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Flavivirus premembrane protein cleavage and spike heterodimer secretion require the function of the viral proteinase NS3

(flavivirus vaccine/serine protease/vaccinia virus recombinants/signal peptidase)

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ABSTRACT **Flavivirus protein biosynthesis involves the proteolytic processing of a single polyprotein precursor by host- and virus-encoded proteinases. In this study, the requirement for the proteolytic function of the viral proteinase NS3 for correct processing of a polyprotein segment encompassing the Murray Valley encephalitis virus structural proteins is shown. The NS3-mediated cleavage in the structural polyprotein region presumably releases the capsid protein from its membrane anchor and triggers the appearance of the premembrane (prM) protein. This suggests that cleavage of prM by signal peptidase in the lumen of the endoplasmic reticulum is under control of a cytoplasmic cleavage catalyzed by a viral proteinase. The function of the viral proteinase is also essential for secretion of flaviviral spike proteins when expressed from cDNA via vaccinia virus recombinants or in COS cell transfections. This has important implications for the design of flavivirus subunit vaccines.**

The Flaviviridae are a family of mainly arthropod-borne viruses, many of which have been associated with disease in humans and domestic animals (for review, see ref. 1). The four serotypes of dengue virus, Japanese encephalitis virus, and yellow fever virus cause millions of cases of disease annually in the tropics and subtropics. Tick-borne encephalitis virus is endemic in northern and eastern Europe and Asia, causing thousands of human cases every year. Murray Valley encephalitis virus (MVE) and Saint Louis encephalitis virus are responsible for sporadic epidemic outbreaks of encephalitis in humans in Australia and North America, respectively. A highly successful live attenuated vaccine for yellow fever and killed vaccines for Japanese encephalitis virus and tick-borne encephalitis virus exist. In recent years, there has been renewed interest, using recombinant DNA approaches (2), in flavivirus vaccines, especially against the dengue viruses and Japanese encephalitis virus.

Several groups have constructed recombinant vaccinia virus (VV) and baculovirus vectors encoding flaviviral antigen but frequently failed to induce immunity and protection in animal models. Good immunity and protection correlated with secretion of the recombinant flaviviral spike proteins (3–8), whereas constructs were poor immunogens when the spike proteins were internally sequestered (3, 4, 7, 9–13). In this paper, a mechanism is provided that accounts for the complete proteolytic processing of the flaviviral structural proteins, when expressed from cDNA, and the secretion of the spike protein heterodimers.

Flaviviruses are enveloped, positive-strand RNA viruses. They encode a single polycistronic message, which is translated into a polyprotein precursor. Co- and posttranslational processing gives rise to three structural proteins—the core (C), the precursor to the membrane (prM), and the envelope (E) proteins—and seven nonstructural (NS) proteins (for reviews, see refs. 14–16). Flaviviruses replicate in close association with the membranes of the endoplasmic reticulum (ER). The structural proteins prM and E, and NS1 and possibly NS4B proteins are translocated into the lumen of the ER, where signal peptidase can mediate their N-terminal cleavages (17–20). The cytoplasmic cleavages at the junctions of NS2A–2B, NS2B–3, NS3–4A, and NS4B–5 are catalyzed by the virally encoded serine proteinase NS3. The proteolytic function of NS3 requires NS2B as a cofactor and cleaves after two basic amino acids, usually followed by a short side chain residue (21–26).

Four additional proteolytic events take place in flavivirus protein biosynthesis. Cellular methionine amino peptidase removes the initiation methionine from the N terminus of C protein (14, 15), the NS1–2A junction is cleaved by an unknown cellular or viral proteinase in the lumen of the ER (27), prM is processed late during virus maturation by a trans-Golgi enzyme to generate the membrane (M) protein present only in extracellular virus particles (15, 28), and finally a C-terminal hydrophobic sequence is removed from the C protein (19, 29). This membrane-spanning domain functions as translocation signal for prM and anchors the C protein to the ER membrane (anchored C protein). The cleavage occurs at a site consistent with that recognized by the viral proteinase NS3, and the presence of this cleavage site is conserved in all flaviviruses (14). Accordingly, NS3 has been implicated in the cleavage of anchored C protein. Here, I show that the proteolytic function of NS3 is required for the signal peptidase-mediated cleavage of prM protein and subsequent E/prM spike heterodimer secretion. A model for the coordinated cleavages of anchored C and prM proteins is discussed.

