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Cell-Associated West Nile Flavivirus Is Covered with E+Pre-M Protein Heterodimers Which Are Destroyed and Reorganized by Proteolytic Cleavage during Virus Release

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Flaviviruses are enveloped viruses which accumulate in cellular vacuoles prior to release. The membrane of cell-associated virus contains the proteins pre-M and E. During release of virus the pre-M protein is cleaved, and only its carboxy-terminal segment remains associated with the virus as M protein. Studies of the association of membrane proteins of intracellular and extracellular particles of West Nile virus show that in cell-associated virus the pre-M and E proteins are present as E+pre-M heterodimers. Cleavage of pre-M during release leads to dissociation of the heterodimers: the amino-terminal region of the pre-M protein is lost from the virus, whereas the proteins M and E remain associated with the viral membrane as separate molecules. The E protein of extracellular virus has a tendency to oligomerize into trimers, and both E-protein monomers and trimers are present on extracellular virions. We have prepared partially purified extracellular virus without loss of viral infectivity. These preparations contain approximately 600 physical particles for each PFU. Since purification of cell-associated virus results in significant loss of PFU, an inactivation of virus may occur during this procedure. Preparations of cell-associated virus contained approximately 40,000 physical particles for each PFU.

The flaviviruses comprise about 60 members, many of which are important human pathogens (14). The virus particles consist of an isometric ribonucleoprotein core containing an infectious single-stranded genomic RNA molecule and a lipid envelope. Flaviviruses accumulate in intracellular vacuoles and are released after fusion of the vacuoles with the cellular surface membrane. The membrane of cell-associated flaviviruses contains the two proteins pre-M and E of 22 and 50 kilodaltons (kDa) molecular mass, respectively. During or shortly after release of virus particles the pre-M protein is cleaved, and only the carboxy-terminal part of this protein remains associated to the extracellular virus as the M protein of 8 kDa molecular mass (for recent reviews, see references 7 and 13).

We have studied the structure of the membrane proteins of the West Nile (WN) flavivirus on three different levels. (i) We have determined the primary structures of the proteins E, pre-M, and M (2, 11). (ii) We have shown that all cysteine residues present in the viral membrane proteins are involved in the formation of intramolecular disulfides, and we have determined the structure of the six disulfides present in the E protein (5). (iii) We have examined the effect of proteolytic cleavage on the structure of the membrane proteins of intact virus (12). These analyses have shown that an E-protein trimer remains associated with the surfaces of the virus particles after proteolytic treatment, whereas the proteins pre-M and M are destroyed. In addition, two regions of the E protein in these trimers are exposed to proteolytic attack (12).

Since the only proteins on the surface of the protease-treated WN virus are E-protein trimers, we attempted to isolate these trimers as a homogeneous complex for further analyses. Protease-treated virus was disassembled in the presence of the nonionic detergent octylglucoside, and the resulting complexes were fractionated on sucrose density gradients. By these means it was possible to isolate the E-protein trimer from the membrane of protease-treated virus, and in addition we could separate a number of different protein complexes present in the membrane of intact extracellular and intracellular virus.

MATERIALS AND METHODS

Propagation of BHK cells, infection of BHK cells with WN virus, and plaque assay of WN virus were performed as described previously (10).

Purification of extracellular WN virus. Two liters of growth medium were harvested from infected BHK cells at 30 h postinfection and clarified by centrifugation at 20,000 × *g* for 15 min. Virus was pelleted by centrifugation for 4 h at 19,000 rpm in a no. 19 Beckman rotor and suspended in 6 ml of TNE buffer containing 10 mM Tris, 150 mM NaCl, and 1 mM EDTA, pH 8.0, by homogenization with a tight-fitting Dounce homogenizer at 4°C. After removal of insoluble material by a 5-min centrifugation at 3,000 × *g*, the virus suspension was centrifuged to equilibrium in six preformed linear glycerol-tartrate gradients composed of 45% (wt/wt) potassium tartrate in TNE and 30% (vol/vol) glycerol in TNE (6) at 4°C by using an SW41 Beckman rotor. Virus was pooled, dialyzed against PNE buffer containing 40 mM potassium phosphate, 150 mM NaCl, and 1 mM EDTA, pH 8.0, at 4°C, concentrated to a volume of about 4 ml by covering the dialysis bags with Sephadex G-200, and stored at −80°C after addition of glycerol to a 2% (vol/vol) final concentration.

