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Maturation of Japanese Encephalitis Virus Glycoproteins Produced by Infected Mammalian and Mosquito Cells

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The Japanese encephalitis virus (JE) structural glycoprotein (E) and two nonstructural glycoproteins (NS1 and NS1′) were processed differently by JE-infected vertebrate and invertebrate cell lines. All three proteins were released slowly ($t_{1/2} > 6$ hr) from JE-infected monkey cells (Vero cells). Mosquito cell lines released E at a similar rate ($t_{1/2} > 8$ hr), while NS1 and NS1′ were retained in an undegraded form in the cell layer. The proteolytic processing of the three proteins appeared identical in both cell types, but some differences in N-linked glycosylation were observed. E, NS1, and NS1′ found within the infected cells of both types contained high-mannose oligosaccharide groups for more than 8 hr after synthesis. Additional sugar residues were added to the single E protein oligosaccharide group prior to release from Vero cells, while sugar residues were trimmed from the E protein oligosaccharide group prior to release from mosquito cells. The forms of NS1 and NS1′ found in the culture fluid of infected Vero cells contained one complex and one high-mannose oligosaccharide. All three glycoproteins released from JE-infected Vero cells were associated with extracellular particles, the virion in the case of E and a low density particle in the case of NS1 and NS1′. Furthermore, E, NS1, and NS1′ exhibited amphipathic properties in Triton X-114 extraction experiments. Taken together, these results suggest that both the structural (E) and nonstructural (NS1 and NS1′) glycoproteins were accumulated within the secretory pathway of the infected Vero cells, assembled into particles, and then released into the extracellular fluid. © 1989 Academic Press, Inc.

INTRODUCTION

Japanese encephalitis virus (JE) is one of the most important human pathogens of the family *Flaviviridae* (Shope, 1980; Monath, 1986). Recently, extensive information concerning flavivirus gene expression has been deduced from analysis of partial or complete nucleotide sequence data from a large number of flaviviruses. These genomic sequence data and limited protein sequence information have provided strong evidence for cotranslational processing of flavivirus protein products translated from a single, long open reading frame (Rice *et al.*, 1986).

The flavivirus E protein is 50 to 55 kDa in size. It is the site of binding of neutralizing antibodies (Roehrig, 1986) and is glycosylated in some but not all viruses (Wright, 1982; Wengler *et al.*, 1985; Deubel *et al.*, 1987). Work by Winkler *et al.* (1987) has suggested that E produced by avian cells infected with tick-borne flaviviruses contains a single high-mannose oligosaccharide while E produced by avian cells infected with mosquito-borne flaviviruses has a single complex oligosaccharide. These results seem to conflict with the work of Stephenson *et al.* (1987) that show that the E protein produced by tick-borne encephalitis virus infected porcine cells can be radiolabled with radioactive mannose, glucosamine, galactose, and fucose. N-terminal sequence data obtained from the JE E protein has shown that the processing site that releases E from the viral translation product is a signalase-like processing site that is highly conserved among the sequenced flaviviruses (McAda *et al.*, 1987; Mason *et al.*, 1987b).

The NS1 glycoprotein is a major viral antigen that is present in infected cells, in the extracellular fluid of infected cell cultures, and in the serum of infected animals (Russell *et al.*, 1980). This protein was originally designated the soluble complement-fixing antigen, or SCF, and elicits most of the complement-fixing antibody produced by infected animals (Russell *et al.*, 1980). The understanding of this important viral immunogen has been further expanded by demonstration that this protein exists as a dimer that is resistant to reduction and SDS treatment, but not heating (Winkler *et al.*, 1988). In addition, endoglycosidase studies have shown that NS1 produced in dengue 2-infected hamster cells contains a mixture of high-mannose and complex carbohydrates (Winkler *et al.*, 1988), and radiolabeling experiments have shown that the NS1 protein produced in tick-borne encephalitis virus-infected porcine cells contains mannose, glucosamine, galactose, and fucose (Stephenson *et al.*, 1987). Little is known of the function of NS1 in viral replication. However, this protein may serve a role in flavivirus assembly which ultrastructural studies have identified as a multistep process that occurs in the Golgi and endoplasmic reticulum complex of infected cells of both mammalian

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(Leary and Blair, 1980; Ishak *et al.*, 1988) and mosquito (Ng, 1987; Hase *et al.*, 1987a,b) origin.

Several studies have suggested that NS1 could be pursued as a flavivirus vaccine. Results with yellow fever virus (YF) showed that monoclonal antibodies (MAbs) specific for NS1 were capable of passively protecting animals from a lethal YF challenge (Schlesinger *et al.*, 1985; Gould *et al.*, 1986). Furthermore, direct immunization of animals with NS1 purified from YF-infected cells elicited a protective immune response to YF (Schlesinger *et al.*, 1985), and immunization with NS1 purified from dengue type 2-infected cells provided partial protection from challenge with dengue 2 (Schlesinger *et al.*, 1987). Although the mechanism of action of both direct immunization and passive protection was unclear, the protected animals showed a postchallenge seroconversion to other viral proteins, indicating that viral replication had taken place (Schlesinger *et al.*, 1985, 1987). These findings suggest that a humoral immune response to NS1 protected the host by attenuating virus replication, probably by *in vivo* lysis of infected cells. However, the time of NS1 appearance on the infected cell surface and the *in vivo* effectiveness of antibody-mediated lysis of infected cells remain unclear.

JE genomic sequence data have shown that NS1 follows E in the viral translational open reading frame (McAda *et al.*, 1987; Mason *et al.*, 1987a). Like E, the N-terminus of NS1 appears to be generated by a cleavage at a signalase-like processing site that is highly conserved among the flaviviruses (Rice *et al.*, 1986). The C-terminus of NS1 has not been clearly identified. In the case of JE, two different NS1 proteins have been identified (Mason *et al.*, 1987a). One of the NS1 proteins present in JE-infected cells has an estimated molecular mass of 42 kDa, similar to the protein observed in cells infected with other flaviviruses. The second NS1 protein has been defined as NS1′ and has only been identified in JE-infected cells. NS1′ has an estimated molecular mass of 58 kDa and appears to share N-terminal antigenic determinants with the 42-kDa NS1, but contains unique C-terminal antigenic determinants derived from the ns2a region of the JE genome (Mason *et al.*, 1987a).

