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Posttranslational Signal Peptidase Cleavage at the Flavivirus C-prM Junction In Vitro

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We have investigated the cleavages at the flavivirus capsid-prM protein junction in vitro. When expressed in the absence of the flavivirus proteinase, capsid and prM, which are separated by an internal signal sequence, exist as a membrane-spanning precursor protein. Here we show the induction of posttranslational signal peptidase cleavage of prM by trypsin cleavage of a cytoplasmic region of this precursor protein.

Flavivirus replication and assembly takes place in close association with the membranes of the endoplasmic reticulum (ER). The flavivirus genomic plus-stranded RNA molecule is translated into a single polyprotein with multiple ER membrane-spanning domains (reviewed in reference 4). This precursor polyprotein is proteolytically processed by viral and host proteinases into three structural proteins and at least seven nonstructural proteins. The structural proteins (capsid [C] protein, precursor to membrane [prM] protein, and envelope [E] protein) are encompassed in this order by the NH_2-terminal one-fourth of the polyprotein. A signal sequence at the junction of C and prM and consecutive stop-transfer/signal sequences at the junctions of prM and E and of E and NS1 direct the ER membrane topology of the flavivirus structural polyprotein region. This results in cytoplasmic location of the C protein and luminal topology of the virion transmembrane proteins, prM and E. It also provides a mechanism for proteolytic cleavages at the NH_2 termini of prM and E mediated by signal peptidase on the luminal side of the ER membrane (9, 17, 20, 24). An additional cleavage at the COOH terminus of the C protein completes the proteolytic processing events in the flavivirus structural polyprotein region that take place in the ER. The internal signal sequence at the junction of C and prM (the translocation signal for prM) is cleaved from the COOH terminus of the C protein by the viral proteinase complex NS2B-3 (1, 20, 29), which also catalyzes the other cytoplasmic proteolytic cleavages of the flavivirus polyprotein (3, 6, 8, 21, 26).

The kinetics of signal peptidase cleavage at the C-prM junction are unexpected in view of the notion that signal peptidase cleavage is a rapid cotranslocational event (22). Several groups have noted, when using recombinant expression of cDNA from different flaviviruses encompassing the C-prM region, that the signal peptidase cleavage of prM is inefficient and that coexpression of functional NS2B-3 proteinase greatly enhances the generation of mature prM (1, 16, 28, 30). This has led one of us to suggest that the signal peptidase cleavage at the NH_2 terminus of prM is dependent on prior removal of the C protein from the transmembrane domain by the viral proteinase (cleavage of anchored C) (16). An alternative interpretation proposes that the interaction of NS2B as part of the active flavivirus proteinase complex with regions at the COOH terminus of anchored C renders the signal peptidase site at the C-prM junction accessible for cleavage (30). This suggestion is based largely on the observation that substitutions at the NS2B-3 cleavage site in anchored C which apparently reduce or abolish proteolytic processing at this site do not inhibit luminal cleavage of prM by signal peptidase if the active viral proteinase complex is also present. According to this second model the upregulation of signal peptidase cleavage at the amino terminus of prM and the processing of the carboxy terminus of C are distinct and independent functions of the viral NS2B-3 proteinase complex.

The modulation of a signal peptidase cleavage site from a cryptic to a cleavable conformation dependent on cytoplasmic, most likely proteolytic, processing events would present a novel mechanism for the regulation of viral protein biosynthesis. Prerequisites for this mechanism are the following assumptions, which have so far not been corroborated: (i) the increase in signal peptidase cleavage efficiency in the presence of the viral proteinase is not due to pretranslocational cleavage by the NS2B-3 complex exposing the prM signal sequence as an amino-terminal translocation signal (a possibility discussed in references 1 and 18), (ii) the covalent association of the C protein with the internal signal sequence of prM influences the positioning of the membrane-spanning region in the lipid bilayer and, in turn, the accessibility of the signal peptidase cleavage site, (iii) signal peptidase can cleave posttranslationally at the C-prM junction, and (iv) the covalent cytoplasmic attachment of C to the internal signal sequence is the only factor causing delayed signal peptidase cleavage at the NH_2 terminus of prM.