Preparation of fully infectious, partially purified extracellular virus. Growth medium was harvested from infected BHK cells at 22 h postinfection and clarified by centrifugation at 20,000 × *g* at 4°C for 15 min, and the virus was pelleted from the supernatant by centrifugation at 19,000 rpm at 4°C in a no. 19 Beckman rotor. The resulting pellet was suspended immediately at 4°C in PNE buffer by intermittent homogenization for about 30 min, using a tight-fitting Dounce

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homogenizer. Buffer (1 ml) was used for the virus recovered from 100 ml of growth medium during this step. Insoluble material was removed by centrifugation for 5 min at 3,000 × *g*. The virus suspension was used immediately for plaque titrations, sucrose density gradient centrifugation, and protease treatment. Plaque titrations of the growth medium and of the virus concentrate showed that the 100-fold-concentrated virus suspension regularly had a titer that was 100-fold that of the growth medium used for virus preparation (typical titers were 2×10^{10} PFU/ml versus 2×10^{8} PFU/ml). No significant loss or inactivation of plaque-forming virus therefore occurred during the preparation of this concentrate.

Isolation of cell-associated WN virus. BHK cells were harvested at 30 h postinfection, suspended in TNE buffer, and lysed by freezing and thawing followed by sonication. A postmitochondrial fraction was prepared from this homogenate, protamine sulfate was added to this fraction to a final concentration of 2 mg/ml, and the precipitate was removed by centrifugation as described by Shapiro et al. (8). The supernatant containing the virus particles was subjected to equilibrium centrifugation in preformed glycerol-tartrate gradients and processed further as described above for the purification of extracellular virus.

Fractionation of membrane protein complexes by gradient centrifugation. Three types of virus concentrates were used in these experiments: purified extracellular virus, purified cell-associated virus, and concentrates of fully infectious, partially purified extracellular virus. All virus preparations were in PNE buffer. In a typical experiment, between 100 and 600 μl of virus concentrate was adjusted to a final volume of 900 μl by PNE buffer, incubated at 22°C for 5 min, and further incubated at 22°C for 10 min after addition of 100 μl of 20% (wt/vol) octylglucoside. The material was then loaded onto a linear 5 to 20% sucrose density gradient in PNE buffer containing 2% (wt/vol) octylglucoside and centrifuged for 20 h at 20°C in an SW41 Beckman rotor. In a number of experiments, virus was digested by proteases prior to octylglucoside dissociation and gradient centrifugation. In these experiments, the virus was incubated in PNE in the presence of 0.4 mg of chymotrypsin per ml at 37°C for 30 min or in the presence of 0.1 mg of trypsin per ml at 37°C for 5 min followed by inactivation of the protease by addition of phenylmethylsulfonyl fluoride to a final concentration of 1 mM. The virus was then processed as described above for untreated virus. Gradients were fractionated, and the proteins present in each fraction were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under a variety of experimental conditions which are described below.