The studies presented in this paper were designed to elucidate the biosynthesis of the E, NS1, and NS1′ antigens produced by JE-infected cells of vertebrate and invertebrate origin. These results have added the following information about JE glycoprotein biosynthesis: (1) E was released very slowly ($t_{1/2} > 6$ hr) from both vertebrate and invertebrate cells. (2) NS1 and NS1′ were only released from vertebrate cells. (3) The proteolytic processing and addition of N-linked oligosaccharides to the viral glycoproteins were similar in both vertebrate and invertebrate cells. (4) NS1 and NS1′ found in the culture fluid of vertebrate cells were in a particulate form.

MATERIALS AND METHODS

Cell lines and virus

Vero cells (African green monkey cell line) were grown at 37° in Eagle's minimal essential medium (MEM) supplemented with 5% heated fetal bovine serum (FBS) and antibiotics. The C6/36 cell line (*Aedes albopictus* cell line) (Igarashi, 1978) was grown at 28° as described by Tesh (1979). The TRA284 (*Toxorhynchites amboinensis* cell line) (Kuno, 1981) and the AP61 (*Aedes pseudoscutellaris* cell lines) (Varma *et al.*, 1974) cell lines were grown at 28° in MM/VP12 medium containing antibiotics and 10% FBS (Varma *et al.*, 1974). The virus used in all experiments was a clarified culture fluid prepared from C6/36 cells infected with a passage 55 suckling mouse brain suspension of the Nakayama strain of JE. Virus titers were determined on Vero cell monolayers as described by Tesh and Duboise (1987).

Infection with JE and radiolabeling

C6/36 and Vero cell monolayers prepared in 24- or 35-mm-diameter dishes and inoculated with virus at a 10:1 m.o.i. diluted in maintenance medium (same components as growth medium described above, except containing less FBS: 1%, Vero cells; 2%, C6/36 cells). Following 1 hr of virus absorption at the growth temperature, the virus inoculum was removed and the cells were incubated in maintenance medium for 24 hr at the appropriate temperature.

At 24 hr postinfection, the medium was removed and replaced with warm methionine (Met)-free maintenance medium containing 100 μCi/ml of ^{35}S-Met. The cells were incubated for 1 or 3 hr at the growth temperature and harvested as described below or rinsed two times with warm maintenance medium containing 10 times the normal amount of unlabeled Met, and incubated in warm 10× Met maintenance medium for 1 to 24 hr.

The TRA284 and AP61 cell monolayers were infected as described above except that all procedures were performed in the MM/VP12 growth medium. The infected cells were labeled for 24 hr in MM/VP12 growth medium containing 100 μCi/ml of ^{35}S-Met.

No cytopathic effect was observed in any of the cell lines during the time course of the experiments.

Radioimmunoprecipitations and polyacrylamide gel electrophoresis

Following radiolabeling, the cell layers were placed on ice. The culture fluids were removed, clarified by

centrifugation at 6700 *g* for 5 min at 4°, mixed with $\frac{1}{3}$ volume of buffer S (40 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl, 20 m*M* EDTA, 4% sodium deoxycholate (Gallard-Schlessinger), 4% Triton X-100 (Sigma), 0.4% sodium dodecyl sulfate (SDS; Biorad)) containing a 4X concentration of protease inhibitors (1X inhibitor concentration = 1 m*M* phenylmethylsulfonyl fluoride (PMSF; Sigma), 10 μg/ml of aprotinin (Sigma), 2 μg/ml of leupeptin (Sigma), 2 μg/ml benzamidine (Sigma), 10 μg/ml TLCK (Sigma)), and frozen at −70° until use. The cell layers were rinsed with ice-cold phosphate-buffered saline (pH 7.4), incubated in buffer A (10 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl, 5 m*M* EDTA, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS) containing protease inhibitors for 15 min on ice, scraped from the dish, clarified by centrifugation for 20 min at 13,000 *g* at 4°, and frozen at −70° until use.

Two MAbs, one specific for the E protein (hybridoma J3-11B9), and the other specific for the NS1 and NS1′ proteins (hybridoma D2-7E11) were used to prepare the radioimmunoprecipitates (RIPs). Hybridoma J3-11B9 was raised against JE and has been described (Hybridoma 5, Table 1; Mason *et al.* 1987a). Hybridoma D2-7E11 was raised against dengue type 1 and has been found to react with the NS1 protein of dengue type 1, the NS1 and NS1′ proteins of JE, and *Escherichia coli* fusion proteins that contain the N-terminus of the dengue type 1 NS1 protein (Zuegel and Mason, unpublished).

RIPs were prepared by a modification of the method of Chen and Huang (1986). Samples were mixed with MAb containing ascitic fluid and incubated overnight at 4°. One-tenth milliliter of an 8% suspension of fixed *Staphylococcus aureus* bacteria (The Enzyme Center, Malden MA) diluted in buffer A was added to each sample, and the samples were rotated slowly for >90 min at 4°. The bacteria were pelleted by microcentrifugation, washed two times with buffer A containing 0.5 *M* NaCl and three times with buffer A. The bound viral antigens were released from the bacterial pellet by heating for 15 min at 68° in SDS gel electrophoresis sample buffer containing 50 m*M* dithiothreitol (Sigma). The proteins were resolved on 7.5% polyacrylamide gels in the presence of SDS (Laemmli, 1970), fixed, incubated in Autofluor (National Diagnostics), dried, and autoradiographed. The molecular masses of the labeled proteins were determined by coelectrophoresis with chicken ovalbumin (42.7 kDa, Bio-Rad) and bovine serum albumin (66.2 kDa, Bio-Rad).

Quantitation of radiolabeled proteins

The amount of radiolabeled antigen present in specific RIPs was determined by excising the specific bands from the dried gels and incubation in Fluorosol (National Diagnostics) followed by liquid scintillation counting. Data from time course experiments were expressed as the percentage of each radiolabeled protein (E, NS1, or NS1′) recovered in the culture fluid at each time point following the pulse labeling. This method of data presentation was selected to overcome the well-to-well variability in cell number, effective m.o.i., and radiolabeling efficiency.

Data from the sucrose density gradients were expressed for each individual protein (E, NS1, and NS1′) as the percentage of cpm recovered in the RIP of each fraction relative to the sum of the cpm recovered from the RIPs of all of the fractions that contained labeled proteins that could be detected on overexposed autoradiograms.

Tunicamycin treatment

JE-infected Vero cell layers were incubated in tunicamycin (Boehringer-Mannheim) at concentrations of 1 or 5 μg/ml for 2.5 hr prior to labeling, and maintained in the same concentration of tunicamycin throughout a 1-hr pulse labeling with ^{35}S-Met and a 6-hr chase.

