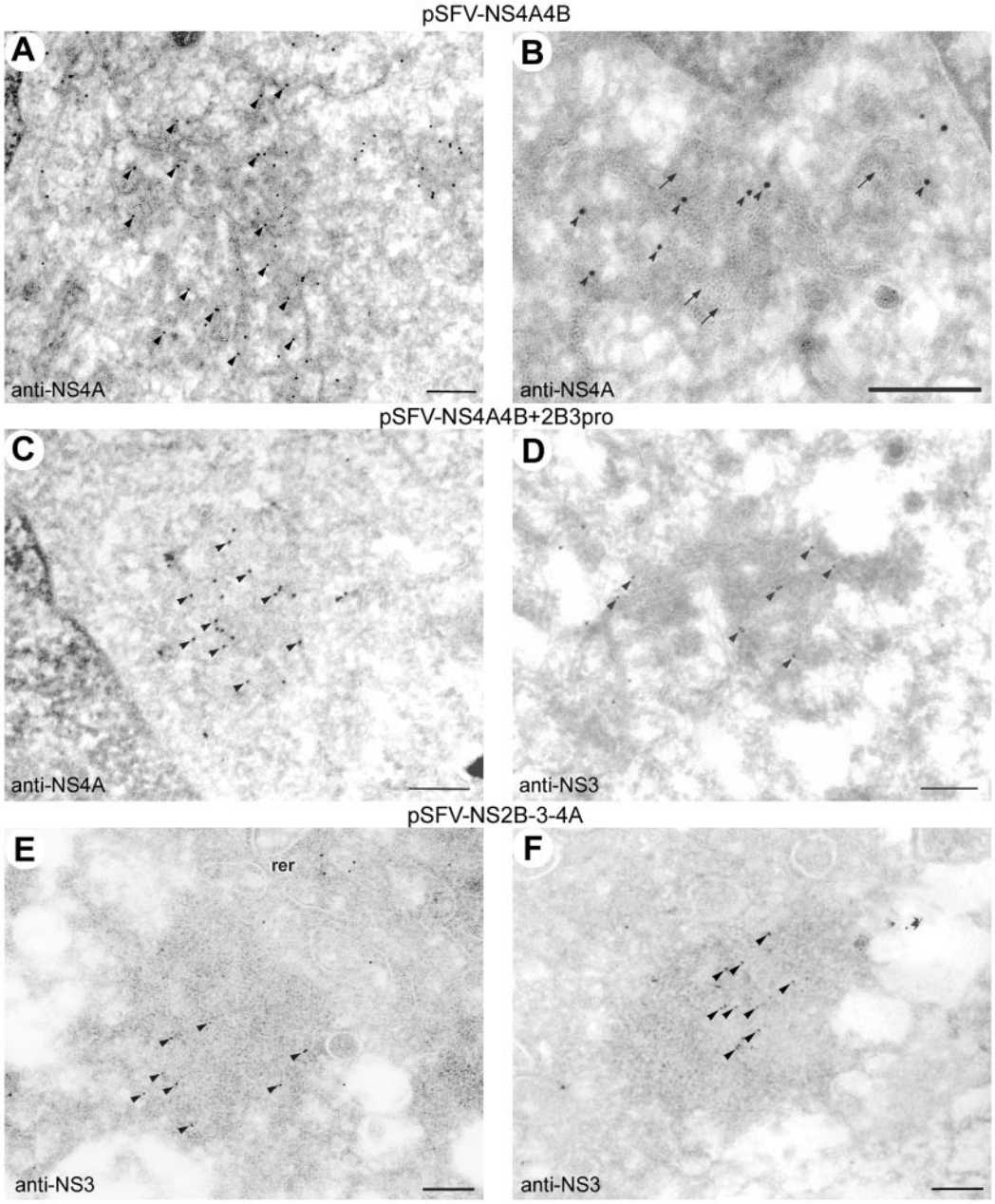

FIG. 5. Formation of characteristic flavivirus CM/PC membrane structures upon expression of NS4A4B plus 2B3pro or NS2B-3-4A. Cryosections from transfected BHK cells were immunolabeled with antibodies to NS4A (A to C) or NS3 (D to F). Membrane structures visually analogous to those observed during flavivirus infection were induced during expression of NS4A-4B plus NS2B-3pro (C and D) or NS2B-3-4A (E and F). Similar membrane rearrangements were also observed during expression of NS4A-4B (A and B); however, these latter structures resembled loose chains of vesicles that may represent precursor structures to those observed in the other panels. Arrowheads indicate gold particles binding to the anti-NS3 or anti-NS4A antibodies, and the arrows indicate the individual vesicles within the induced membrane chains. Bars, 200 nm.

