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NS2B-3 Proteinase-Mediated Processing in the Yellow Fever Virus Structural Region: In Vitro and In Vivo Studies

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Several of the cleavages required to generate the mature nonstructural proteins from the flaviviral polyprotein are known to be mediated by a complex consisting of NS2B and a serine proteinase domain located in the N-terminal one-third of NS3. These cleavages typically occur after two basic residues followed by a short side chain residue. Cleavage at a similar dibasic site in the structural region is believed to produce the C terminus of the virion capsid protein. To study this cleavage, we developed a cell-free *trans* cleavage assay for yellow fever virus (YF)-specific proteolytic activity by using a substrate spanning the C protein dibasic site. Cleavage at the predicted site was observed when the substrate was incubated with detergent-solubilized lysates from YF-infected BHK cells. NS2B and the NS3 proteinase domain were the only YF-specific proteins required for this cleavage. Cell fractionation studies demonstrated that the YF-specific proteolytic activity was membrane associated and that activity could be detected only after detergent solubilization. Previous cell-free studies led to a hypothesis that processing in the C-prM region involves (i) translation of C followed by translocation and core glycosylation of prM by using an internal signal sequence, (ii) signalase cleavage to produce a membrane-anchored form of the C protein (anchC) and the N terminus of prM, and (iii) NS2B-3-mediated cleavage at the anchC dibasic site to produce the C terminus of the virion C protein. However, the results of in vivo transient-expression studies do not support this temporal cleavage order. Rather, expression of a YF polyprotein extending from C through the N-terminal one-third of NS3 revealed that C-prM processing, but not translocation, was dependent on an active NS2B-3 proteinase. This suggests that signalase-mediated cleavage in the lumen of the endoplasmic reticulum may be dependent on prior cleavage at the anchC dibasic site. Possible pathways for processing in the C-prM region are outlined and discussed.

Yellow fever virus (YF) is the prototype member of the flavivirus genus of *Flaviviridae*, a family which also includes the pestivirus (17) and hepatitis C virus (15) genera. The flaviviruses are enveloped viruses (for reviews, see references 5, 10, 45, and 56) with a single-stranded positive-sense RNA molecule of about 11 kb that contains a single, long open reading frame (ORF). The polyprotein is processed by virus- and host-encoded proteinases to produce the structural and nonstructural (NS) proteins (46). The flavivirus gene order has been determined to be 5′-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3′. The flaviviral structural proteins, the capsid (C), membrane (M), and envelope (E) proteins, are derived from precursors encoded in the 5′ quarter of the genome.

Cleavages occurring at the 2A/2B, 2B/3, 3/4A, and 4B/5 sites have been shown to be mediated by a complex consisting of NS2B and a trypsin-like serine proteinase domain residing in the N-terminal one-third of NS3 (1, 9, 14, 20, 40, 54, 59). In addition, NS2B-3-mediated cleavage near the C terminus of NS4A has been shown to be necessary for generation of the N terminus of NS4B (27). These cleavage sites are highly conserved and usually include a pair of basic residues at the P2 and P1 positions, with a short side chain residue at the P1′ position (normally Gly or Ser). Cleavages generating the amino termini of prM (a precursor to the mature structural protein M), E, NS1, and NS4B are thought to be mediated by host signal peptidase (19, 27, 31, 37, 47), with the hydrophobic regions preceding these sites directing translocation into the lumen of the endoplasmic reticulum (ER).

The sequence of the mature capsid protein has been shown to terminate at a conserved dibasic site for two flaviviruses, Kunjin virus (51) and West Nile virus (WN) (37). The signalase cleavage site that generates the N terminus of prM is located 14 to 22 residues (depending on the virus) C-terminal to this potential dibasic cleavage site. The intervening segment between the dibasic site and the signalase site consists mainly of hydrophobic residues which are capable of serving as an internal signal sequence for translocation of prM into the ER (31, 47). According to one model (37), the signal sequence preceding prM directs translocation of prM into the ER. Cotranslational cleavage by signal peptidase generates an anchored form of the capsid, denoted anchC, which is bound to the membrane by the hydrophobic residues at its C terminus. NS2B-3-mediated cleavage occurs later in infection at the conserved dibasic site to generate the truncated, mature form of the capsid found in virus particles, denoted virC. This view has recently been challenged by evidence that the anchC/prM signalase cleavage cannot occur efficiently in the absence of an active NS2B-3 proteinase, suggesting that cleavage at the dibasic site may be a prerequisite to signalase cleavage (28, 57).

Here we report the development of a cell-free *trans* cleavage assay for the YF NS2B-3 proteinase. By using a substrate containing the C protein dibasic site, direct evidence was obtained for NS2B-3 proteinase-mediated cleavage at the predicted site. Finally, in vivo expression of various YF polyproteins demonstrated that C-prM processing requires an active NS2B-3 proteinase. This is consistent with other flavivirus studies (28, 57) and suggests alternative models for processing in the C-prM region.

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MATERIALS AND METHODS

Cells lines and virus. Maintenance of YF 17D stocks and growth of BHK-21, SW-13, and BSC-40 monolayers have been described previously (11).

Vaccinia virus recombinants. Vaccinia virus recombinants expressing YF NS2B (vNS2B), $NS2B3_{181}$ (v$NS2B3_{181}$), and $NS3_{181}$ (v$NS3_{181}$) have been described previously (13). YF-specific products were expressed by coinfection with vTF7-3, a vaccinia virus recombinant expressing T7 RNA polymerase (21). Proper expression of YF-specific antigens was verified by metabolic labeling and immunoprecipitation with appropriate region-specific antisera (12).