Endoglycosidase treatment

Endoglycosidase digestion studies were performed on RIPs prepared from cell layers or culture fluids following a 1-hr pulse label and an 8-hr chase. The precipitated proteins were eluted from the *Staphylococcus aureus* pellet by boiling for 5 min in 1% SDS, 50 m*M* Tris–HCl, pH 6.8, 2 m*M* EDTA. Samples were digested with endoglycosidase H (endo H) by a slight modification of the method of Rose and Bergmann (1983). Digests were performed in buffers containing protease inhibitors in the presence or absence of 3 mU of endo H (Boehringer-Mannheim). Samples were digested with glycopeptidase F (PNGase F) by a modification of the method of Bangs *et al.* (1986). Digests were carried out in buffers containing 5 m*M* *o*-phenanthroline (Sigma) and protease inhibitors, in the presence or absence of 0.4 U of PNGase F (Boehringer-Mannheim).

10–45% Sucrose density gradients

One milliliter of clarified culture fluid obtained from radiolabeled, JE-infected Vero cells was applied to a 10 to 45% continuous sucrose gradient (w/w, prepared with TN buffer; 10 m*M* Tris–HCl, pH 7.5, 100 m*M* NaCl) and centrifuged for 2.5 hr at 25,000 rpm in a Sorvall TH641 rotor at 4°. Fractions were collected and portions were diluted with buffer S, frozen at −70°, and used to prepare RIPs as described above.

5–20% Sucrose density gradient

Clarified culture fluid (250 μl) obtained from radiolabeled, JE-infected Vero cells was applied to a 5–20%

continuous sucrose gradient (w/w, prepared with TN buffer) underlayed with a 65% sucrose cushion (w/w, prepared with TN buffer). Triton X-100 (1 mg/ml) was added to a portion of the culture fluid and a 250-μl sample was applied to an identical sucrose gradient prepared using sucrose solutions containing 1 mg/ml Triton X-100. Both gradients were centrifuged for 20 hr at 35,000 rpm in a Sorvall TH641 rotor at 4°, and fractions were collected and analyzed as described above. The positions of marker proteins (horse heart cytochrome *c*, 2.1S; bovine serum albumin, 4.7S; and murine IgM, 18S) and sucrose concentrations were determined by analysis of gradients prepared and run under identical conditions.

Triton X-114 extraction

JE-infected Vero cells were labeled for 3 hr in the presence of 100 μCi ^{35}S-Met and then chased for 6 hr in the presence of a 10X concentration of unlabeled Met. Clarified culture fluids were diluted with $\frac{1}{3}$ volume of 8% Triton X-114 (Sigma), 200 m*M* Tris–HCl, pH 7.5, containing a 4X concentration of protease inhibitors. PBS-rinsed monolayers were suspended in 2% Triton X-114, 50 m*M* Tris–HCl, pH 7.5, containing protease inhibitors.

Hydrophilic proteins were separated from amphiphilic proteins by a modification of the method of Bordier (1981). The aqueous and detergent phases were separated by warming to 37° in a water bath, and centrifugation at 2000 *g* at 37°. The aqueous phase was removed and saved for analysis, and the detergent pellet was dissolved in 500 μl of ice-cold buffer E (10 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl, 5 m*M* EDTA), incubated for 2 min on ice, rewarmed, and recentrifuged as described above. The detergent phase was reextracted twice and the aqueous phase washes were discarded. The washed detergent phase was diluted with buffer E and saved for analysis.

RESULTS

Synthesis and release of E, NS1, and NS1′ from JE-infected Vero cells

Amounts of radiolabeled E, NS1, and NS1′ found in the cell monolayer and culture fluid of JE-infected Vero cells were examined at various times following a 1-hr pulse of ^{35}S-Met (Fig. 1). The bands identified as E, NS1, and NS1′ in these autoradiograms (Figs. 1A and 1B) could not be immunoprecipitated from radiolabeled uninfected cells, and the radiolabeled material present in the dye front of the gels was found in variable quantities in RIPs prepared from cell lysates of both infected and uninfected cells, using both virus-specific and nonimmune ascitic fluids (results not shown). Quantitation of the amount of labeled protein present in the gels used to prepare the autoradiograms shown in Figs. 1A and 1B shows that E and NS1 were released from Vero cells with a $t_{1/2} > 6$ hr (Fig. 1C). The autoradiogram in Fig. 1B shows that NS1′ was less abundant than NS1 in the infected cells. However, NS1′ was not merely a short-lived precursor to NS1 since NS1′ persisted in infected cells for 24 hr following synthesis. Furthermore, NS1 was far more abundant than NS1′ in the extracellular culture fluid (Figure 1B) and NS1 appeared more rapidly in the culture fluid than NS1′ (Fig. 1C) suggesting that either the NS1 protein was more efficiently released than NS1′, or that NS1′ was degraded to NS1 during or following release into the extracellular fluid. Finally, the autoradiogram in Fig. 1B shows that the extracellular forms of NS1 and NS1′ were larger in apparent molecular mass than their cellular counterparts (Fig. 1B, also see Figs. 4 and 5B for side-by-side comparisons).

Synthesis and release of E, NS1, and NS1′ from JE-infected mosquito cells

Amounts of radiolabeled E, NS1, and NS1′ found in the cell monolayer and culture fluid of JE-infected C6/36 cells were examined at various times following a 1-hr pulse of ^{35}S-Met (Fig. 2). Quantitation of the amount of labeled E protein present in the gel used to prepare the autoradiogram shown in Fig. 2A shows that E was released from C6/36 cells with a $t_{1/2} > 8$ hr (Fig. 2C), a time course similar to the release of E from Vero cells (Fig. 1C). Interestingly, no forms of NS1 or NS1′ were found in the culture fluid of JE-infected C6/36 cells (Figs. 2B and 2C). Rather, NS1 *and* NS1′ remained in the cell monolayer and no changes in the relative amounts of radioactivity associated with each of these proteins were detected during the 24-hr chase period (Figs. 2B, C). The absence of NS1 and NS1′ from the culture fluid obtained from two other mosquito cell lines (Fig. 3) indicated that the inability to release NS1 and NS1′ was a common feature of mosquito cell lines.

Analysis of N-linked oligosaccharide groups

Tunicamycin treatment and endoglycosidase digestion were employed to define the N-linked oligosaccharide components of the JE glycoproteins.