NS4A-4B cassette with NS2B-3pro (providing viral protease) enabled the nuclear translocation of NS4B with some cytoplasmic foci also visible (Fig. 4i). Expression of NS2B-3pro alone showed only diffuse cytoplasmic reticular staining when labeled with anti-NS3 (Fig. 4j) and anti-NS2B (data not shown) antibodies.

These results clearly indicate that expression of the individual KUN proteins led to rather diverse staining patterns within the cytoplasm. The NS4A-4B polyprotein with or without the protease NS2B-3pro, and the KUN NS2B-3-4A polyprotein induced large cytoplasmic foci characteristic of flavivirus infection. Expression of NS4A and NS4A(−2K) as individual proteins induced some smaller foci dispersed within the cytoplasm. The IF data also confirmed our previous data showing translocation of NS4B into the nucleus but only after its cleavage from NS4A.

Ultrastructural changes induced by flavivirus polyproteins. To extend the analysis of the observed subcellular distribution

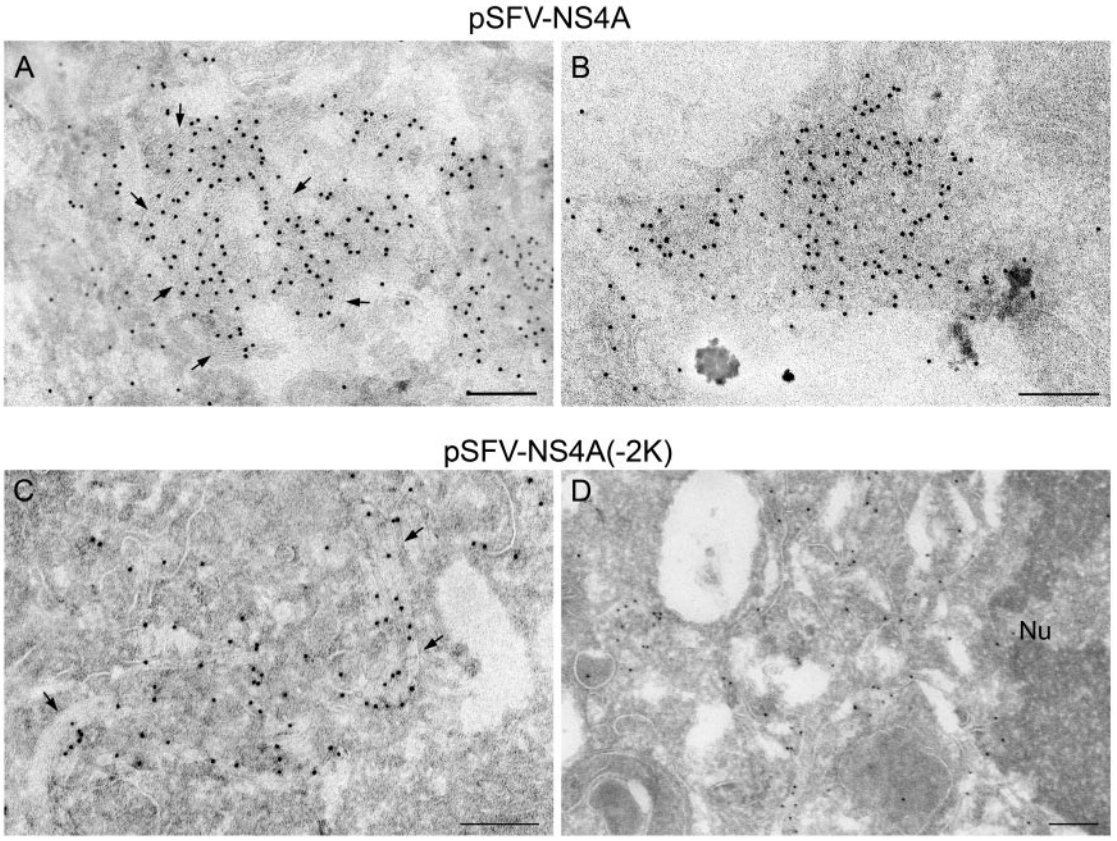

FIG. 6. NS4A proteins with or without the C-terminal 2K peptide can induce the formation of characteristic flavivirus CM/PC membrane structures in Vero cells. Cryosections from Vero cells electroporated with construct pSFV-NS4A or pSFV-NS4A(−2K) were immunolabeled with antibodies to NS4A (A to D) and visualized with 10 nm protein A gold. Membrane structures visually analogous to those observed during flavivirus infection were induced; however, these structures appeared subtly different with observed striations (arrows in panels A and C). Nu, nucleus. Bars, 200 nm.