Analyses of proteins and protein complexes by SDS-PAGE. Proteins present in sucrose density gradient fractions were analyzed as follows. To an aliquot of each fraction, trichloroacetic acid (TCA) was added to a final concentration of 20%, and after 15 min of incubation at 0°C the precipitated protein was recovered by centrifugation (15 min, 4°C, 15,000 × *g*). The TCA was removed by two washes of the pellet with ethanol-diethylether (1:1). The dry pellet was then solubilized in sample buffer and subjected to analytical SDS-PAGE on slab gels containing 12.5% acrylamide in the buffer system of Laemmli (4). The influence of proteases on the integrity of the fractionated proteins was assayed as follows. To an aliquot of each gradient fraction was added either chymotrypsin (50 μg/ml final concentration) or trypsin (25 μg/ml final concentration). After incubation at 37°C for 30 min (for chymotrypsin) or 5 min (for trypsin), the protease was inactivated by addition of phenylmethylsulfonyl fluoride to a 2 mM final concentration, and the resulting material was precipitated with TCA and analyzed by SDS-PAGE as described above. Complexes of proteins purified by sucrose density gradient centrifugation were analyzed by chemical cross-linking with DTSSP, a homobifunctional reagent which cross-links lysine residues. Since the cross-links contain a disulfide bond, they can be broken by reduction (9). Complexes were revealed by one-dimensional SDS-PAGE. An aliquot of each gradient fraction was adjusted to a final DTSSP concentration of 1.25 mM by using a stock solution of 25 mM DTSSP freshly prepared in water. After incubation at 22°C for 10 min, the cross-linking reaction was stopped by addition of lysine to a final concentration of 10 mM, and the resulting material was subjected to TCA precipitation and analytical SDS-PAGE in the absence of mercaptoethanol as described above. The protein constituents present in cross-linked complexes were analyzed by two-dimensional SDS-PAGE. An aliquot of a gradient fraction containing the protein complex was subjected to DTSSP cross-linking followed by TCA precipitation and SDS-PAGE in the absence of mercaptoethanol as described above. The gel lane containing the complex was cut out and incubated in 200 ml of the SDS-PAGE spacer gel buffer containing 1% SDS and 5% mercaptoethanol for 45 min at room temperature with continuous shaking. The gel lane was then inserted into a second-dimension slab gel and subjected to a second SDS-PAGE fractionation. Since the mercaptoethanol present in the first-dimension gel lane inhibited the polymerization of the acrylamide in the standard spacer gel, the second-dimension spacer gel consisted of 1% agarose in spacer gel buffer containing 0.1% SDS and 5% mercaptoethanol to further cleave the DTSSP cross-links. Gels of the same composition (12.5% acrylamide, 0.3% bisacrylamide) were used for both the first- and second-dimension analyses. Proteins which are not cross-linked by DTSSP migrate onto a position on a diagonal line on the second-dimension gel. Proteins were visualized after SDS-PAGE by staining the gels with Coomassie brilliant blue R 250 or by using the improved silver-staining procedure of Blum et al. (1).

RESULTS

Membrane protein complexes present in purified extracellular WN virus. WN virus particles were disassembled by octylglucoside followed by centrifugation through a sucrose density gradient containing octylglucoside. The viral core was not disassembled and sedimented into the pellet, whereas the viral membrane proteins sedimented into different positions, as can be seen from the SDS-PAGE analysis of such a gradient (Fig. 1A). The gradient was divided into three zones: zone 1, comprising fractions 5, 6, and 7, contained a fast-sedimenting species of E protein; zone 2, comprising fractions 9, 10, 11, and 12, contained a slow-sedimenting species of E and all pre-M molecules; and zone 3, comprising fractions 13, 14, and 15, contained all M-protein molecules. The amount of E protein present in the pellet was somewhat variable in this type of experiment (compare Fig. 1 and 2) but had no influence on the pattern of proteins in the gradient. We have shown earlier that treatment of intact WN virus with chymotrypsin or trypsin destroys M and pre-M, whereas an E-protein trimer containing characteristic breaks remains associated to the viral membrane. The E-protein molecules present in this complex are cleaved by chymotrypsin in the carboxy-terminal region, leading to the generation of a shortened E protein of 42 kDa apparent molecular mass (fr 42). The same region of the E