Tunicamycin treatment of JE-infected Vero cells reduced the quantity of glycoprotein synthesized, dramatically reduced the amount of E released, and abolished release of NS1 and NS1′ (Fig. 4). Furthermore, treatment with tunicamycin appeared to alter the ratio of NS1 to NS1′ found within the monolayer, possibly reflecting a change in proteolytic processing, or a differential increase in the degradation of the deglycosylated form of NS1 relative to the deglycosylated form

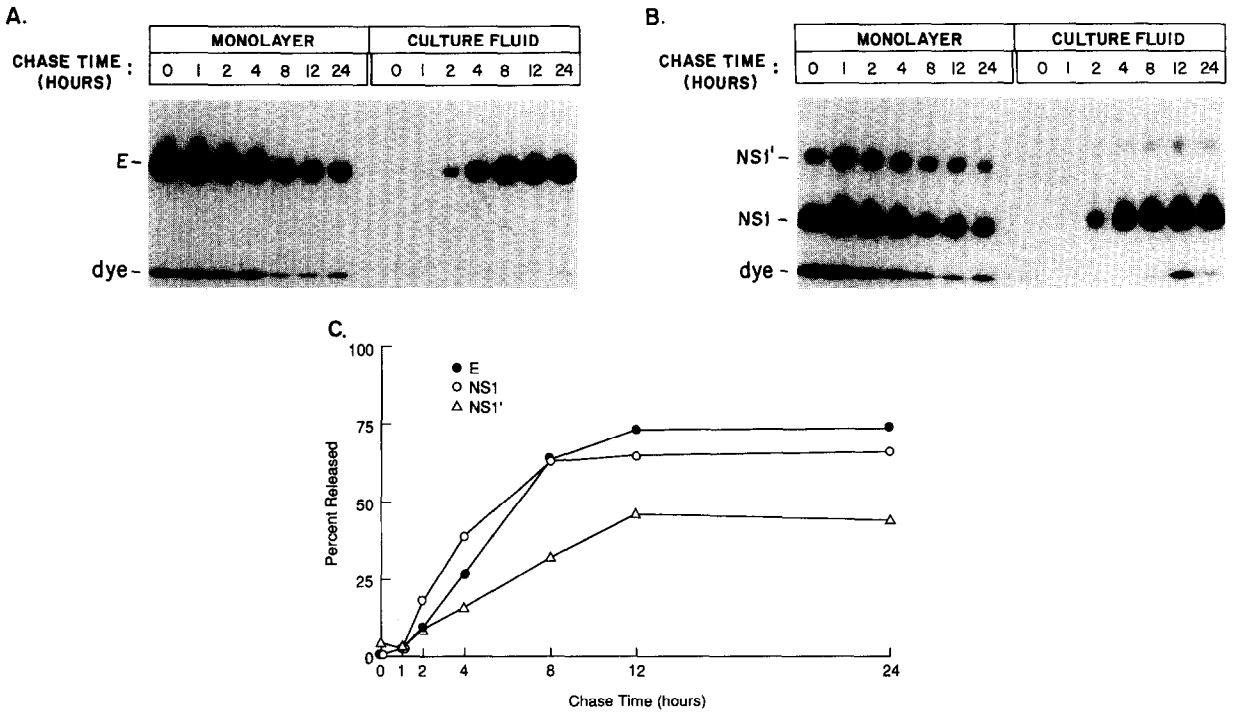

FIG. 1. Synthesis and release of E, NS1, and NS1′ in JE-infected Vero cells. JE-infected Vero cells were pulse-labeled for 1 hr with ^{35}S-Met and then chased for 0, 1, 2, 4, 8, 12, or 24 hr. Equal fractions of the cell monolayer and culture fluid obtained at each time point were immunoprecipitated with MAbs, subjected to electrophoresis, and autoradiographed (see under Materials and Methods). (A) Immunoprecipitates prepared with the MAb specific for E. (B) Immunoprecipitates prepared with the MAb specific for NS1 and NS1′. (C) Kinetics of release of E, NS1, and NS1′ from Vero cells. The data shown in (C) were based on the cpm of ^{35}S-protein recovered from the gels shown in (A) and (B) (see under Materials and Methods). (●) E; (○) NS1; (△) NS1′.

of NS1′. Tunicamycin treatment resulted in about a 50% reduction in the levels of NS3 present in infected cells under these conditions (results not shown), suggesting that the dramatic effect on the viral glycoproteins was not a nonspecific protein synthesis effect. The observed differences in molecular mass between the normal glycoprotein forms and the forms produced in the presence of tunicamycin (Fig. 4) suggested the presence of two N-linked oligosaccharide groups on NS1 and NS1′ and a single group on E, consistent with the cDNA-derived amino acid sequence data for JE (McAda *et al.*, 1987) and endoglycosidase studies described below. The low molecular mass polypeptide (approximately 42 kDa) observed in lanes 3 and 5 of Fig. 4 was probably a degradation product of the E protein produced by the tunicamycin-treated cells.

Endoglycosidase treatment was used to analyze the N-linked oligosaccharide groups present on the cell-associated and extracellular forms of E, NS1, and NS1′ (Fig. 5). These studies compared the susceptibility of the different glycoproteins to endo H which is specific for high-mannose carbohydrates (Tarentino *et al.*, 1978) and PNGase F which cleaves all N-linked carbohydrate groups (Tarentino *et al.*, 1985). Proteins obtained from tunicamycin-treated Vero cells served as standards for complete deglycosylation (Fig. 5).

The 51-kDa form of E found in both Vero and C6/36 cells was predominantly the high-mannose type (see Fig. 5A, lanes 2, 3, 10, 11). In the case of the Vero cells, a small fraction of the cell-associated form of E appeared to have been processed to either a complex or hybrid type since it was resistant to endo H digestion (Fig. 5A, lanes 2, 3).