Plasmid constructions. For construction of the C-prM expression vector, YF cDNA from nucleotides 109 to 973 was amplified by PCR (from pYF5′3′IV [42]) to create a cassette with 5′-flanking *Xba*I and *Bst*EII sites, followed by the C-prM coding sequence, a UAA termination codon, and a 3′-flanking *Xba*I site. PCR products were digested with *Xba*I and cloned into *Xba*I-digested pH3′2J1 (22). pBS/C-prM was created by cloning the C-prM cassette from pH3′2J1/C-prM into the *Xba*I site of the expression vector pBluescript II SK (−) (Stratagene). PCR amplification of pH3′2J1/C-prM was used to generate fragments with a 5′ terminus identical to the C-prM cassette described above, with termination codons following either residue 101 (virC) or 121 (anchC), and 3′-flanking *Xba*I sites. PCR products were digested with *Xba*I and cloned into *Xba*I-digested pBluescript II SK (−). All sequences derived by PCR were confirmed by sequencing. Sense transcripts are produced from the T7 promoter for pBS/C-prM and pBS/anchC or the T3 promoter for pBS/virC. To produce an N-terminally truncated form of C, a 209-bp *Bam*HI-*Sau*3AI fragment was deleted from pBS/anchC to create pBS/anchC.3. This construct lacks the YF initiation codon and should initiate translation at the third AUG of the YF ORF (YF nucleotide 329; C residue 71).

pETBS/YF/C-$NS3_{181}$ (also called pETBS/YF/S/NS1-$NS3_{181}$), which encodes a polyprotein extending from the N terminus of C through the first 181 residues of NS3, was derived by inserting the 1,292-bp *Nsi*I-*Mlu*I fragment (YF nucleotides 1657 to 2948) of YFM5.2 (42) and the 1,560-bp *Xba*I-*Nsi*I fragment (YF nucleotides 109 to 1657) from pBSIISK(+)YFS into *Xba*I-*Mlu*I-digested pETBS/YF/sigNS1-$NS3_{181}$ (36). pETBS/YF/C-$NS3_{181}$(S-138→A) (also called pETBS/YF/S/NS1-NS3*.a) was created by replacing the 349-bp *Xba*I-*Bam*HI fragment from pETBS/YF/NS1-$NS3_{181}$(S-138→A) (36) with the 2,624-bp *Xba*I-*Bam*HI fragment from pETBS/YF/C-$NS3_{181}$.

In vitro transcription and cell-free translation. We synthesized 5′-capped RNA transcripts (44) by using T7 RNA polymerase for pBS/anchC, pBS/anchC.3, and pBS/C-prM or T3 RNA polymerase for pBS/virC. Rabbit reticulocyte lysates (Promega) were used for cell-free translation experiments as specified by the manufacturer. Translations conducted in the presence of membranes were performed with 4 eq of canine pancreatic microsomal membranes (Promega) for 50-μl translation reactions. Proteins were labeled by inclusion of either [^{35}S]Met or [^{3}H]Leu (Amersham) at a final concentration of 1 μCi/μl.

Preparation of cell extracts. Unless otherwise specified, SW-13 monolayers were mock infected or infected with YF 17D at a multiplicity of infection of 10 and incubated at 37°C. After 36 h, the monolayers were washed with phosphate-buffered saline and lysed with 0.5% Triton X-100 in buffer A (150 mM NaCl, 50 mM Tris [pH 7.5], 1 mM EDTA, 20 μg of phenylmethylsulfonyl fluoride per ml) (11); lysates prepared in the presence or absence of phenylmethylsulfonyl fluoride exhibited indistinguishable activity in the cleavage assay. For extracts prepared from monolayers infected with various vaccinia virus recombinants, BSC-40 monolayers were infected with vTF7-3 and the specified recombinants at a multiplicity of infection of 10 each and lysed, as above, at 6.5 h postinfection.

A crude separation was used to determine if the proteinase activity was cytosolic or membrane associated. After 36 h, YF-infected SW-13 monolayers were washed with PBS and scraped into buffer A. Cells were collected by centrifugation at 10,000 × *g* for 5 min. The pellet was resuspended in buffer A and subjected to three cycles of successive freezing (dry ice-ethanol bath) and thawing (room temperature). The crude lysate was fractionated by centrifugation at 16,000 × *g* for 15 min. The pellet (P16), which contained the nuclei, cytoskeleton, and cytoplasmic membrane organelles, is referred to as the membrane fraction; the supernatant (S16) is referred to as the soluble fraction. The pellet was resuspended in buffer A. Samples were assayed for proteinase activity either directly or following addition of Triton X-100 to a final concentration of 0.5%.

Standard proteinase assay. Radiolabeled anchC.3 substrate was produced by translation of 200 ng of transcript RNA in a final volume of 6 μl. Translations were terminated by addition of DNase-free RNase (Boehringer Mannheim) to 0.01 U/μl. The standard proteinase assay consisted of 0.6 μl of this translation reaction mixed with detergent-solubilized cell lysates (amount equivalent to 2×10^4 cells) in a final volume of 7 to 12 μl. For cell lysates separated into soluble and membrane fractions, the equivalent of 10^5 cells was used in each assay. Unless otherwise indicated, reactions were incubated at 30°C for 80 min and terminated by the addition of sodium dodecyl sulfate (SDS) to 1%.

N-terminal sequence analysis of radiolabeled cleavage products. anchC.3 transcripts were translated in the presence of either [^{35}S]Met or [^{3}H]Leu to generate radiolabeled cleavage substrates (see above). After incubation with extracts containing active YF proteinase, cleavage products were resolved by SDS polyacrylamide gel electrophoresis (PAGE) with the Tricine system (see below) and electroblotted onto Immobilon-P polyvinylidene difluoride membrane (Millipore) (34). The transferred peptide was extracted from the polyvinylidene difluoride membrane with 100% trifluoroacetic acid and covalently attached to a Sequelon-AA membrane (Millipore) (2). Sequence analysis was performed on an ABI model 470 A sequencer (11).

In vivo transient-expression assay. The hybrid T7-vaccinia virus system, as used for expression of radiolabeled YF polyproteins from transfected plasmids in BHK-21 cells, has been described previously (27). In experiments in which vaccinia virus recombinants were used in conjunction with transfected plasmids, cells were coinfected with v$NS2B3_{181}$ and vTF7-3 at a multiplicity of infection of 10 each prior to DNA transfection.