by IF, BHK cells transfected with SFV replicon-expressing KUNV genes/gene cassettes were fixed for cryosectioning and immunolabeling (22, 23). Immunogold labeling of NS4A-4B-, NS4A-4B plus 2B-3pro-, and NS2B-3-4A-transfected cells with anti-NS4A antibodies revealed rearrangement of intracellular membranes (Fig. 5A to C). Cryosections of NS4A-4B-transfected cells showed large membrane structures strongly immunogold labeled with NS4A antibodies (Fig. 5A and B). The arrangement of these large membrane clusters did not seem to be as tight as the arrangement characteristic for the KUNV-induced CM/PC membranes as observed previously (22, 25, 27, 38). Small regions of tightly arranged membranes (Fig. 5B), which resemble the flavivirus CM/PC structures, appeared to be connected by chains of long reticular membranes that contained small apparent vesicles of ~10 nm. These enclosed structures appeared to be vesicular, as we did not observe any structures that could represent tubules cut transversely or longitudinally. Serial sections also suggested that these membranes were vesicular in morphology. More interestingly, cryosections of NS4A-4B plus 2B-3pro-transfected cells immunolabeled with antibodies to NS4A (Fig. 5C) and to NS3 (Fig. 5D) showed labeling of membrane structures that closely resemble CM structures detected by both antibodies in KUNV-infected Vero cells (25, 38). Within these larger more dense membrane structures, the strings of reticular structures, as observed with the NS4A-4B construct, were absent throughout. In addition, NS2B-3-4A cryosections immunolabeled with anti-NS3 (Fig. 5E and 4F) also specifically identified large membrane arrays visually similar to KUNV CM. This last observation suggested that the KUNV protein required for induction of the CM (and possibly PC) lies within the KUNV coding sequence of NS2B-3-4A and discounts a role for NS4B in membrane induction.

To further define the minimal requirement for membrane induction within the KUNV polyprotein region, we immunolabeled cryosections from BHK cells transfected with SFV replicons encoding KUNV NS4A and NS4A(−2K) genes. Our numerous attempts to detect positive staining after labeling cryosections of transfected BHK cells with NS4A antibodies failed for unknown reasons. To overcome this predicament, we chose to electroporate Vero cells with these constructs. In this case, both NS4A and NS4A(−2K) were efficiently identified on prepared cryosections and were observed to accumulate and cluster on membrane structures within the cytoplasm analogous to those observed during virus infection (Fig. 6) (25). The morphology of the induced membrane structures appeared subtly different, as a more striated appearance within the membrane structures was observed (Fig. 6A, B, and C). The mem-