MATERIALS AND METHODS

Plasmid Constructions. MVE cDNA spanning from nucleotide 8 in the 5′ untranslated region to nucleotide 4230 was isolated as a *Bam*HI/*Hpa* I fragment from p2/1/22 (30) and subcloned into pBCB07 (31) digested with *Hin*cII and *Bam*HI to generate the VV recombination plasmid pBCB-STR. The same region of the MVE genome was subcloned as *Bgl* II/*Hpa* I fragment from p2/1/22 into the eukaryotic expression vector pcDNAI (Invitrogen, San Diego) cut with *Bam*HI and *Eco*RV to generate plasmid pcDNA-STR.

The C protein deletion plasmid pcDNAΔC has most of the cytoplasmic domain of C from Pro-5 to Lys-104 deleted. For *in vitro* mutagenesis, a *Hin*dIII fragment containing nucleotides 4–472 of the MVE genome was subcloned from p2/1/22 into M13mp19. Mutagenesis was as described (32) using the 40-mer 5′-GATGTTTCACTGCCACCTCTTTTTTTAGA-

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Abbreviations: MVE, Murray Valley encephalitis virus; ER, endoplasmic reticulum; VV, vaccinia virus; NS, nonstructural; C, prM, E, and M proteins, core, precursor to membrane, envelope, and membrane proteins.

CATTTGAAATC-3'. The mutated insert was sequenced and exchanged with the corresponding *Hin*dIII fragment in pcDNA-STR.

To destroy the proteolytic function of NS3, Ser-135 of the catalytic triad was substituted by threonine. A 1318-bp *Hin*dIII fragment, spanning nucleotides 3990–5303 of the MVE genome, was subcloned from plasmid pcDNA-NS (24) into M13mp18. The oligonucleotide 5'-GGATCCTGTAGTTC-CAAT-3' was used for *in vitro* mutagenesis. The *Hin*dIII region containing the mutated cDNA was sequenced and exchanged with the corresponding part in plasmid pcDNA-NS, creating plasmid pcDNA-NS3mut^S.

Recombinant VV-STR. Homologous recombination using VV-WR DNA, pBCB-STR DNA, and VV-ts7 as helper virus was as described (33).

Virus Stocks. MVE (strain 1-51) and Kunjin virus (strain 61C) were prepared as 10% suspensions of infected suckling mouse brain in Hanks' balanced salt solution containing 20 mM Hepes (pH 8.0) and 0.2% bovine serum albumin. VV stocks were crude CV1 cell lysates.

COS Cell Transfection, Metabolic Labeling, and Immunoprecipitation. The method for COS cell transfection has been described in detail elsewhere (24). At 48 h after transfection, cells were washed twice with phosphate-buffered saline and starved with methionine-free Eagle's minimal essential medium (EMEM) for 0.5 h. The cells were then pulse-labeled for 6 h with methionine-free EMEM containing [^{35}S]methionine (1004 Ci/mmol; 1 Ci = 37 GBq; ICN Radiochemicals) at a final concentration of 250 μCi/ml in a vol of 1.5 ml. Culture fluids were clarified by centrifugation at 15,000 × *g* for 10 min, and cell monolayers were solubilized in Nonidet P-40 buffer (33). Infection of COS cell monolayers with MVE was at a multiplicity of 20 plaque-forming units/ml and metabolic labeling 24–36 h after infection with [^{35}S]methionine at 100 μCi/ml.

Immunoprecipitation using anti-MVE or anti-Kunjin virus hyperimmune ascitic fluid or anti-E monoclonal antibody 4B3B6 (34) was as described (33).

Pulse labeling of VV and MVE- or Kunjin virus-infected CV1 cells grown in 175-cm^2 flasks was at 4 or 24 h after infection, respectively, for a period of 12 h in methionine-free EMEM containing 1% fetal calf serum, 1/20th the normal concentration of methionine, and 25 μCi of [^{35}S]methionine in a final vol of 25 ml.