Immunoprecipitation. Lysates of BHK-21 monolayers transfected with C-$NS3_{181}$ derivatives were prepared by solubilization with SDS and immunoprecipitated as described previously (11), using 2 μl of rabbit antiserum raised against either a fusion protein containing residues 1 to 94 of the YF capsid protein (kindly provided by M. Bouloy, Institute Pasteur, Paris, France) or a YF C-prM polyprotein, which has been shown to react with only prM and M (12). Lysates of BHK-21 monolayers transfected with pBS/C-prM were prepared by solubilization in a nondenaturing buffer and immunoprecipitated with 3 μl of the prM-specific antisera as described previously (13). Nondenaturing protocols were found to be somewhat more

effective for immunoprecipitation of C-containing peptides, although they produced a significantly greater nonspecific background.

Gel electrophoresis. The small peptides produced by the in vitro cleavage assay were resolved by SDS-PAGE with the Tricine system (49) and with 16.5% acrylamide (acrylamide/bisacrylamide ratio, 15.5:1) in the separating portion of the gel. All other gels were conventional SDS-PAGE gels (26) with 15% acrylamide (acrylamide/bisacrylamide ratio, 37.5:1). After electrophoresis, gels were treated for fluorography by a variation of the technique described by Chamberlain (8). Gels were fixed for 30 min in 10 volumes of 25% ethanol–10% acetic acid, washed twice with distilled-deionized H_2O (15 min per wash), and shaken for 30 to 45 min in 10 volumes of 1 M sodium salicylate–50 mM sodium phosphate (pH 7.0)–5% glycerol. Gels were dried for 4 h under vacuum at 60 to 70°C before being exposed to preflashed X-ray film (Kodak X-Omat) at −70°C.

RESULTS

Substrate for assay of proteolytic activity. The goal of this study was to develop an in vitro *trans*-cleavage assay for the YF NS2B-3 proteinase. Although NS2B-3-mediated cleavage at the anchC dibasic site had not been demonstrated, we focused on this site since it seemed likely that it could be cleaved efficiently in *trans*, as least in vivo. Cell-free translation of a transcript encoding anchC led to a complex pattern of translation products, presumably owing to internal initiation at two downstream AUG codons. Preliminary *trans*-cleavage assays suggested that the smallest anchC translation product was cleaved most efficiently by the YF proteinase. To simplify the pattern of potential substrates and cleavage products, the N-terminal portion of the C coding region was deleted to produce a transcription construct encoding an amino-terminally truncated substrate, designated anchC.3, which initiates at the third Met codon of the YF ORF to produce a 51-residue peptide containing the anchC cleavage site (Fig. 1A).

Sources of proteolytic activity for in vitro studies. The active YF NS2B-3 proteinase is a complex consisting of the hydrophobic NS2B protein and the NS3 serine proteinase domain (1, 9, 13, 14, 20, 27, 40). Given the likely membrane association of the proteinase (45, 55), we began by assaying crude Triton X-100-solubilized extracts from YF-infected cells. Lysates derived from YF-infected cells, but not from mock-infected cells, were capable of cleaving the substrate into two products with the expected sizes of 31 residues (predicted molecular mass, 3.6 kDa) and 20 residues (predicted molecular mass, 2.1 kDa). The substrate and the larger cleavage product migrate more slowly than predicted, perhaps owing to their basic character. Lysates were most active when prepared from cells that had been infected for longer than 24 h, coinciding with the peak of virus release (Fig. 2). Lysates prepared in the absence of detergent, by using a freeze-thaw protocol, were inactive in the same assay (data not shown). However, when freeze-thaw lysates were separated into crude membrane and soluble fractions and then subsequently solubilized with Triton X-100, proteolytic activity could be recovered from the membrane fraction (Fig. 3, lane 5) but not the soluble fraction (lane 3). Several detergents were tested as solubilizing agents; the greatest activity was recovered from cells lysed with 0.5% Triton X-100, 1% *n*-octylglucoside, 1% *n*-dodecyl-β-D-maltoside, 0.5% Thesit, or 0.5% isotridecylpoly(ethyleneglycol-ether)$_n$; no activity was recovered from cells lysed with 1% octanoyl-*N*-methylglucamide (MEGA-8) or 1% *N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate; moderate activity was obtained from lysates prepared with 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1% 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO), or 0.5% Triton X-114 (unpublished observations). Lysates prepared with Triton X-100 con-