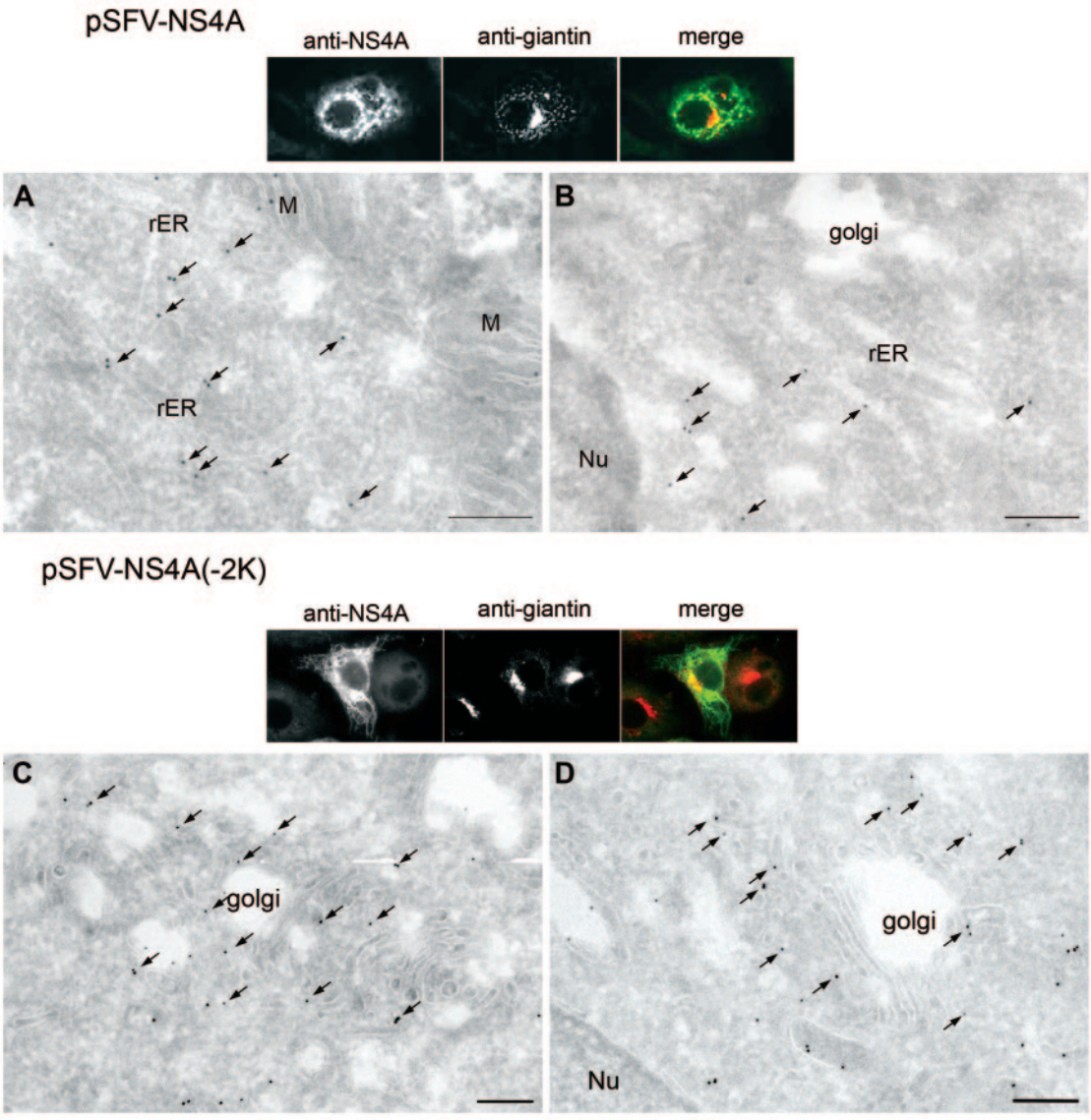

FIG. 7. Localization of the mature NS4A protein to the Golgi apparatus early after transfection of Vero cells with the NS4A(−2K) construct. Electroporated cells were fixed at 6.5 h for cryosectioning or at 23 h postelectroporation for IF analysis. Localization of the NS4A proteins (visualized with Oregon green) was compared by immunolabeling to that of the recognized Golgi marker Giantin (visualized with Texas Red). Cryosections revealed an accumulation of gold-labeled anti-NS4A antibodies within visually recognized Golgi bodies only after transfection with the NS4A(−2K) construct (C and D) but not for the NS4A construct (A and B). Arrows indicate the gold-labeled anti-NS4A antibodies Abbreviations: Golgi, Golgi apparatus; Nu, nucleus; rER, rough endoplasmic reticulum. Bars, 200 nm.

brane structures in cells expressing NS4A with the 2K domain more closely resembled membrane structures observed in KUNV-infected cells, while membrane rearrangements in cells expressing NS4A without the 2K domain were less prominent (compare Fig. 6A and B with Fig. 6C and D). An additional observation was that the NS4A(−2K) protein also displayed some specific immunolabeling within the Golgi apparatus (Fig. 7C and D), in contrast to NS4A containing 2K that had negligible association with Golgi membranes but rather localized to endoplasmic reticulum membranes (Fig. 7A and B). This targeting to Golgi membranes was observed both early and late by IF but was more prominently observed by cryoimmunoelectron microscopy at earlier time points (Fig. 7, compare IF and electron microscopy panels), presumably before membrane induction had occurred. The Golgi localization is an intriguing observation, as the site of flavivirus RNA replication appears to contain membrane and proteins of a *trans*-Golgi origin (22).

The results establish that cleavages at the NS4A-4B junction are key events in the following: (i) the induction of large cytoplasmic membranes (CM/PC) by NS4A containing the C-terminal domain (2K) and, to a minor extent, by NS4A(−2K); (ii) the redistribution of NS4A(−2K) to the Golgi apparatus, from which the membranes within the replication complex are derived (22); and (iii) the enabling of NS4B migration to the nucleus.