The E protein recovered in the culture fluids of both vertebrate and invertebrate cell types was not sensitive to endo H (Fig. 5A, lanes 6, 7, 14, 15), indicating that a late-stage oligosaccharide processing event had occurred. In the case of E released by Vero cells, the lack of sensitivity to endo H (Fig. 5A, lanes 6, 7) was accompanied by a barely detectible increase in its observed molecular mass (Fig. 5A, lanes 2, 4 versus 6, 8; Fig. 4, lanes 1 and 2). In contrast, mosquito cells released forms of E that were smaller in molecular mass than their cellular counterparts (Fig. 3, lanes 1–4; Fig. 5A, lanes 10, 12 versus 14, 16). These results are consistent with the findings of Hsieh and Robbins (1984) that showed that Sindbis virus envelope glycoprotein oligosaccharides that were processed to a complex form in mammalian cells were trimmed to a $Man_3GlcNAc_2$ glycan in mosquito cells, which are very poor substrates for endo H (Tarentino *et al.*, 1978). The autoradiogram in Fig. 5A (as well as side-by-side compari-

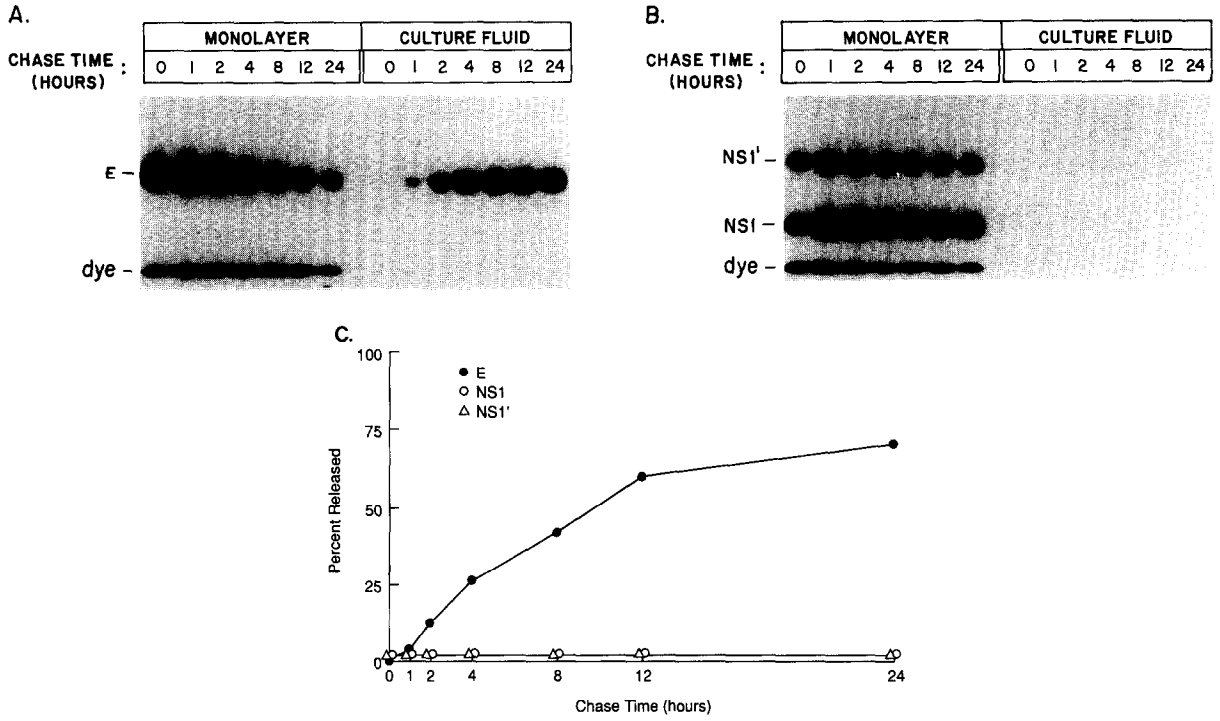

FIG. 2. Synthesis and release of E, NS1, and NS1′ in JE-infected C6/36 cells. JE-infected C6/36 cells were pulse-labeled for 1 hr with ^{35}S-Met and then chased for 0, 1, 2, 4, 8, 12, or 24 hr. Equal fractions of the cell monolayer and culture fluid obtained at each time point were immunoprecipitated with MAbs, subjected to electrophoresis, and autoradiographed (see under Materials and Methods). (A) Immunoprecipitates prepared with the MAb specific for E. (B) Immunoprecipitates prepared with the MAb specific for NS1 and NS1′. (C) Kinetics of release of E, NS1, and NS1′ from C6/36 cells. The data shown in (C) were based on the cpm of ^{35}S-protein recovered from the gels shown in (A) and B) (see under Materials and Methods). (●) E; (○) NS1; (△) NS1′.

sons, not shown) reveals that the extracellular form of the JE E protein produced by C6/36 cells was smaller than the protein produced by Vero cells, consistent with published findings for E of dengue type 2 virus (Smith and Wright, 1985).

NS1 and NS1′ found in both Vero and C6/36 cell monolayers contained high-mannose oligosaccharides since treatment with either endo H or PNGase F reduced them to the size of the proteins obtained from tunicamycin-treated cells (42 and 52 kDa; Fig. 5B,

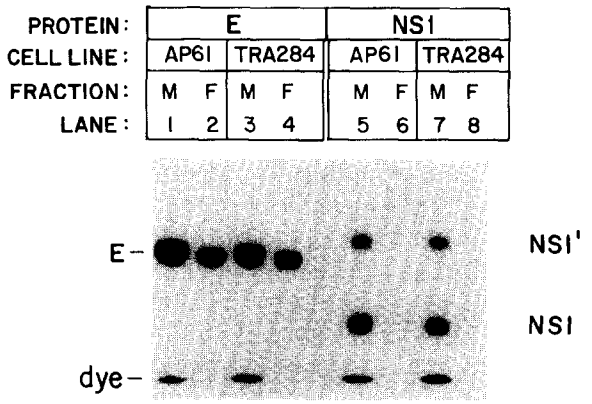

FIG. 3. Synthesis and release of E, NS1, and NS1′ in JE-infected AP61 and TRA284 cells. Samples of JE-infected AP61 and TRA284 cell monolayers (M) or culture fluids (F) were prepared from infected cells labeled for 24 hr with ^{35}S-Met. Equal fractions of each sample were immunoprecipitated with MAbs specific for E or NS1 and NS1′, subjected to electrophoresis, and autoradiographed.

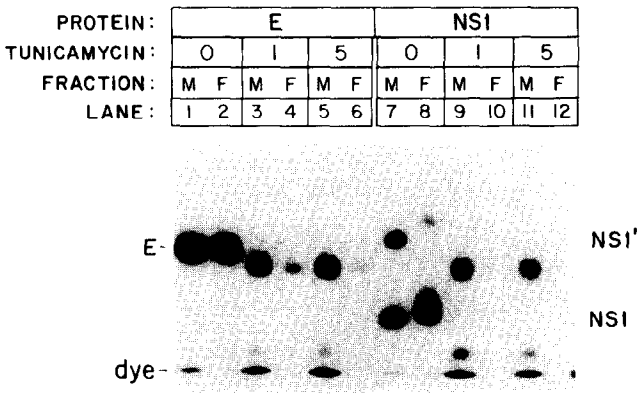

FIG. 4. Effect of tunicamycin treatment on synthesis and release of E, NS1, and NS1′ from JE-infected Vero cells. Samples of JE-infected Vero cell monolayers (M) or culture fluids (F) were prepared from radiolabeled cultures treated with 0, 1, or 5 μg/ml of tunicamycin (see under Materials and Methods). Equal fractions of each sample were radioimmunoprecipitated with MAbs specific for E or NS1 and NS1′, subjected to electrophoresis, and autoradiographed.