Isolation of prM and N-Terminal Radiosequencing. CV1 cells grown in a 175-cm^2 flask were double infected with VV-STR and VV-NS at a multiplicity of 5. The cells were labeled with [^{35}S]methionine for 12 h and solubilized in Nonidet P-40 buffer, nuclei were pelleted, and the prM/E heterodimer was immunoprecipitated with anti-E monoclonal antibody 4B3B6 and protein A-Sepharose. Immunocomplexes were suspended in 1.5% SDS containing 5% 2-mercaptoethanol, boiled for 5 min, and loaded on a Bio-Rad Prep cell. Electrophoresis through a SDS/5% polyacrylamide stacking gel and 12% resolving gel and elution of prM were as specified by the supplier. The prM-containing fractions were lyophilized and sequenced on an Applied Biosystems protein sequencer (model 420A).

RESULTS

The Structural Region of a Flaviviral Polyprotein Is Incompletely Cleaved When Expressed from cDNA. The flaviviral structural polyprotein region C–prM–E constitutes the N-terminal one-quarter of the polyprotein precursor. The model for biosynthesis of the structural proteins envisages that a hydrophobic segment at the C terminus of the C protein functions as signal sequence for translocation of prM into the lumen of the ER. Chain translocation would be terminated by the first of two hydrophobic sequences at the prM–E junction and reinitiated by the second stretch of hydrophobicity. Similarly, two stretches of hydrophobicity at the C terminus of E would also function as stop-transfer and signal sequences. Signal peptidase cleavages are thus predicted to occur at the N termini of prM, E, and NS1, releasing membrane-anchored C, prM, and E from the nascent polyprotein chain.

When a segment of the MVE polyprotein spanning from the N terminus of C protein to Val-1410 at the start of NS2B was expressed in CV1 cells via a recombinant VV (VV-STR) and infected cell lysates were subjected to immunoprecipitation with anti-MVE antibodies, only the E protein was efficiently recovered, whereas prM was not or was only barely apparent (Fig. 1*A*, lane 1). Both spike proteins were efficiently precipitated from MVE-infected cell lysates (lane 4). Monoclonal anti-E antibody 4B3B6, which precipitates both components of the spike heterodimer (Fig. 1*B*, lane 4), also failed to recover prM from VV-STR-infected cell lysates (lane 1). The absence of prM in VV-STR-infected cells was surprising, since signal peptidase cleavage is thought to take place cotranslationally (35) and should have released both spike proteins from the polyprotein in an equimolar ratio.

Incomplete proteolytic processing of a polyprotein segment spanning from C to NS2B of a second flavivirus, Kunjin virus, was also observed when expressed via a VV recombinant (VV-1031; ref. 36). E but not prM protein was immunoprecipitated from VV-1031-infected cells (Fig. 2, lane 2), whereas prM and E were efficiently recovered from Kunjin virus-infected cell lysates (lane 4).

Transcleavage by the Viral Proteinase NS3 in the Structural Polyprotein Region. The incomplete proteolytic processing of the structural region of the flaviviral polyprotein, when expressed from cDNA, suggests that an additional, virally encoded, component is needed in the cleavage events. The viral proteinase NS3 is thought to cleave the C protein at a conserved NS3 proteinase cleavage consensus site from its C-terminal membrane anchor. To test whether NS3 is involved in the biosynthesis of the flaviviral structural proteins, double infections were performed with VV-STR and VV-NS (a VV recombinant encoding a polyprotein segment of MVE spanning from NS2A to the C terminus of NS5; ref. 24). The expression of the nonstructural proteins in double-infected cells resulted in the appearance of a protein with electrophoretic mobility identical to that of viral prM (Fig. 1*A*, lane 3). This protein was coimmunoprecipitated with the anti-E monoclonal antibody 4B3B6, suggesting that it formed a multimeric association with the E glycoprotein (Fig. 1*B*, lane 2). Finally, N-terminal amino acid sequencing confirmed its identity as prM (see below). PrM synthesized in MVE-infected and in VV-STR and VV-NS double-infected cells migrated as a doublet, which in pulse–chase experiments shifted to the faster-migrating form, consistent with maturation of a single carbohydrate moiety (data not shown).

Double infections were also performed with VV-STR and VV-NS3/T (a VV recombinant expressing NS2B and NS3 only; ref. 24). Again, efficient generation of prM was observed (data not shown), suggesting that the proteolytic function of NS3, probably mediating cleavage of anchored C protein, is a requirement for the N-terminal cleavage of prM.