To address these issues, we prepared crude microsomes from CV1 (American Type Culture Collection designation) cells infected with a vaccinia virus recombinant expressing the entire Murray Valley encephalitis virus (MVE) structural region and a segment of the nonstructural polyprotein region from C to NS2B (VV-STR [16]). As shown previously, only minimal signal peptidase-mediated processing at the C-prM junction is seen in the translation products from this construct (16). Cells were labelled 4.5 h after infection with L-[^{35}S]methionine and L-[^{35}S]cysteine (PRO-MIX [^{35}S] cell labelling mix; Amersham) for 30 min, and microsomal vesicles were prepared by Dounce homogenization and centrifugation through a 10% (wt/wt) sucrose cushion and resuspended in Tris-buffered saline (0.05 M Tris-HCl [pH 7.5], 0.15 M NaCl) (2). Microsome preparations solubilized with 0.8% Nonidet P-40 and analyzed by immunoprecipitation with anti-MVE hyperimmune ascitic fluid followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16) reveal only a very faint band corresponding to prM, which contrasts with the efficient generation of the E protein (Fig. 1, lane 2).

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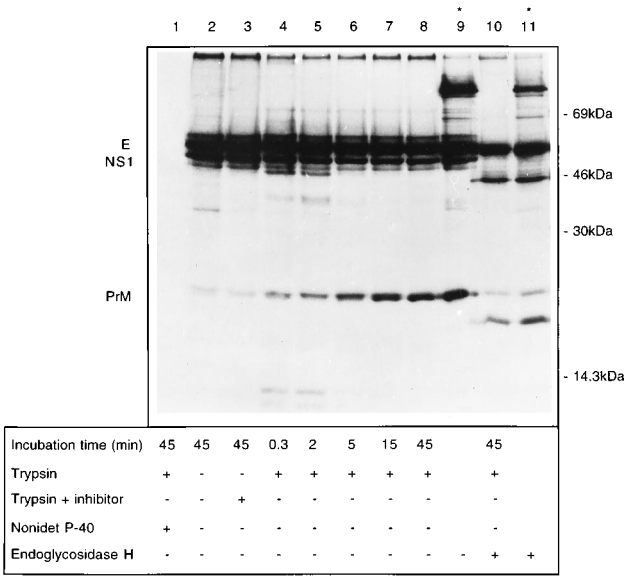

FIG. 1. Posttranslational processing of the MVE structural polyprotein segment in microsomal membranes following treatment with trypsin. Microsomes prepared from VV-STR-infected CV1 cells as described in the text were incubated for the indicated periods at 37°C with 0.4 mg of trypsin per ml plus 0.5% Nonidet P-40 (lane 1), no treatment (lane 2), trypsin premixed with soybean trypsin inhibitor (lane 3), or 0.4 mg of trypsin (lanes 4 to 8 and 10) per ml. CV1 cells doubly infected with VV-STR and VV-NS were labelled for 30 min and lysed with buffer containing 1% Nonidet P-40 (lanes 9 and 11). Products obtained after immunoprecipitation with anti-MVE mouse ascitic fluid were treated with endoglycosidase H (lanes 10 and 11) or left untreated (lanes 1 to 9) and separated by SDS–12% PAGE in parallel with molecular weight standards (positions shown on the right). Positions of glycosylated E, NS1, and prM bands are indicated on the left.