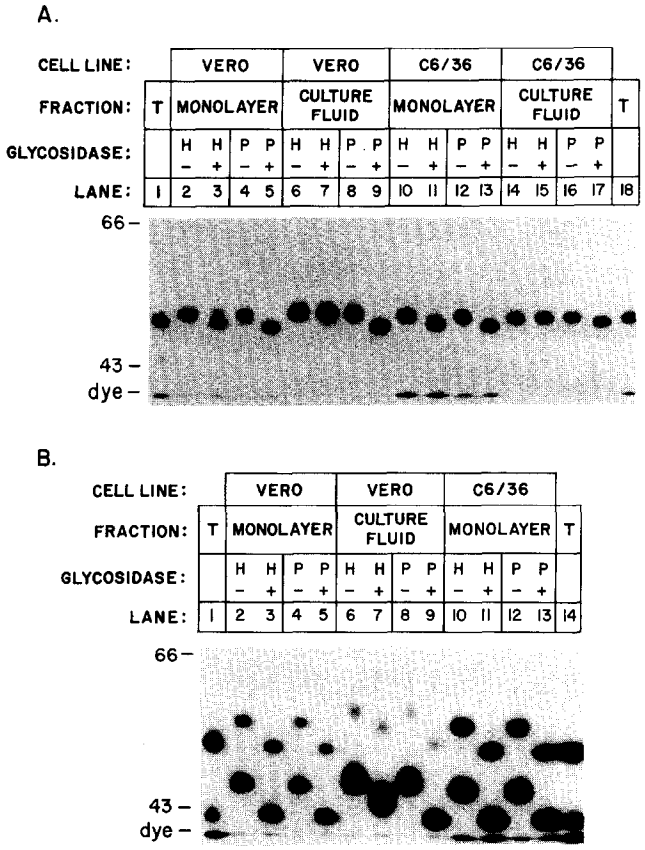

FIG. 5. Endoglycosidase treatment of the cell-associated and extracellular forms of E, NS1, and NS1′ derived from Vero and C6/36 cells. RIPs prepared from cell monolayers or culture fluids prepared from JE-infected cultures pulse-labeled with ^{35}S-Met for 1 hr and then chased for 8 hr were digested with endo H (H) or PNGase F (P) (see under Materials and Methods). (A) RIPs prepared with the MAb specific for E. (B) RIPs prepared with the MAb specific for NS1 and NS1′. For each RIP, samples were mock-digested (–) or digested (+) with each enzyme and then subjected to electrophoresis and autoradiographed. The samples electrophoresed in the lanes at the edges of each gel were undigested samples prepared by radioimmunoprecipitation of samples from the cell layers of tunicamycin-treated JE-infected Vero cells (T; see Fig. 4). The positions of the molecular weight standards, bovine serum albumin (66 kDa), and ovalbumin (43 kDa) are shown on the left side of each autoradiogram.

lanes 1–5, 10–14). Furthermore, Fig. 5B shows that NS1 and NS1′ found in JE-infected Vero cells (46 and 56 kDa) were similar in molecular mass to NS1 and NS1′ found in JE-infected C6/36 cells (Fig. 5B, lanes 2, 4 versus 10, 12).

NS1 and NS1′ released by Vero cells were larger in molecular mass (47 and 58 kDa; Fig. 5B, lanes 6, 8) than their cellular counterparts (46 and 56 kDa; Fig. 5B, lanes 2, 4; see also Fig. 4, lanes 7, 8 for side-by-side comparison). The presence of a high-mannose group on the extracellular forms of NS1 and NS1′ was revealed by endo H digestion (Fig. 5B, lanes 6, 7). The finding that treatment with PNGase F was required to completely remove the N-linked oligosaccharides from these forms of NS1 and NS1′ (Fig. 5B, lanes 6–9) showed that in addition to the high-mannose oligosaccharide, the extracellular forms of NS1 and NS1′ contained one larger, presumably complex oligosaccharide. The finding that PNGase F digestion of the cellular and extracellular forms of NS1 and NS1′ produced proteins of the same molecular mass (Fig. 5B, lanes 5, 9) suggested that the differences in molecular mass observed between the cellular and released forms of NS1 and NS1′ resulted exclusively from modification of the N-linked oligosaccharides.

Physical studies

Sucrose density gradient ultracentrifugation and detergent solubility studies were undertaken to define the physical form of the JE glycoproteins in the extracellular fluid. As expected, the majority of the radiolabeled E protein was present in the 10–45% sucrose density gradient fractions that contained the infectious virion (Fig. 6). Some of the radiolabeled E protein was also present in the position of the slowly sedimenting hemagglutinin (fractions 14 and 15) (Russell *et al.*, 1980). NS1 and NS1′ remain at the top of this type of sucrose gradient (Fig. 6), consistent with their initial description as soluble complement-fixing antigen (Russell *et al.*, 1980).

The original description of NS1 as SCF may be misleading since dengue 2 NS1 (Winkler *et al.*, 1988) and JE NS1 and NS1′ (results not shown) can be readily removed from extracellular culture fluid by ultracentrifugation. Furthermore, although JE NS1 and NS1′ remain at the top of sucrose gradients used to purify the virus particles (Fig. 6), these glycoproteins readily enter 5–20% sucrose gradients (Fig. 7A). To investigate the possibility that these forms of NS1 were associated with membranous particles, the sedimentation properties of NS1 and NS1′ found in culture fluid were examined in the presence of 1 mg/ml Triton X-100 (Fig. 7B). As expected, the addition of Triton X-100 dramatically altered the sedimentation of E by releasing it from the virion (Figs. 7A and 7B). In addition, the distributions of NS1 and NS1′ were markedly changed (Figs. 7A and 7B), in a manner consistent with their presence in membrane containing vesicles (Helenius and Simons, 1975). Interestingly, in the presence of Triton X-100 NS1 and NS1′ co-sedimented, ahead of E, consistent with their presence in dimers or multimers of NS1 and NS1′ (Fig. 7B). The existence of SDS resistant, heat labile, dimers of NS1 has been reported for dengue 2 (Winkler *et al.*, 1988), and similar dimers that contain both NS1 and NS1′ have been observed in the cell layers and the culture fluids of JE-infected cells (results not shown).