MVE Proteinase NS3 Can Cleave in Trans in the Structural Region of a Heterologous Flavivirus. The NS3 proteinase domain is highly conserved between flaviviruses at the residues surrounding the putative catalytic triad, as are the cleavage sites recognized by the viral proteinase (14). To test whether MVE NS3 could catalyze a cleavage in the structural polyprotein region of Kunjin virus required for generation of prM, double infections with VV-1031 and VV-NS were performed. In the presence of the MVE nonstructural proteins, a doublet comigrating with Kunjin viral prM was clearly produced (Fig. 2, lanes 3 and 4). The same double

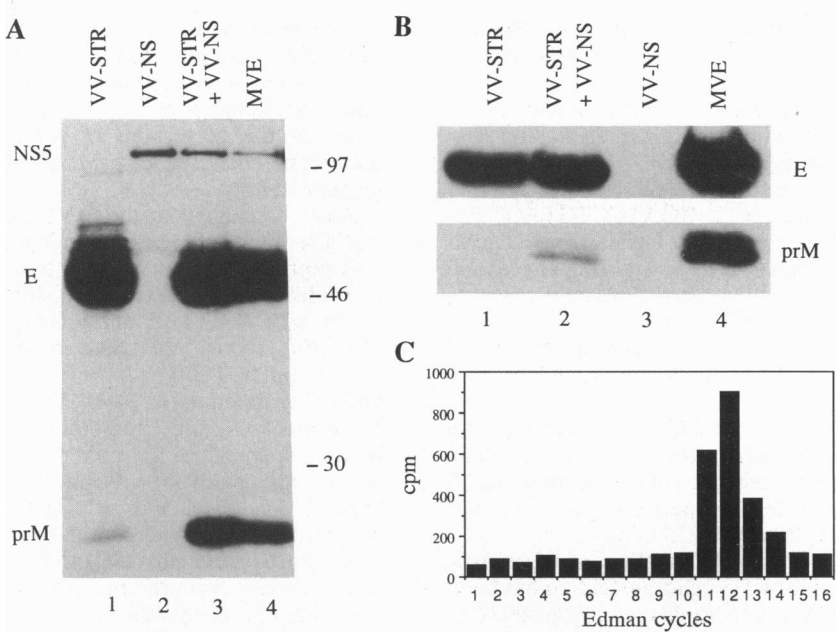

FIG. 1. Correct processing of MVE prM, expressed from cDNA, requires the function of the NS proteins. CV1 cells in 175-cm^2 flasks were infected with VV recombinants or MVE at multiplicities of 5 or 10 plaque-forming units per cell, respectively. In double infections, equal multiplicities (5 plaque-forming units per cell) of both viruses were used. MVE-specific polypeptides were immunoprecipitated with anti-MVE hyperimmune ascitic fluid (*A*) or anti-E monoclonal antibody 4B3B6 (*B*) and were analyzed by electrophoresis on SDS/12% polyacrylamide gels. Positions of size standards (kDa) are indicated in *A*, and MVE-specific proteins NS5, E, and prM are identified. N-terminal radiosequence analysis of prM immunoisolated with monoclonal antibody 4B3B6 from [^{35}S]methionine-labeled and VV-STR and VV-NS double-infected CV1 cells is shown in *C*. Peaks of radioactivity were detected at positions 11 and 12.

band was not detected in immunoprecipitates of cells infected only with VV-1031 (lane 2) or VV-NS (lane 1). Identical results were obtained in double infections with VV-1031 and VV-NS3/T, suggesting that the MVE proteinase can mediate a cleavage in trans in the structural region of the polyprotein of Kunjin virus, which triggers the production of prM.