DISCUSSION

In this study, we have sought to identify the West Nile virus protein(s) required to induce membrane structures characteristic of flavivirus infection. For this purpose, we chose the SFV replicon expression system. The SFV replicon-based expression does have some advantages as mentioned earlier, but also some disadvantages, such as rapid cytopathic effect, shutoff of host cell protein synthesis, and the presence of membranous structures induced by SFV replication (9). However, we are confident that the changes in membrane rearrangements described in transfected cells are highly specific for the expressed KUNV proteins and are quite distinct from the membrane changes induced by SFV (Fig. 2). Thus, we have now shown that KUNV ns protein NS4A is the key protein responsible for the induction of membrane structures that appear visually similar to the CM/PC membranes present at the latter stages of KUNV infection.

We have also shown a strict requirement for the KUNV protease NS2B-3pro to cleave the cytoplasmic domain of NS4A at the 4A-2K site prior to the postulated cleavage by cellular signalase downstream to release the mature NS4B protein from the 2K domain. This was demonstrated in Fig. 3, wherein expression of the NS4A-4B polyproteins lead to its accumulation, with neither mature NS4A nor NS4B being released. However, upon coexpression of the protease NS2B-3pro, the individual NS4A and NS4B proteins could be clearly identified. Our results confirm the earlier studies with YFV and dengue virus (DENV) where similar observations have been made using a vaccinia virus expression system (17, 31). In retrospect, both our results with SFV replicon-directed expression of KUNV proteins and the results with expression of YFV/DENV proteins by vaccinia virus vectors are in apparent conflict with the only published data on the determination of C terminus of flavivirus NS4A translated in KUNV-infected cells (34). In that report, the authors gel purified KUNV NS4A from an SDS–phosphate–12% polyacrylamide gel to perform N- and C-terminal sequencing. Those results clearly indicated that the purified NS4A contained the "2K" domain, suggesting that during virus infection of cells, the NS2B-3-mediated cleavage of the NS4A "2K" domain had not occurred in the great majority of the NS4A population. In support of these latter findings, Cahour et al. (3) demonstrated that cleavage at the DENV NS4A-NS4B junction could occur in the absence of protease when expressed from recombinant vaccinia virus, suggesting a potential signalase cleavage without the prerequisite viral protease cleavage. However, the authors additionally showed that, within an uncleaved polyprotein consisting of NS3-NS4A-NS4B-NS5, the cleavage between NS4A and NS4B was in fact inhibited (3). It is possible that processing of flavivirus precursors during normal virus infection may differ from that occurring when they are expressed from other vectors in the absence of other normally coexpressed viral products. Alternatively, an NS4A protein intermediate with an uncleaved 2K domain may be more abundant than NS4A protein lacking 2K domain in KUNV-infected cells. Certainly most of KUNV NS4A (presumably with an intact "2K" domain) was separable from the relatively small amount of NS4A [possibly as NS4A(−2K)] present in the heavy membranes that sedimented with the active replication complex (4, 5). More experimental data are required to conclusively establish which is the predominant NS4A species in flavivirus-infected cells and to determine which NS4A species is involved in the replication of flavivirus RNA (25, 40).

In any case, it is apparent that the NS4A-4B polyprotein precursor is involved in rearranging internal host cell membranes. Expression data presented here indicate that the NS4A-4B polyprotein is intimately associated with internal membranes and appears to have the capacity to modify these membranes into apparent chains of reticulum-containing vesicles of approximately 10 nm in diameter (Fig. 5A and B). Upon closer inspection of some cryosections from KUNV-infected cells, similar ultrastructural morphology can be seen (data not shown); however, due to the amorphous and irregular nature of membrane induction during infection, these structures are not always readily observed. In addition, the "strings" of the NS4A-4B-induced membranes appear to be more relaxed and are not associated or tightly bound together. Taking the above into account, we suggest that the uncleaved NS4A-4B acts as a precursor for initiation of membrane induction by bringing the required membranes together, perhaps through the dimer-associated interaction of NS4A (25). The condensing of the membranes to form CM/PC structures then appears to require either the cleavage of NS4A from NS4B by the NS2B-3 protease or the presence of the protease itself (Fig. 5). We tend to favor the former, as expression of NS2B-3-4A without NS4B induced membrane arrays consistent with that observed during flavivirus infection; however, the possible effect on membrane induction by NS2B-3pro protease-mediated cleavage of the "2K" at the C terminus of NS4A in the NS2B-3-4A polyprotein cannot be excluded.

The precise role of 2K in membrane induction, if any, is not entirely clear, as the expression of NS4A without 2K could also initiate membrane rearrangements, although to a lesser extent than that observed for NS4A containing 2K. This is obviously in contrast with observations made upon expression of the HCV NS4B protein, whereupon flavivirus-like CM/PC membrane structures were clearly observed (8, 15). Interestingly, both HCV and KUNV NS4B proteins localize to apparently induced perinuclear ER membranes with unknown functions during replication (11, 12, 37). Some striking contrasts are the observations that the KUNV NS4B translocates to the nucleus (37; this study), whereas HCV NS4B does not. Therefore, one would suspect that the roles of NS4B between these two virus genera are distinctly different.