The hydrophobic properties of the JE glycoproteins were examined using Triton X-114 phase separation

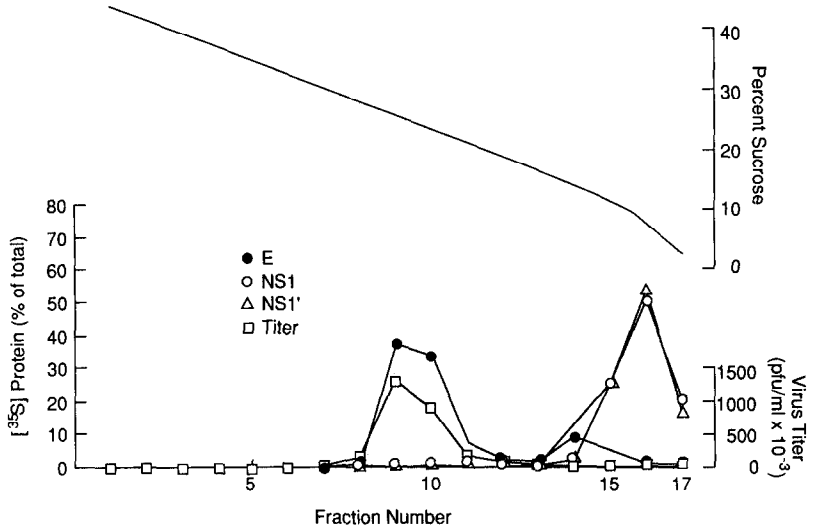

FIG. 6. Position of E, NS1, NS1', and infectious virion in a 10–45% sucrose gradient. Culture fluid from ^{35}S-Met-labeled, JE-infected Vero cells was subjected to centrifugation for 2.5 hr at 25,000 rpm in a 10–45% sucrose gradient. Fractions were collected and the percentage of each protein recovered in the fractions was determined as described under Materials and Methods. (●) E; (○) NS1; (△) NS1'; (□) virus titer.

experiments (Bordier, 1981). These studies showed that all three glycoproteins (both cellular and extracellular forms of E, NS1, and NS1') obtained from Vero cells remained in the detergent phase during extraction (Fig. 8), consistent with the possibility that all of these proteins contained membrane-binding domains.

DISCUSSION

Synthesis of JE glycoproteins requires cotranslational proteolytic processing. Although the N-termini of E, NS1, and NS1' and the C-terminus of E are probably formed by cleavage at signalase sites conserved among the flaviviruses (McAda *et al.*, 1987; Mason *et al.*, 1987a), the proteolytic cleavage sites that produce the C-termini of NS1 and NS1' have not been identified. On the time scale of the pulse-chase studies presented here, NS1' was a major viral product, not merely a short-lived intermediate in the production of NS1. This result suggests that alternative, rather than sequential, proteolytic cleavages are utilized in this region of the JE polyprotein. Several protease cleavage sites have recently been identified in this region of the Kunjin virus polyprotein by sequencing the N-terminus of the NS2A and NS2B proteins (Speight *et al.*, 1988). Although homologous cleavage sites can be identified in the deduced JE protein sequence (McAda *et al.*, 1987), the predicted characteristics of the resulting protein products appear inconsistent with the properties of the JE NS1 and NS1' described in this paper. A JE NS1 protein with a C-terminus produced by the cleavage site defined by the N-terminus of the Kunjin NS2A protein would consist of 352 amino acid residues (deglycosylated molecular mass of 40 kDa) and would lack any significant linear hydrophobic domains (Adams and Rose, 1985), inconsistent with the observed amphipathic properties of the JE NS1 protein. A combined JE NS1-NS2A polypeptide with a C-terminus produced by the cleavage site defined by the N-terminus of the Kunjin NS2B protein would consist of 579 amino acid residues and have an unglycosylated molecular mass of 65 kDa, much larger than the observed size of the enzymatically deglycosylated NS1' protein (determined to be 52 kDa, see under Results).

E, NS1, and NS1' acquired N-linked oligosaccharides in both Vero and C6/36 cells, but the processing and release of these glycoproteins was different in mammalian and mosquito cells. The results presented here demonstrate that one N-linked glycosylation site on E and two shared by NS1 and NS1' were utilized during glycoprotein synthesis in JE-infected Vero and C6/36 cells, and in both cell types, all three glycoproteins retained high-mannose oligosaccharides long after synthesis. The oligosaccharide present on E synthesized by Vero cells was processed to an endo H-resistant, presumably complex form, immediately prior to release. In contrast, the oligosaccharide present on E released by mosquito cells had been trimmed to a smaller, endo H-resistant form. Vero cells released both NS1 and NS1', although NS1 was released more rapidly, and in higher amounts. Furthermore, the extracellular forms of NS1 and NS1' each contained one oligosaccharide that was processed to a larger, presumably complex form, and one high-mannose oligosaccharide. Surprisingly, no forms of NS1 or NS1' were detected in the culture fluid of JE-infected mosquito cells. The inability of mosquito cells to produce com-