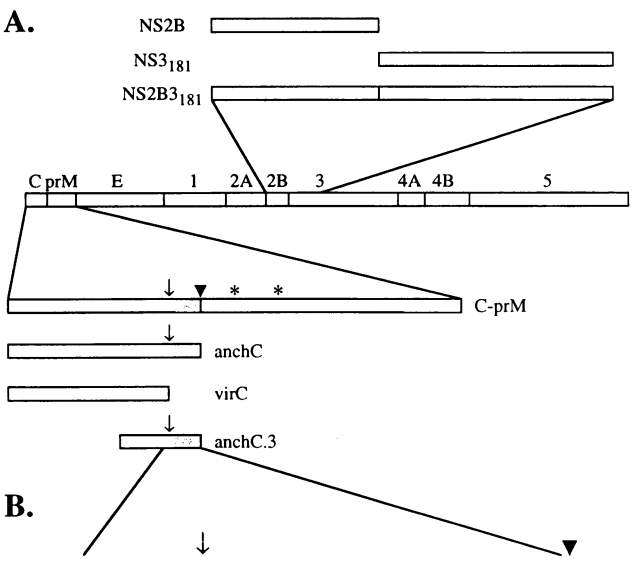

FIG. 1. (A) Diagram of constructs used to express YF proteinase components and substrates. The entire YF polyprotein is shown in the center, with nonstructural proteins NS1 to NS5 denoted by their numbers. Vaccinia virus recombinants vNS2B, vNS3$_{181}$, and vNS2B3$_{181}$, which express the corresponding YF polypeptides (containing two extra N-terminal residues), are indicated at the top. Constructs for in vitro transcription-translation of the C-prM region are shown below the YF polyprotein diagram. Genes for C-prM, anchC, and virC are designed to initiate at the authentic initiation codon of the YF ORF. The anchC.3 construct is engineered to initiate at the third in-frame Met codon in the YF ORF. The C termini are positioned at the prM/E signalase cleavage site (C-prM), at the C/prM signalase cleavage site (anchC and anchC.3), or at the dibasic, putative NS2B-3-processed cleavage site (virC). The dibasic cleavage site is indicated by ↓, the signalase cleavage site is indicated by ▼, and probable N-linked glycosylation sites are indicated by *; the hydrophobic regions are shaded. (B) Sequence alignment of the dibasic capsid cleavage sites from several members of the flavivirus family. The seven residues preceding the dibasic cleavage site, the hydrophobic span between the dibasic and signalase cleavage sites, and the N-terminal three residues of prM are shown. Abbreviations and references are as follows: YF, yellow fever virus (43); DEN1, dengue type 1 virus (32); DEN2, dengue type 2 virus (23); DEN3, dengue type 3 virus (38); DEN4, dengue type 4 virus (60); JE, Japanese encephalitis virus (52); MVE, Murray Valley encephalitis virus (18); SLE, St. Louis encephalitis virus (53); WN, West Nile virus (7); KUN, Kunjin virus (16); TBE, tick-borne encephalitis virus (29); LGT, Langat virus (30); LI, Louping ill virus (50).

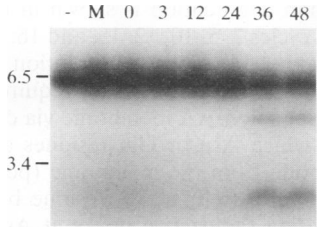

FIG. 2. Proteinase assay of YF-infected SW-13 cell lysates. Cell-free-translated anchC.3 (migrating at 6.5 kDa) was incubated alone (−) or with Triton X-100 lysates from uninfected (M) or YF-infected cells harvested at the indicated times postinfection (in hours). Products were separated by using a Tricine gel system as described in Materials and Methods. By 60 h postinfection (not shown), proteolytic activity was greatly diminished, probably because a significant fraction of the cells had detached from the dishes. The sizes of molecular mass standards (in kilodaltons) are indicated on the left.

centrations from 0.05 to 2% were all active, although the greatest activity was generated at concentrations of 0.1% and higher.

Proteolytic products could be observed when the assay was conducted at temperatures ranging from 15 to 37°C but not at 40°C or higher. At 30°C, products could be visualized after incubation times as short as 1 min; however, we have never observed complete substrate cleavage, even after extended incubations. Repeated freezing (at −80°C) and thawing (at 25°C) of Triton X-100 lysates from YF-infected cells demonstrated no appreciable loss of proteolytic activity. Surprisingly, the general serine proteinase inhibitor phenylmethylsulfonyl fluoride had no discernible effect on proteolysis when included in either the cell lysis buffer or the in vitro assay at a concentration of 20 μg/ml.

Both NS2B and NS3 are required for capsid cleavage. As mentioned above, NS2B and the NS3 serine proteinase domain are believed to form a complex which is responsible for cleavage at the anchC dibasic site. To test this hypothesis, we constructed three vaccinia virus recombinants expressing portions of the YF genome under control of the bacteriophage T7 promoter (Fig. 1A). The first recombinant, vNS2B3$_{181}$, contains the YF coding region encompassing NS2B and the first 181 residues of NS3, which includes the serine proteinase

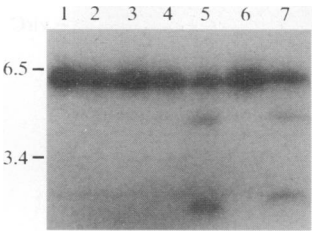

FIG. 3. Proteinase assay of membrane and soluble fractions from YF-infected SW-13 cells. Cell-free-translated anchC.3 substrate was incubated alone (lane 1), with the soluble fraction of a YF-infected cell lysate (lanes 2 and 3), with the membrane fraction of a YF-infected cell lysate (lanes 4 and 5), or with a Triton X-100 lysate of mock-infected (lane 6) or YF-infected (lane 7) SW-13 cells and run on a Tricine gel system as described in Materials and Methods. Soluble and membrane fractions were used for this assay either directly (lanes 2 and 4, respectively) or following addition of Triton X-100 to 0.5% (lanes 3 and 5, respectively). The sizes of molecular mass standards (in kilodaltons) are indicated on the left.

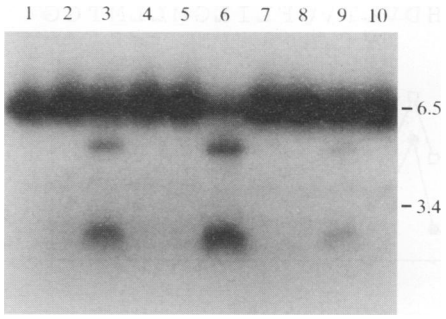

FIG. 4. Proteinase assay of recombinant vaccinia virus-infected BSC-40 or YF-infected SW-13 cell lysates. Cell-free-translated anchC.3 substrate was incubated alone or with Triton X-100 lysates from uninfected cells or cells infected with YF or various vaccinia virus recombinants and separated with the Tricine gel system, as described in Materials and Methods. Lanes: 1, substrate incubated in the absence of a cell lysate; 2, mock-infected SW-13 cell lysate; 3, YF-infected SW-13 cell lysate; 4, mock-infected BSC-40 cell lysate; 5, vTF7-3-infected BSC-40 cell lysate; 6, vNS2B3$_{181}$-infected BSC-40 cell lysate; 7, vNS2B-infected BSC-40 cell lysate; 8, vNS3$_{181}$-infected BSC-40 cell lysate; 9, cell lysate from BSC-40 cells coinfected with vNS2B and vNS3$_{181}$; 10, vNS2B lysate mixed in vitro with vNS3$_{181}$ lysate. The sizes of molecular mass standards (in kilodaltons) are indicated on the right.

domain. This truncated polyprotein has been shown, by using in vivo transient-expression assays, to be capable of autocatalytic cleavage at the 2B/3 site as well as *trans* cleavage at the other dibasic sites in the YF nonstructural region (1, 9, 13, 20, 27). Two additional recombinants, vNS2B and vNS3$_{181}$, which express the NS2B and NS3$_{181}$ regions separately, were constructed.