We know that the CM/PC are not induced until the end of the latent period (25, 38). This biogenesis corresponds with the exponential increase in viral RNA and, subsequently, virus replication. This intimate association of many factors makes it difficult to clearly dissect one event from the other and also to pinpoint certain events responsible for subsequent changes. We propose that once a certain concentration of the NS4A-4B intermediate is reached, membrane rearrangement is initiated. We have shown (Fig. 3) that cleavage at the NS4A-2K junction must occur in our expression vector system before signalase cleavage can then liberate 2K from NS4B, yet both NS4A(−2K) and NS4A(+2K) proteins (Fig. 6) as well as uncleaved NS4A-4B (Fig. 5) appear to have the ability to rearrange intracellular membranes in their own right. It would be of interest to determine whether an accumulation of NS4A-4B

polyprotein early during the infection cycle is required for initiation of membrane induction to form CM/PC or, alternatively, to analyze similar lysates from some KUNV replicon cell lines in which membrane induction does not occur (26).

This regulated cleavage at the NS4A-4B junction shares a great similarity to that proposed for the cleavage of the core protein from prM during MVEV assembly. In that case, it was proposed that a coordinated cleavage regimen exists, whereby release of the mature core and prM proteins is regulated by an initial cleavage via the viral protease NS2B-3 at the cytosolic C terminus of core followed by generation of N terminus of prM by host signalase within the lumen of the ER (1, 16, 35). When this series of events was altered by promoting earlier cleavage within the lumen of ER by host signalase, MVEV nucleocapsid incorporation into assembling virions was dramatically impaired (20). This crucial and precise regulation of cleavage events highlights the importance of timing of mature protein production and/or release during flavivirus replication and suggests that the RNA replication, protein translation, and virus assembly are tightly controlled. Why the apparent regulation of cleavage at the "4A/2K" site coincides with membrane induction and what effect(s) this may have on virus replication and/or virus-cell interactions is not clear. Interestingly, recent studies with flaviviruses showed that viral protease-mediated cleavage of NS4A-4B polyprotein intermediate was essential for ensuring the function of released NS4A and NS4B proteins in inhibition of interferon signaling, while uncleaved NS4A-4B intermediate did not inhibit interferon signaling (29). It is tempting to speculate that membrane rearrangements induced by the viral protease-mediated cleavage at the NS4A-2K site and the ability of flaviviruses to inhibit cellular antiviral response may be linked.

A significant observation is the that the NS4A(−2K) protein appears to traffic to and accumulate within the Golgi apparatus (Fig. 7). Previously, we have observed that the intracellular site of flavivirus RNA synthesis is derived from *trans*-Golgi membranes (22), and the data revealed here may indicate that NS4A may play a role in targeting the replication complex to the site of viral RNA replication, perhaps through association with NS1, as proposed previously (39). Thus, we could speculate that NS4A may have two roles during virus replication: one involving initiation of membrane induction when the majority of NS4A is still associated with 2K (and possibly NS4B), and the other as a minor but important NS4A(−2K) population to target the replication complex to Golgi membranes following the cleavage from the polyprotein. Our future experiments will involve deletion analysis of the NS4A protein to identify the Golgi localization motif and its role when mutated in the KUNV replicon and infectious clone.

It is now well established that rearrangement of internal host cell membranes is a common feature of positive-strand RNA viruses which infect mammals and plants. However, their precise roles in the virus propagation and/or virus-host interactions need to be clarified further. We are currently investigating the role of host cell proteins in this process, and with the use of green fluorescent protein fusion constructs of both viral and host proteins, we hope to visualize the biogenesis of these membrane structures over real time.