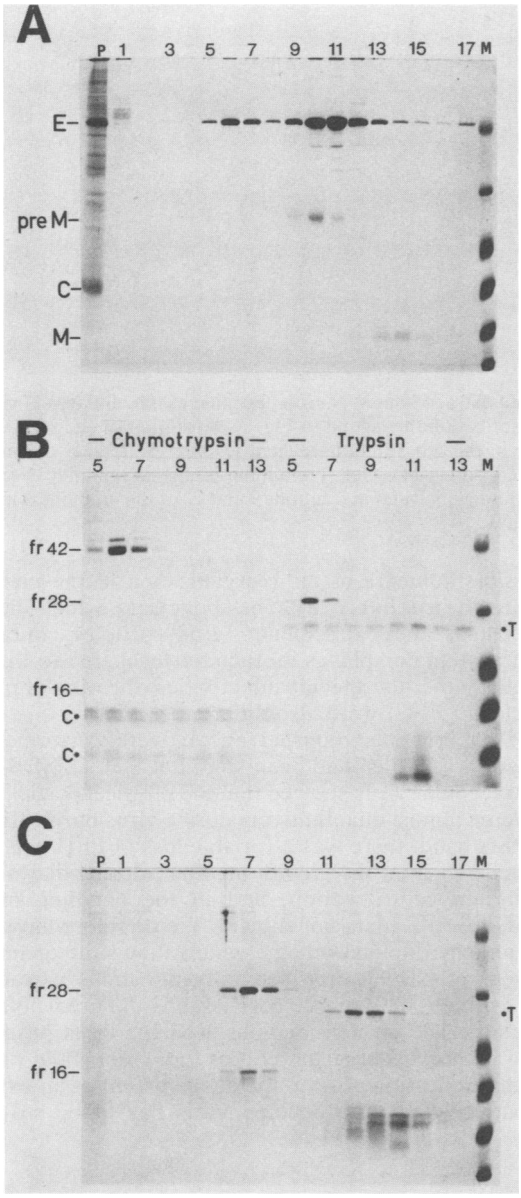

FIG. 1. Analyses of membrane protein complexes of extracellular WN virus. (A) Virus was dissociated by octylglucoside and subjected to sucrose density gradient centrifugation (20 h, Beckman SW41 rotor, 20°C) in the presence of octylglucoside. An aliquot of the pellet (lane P) and of each fraction was subjected to SDS-PAGE. In all gradients presented, fraction 1 contains the fast-sedimenting material and fraction 17 contains the material from the top. (B) Aliquots of fractions 5 to 13 of the gradient shown in panel A were incubated with chymotrypsin or trypsin precipitated by TCA, and analyzed by SDS-PAGE. The protease can be detected in the stained gel (bands C* and *T). The E-protein fragments of 42, 28, and 16 kDa are indicated. (C) Extracellular virus was treated with trypsin and fractionated by centrifugation as described for panel A. An aliquot of each fraction was subjected to TCA precipitation and analyzed by SDS-PAGE. The band corresponding to trypsin is indicated by *T. The tryptic E-protein fragments fr 28 and fr 16 are indicated. Gels contained 12.5% acrylamide and were stained with Coomassie blue. Marker proteins (ovalbumin, α-chymotrypsinogen, β-lactoglobulin, lysozyme, bovine trypsin inhibitor, and insulin) are fractionated in lanes M. See Materials and Methods for further details.

protein is susceptible to cleavage by trypsin, but the resulting 42-kDa fragment is further accessible to a second cleavage by trypsin and is cleaved into the molecules fr 16 and fr 28 (12). It should be possible to determine which of the E-protein-containing fractions contained the trimers by subjecting gradient fractions to treatment with either chymotrypsin or trypsin followed by SDS-PAGE. These analyses (Fig. 1B) showed that the fast-sedimenting E protein present in zone 1 has the same reactivity towards chymotrypsin or trypsin as was identified for the E-protein trimers and that the slow-sedimenting E protein present in zone 2 is degraded. Further evidence that the E protein present in zone 1 represents the trimer comes from the experiment shown in Fig. 1C. Here, purified virus was digested with trypsin, and then gradient analysis of the membrane protein complexes was performed. Only a single protein complex which sedimented into zone 1 survived the protease treatment. This complex is composed of the E-protein fragments fr 28 and fr 16, which are characteristic for the trypsin-treated E-protein trimer. The trypsin-treated E-protein complex sedimented slightly more slowly than did the intact trimer (Fig. 1A and C and unpublished data).

The protein associations revealed by the gradient procedure appear to reflect the association of the proteins in the viral membrane (Fig. 1). This is evident because the same pattern of protease resistance was obtained by fractionation of surface proteins followed by protease treatment of the isolated complexes (Fig. 1B) as was obtained by protease treatment of intact virus followed by centrifugation (Fig. 1C).