To investigate whether posttranslational removal of the C protein would trigger a signal peptidase cleavage on the luminal side of the ER to generate prM, microsomes from VV-STR-infected cells were incubated with 0.4 mg of trypsin (Boehringer Mannheim) per ml at 37°C. There are seven basic amino acids near the COOH terminus of the MVE C protein which constitute potential trypsin cleavage sites that may be accessible to the protease, given that this region is recognized by the flavivirus proteinase. Trypsin digestions were terminated by the addition of soybean trypsin inhibitor (Boehringer Mannheim) to 4 mg/ml at various times after trypsin addition; then, membranes were solubilized and immune precipitates were analyzed by SDS-PAGE. Figure 1 (lanes 4 to 8) shows the appearance of a band which has the electrophoretic mobility of glycosylated prM. The appearance of this band is dependent on the action of trypsin, as the intensity of the band increases with the length of incubation with trypsin. A control reaction in which microsomes were incubated with premixed trypsin and soybean trypsin inhibitor revealed a very low level of prM production (Fig. 1, lane 3), comparable to that seen when no enzyme is added. The E protein and newly formed prM protein were protected from protease digestion by the microsomal membranes; inclusion of detergent (Nonidet P-40 to 0.5%) during trypsin digestion resulted in proteolysis of the luminal contents of the vesicles (Fig. 1, lane 1). We obtained similar results with microsomes isolated from COS-7 cells transfected with a plasmid construct (pcDNA-STR [16]) containing the identical MVE coding region (data not shown). The microsomal cleavage assays consistently generated a significant amount of luminal prM following trypsin treatment, with the molar ratio of prM to E ranging from 20 to 40% according to densitometric examination of the autoradiograms. Accordingly, the C-prM precursor is predominantly integrated into the ER membrane and constitutes a suitable substrate for signal peptidase processing following proteolytic removal of a cytoplasmic region.

Double infection of CV1 cells with VV-STR and VV-NS (a vaccinia virus recombinant encoding an MVE polyprotein segment from NS2A to the carboxy terminus of NS5 [15]) results in production of authentic signal peptidase-cleaved prM (16). The putative prM generated after trypsin treatment of microsomes from VV-STR-infected cells comigrates with prM from this source (compare lanes 8 and 9 in Fig. 1), and the two molecules exhibit similar sensitivities to endoglycosidase H (Fig. 1, lanes 10 and 11).

To ascertain that the molecule with the electrophoretic mobility of prM generated following trypsin treatment of microsomes from VV-STR-infected cells is authentic prM, NH_2-terminal sequence analysis was performed. Microsomes were prepared as described above from VV-STR-infected CV1 cells labelled 4.5 h after infection with L-[^{35}S]methionine and L-[^{35}S]cysteine (Expre^{35}S^{35}S protein labelling mix; Du Pont) for 30 min. The microsome preparation was treated with 0.4 mg of trypsin per ml for 30 min, digestion was terminated by the addition of soybean trypsin inhibitor to 4 mg/ml, and the membranes were solubilized and antigens were immunoprecipitated as described above. The radioactively labelled immunoprecipitate was subjected to electrophoresis on an SDS-polyacrylamide tube gel, and the putative prM molecule was collected as described elsewhere (16). Consecutive cycles of Edman degradation revealed that radiolabelled residues were present in fractions 11 and 12 (Fig. 2B), in agreement with the presence of methionine at these positions in the predicted NH_2-terminal sequence of MVE prM (Fig. 2A). Examination of the sequence of the transmembrane region showed two methionine residues separated by three amino acids; the absence of this pattern in