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REFERENCES

1. **Amberg, S. M., and C. M. Rice.** 1999. Mutagenesis of the NS2B-NS3-mediated cleavage site in the flavivirus capsid protein demonstrates a requirement for coordinated processing. J. Virol. **73:**8083–8094.
2. **Bienz, K., D. Egger, and L. Pasamontes.** 1987. Association of polioviral proteins of the P2 genomic region with the viral replication complex and virus-induced membrane synthesis as visualized by electron microscopic immunocytochemistry and autoradiography. Virology **160:**220–226.
3. **Cahour, A., B. Falgout, and C. J. Lai.** 1992. Cleavage of the dengue virus polyprotein at the NS3/NS4A and NS4B/NS5 junctions is mediated by viral protease NS2B-NS3, whereas NS4A/NS4B may be processed by a cellular protease. J. Virol. **66:**1535–1542.
4. **Chu, P. W., and E. G. Westaway.** 1992. Molecular and ultrastructural analysis of heavy membrane fractions associated with the replication of Kunjin virus RNA. Arch. Virol. **125:**177–191.
5. **Chu, P. W., E. G. Westaway, and G. Coia.** 1992. Comparison of centrifugation methods for molecular and morphological analysis of membranes associated with RNA replication of the flavivirus Kunjin. J. Virol. Methods **37:**219–234.
6. **Chua, J. J., M. M. Ng, and V. T. Chow.** 2004. The non-structural 3 (NS3) protein of dengue virus type 2 interacts with human nuclear receptor binding protein and is associated with alterations in membrane structure. Virus Res. **102:**151–163.
7. **Coia, G., M. D. Parker, G. Speight, M. E. Byrne, and E. G. Westaway.** 1988. Nucleotide and complete amino acid sequences of Kunjin virus: definitive gene order and characteristics of the virus-specified proteins. J. Gen. Virol. **69:**1–21.
8. **Egger, D., B. Wolk, R. Gosert, L. Bianchi, H. E. Blum, D. Moradpour, and K. Bienz.** 2002. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. J. Virol. **76:**5974–5984.
9. **Froshauer, S., J. Kartenbeck, and A. Helenius.** 1988. Alphavirus RNA replicase is located on the cytoplasmic surface of endosomes and lysosomes. J. Cell Biol. **107:**2075–2086.
10. **Gazina, E. V., J. M. Mackenzie, R. J. Gorrell, and D. A. Anderson.** 2002. Differential requirements for COPI coats in formation of replication complexes among three genera of Picornaviridae. J. Virol. **76:**11113–11122.
11. **Gretton, S. N., A. I. Taylor, and J. McLauchlan.** 2005. Mobility of the hepatitis C virus NS4B protein on the endoplasmic reticulum membrane and membrane-associated foci. J. Gen. Virol. **86:**1415–1421.
12. **Hugle, T., F. Fehrmann, E. Bieck, M. Kohara, H. G. Krausslich, C. M. Rice, H. E. Blum, and D. Moradpour.** 2001. The hepatitis C virus nonstructural protein 4B is an integral endoplasmic reticulum membrane protein. Virology **284:**70–81.
13. **Khromykh, A. A., A. N. Varnavski, and E. G. Westaway.** 1998. Encapsidation of the flavivirus kunjin replicon RNA by using a complementation system providing Kunjin virus structural proteins in trans. J. Virol. **72:**5967–5977.
14. **Khromykh, A. A., and E. G. Westaway.** 1994. Completion of Kunjin virus RNA sequence and recovery of an infectious RNA transcribed from stably cloned full-length cDNA. J. Virol. **68:**4580–4588.
15. **Konan, K. V., T. H. Giddings, Jr., M. Ikeda, K. Li, S. M. Lemon, and K. Kirkegaard.** 2003. Nonstructural protein precursor NS4A/B from hepatitis C virus alters function and ultrastructure of host secretory apparatus. J. Virol. **77:**7843–7855.
16. **Lee, E., C. E. Stocks, S. M. Amberg, C. M. Rice, and M. Lobigs.** 2000. Mutagenesis of the signal sequence of yellow fever virus prM protein: enhancement of signalase cleavage in vitro is lethal for virus production. J. Virol. **74:**24–32.
17. **Lin, C., S. M. Amberg, T. J. Chambers, and C. M. Rice.** 1993. Cleavage at a novel site in the NS4A region by the yellow fever virus NS2B-3 proteinase is a prerequisite for processing at the downstream 4A/4B signalase site. J. Virol. **67:**2327–2335.
18. **Linstedt, A. D., and H. P. Hauri.** 1993. Giantin, a novel conserved Golgi membrane protein containing a cytoplasmic domain of at least 350 kDa. Mol. Biol. Cell **4:**679–693.
19. **Lobigs, M.** 1992. Proteolytic processing of a Murray Valley encephalitis virus non-structural polyprotein segment containing the viral proteinase: accumulation of a NS3-4A precursor which requires mature NS3 for efficient processing. J. Gen. Virol. **73**(Pt 9)**:**2305–2312.
20. **Lobigs, M., and E. Lee.** 2004. Inefficient signalase cleavage promotes efficient nucleocapsid incorporation into budding flavivirus membranes. J. Virol. **78:** 178–186.
21. **Mackenzie, J.** 2005. Wrapping things up about virus RNA replication. Traffic **6:**967–977.
22. **Mackenzie, J. M., M. K. Jones, and E. G. Westaway.** 1999. Markers for trans-Golgi membranes and the intermediate compartment localize to in-