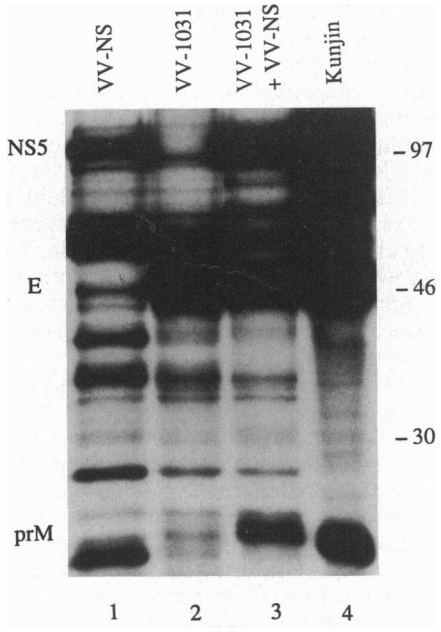

FIG. 2. Processing of a Kunjin virus structural polyprotein segment to generate prM is triggered in the presence of MVE NS proteins. CV1 cells in 80-cm^2 flasks were infected with VV-NS (encoding a MVE polyprotein segment from NS2A to NS5), VV-1031 (encoding a Kunjin virus polyprotein segment from C to NS2B), or double-infected with both recombinants at multiplicities of 5 plaque-forming units per cell. Kunjin virus infection was at a multiplicity of 10 plaque-forming units per cell. Cell lysates were incubated with anti-Kunjin virus hyperimmune ascitic fluid and immunoprecipitates were analyzed by electrophoresis on a SDS/12% polyacrylamide gel. Positions of size standards (kDa) are indicated, and bands corresponding to MVE and Kunjin virus NS5 and to Kunjin virus E and prM are indicated.

Signal Peptidase Probably Cleaves at the N Terminus of prM Following NS3 Proteinase-Mediated Processing in the Structural Polyprotein Segment. The N termini of West Nile virus and Kunjin virus prM have been defined and are consistent with a signal peptidase cleavage following the membrane-spanning segment of anchored C protein (19, 37). To verify that MVE prM expressed from cDNA is cleaved by signal peptidase following the putative NS3-mediated cleavage of anchored C protein, N-terminal sequence analysis of prM was performed. [^{35}S]Methionine-labeled spike heterodimers were immunoisolated from VV-STR and VV-NS double-infected cells and prM purified by electrophoresis on a SDS/polyacrylamide tube gel. Consecutive cycles of Edman degradation revealed that methionine was present at residues 11 and 12 from the N terminus of prM (Fig. 1*C*), consistent with cleavage at the predicted signal peptidase site.

Mutation in the Proteolytic Domain of NS3 Abolishes prM Cleavage. In COS cells transiently transfected with the eukaryotic expression vector pcDNA-STR, which encodes the same MVE cDNA fragment as VV-STR, prM was not detectable in immunoprecipitations, whereas the E protein was efficiently synthesized (Fig. 3*A*, lane 2). When the MVE NS polyprotein segment encoded by a second expression vector (pcDNA-NS; ref. 24) was provided in cotransfection, prM was efficiently generated (lane 9). Correct processing of prM was equally efficient when a construct expressing only NS2B and NS3 (pcDNA-NS3/T; ref. 24) was used for cotransfection. Deletion of the cytoplasmic region of the C protein also resulted in the generation of prM (lane 3). Thus, in both eukaryotic expression vector and recombinant VV expression, NS3 was required for processing of the MVE structural polyprotein segment.

To corroborate that the proteolytic function of NS3 triggered prM processing, a serine to threonine substitution was