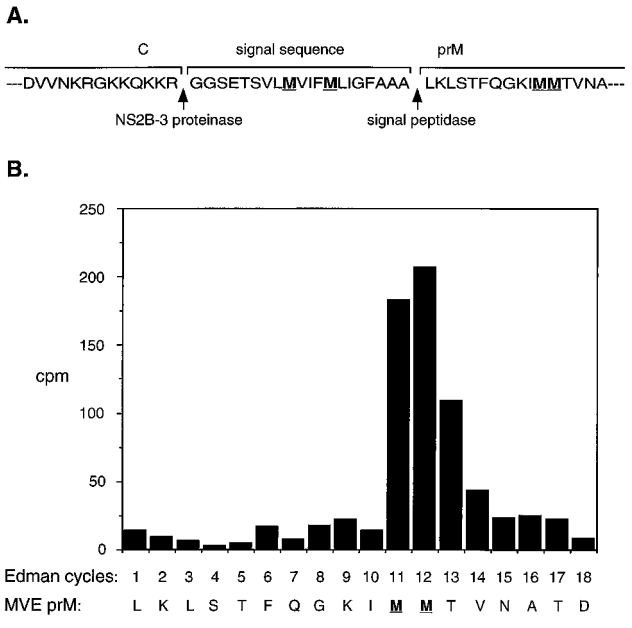

FIG. 2. Sequence analysis of prM from trypsin-treated microsomes of VV-STR-infected cells. (A) Deduced amino acid sequence of the MVE polyprotein encoded by VV-STR from residue 93 of C to residue 16 of prM. Cleavage sites at the C-prM junction for NS2B-3 proteinase and signal peptidase are indicated. (B) Counts per minute detected in fractions obtained by Edman degradation of isolated prM from trypsin-treated microsomes. The NH_2-terminal sequence of MVE prM (7) is shown for comparison.

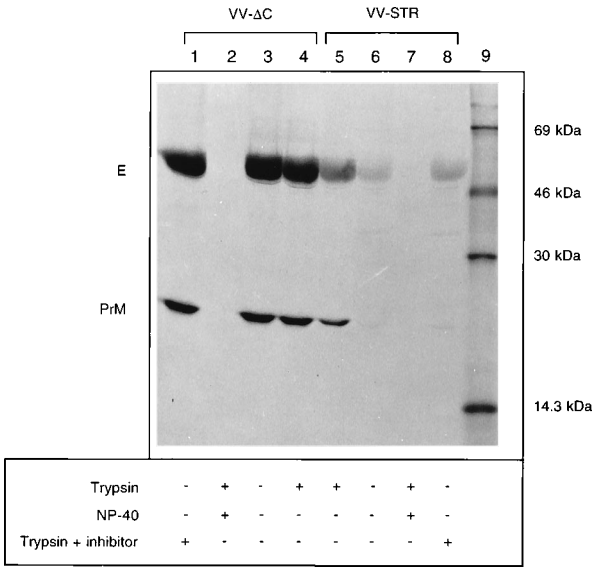

FIG. 3. Coimmunoprecipitation of prM and E following treatment with trypsin of microsomal membranes from VV-STR-infected cells. Microsomes prepared from CV1 cells infected with VV-ΔC (lanes 1 to 4) or VV-STR (lanes 5 to 8) as described in the text were incubated with 0.4 mg of trypsin (lanes 4 and 5) per ml, 0.4 mg of trypsin per ml plus 0.5% Nonidet P-40 (lanes 2 and 7), premixed trypsin plus soybean trypsin inhibitor (lanes 1 and 8), or no treatment (lanes 3 and 6). Products obtained after immunoprecipitation with monoclonal antibody M2-6D12 were separated by SDS–12% PAGE in parallel with molecular weight standards (lane 9). Positions of E and prM bands are indicated.

the fractions released by Edman degradation confirms that prM which has not been cleaved by signal peptidase does not contribute significantly to the partially purified product identified as prM.

To investigate whether prM produced by posttranslational signal peptidase cleavage can contribute to prM-E heterodimers, trypsin-digested products were immunoprecipitated with monoclonal antibody M2-6D12 (10). This antibody fails to efficiently precipitate E in the absence of correctly processed prM (data not shown). Microsomes prepared from CV1 cells infected with VV-ΔC (16) or VV-STR and labelled with L-[^{35}S]methionine and L-[^{35}S]cysteine (PRO-MIX) were treated with trypsin (0.4 mg/ml) or premixed trypsin and soybean trypsin inhibitor for 45 min at 37°C. Membranes were solubilized by the addition of Nonidet P-40 to 0.8%. Complexes were collected after immunoprecipitation with monoclonal antibody M2-6D12 and analyzed by SDS-PAGE (Fig. 3). The monoclonal antibody immunoprecipitated both prM and E from microsomes prepared from cells infected with VV-ΔC (resulting in generation of prM [16]) regardless of the presence (lane 4) or absence (lanes 1 and 3) of trypsin. However, for microsomes containing VV-STR translation products the appearance of E and prM after immunoprecipitation with the monoclonal antibody was considerably enhanced by trypsin digestion (Fig. 3; compare lane 5 with lanes 6 and 8). This suggests that the prM molecules generated as a result of trypsin digestion are able to associate with E posttranslationally, resulting in coimmunoprecipitation of the two molecules. Lanes 2 and 7 are controls showing that trypsin treatment in the presence of detergent resulted in digestion of the structural proteins. Faint bands corresponding to prM and E were visible in samples derived from microsomes of VV-STR-infected cells not treated with trypsin or treated with trypsin plus inhibitor (Fig. 3, lanes 6 and 8), in agreement with the low level of correct proteolytic processing that occurs at the C-prM junction. A faint band of approximately 35 kDa is also apparent in these lanes and may constitute the C-prM precursor. The precipitation of a putative C-prM precursor with the monoclonal antibody may be nonspecific or the result of a weak interaction of a minor fraction of C-prM with E generating the epitope for the antibody.