duced membranes with distinct replication functions in flavivirus-infected cells. J. Virol. **73:**9555–9567.
23. **Mackenzie, J. M., M. K. Jones, and P. R. Young.** 1996. Immunolocalization of the dengue virus nonstructural glycoprotein NS1 suggests a role in viral RNA replication. Virology **220:**232–240.
24. **Mackenzie, J. M., M. K. Jones, and P. R. Young.** 1996. Improved membrane preservation of flavivirus-infected cells with cryosectioning. J. Virol. Methods **56:**67–75.
25. **Mackenzie, J. M., A. A. Khromykh, M. K. Jones, and E. G. Westaway.** 1998. Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A. Virology **245:**203–215.
26. **Mackenzie, J. M., A. A. Khromykh, and E. G. Westaway.** 2001. Stable expression of noncytopathic Kunjin replicons simulates both ultrastructural and biochemical characteristics observed during replication of Kunjin virus. Virology **279:**161–172.
27. **Mackenzie, J. M., and E. G. Westaway.** 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.
28. **Magliano, D., J. A. Marshall, D. S. Bowden, N. Vardaxis, J. Meanger, and J. Y. Lee.** 1998. Rubella virus replication complexes are virus-modified lysosomes. Virology **240:**57–63.
29. **Munoz-Jordan, J. L., M. Laurent-Rolle, J. Ashour, L. Martinez-Sobrido, M. Ashok, W. I. Lipkin, and A. Garcia-Sastre.** 2005. Inhibition of alpha/beta interferon signaling by the NS4B protein of flaviviruses. J. Virol. **79:**8004–8013.
30. **Ng, M. L.** 1987. Ultrastructural studies of Kunjin virus-infected Aedes albopictus cells. J. Gen. Virol. **68:**577–582.
31. **Preugschat, F., and J. H. Strauss.** 1991. Processing of nonstructural proteins NS4A and NS4B of dengue 2 virus in vitro and in vivo. Virology **185:**689–697.
32. **Restrepo-Hartwig, M. A., and P. Ahlquist.** 1996. Brome mosaic virus helicase- and polymerase-like proteins colocalize on the endoplasmic reticulum at sites of viral RNA synthesis. J. Virol. **70:**8908–8916.
33. **Schlegel, A., T. H. Giddings, Jr., M. S. Ladinsky, and K. Kirkegaard.** 1996. Cellular origin and ultrastructure of membranes induced during poliovirus infection. J. Virol. **70:**6576–6588.
34. **Speight, G., and E. G. Westaway.** 1989. Positive identification of NS4A, the last of the hypothetical nonstructural proteins of flaviviruses. Virology **170:**299–301.
35. **Stocks, C. E., and M. Lobigs.** 1998. Signal peptidase cleavage at the flavivirus C-prM junction: dependence on the viral NS2B-3 protease for efficient processing requires determinants in C, the signal peptide, and prM. J. Virol. **72:**2141–2149.
36. **van der Meer, Y., E. J. Snijder, J. C. Dobbe, S. Schleich, M. R. Denison, W. J. Spaan, and J. K. Locker.** 1999. Localization of mouse hepatitis virus nonstructural proteins and RNA synthesis indicates a role for late endosomes in viral replication. J. Virol. **73:**7641–7657.
37. **Westaway, E. G., A. A. Khromykh, M. T. Kenney, J. M. Mackenzie, and M. K. Jones.** 1997. Proteins C and NS4B of the flavivirus Kunjin translocate independently into the nucleus. Virology **234:**31–41.
38. **Westaway, E. G., J. M. Mackenzie, M. T. Kenney, M. K. Jones, and A. A. Khromykh.** 1997. Ultrastructure of Kunjin virus-infected cells: colocalization of NS1 and NS3 with double-stranded RNA, and of NS2B with NS3, in virus-induced membrane structures. J. Virol. **71:**6650–6661.
39. **Westaway, E. G., J. M. Mackenzie, and A. A. Khromykh.** 2003. Kunjin RNA replication and applications of Kunjin replicons. Adv. Virus Res. **59:**99–140.
40. **Westaway, E. G., J. M. Mackenzie, and A. A. Khromykh.** 2002. Replication and gene function in Kunjin virus. Curr. Top. Microbiol. Immunol. **267:**323–351.