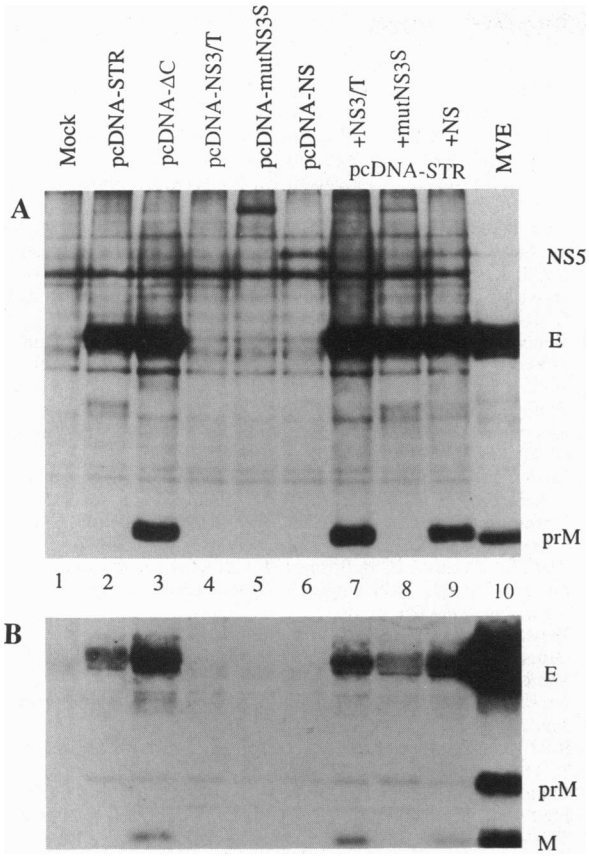

FIG. 3. Proteolytic processing and secretion of MVE spike proteins in transiently transfected COS cells. Confluent COS cell monolayers in 50-mm^2 dishes were transfected with 5 μg of plasmid DNA. In cotransfections, 5 μg of DNA of each plasmid was used. MVE-specific proteins were immunoprecipitated with anti-MVE hyperimmune ascitic fluid from cell lysates (*A*) or culture fluids (*B*). Immunoprecipitates were analyzed by electrophoresis on 12% (*A*) or 15% (*B*) SDS/polyacrylamide gels.

introduced at position 135 of the catalytic triad in NS3 (pcDNA-mutNS3^S). This change completely abolished processing of prM in cotransfections (Fig. 3*A*, lane 8). As expected, the NS3 proteinase mutant was also deficient in cis cleavages at the junctions of NS2B–NS3, NS3–NS4A, and NS4B–NS5 of the nonstructural polyprotein segment resulting in formation of a polyprotein of ≈200 kDa (lane 5).

Secretion of Flaviviral Spike Proteins Requires the Proteolytic Function of NS3. Secretion of the MVE spike proteins, when expressed from cDNA, was examined in COS cell transfections. Immunoprecipitations of MVE-specific proteins from culture fluids of cells transfected with pcDNA-STR revealed some E but no prM or M proteins (Fig. 3*B*, lane 2). Transfection with the C protein deletion construct resulted in greatly increased secretion of the E glycoprotein and the appearance of an 8-kDa band, corresponding to the viral M protein, the mature extracellular form of prM (lanes 3 and 10). Transfections with pcDNA-STR and pcDNA-ΔC were of similar efficiencies, which was reflected by the detection of similar amounts of E in cell lysates (Fig. 3*A*).

Cotransfection of COS cells with pcDNA-STR and pcDNA-NS3/T or pcDNA-NS resulted in the secretion of M protein (Fig. 3*B*, lanes 7 and 9), which was not detected when the NS3 proteinase mutant was used (lane 8). Again, considerably more E protein was secreted in the presence of functional than defective viral proteinase. The maturation cleavage of prM to M, which probably occurs in the trans Golgi, suggests that exocytosis of the spike proteins followed the same route as in virus-infected cells. The results clearly show that a trans cleavage in the structural polyprotein region mediated by the proteinase domain in NS3 triggered secretion of the spike proteins.

DISCUSSION

Several groups have constructed recombinant vaccinia or baculovirus expression vectors encoding flaviviral structural polyprotein segments as candidate subunit vaccines. The constructs were generally poorly immunogenic unless part or the entire C protein was deleted (see Introduction). Interestingly, C protein deletion resulted in secretion of the immunodominant flaviviral spike protein E in heterodimeric association with prM in the form of empty viral envelopes, similar to the noninfectious slow-sedimenting hemagglutinin particles from flavivirus-infected cells (5–7). Here the requirement of the presence of the flaviviral proteinase NS3 for correct proteolytic processing of prM and secretion of flaviviral spikes is demonstrated.

When the entire structural region of the MVE polyprotein including NS1 and NS2A was expressed via a VV recombinant or by COS cell transfection with a eukaryotic expression plasmid, the E glycoprotein was efficiently synthesized, whereas prM could not or could only barely be detected. Coinfections or transfections with a second construct encoding the viral proteinase NS3 and its cofactor NS2B gave rise to efficient production of prM. It also resulted in secretion of the spike heterodimer prM/E, which underwent maturation cleavage of prM to M prior to release into the extracellular medium. The production of prM and spike protein secretion also occurred when the C protein was deleted. The data demonstrate the importance of a NS3 proteinase-mediated cleavage in the structural polyprotein region for the proteolytic processing, assembly, and secretion of the flaviviral spike heterodimer. The viral proteinase most likely cleaves at the NS3 cleavage consensus site at the C terminus of anchored C as originally suggested by Rice *et al.* (15). However, no direct evidence for anchored C cleavage was obtained since the membrane-bound or free C protein could not be immunoprecipitated, a problem reported by others (38). The putative NS3-mediated cleavage of anchored C protein was monitored by the appearance of prM and supported by the findings that (*i*) NS2B and NS3 alone were sufficient to trigger this event in trans and (*ii*) that a mutation in the catalytic triad of the NS3 proteinase domain abolished the appearance of prM.