An analysis of the association of the proteins fractionated by centrifugation by using cross-linking is shown in Fig. 2. Proteins in each gradient fraction were analyzed by SDS-PAGE either directly (Fig. 2A) or after cross-linking with the homobifunctional reducible cross-linker DTSSP (Fig. 2B). The E protein present in zone 1 was cross-linked into a high-molecular-weight complex which did not enter the gel. The major complex generated under these conditions had a molecular mass of about 150 kDa (data not shown). The majority of the E-protein molecules present in zone 2 were not cross-linked into oligomers and therefore presumably are monomeric molecules. These molecules migrate more heterogenously after cross-linking, possibly because intramolecular cross-links influence the unfolding and SDS-binding capacity of the resulting protein. A further effect of the cross-linking reaction is the appearance of a protein complex in fractions 9 and 10 which had an apparent molecular mass of about 70 kDa. This complex was generated in those fractions which contained both E and pre-M, and the amount of pre-M of 22 kDa molecular mass was greatly reduced after DTSSP treatment. These data suggest that 70-kDa material might represent a heterodimer, containing one molecule each of E and pre-M, which sediments slightly faster than the monomeric E protein and cannot be separated from the latter by the centrifugation procedure used. The material present in the gradient fraction containing this presumed complex was therefore subjected to a two-dimensional diagonal SDS-PAGE analysis (Fig. 3). It can be seen that the p70 complex (which was not completely cleaved under the experimental conditions used) contained E and pre-M protein and therefore represents an E+pre-M heterodimer. The amount of monomeric M protein was reduced after DTSSP treatment of the isolated fraction (Fig. 2), but we have been unable to identify a specific oligomer of this molecule (data not shown).

Membrane protein complexes present in purified intracellu-

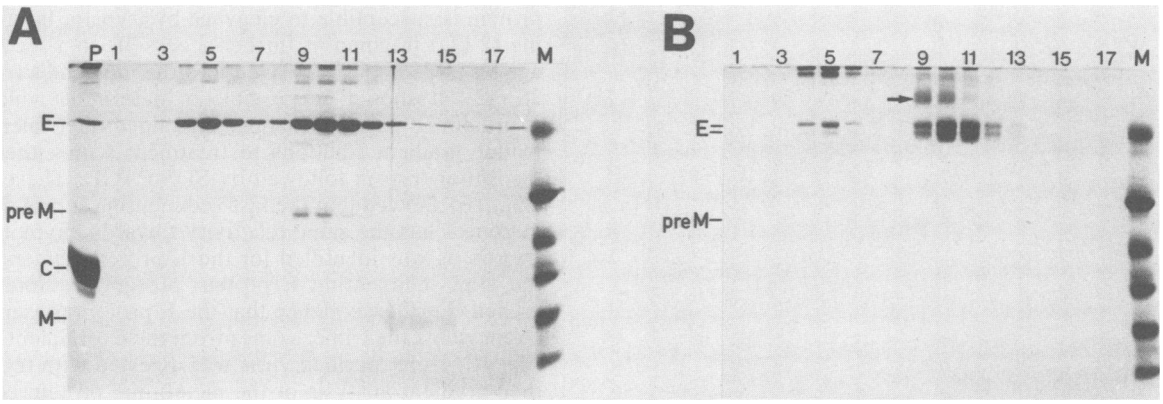

FIG. 2. Chemical cross-linking of the membrane protein complexes of purified extracellular WN virus. Purified extracellular WN virus was dissociated by octylglucoside and fractionated by gradient centrifugation as described in the legend to Fig. 1. An aliquot of each fraction was subjected to SDS-PAGE either directly (A) or after incubation in the presence of the cross-linking reagent DTSSP (1.25 mM, 10 min, 22°C) (B). The marker proteins described in the legend to Fig. 1 were also included (lanes M). Gels containing 12.5% acrylamide were used. Coomassie blue-stained gels are shown. A protein complex (revealed by cross-linking analysis) in fractions 9 and 10 of the gradient is indicated by the arrow in panel B. The constituents of this complex are analyzed in Fig. 3.

lar WN virus particles. Since cell-associated viruses contain pre-M rather than M protein, the data described above suggest that the E+pre-M heterodimer on the surface of extracellular WN virus may represent a small amount of such a complex which survived from intracellular virus. An analysis of the protein complexes present in purified cell-associated WN virus is shown in Fig. 4. The proteins E and pre-M cosedimented in zone 2 (Fig. 4A). The fact that no M protein was detectable shows that intracellular virus was analyzed. Fast-sedimenting E-protein trimers were not found in intracellular virus. Each gradient fraction was subjected to cross-linking followed by analytical SDS-PAGE (Fig. 4B). It can be seen that the proteins E and pre-M were efficiently cross-linked into a 70-kDa complex similar to that seen in Fig. 2. Two-dimensional gel electrophoresis showed that this complex contains E and pre-M exactly as described above for the corresponding complex isolated from extracellular virus (data not shown). These data show that cell-associated virus predominantly contains a single protein-containing surface structure, namely an E+pre-M heterodimer.