Proteolytic assays were performed with Triton X-100 lysates of BSC-40 cells infected with vTF7-3 alone (a vaccinia virus recombinant expressing bacteriophage T7 RNA polymerase) or coinfected with the various vaccinia virus-YF recombinants. Figure 4 shows the results of one such experiment. Lysates from cells infected with vTF7-3 alone or coinfected with vTF7-3 and either vNS2B or vNS3$_{181}$ did not contain detectable proteolytic activity (lanes 5, 7, and 8, respectively). The lysate from vNS2B3$_{181}$-infected cells was active, as evidenced by the appearance of the two characteristic cleavage products and a concomitant decrease in the amount of substrate (lane 6). Lysates from cells coinfected with both vNS2B and vNS3$_{181}$ were also active but to a lesser degree (lane 9), demonstrating that although both moieties are necessary, they need not be expressed as a polyprotein for activity. The reduced activity might reflect a lower efficiency of NS2B-NS3$_{181}$ complex formation when the subunits are not derived from a common precursor. The need for both proteins is consistent with studies of the dibasic cleavages in the nonstructural region, which show an absolute requirement for both NS2B and the N-terminal one-third of NS3 (1, 6, 9, 13, 20, 40, 59). Mixing vNS2B- and vNS3$_{181}$-infected BSC-40 lysates did not reconstitute activity (lane 10), which could be due to dilution by lysis buffer, reduction of protein concentrations, and inefficient complex formation.

In vitro cleavage occurs at the predicted dibasic site. Cleavage at the anchC.3 dibasic site would yield peptides of 31 and 20 residues, with the smaller peptide representing the C-terminal segment of the substrate. To confirm that the observed proteolysis was occurring at the predicted, conserved dibasic site, we performed N-terminal amino acid sequencing on the smaller of the two products. The radioactivity from each

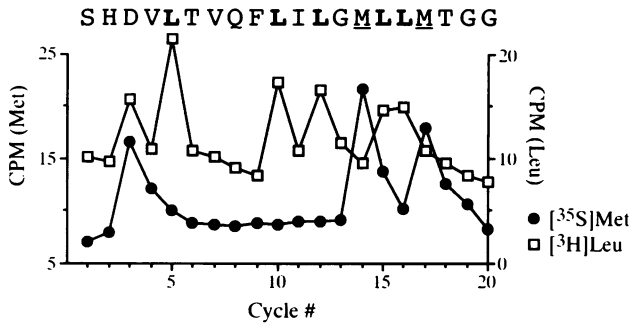

FIG. 5. N-terminal amino acid sequencing of the faster-migrating cleavage product from the anchC.3 proteinase assay. The anchC.3 substrate was labeled with either [^{35}S]Met or [^{3}H]Leu. The radioactivity recovered at each cycle is shown. The amino acid sequence of the YF polyprotein from residues 102 to 121 (immediately following the conserved dibasic site in the capsid protein), as predicted by its cDNA sequence, is shown (the two Met residues are underlined, and the five Leu residues are in boldface type). The chemistry of the covalent attachment of the sequencing substrate to the membrane support generates peaks at positions where Asp (D) and Glu (E) residues are found (see Results).

cycle of the Edman degradation is shown in Fig. 5. [^{3}H]Leu peaks are seen in cycles 3, 5, 10, 12, 15, and 16; [^{35}S]Met peaks are seen in cycles 3, 14, and 17. Retention of the 20-mer peptide during solid-phase sequencing required covalent attachment to the Sequelon-AA membrane via carboxyl groups. Hence, cycles in which Asp or Glu residues are cleaved can release some peptide from the membrane (peptide bound to the membrane by an Asp or a Glu residue but not by its C terminus), generating radioactive peaks at Asp or Glu positions in the sequence. Given that position P3′ from the predicted cleavage site is an Asp residue, these data unambiguously define the cleavage site to be the conserved dibasic site, between residues Arg-101 and Ser-102 of the YF polyprotein.

Transient expression of C-NS3$_{181}$ and cell-free expression of C-prM. To examine the role of the NS2B-3 proteinase in structural-region processing in vivo, we created two constructs. The first, C-NS3$_{181}$, expressed a YF polyprotein predicted to initiate at the first Met of the ORF and extend through the NS3 serine proteinase domain (the N-terminal 181 residues). The second construct, C-NS3$_{181}$ (Ser-138→Ala), was identical except for an Ala substitution for NS3 residue Ser-138, which is believed to be the active-site nucleophile. This substitution has previously been demonstrated to completely inactivate cleavages occurring at the dibasic sites in the nonstructural region (9, 14, 27). As expected, lysates of cells expressing the