During the course of this study Yamshchikov and Compans (38) reported that the presence of NS2B–NS3 in an expression cassette spanning from C protein to residue 243 of NS3 of the West Nile virus polyprotein was crucial for efficient proteolytic processing of prM and spike heterodimer secretion. However, they did not observe prM processing and spike heterodimer secretion in double infections with the structural polyprotein region and the viral proteinase domain on separate coding units, in contrast to the evidence for a bimolecular cleavage shown here. Sato *et al.* (39), using a construct spanning from residue 62 in the C protein to residue 59 in NS1 of the Japanese encephalitis virus polyprotein, observed the production of prM and extracellular release of E, which was enhanced in coinfections with a second VV recombinant expressing NS2B and NS3. The authors of both reports also propose a NS3 proteinase-mediated cleavage of anchored C protein. These studies and the results presented here thus provide an explanation for the failure to observe prM cleavage and spike heterodimer secretion in numerous vaccinia and baculovirus expression studies of flaviviral structural polyprotein segments (see Introduction) and underline the importance of correct polyprotein processing in the design of flavivirus subunit vaccines.

In *in vitro* translation/translocation experiments of dengue virus and yellow fever virus structural proteins, the N-terminal signal peptidase cleavage of prM was also inefficient, although some prM could be detected (18, 20). A stable C–prM precursor protein accumulated, which was probably identical to protein NVX found in flavivirus-infected cells (16). *In vitro* translation/translocation of West Nile virus structural proteins showed relatively efficient processing of prM, but the C–prM precursor could always be seen (19). The latter study also identified anchored C protein, which underwent no further cleavage in the absence of the viral proteinase.

Here the requirement of the putative cytoplasmic cleavage of anchored C protein by the flaviviral proteinase for efficient signal peptidase cleavage at the luminal side of the ER membrane is clearly demonstrated. The catalytic domain of signal peptidase is located on the luminal side of the ER membrane within or just outside the lipid bilayer, and the correct positioning of the signal peptide with respect to the protease is important for processing (for a review, see ref. 40). Thus, alterations at the N terminus of the signal peptide (41) or in the length of the hydrophobic domain (42) can influence processing probably by shifting the signal peptide in the membrane. The cytoplasmic domain preceding an internal signal can also affect signal peptidase cleavage. The asialoglycoprotein receptor (43) and the invariant chain of the class II histocompatibility antigens (44) are transmembrane proteins with internal uncleaved signal sequences and cryptic signal peptidase sites, which are cleaved only upon removal of the preceding hydrophilic cytoplasmic segment. I suggest a similar sequence of events for processing of the flavivirus-anchored C–prM site, where the removal of the C protein by NS3-mediated cleavage or deletion mutagenesis allows the membrane-spanning segment preceding the signal peptidase site of prM to shift its position toward the luminal side of the ER. This would make the N terminus of prM accessible to signal peptidase cleavage. The C protein is highly positively charged, which could account for its influence on the membrane topology of the membrane-spanning domain of anchored C protein. This model predicts that the cleavage of anchored C protein precedes that of prM by signal peptidase. Pulse–chase experiments of VV-STR- and VV-NS3/T-coinfected cells suggest that both cleavages are rapid events (data not shown). This is consistent with protein biosynthesis in flavivirus-infected cells where no delay in the appearance of prM was noted and the anchored form of C was not found (45).

Polyprotein processing plays a regulatory role in the gene expression of many positive-strand RNA viruses (46, 47). Accordingly, it is of interest to determine whether in flavivirus infections the cleavage of anchored C protein also triggers the processing of prM and has a regulatory function in virus replication. One such function could be in the control of virus assembly, which takes place intracellularly, probably on the membranes of the ER. Flavivirus core formation and budding seem to be tightly linked, since no free cores are found in virus-infected cells (16, 48). The coordinated release of the C protein from its membrane anchor with signal peptidase cleavage of prM, resulting in the formation of transport-competent spike heterodimers, may be important for simultaneous core formation and envelopment.

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