Possible influence of the reorganization of the membrane proteins on virus infectivity. The above data, which show that intracellular and extracellular virus particles contain different protein complexes on their surfaces, led to the question of whether the specific infectivities (the ratio of physical particles to PFU) were also different.

The experiments reported above were performed with purified virus which had been stored at −80°C. SDS-PAGE of extracellular virus showed that the physical particles were recovered almost quantitatively during virus purification. On the other hand, there was a variable loss of PFU when virus was pelleted from the growth medium, during dialysis after equilibrium centrifugation, and in the handling of virus stored at −80°C (data not shown). We therefore have developed an experimental scheme which allows the quantitative recovery of partially purified fully infectious extracellular virus without measurable loss of PFU. Three points are important. The growth medium used for virus purification has to be harvested at the end of the exponential phase of viral multiplication prior to the development of an extensive cytopathic effect, the pelleted virus has to be suspended

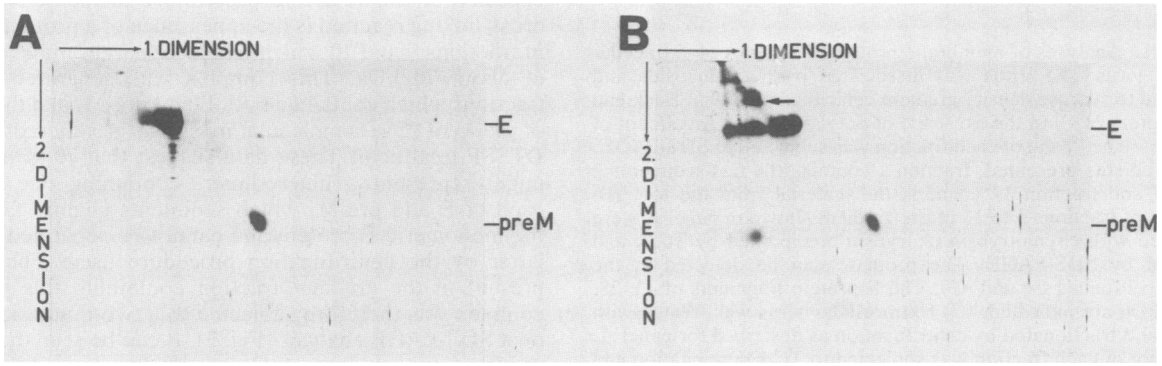

FIG. 3. Two-dimensional SDS-PAGE of a protein complex revealed by chemical cross-linking in the membrane of purified extracellular WN virus. Purified extracellular WN virus was dissociated by octylglucoside and fractionated by gradient centrifugation. The protein distribution is shown in Fig. 2A. Two aliquots were taken from the material present in fraction 10 of this gradient which either were left untreated or were incubated in the presence of 1.25 mM DTSSP for 10 min at 22°C. Both samples were then subjected to two-dimensional SDS-PAGE. Nonreducing conditions of sample preparation were used for the first-dimension separation, which was followed by reduction of cross-links and second-dimension analysis. Gels containing 12.5% acrylamide were used for both separations (see Materials and Methods for details). The silver-stained gels of the untreated material (A) and the cross-linked material (B) are shown. Not all complexes were completely reduced; the remaining cross-linked complex which contained E and pre-M is indicated by an arrow in panel B.