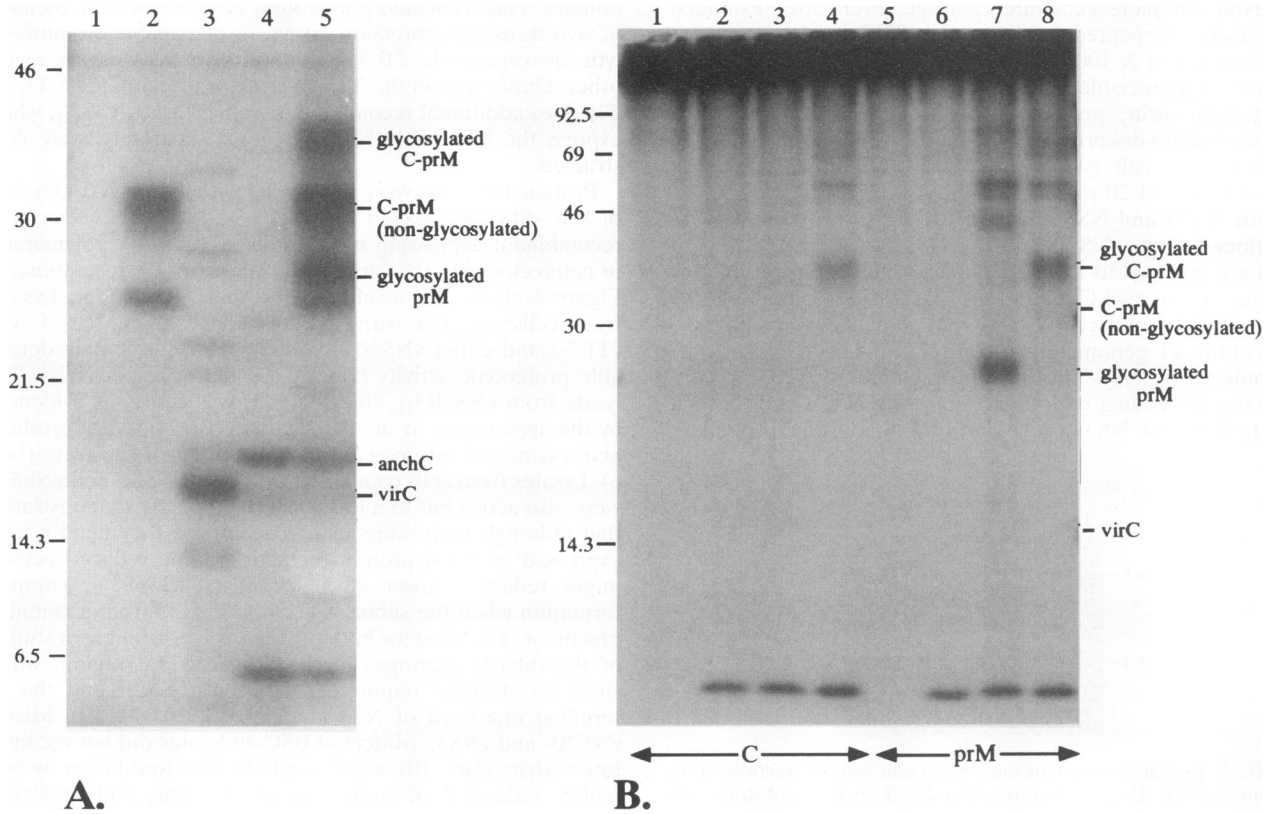

FIG. 6. (A) Cell-free translation of RNA transcripts encoding various portions of the YF structural region. Lanes: 1, no RNA; 2, C-prM; 3, virC; 4, anchC; 5, C-prM in the presence of microsomal membranes. Molecular mass standards (in kilodaltons) are indicated on the left. On the basis of their migration, bands were identified as indicated on the right. Strong initiation at the third AUG of the ORF is thought to generate bands approximately 8 kDa smaller than the primary product (seen most prominently in lanes 2, 4, and 5). (B) Transient expression of YF C-NS3$_{181}$ polyproteins. Antiserum to either C or prM was used for immunoprecipitation of SDS lysates of BHK-21 cells, as indicated below the figure. Lanes: 1 and 5, mock infected; 2 and 6, vTF7-3 alone; 3 and 7, vTF7-3 infection followed by transfection of the pETBS/YF/C-NS3$_{181}$ construct; 4 and 8, vTF7-3 infection followed by transfection with the pETBS/YF/C-NS3$_{181}$(Ser-138→Ala) construct. The sizes of molecular mass markers (in kilodaltons) are indicated on the left. The positions of markers produced by cell-free translation (see panel A) are indicated on the right.

C-NS3$_{181}$ construct demonstrated site-specific cleavage activity with the anchC.3 substrate, whereas lysates from cells expressing C-NS3$_{181}$(Ser-138→Ala) were inactive (data not shown).

Identification of proteins expressed in the transient-expression experiments was based on reactivity with specific antisera and on migration on SDS-PAGE. To generate markers, we constructed several transcription plasmids expressing different portions of the YF structural region (Fig. 1A). Constructs pBS/anchC and pBS/virC express the full-length anchC form and the C-terminally truncated virion C, respectively; pBS/C-prM is designed to express the C-prM polyprotein. Figure 6A shows the results of an in vitro transcription-translation experiment. The full-length products of the virC (lane 3), anchC (lane 4), and C-prM translations (lane 2) are indicated, although major bands thought to be internally initiated or premature termination products are also seen. When C-prM is translated in the presence of microsomal membranes (lane 5), a more slowly migrating band is observed, which is thought to represent glycosylated C-prM (prM is thought to contain two N-linked glycans [12]). In addition, two new bands are seen: one corresponds in size to anchC, and the other corresponds to glycosylated prM, indicating that signalase cleavage has occurred, albeit at low efficiency.

Figure 6B shows the results of immunoprecipitation of SDS lysates of cells transiently expressing these constructs, using antisera specific for either C or prM. The ticks on the right of the gel denote the positions of marker polypeptides produced by cell-free translation (Fig. 6A). The C-NS3$_{181}$ polyprotein was processed as expected. A faint band migrating slightly more slowly than the 14.3-kDa marker was specifically immunoprecipitated with C antiserum (Fig. 6B, lane 3). The capsid protein is often difficult to detect by immunoprecipitation (24, 28, 57) or cell-free translation (31, 47) experiments. Although positive identification was not possible, this band is denoted virC since its migration is very similar to that of virC produced by cell-free translation. The protein immunoprecipitated by prM antisera (lane 7) migrates in the same position as the band identified as glycosylated prM in the cell-free translation experiment. Surprisingly, when the polyprotein containing the Ala-138 substitution was expressed, a larger protein was immunoprecipitated by antisera to either C or prM (lanes 4 and 8). This band migrates at the position of the glycosylated C-prM polyprotein, indicating that translocation of the prM segment has occurred (the position of nonglycosylated C-prM is noted for reference). Neither glycosylated prM nor virC was detected, indicating that the prM signalase cleavage had not occurred. The larger species in lanes 4, 7, and 8 have not been identified. These results suggest that NS2B-3 proteinase-mediated cleavage at the anchC dibasic site is a prerequisite for processing at the prM signalase site.