The data from this and other studies (1, 16, 28, 30) imply that the expression of the flavivirus structural polyprotein region in the absence of the viral proteinase complex NS2B-3 results in the production of a predominantly uncleaved C-prM precursor polypeptide. Here we used protease treatment of microsomes to uncouple the luminal cleavage by signal peptidase at the C-prM junction from its dependence on prior cytoplasmic cleavage of anchored C by the viral proteinase. The generation of large amounts of authentic prM only after trypsin treatment of microsomes establishes the temporal and causal relationship between the two cleavages at the prM junction. It demonstrates that for MVE the viral proteinase can be substituted with a less specific protease and thus that the proteolytic removal of the cytoplasmic domain of the C-prM molecule is sufficient to permit signal peptidase processing at the N terminus of prM. We do not find a requirement for an interaction of NS2B with a region at the COOH terminus of anchored C in the induction of signal peptidase cleavage of prM, as has been suggested for West Nile virus in a study using artificial structural protein constructs resistant to cleavage at the C-prM junction (30). It may be that the binding of the flavivirus proteinase complex at this junction (which in wild-type constructs should catalyze cleavage at the COOH terminus of C) can also alter the positioning of the signal sequence such that luminal processing by signal peptidase can occur. Alternatively, it is possible that there may be slight differences in the processing pathways at the C-prM junction between the different flaviviruses. The microsome experiments described here also demonstrate that prM can be generated by signal peptidase cleavage from the ER membrane-spanning C-prM precursor, and thus, the efficient generation of prM does not require removal of the C domain prior to initiation of translocation (a possibility which has been suggested previously [1, 18]).

The signal peptidase cleavage site at the NH_2 terminus of prM in this C-prM precursor remains cryptic unless a cytoplasmic region is cleaved. This suggests that the covalent association of the C protein influences the position of the signal sequence of prM in relation to signal peptidase on the luminal side of the ER membrane. It has been shown previously that substitutions of residues in the transmembrane or flanking regions of uncleaved signal-anchor sequences (11, 12, 19, 31) or deletion of NH_2-terminal cytoplasmic domains preceding uncleaved signal-anchor sequences (13, 14, 23, 25) can convert these to cleaved signal sequences. The processing at the flavivirus C-prM junction is the first example of posttranslational conversion of a signal peptidase site from an uncleaved to a cleavable conformation in a naturally occurring molecule. Central to this mechanism is the delayed processing by signal peptidase, generally considered to cleave rapidly following initiation of protein translocation (22). The microsome experiments conclusively demonstrate posttranslational removal of a membrane insertion signal by signal peptidase.

It remains to be determined whether the sequence of events resulting in the cleavages at the C-prM junction functions in the regulation of viral protein biosynthesis or is limited to recombinant expression of the flavivirus structural polyprotein regions in the absence of NS2B-3. The evidence for precursors to the structural proteins in flavivirus-infected cells is not con-

clusive (5, 27) and particularly difficult to establish for the latent period of infection when only a low level of virus-specific protein synthesis takes place. If processing at the C-prM junction is dependent on the cytoplasmic accumulation of the viral proteinase complex, the ratio of C-prM precursor to proteolytically processed prM would change during the course of infection. This could be of significance for the intracellular assembly of flavivirus particles thought to take place in the ER. The E protein, which requires dimerization with prM for secretion (16, 28), would be retained in the ER in proximity to the other structural components of the viral particles.

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