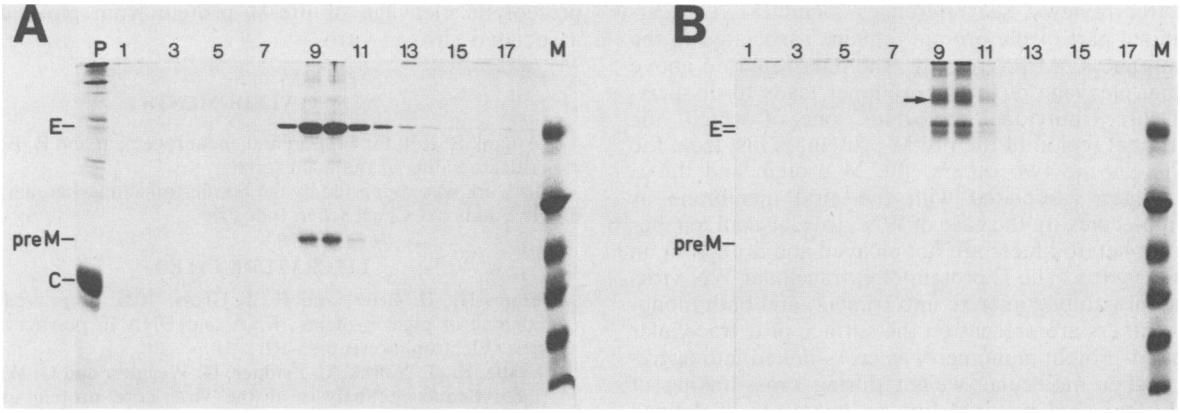

FIG. 4. Chemical cross-linking of the membrane protein complexes of purified cell-associated WN virus separated by gradient centrifugation. Conditions were the same as described for Fig. 2 except that purified cell-associated virus was used. The E+pre-M complex generated in the presence of DTSSP is indicated by an arrow in panel B.

rapidly, and the virus should not be concentrated more than 100-fold (see Materials and Methods for details). SDS-PAGE of 5, 10, and 15 μl of such a virus preparation is shown in Fig. 5 (lanes 4, 5, and 6, respectively). Since this virus preparation had a titer of 2×10^{10} PFU/ml, this corresponds to 1×10^8, 2×10^8, and 3×10^8 PFU per lane, respectively. Intracellular virus was purified by the standard procedure, but the resulting virus preparations were not dialyzed following equilibrium centrifugation and were not stored frozen but were analyzed directly for PFU. This virus concentrate had a titer of 2×10^7 PFU/ml. The virus present in 80, 160, and 240 μl of this concentrate was also subjected to SDS-PAGE (Fig. 5, lanes 1, 2, and 3, respectively). It can be seen from the staining intensities of the E-protein and/or C-protein bands that 10 μl of extracellular virus (lane 5), which contains 2×10^8 PFU, contains a similar amount of proteins E and C to that in 160 μl of cell-associated virus (lane 2) (3.2×10^6 PFU). The ratio of the specific infectivity of extracellular virus to that of intracellular virus therefore is about $(2 \times 10^8)/(3.2 \times 10^6) = 0.62 \times 10^2 = 62$. Defined amounts of known proteins were also fractionated on the gel in order to allow a rough estimation of the specific infectivities of the virus preparations. It can be estimated by visual inspection of the staining intensity that 10 μl of extracellular virus (lane 5) contains approximately 2 μg of E protein, which corresponds to 24×10^{12} protein molecules of 50 kDa molecular mass. Since each WN virus particle contains about 200 E-protein molecules (data not shown), these 24×10^{12} E-protein molecules correspond to approximately 12×10^{10} physical virus particles. These particles are present in 10 μl of extracellular WN containing 2×10^8 PFU. The ratio of physical to infectious particles in the fully infectious extracellular virus therefore is approximately 12×10^{10} physical particles divided by 2×10^8 PFU, which represents a ratio of 600 physical particles per PFU. It can also be estimated that 160 μl of the preparation of intracellular virus (lane 2) contains approximately 2 μg of E protein, which corresponds to 12×10^{10} physical virus particles, each containing 200 E-protein molecules. Since 160 μl of this virus preparation contains 3.2×10^6 PFU, the specific infectivity of the intracellular virus is (12×10^{10} physical particles)/(3.2×10^6 PFU), which represents a ratio of 37,000 physical particles per PFU.