NS2B-3-mediated C/prM cleavage can occur in *trans*. The in vitro cleavage assay clearly demonstrates that cleavage at the anchC dibasic site can occur in *trans*. To examine *trans* cleavage at this site in vivo, we analyzed processing of C-prM, expressed in the presence or absence of NS2B3$_{181}$. Immunoprecipitation of prM-specific proteins demonstrates a protein of the same mobility as glycosylated prM in vNS2B3$_{181}$-infected cells, and a protein of the same mobility as glycosylated C-prM in the absence of vNS2B3$_{181}$ (Fig. 7). Unfortunately, the presence of any nonglycosylated C-prM was obscured by a nonspecific band migrating in this position. Nevertheless, we conclude from this experiment that the NS2B-3 proteinase-dependent mechanism for separating C and prM can occur in *trans*.

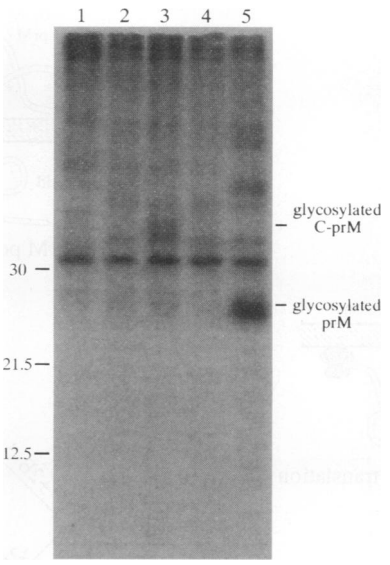

FIG. 7. NS2B-3-proteinase mediated processing of C-prM can occur in *trans*. Lanes: 1, mock infected; 2, vTF7-3 alone; 3, vTF7-3 infection followed by transfection of pBS/C-prM; 4, coinfection with vTF7-3 and vNS2B3$_{181}$; 5, coinfection with vTF7-3 plus vNS2B3$_{181}$ followed by transfection of pBS/C-prM. BHK-21 cells were lysed in a nondenaturing buffer and immunoprecipitated with prM antiserum. The sizes of molecular mass markers (in kilodaltons) are indicated on the left. The positions at which cell-free-translated markers migrate (Fig. 6A) are indicated on the right.

DISCUSSION

This report directly demonstrates NS2B-3-dependent cleavage at the proposed consensus cleavage site, and the in vitro *trans*-cleavage assay provides a means for purification of an active NS2B-3 proteinase which should be key for future biochemical and structural studies on this critical flavivirus enzyme. Although we have been unable to obtain direct evidence that the YF virion capsid protein terminates at the dibasic site, indirect evidence for YF and C-terminal sequence data of virC for WN (37) and Kunjin virus (51) suggests that this is likely to be the case.

Although the capsid protein is one of the least conserved of the flaviviral proteins, it does have some conserved features, including the consensus cleavage site for the virus-encoded NS2B-3 proteinase. Also conserved is the hydrophobicity of the span of residues located between the dibasic site and the signalase cleavage site that generates the N terminus of prM (Fig. 1B). A number of observations have led to the hypothesis that an anchC protein, with the hydrophobic region intact, is separated from the growing polypeptide chain via a cotranslational signalase cleavage at the anchC/prM junction, generating an anchored form of the capsid. The anchC protein would remain anchored in the ER membrane by its hydrophobic C terminus, where it is a substrate for proteolytic cleavage by the NS2B-3 proteinase at the conserved dibasic site. This model has been supported by both cell-free translation studies and studies of recombinants expressing portions of the flavivirus structural region. Cleavage of the anchC/prM signalase site has been shown to be dependent on the signal sequence preceding the N terminus of prM in the absence of the nonstructural coding region (31, 47), whereas evidence that the virion capsid sequence terminates at the dibasic site (37, 51) implied that NS2B-3-mediated cleavage was occurring at some point. Fi-