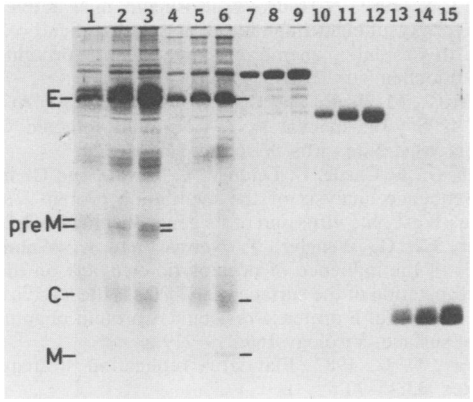

FIG. 5. SDS-PAGE of partially purified extracellular virus and of purified cell-associated virus; 5, 10, and 15 μl of fully infectious, partially purified extracellular virus (containing 1×10^8, 2×10^8, and 3×10^8 PFU) were run on lanes 4, 5, and 6, respectively. Infected cells were used for the preparation of cell-associated virus, with two modifications. Cells were subjected to sonication without prior freezing, and the virus recovered from the equilibrium centrifugation was immediately tested to determine the plaque-forming titer without dialysis and storage at −80°C. On lanes 1, 2, and 3, respectively, 80, 160, and 240 μl of this material (containing 1.6×10^6, 3.2×10^6, and 4.8×10^6 PFU) were analyzed. Lanes: 7, 8, and 9, 2, 4, and 6 μg of bovine serum albumin, respectively; 10, 11, and 12, 2, 4, and 6 μg of ovalbumin; 13, 14, and 15, 2, 4, and 6 μg of chick lysozyme. A photograph of the gel obtained after staining with Coomassie blue R 250 is shown. Localization of the viral structural proteins E, pre-M (=), C, and M are indicated.

The results presented in Fig. 5 indicate that the partially purified, fully infectious extracellular virus is of sufficient purity for use in the analysis of membrane protein complexes. When such virus preparations were analyzed immediately after preparation by the gradient centrifugation procedure without prior storage in the frozen state, this virus gave the same pattern of protein complexes as that found on the membrane of highly purified extracellular virus which had been stored at −80°C (data not shown).

DISCUSSION

The two membrane proteins pre-M and E of intracellular WN virus particles are shown here to be present as a heterodimer. The pre-M protein is cleaved during the release of virus from cells (2, 8). The amino-terminal part of the pre-M protein is lost from the virus in all flaviviruses

analyzed (for reviews, see references 7 and 13). The carboxy-terminal part of the protein remains associated to the viral membrane as M protein (2). The data reported above suggest that cleavage of the heterodimer leads to dissociation into three individual molecules, one of which, the amino-terminal region of the pre-M protein, is lost from the virus, whereas the two others, the M protein and the E protein, remain associated with the viral membrane as separate molecules. In the case of WN virus, a small number of E+pre-M heterodimers are not cleaved and are found on extracellular virus. The E protein of extracellular WN virus has a tendency to oligomerize into trimers, and both monomers and trimers are present on the surface of extracellular virus. The M-protein monomer was cross-linked into aggregates of higher molecular weight during cross-linking of gradient-fractionated material, but an aggregate of defined size could not be identified.

Comparison of the experiments reported above indicated that the results obtained reflect the state of the proteins in the viral membrane. For example, the E-protein trimer which has been isolated as the sole surface component of protease-treated virus (12) is also detected after gradient centrifugation of native extracellular virus and retains, as a purified complex, the characteristic protease cleavage sites which were identified for this structure embedded in the viral membrane. It should also be noted that the complete virus lysate is subjected to density gradient fractionation. Therefore, all subviral complexes can be recovered either from the pellet of the gradient or from its fractions.

As far as we know, this is the first study of the association of the membrane proteins of a cell-associated flavivirus to be reported. Protein association in extracellular virus has been studied in the tick-borne encephalitis virus (3). In the latter analyses, heteropolymeric associations were not detected between the M and E proteins, a finding which is in accordance with the data presented above for WN virus. However, the E protein was present in the tick-borne encephalitis virus membrane, mostly in monomeric and dimeric forms. The state of oligomerization of E protein in the membranes of extracellular flaviviruses therefore may vary between different viruses. Further experiments will be necessary to verify this possibility.

The ratio of physical particles to PFU is about 600 for the fully infectious extracellular virus and is about 60-fold higher in the cell-associated virus. The quantitative recovery of infectious particles of extracellular virus showed that the infectivity of these particles was not impaired. In contrast, during the preparation of cell-associated virus large losses of plaque-forming activity occurred (data not shown). Therefore, the plaque-forming ability of the intracellular virus may be impaired during purification. Unequivocal evidence for a low specific infectivity of cell-associated flaviviruses and for the role of cleavage of pre-M in the conversion of these particles into particles of higher specific infectivity could be obtained if it were possible to activate viral infectivity by proteolytic cleavage of pre-M protein from purified cell-associated virus in vitro.

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