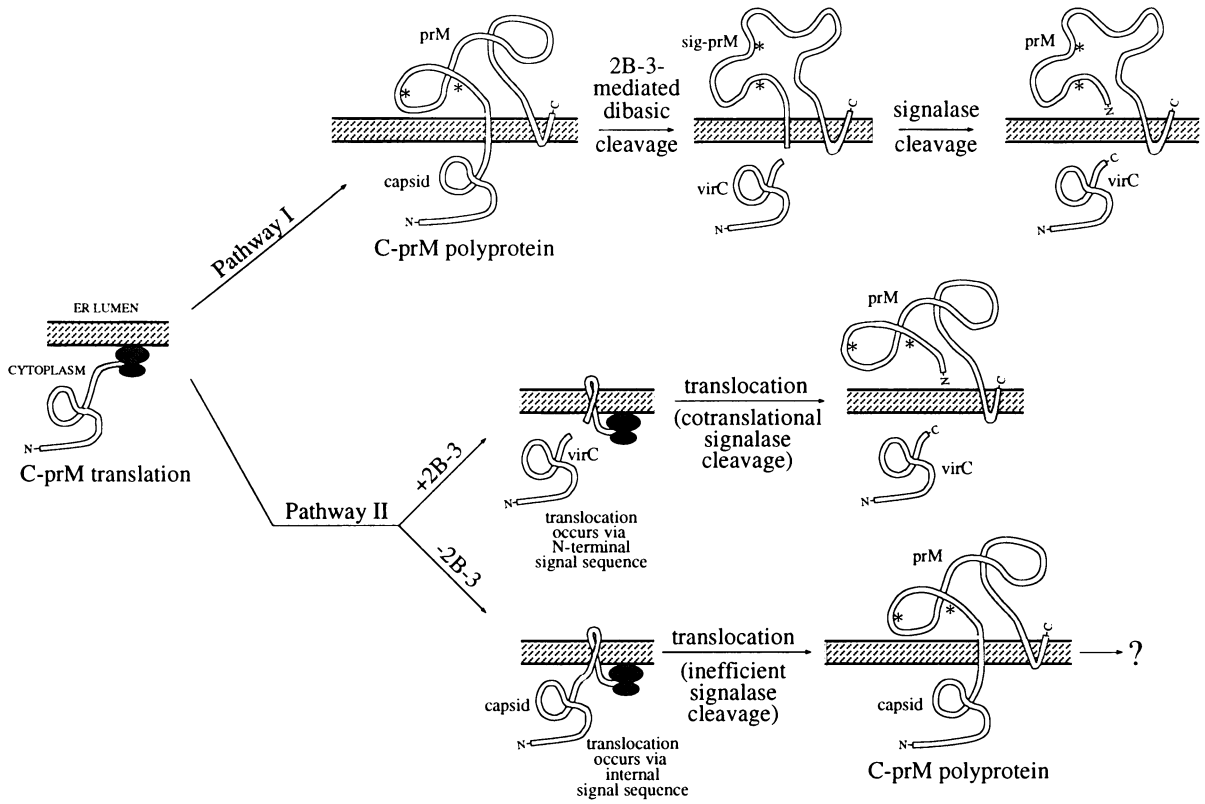

FIG. 8. Possible pathways for C-prM processing. The hydrophobic stretch of amino acids immediately preceding the signalase cleavage site at the N terminus of prM can serve exclusively as an internal signal sequence (the first pathway) or as either an internal or an N-terminal signal sequence (the second pathway), depending on the availability of protease. In the first pathway, NS2B-3-mediated cleavage on the cytosolic side of the polyprotein induces conformational changes on the lumenal side of the membrane which allow posttranslational signalase cleavage to occur. The fate of the signal peptide is unknown. For the second pathway, rapid, perhaps cotranslational, NS2B-3-mediated cleavage at the dibasic site can generate an N-terminal signal sequence which can facilitate translocation and cotranslational signalase cleavage. In the absence of the dibasic cleavage, translocation can occur by using the signal sequence internally; under these circumstances, signalase cleavage does not occur efficiently, generating the C-prM species. A combination of these two pathways might also be possible, in which the C-prM product of the second pathway can serve as a substrate for the first pathway. Hydrophobic stretches of amino acids are shaded, and probable glycosylation sites are marked with an asterisk.

nally, it was observed that the YF capsid protein from mature virions migrated faster on SDS-PAGE than did intracellular capsid protein (12), suggesting that the intracellular form was trimmed prior to incorporation into virions. From these data it was hypothesized that cleavage at the dibasic site occurred after signalase cleavage to generate the truncated form of the capsid (virC), possibly in association with viral maturation.

More recent data have challenged this model. Efficient cleavage of C-prM and secretion of prM-E have been shown to be dependent on an active NS2B-3 proteinase. Although the anchC-prM signalase cleavage has been demonstrated in the absence of the proteinase both in vivo for Japanese encephalitis virus (58), dengue type 2 virus (24), dengue type 4 virus (3, 4, 61), and YF (24) and in vitro for WN (37), dengue type 4 virus (31), and YF (47; also see Fig. 6A), cleavage is usually incomplete, as seen by variable amounts of glycosylated C-prM polyprotein. Vaccinia virus recombinants expressing the prM-E coding region of Japanese encephalitis virus (25, 33) or YF (39) produced secreted forms of the E protein, whereas addition of the C sequence prevented E secretion. Secretion of E from a vaccinia virus recombinant expressing the structural region of Japanese encephalitis virus (excluding the N-terminal half of C) was stimulated by coinfection with a recombinant expressing the NS2B-3 proteinase (48). Cells expressing a WN polyprotein extending from C through the proteolytic domain of NS3 secreted a prM-E complex (57); removal of the proteolytic domain prevented secretion, which could in turn be restored by removal of the cytoplasmic domain of C. Finally, it was shown for Murray Valley encephalitis virus that a substitution at the active-site serine of the NS3 proteinase inactivated prM processing and prM-E secretion (28). The NS2B-3 proteinase, then, appears to play an important role in the proper maturation of the structural proteins. The data presented here demonstrate that although translocation of prM is independent of a functional NS2B-3 proteinase, cleavage of the C-prM polyprotein is proteinase dependent; presumably the NS2B-3-dependent cleavage generates virC by cleavage at the dibasic site and an extended prM species containing an N-terminal signal sequence serves as the substrate for signalase cleavage.

Two possible pathways for C-prM processing present themselves at this time (Fig. 8). In the first, prM is translocated into the lumen of the ER by the internal C-prM signal sequence, leaving the majority of C exposed in the cytosol. Cleavage at the dibasic site might then allow posttranslational signalase cleavage on the lumenal side of the ER membrane to generate

the N terminus of prM. Although the usual model for signalase processing is cotranslational (41), pulse-chase studies performed in vitro for YF (47) and in vivo for dengue type 2 virus (35) suggest that C-prM might be a precursor to prM.

In the second scenario, rapid cleavage at the dibasic site would release the hydrophobic domain preceding the N terminus of prM and allow it to serve as a traditional N-terminal signal sequence, similar to processing at the 4A/4B site (27). The N-terminal signal sequence might be cleaved more efficiently than as a part of the intact C-prM polyprotein. Early in infection, prior to significant accumulation of the nonstructural proteins, cleavage at the dibasic site would be infrequent, generating a membrane-inserted C-prM polyprotein. This product may be a substrate for cleavage, as discussed above; it may perform some as yet unknown function in virus assembly; or it may be a dead-end product. A C-prM species has been observed in dengue type 2 virus-infected cells (35).

Either of these models might characterize an important level of regulation in the viral life cycle. Assembly of viral particles might be coordinated with viral protein and RNA levels by delaying production of the mature virC until substantial accumulation of the nonstructural proteins has been achieved. This type of regulation would be possible only if the dibasic capsid cleavage occurs in *trans*. A *cis* cleavage, in which the viral polyprotein remains associated at the membrane throughout translation and processing, would be insensitive to concentration. An obligate *cis* cleavage at the anchC dibasic site would also disallow the second model of cotranslational C-prM processing, in which NS2B-3-mediated cleavage of the dibasic site generates an N-terminal signal sequence for prM translocation. Although in vitro experiments demonstrate that cleavage at the dibasic site occurs readily in *trans* and in vivo experiments suggest the same, no evidence is available to address how the efficiencies of these types of cleavages might differ in an infected cell. Further studies will be necessary to distinguish between these different pathways of structural-protein processing.

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