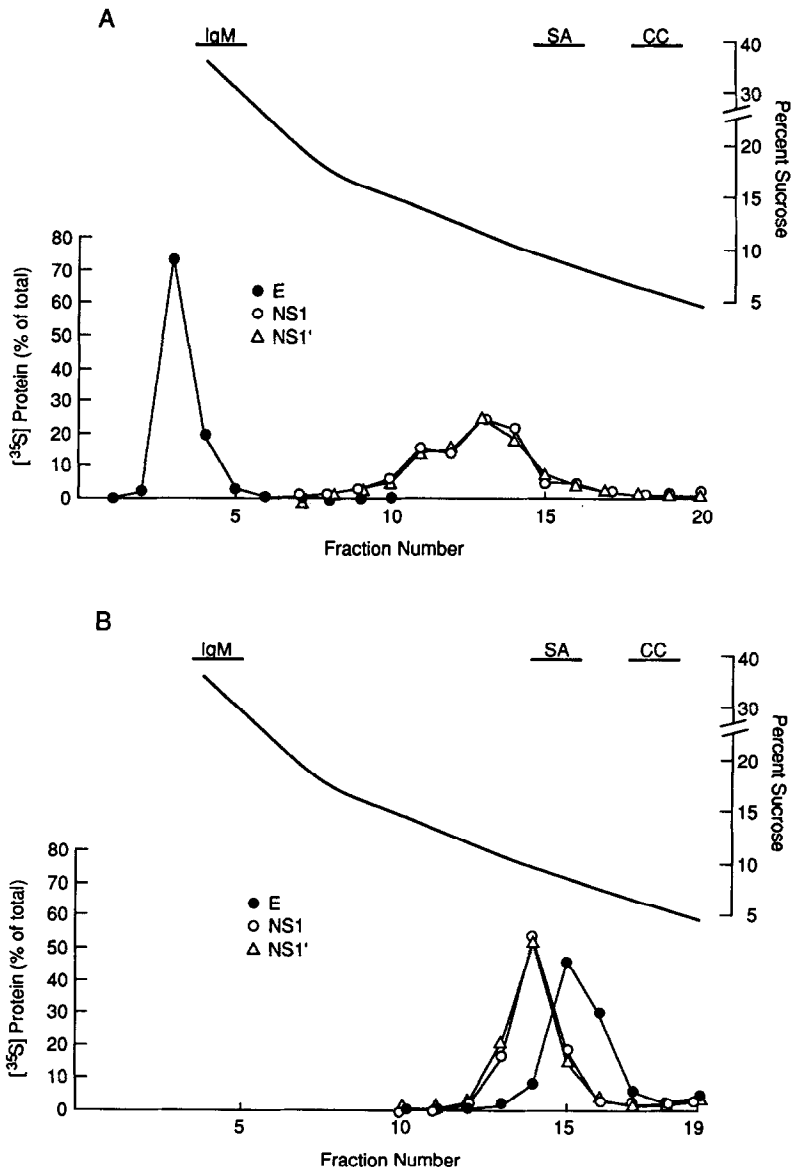

FIG. 7. Position of E, NS1, and NS1′ in 5–20% sucrose gradients prepared in the presence or absence of Triton X-100. Culture fluid from ^{35}S-Met-labeled, JE-infected Vero cells was subjected to centrifugation for 20 hr at 35,000 rpm in 5–20% sucrose gradients in the absence (A) or presence (B) of 1 mg/ml Triton X-100 (see under Materials and Methods). Fractions were collected and the percentage of each protein recovered in the fractions was determined as described under Materials and Methods. (●) E; (○) NS1; (△) NS1′.

plex oligosaccharides (Kornfeld and Kornfeld, 1985; Hsieh and Robbins, 1984) may be related to their inability to release NS1 and NS1′. In addition, the finding that tunicamycin dramatically reduced the appearance of E in the extracellular fluid and abolished release of NS1 and NS1′ from JE-infected mammalian cells may be indicative of the importance of N-linked oligosaccharides in the maturation and release of JE glycoproteins.

E, NS1, and NS1′ were released from JE-infected cells at a very slow rate. The greater than 6-hr time period required for half of the pulse-labeled protein to be released from either mammalian or mosquito cells (Figs. 1C and 2C) is much longer than the $t_{1/2}$ = 34 min required for release of the vesicular stomatitis virus (VSV) G protein from infected cells (Strous and Lodish, 1980). These data provide biochemical evidence for a protracted period of flavivirus glycoprotein synthesis, transport, and/or assembly, consistent with ultrastructural studies (Leary and Blair, 1980; Ng, 1987; Hase *et al.*, 1987a,b; Ishak *et al.*, 1988) and virus synthesis studies (Westaway, 1980). This slow rate of release suggests that these three JE glycoproteins were specifically retained in the cellular secretory pathway (Pfeffer and Rothman, 1987; Wieland *et al.*, 1987), probably to facilitate virion assembly. Although the exact site(s) of retention of E, NS1, and NS1′ within the

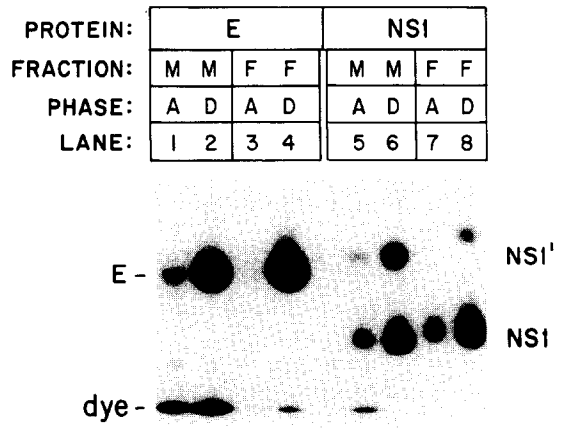

FIG. 8. Radioimmunoprecipitates of Triton X-114-extracted JE-infected cell lysates and culture fluids. Cell monolayers (M) and culture fluids (F) obtained from ^{35}S-Met-labeled, JE-infected Vero cells were suspended in Triton X-114 containing buffers, aqueous and detergent phases were prepared, and viral glycoproteins were immunoprecipitated from equal portions of the aqueous (A) and washed detergent (D) phases, subjected to electrophoresis, and autoradiographed.

endoplasmic reticulum and Golgi of the infected cells has not been established, the finding that the cellular forms of these three glycoproteins retained sensitivity to endo H indicated that they were retained in a compartment that preceded the *trans*-Golgi (Kornfeld and Kornfeld, 1985). This property of retention within the secretory pathway is shared by several other viral proteins (Madoff and Lenard, 1982; Tooze *et al.*, 1987; Paabo *et al.*, 1987).

No role has been defined for the flavivirus NS1 protein(s). The finding that the JE, E, NS1, and NS1′ glycoproteins were co-retained within the secretory pathway of the infected cell provides evidence for a role for the nonstructural glycoproteins in virion formation. Other evidence for the role of the NS1 protein(s) at this intracellular site is provided by the data presented here that indicate the cellular forms of NS1 and NS1′ were identically processed in JE-infected mosquito and mammalian cells, but NS1 and NS1′ were *not* released from mosquito cells. This dramatic difference in viral replication strategy in mosquito and mammalian cells may be related to the differences in pathology induced by flavivirus infection of insect and vertebrate hosts.

The amphipathic nature and the sedimentation properties of the forms of NS1 and NS1′ found in Vero cell culture fluid suggests that these nonstructural glycoproteins were released from infected cells in a membrane-associated particulate form. The release of the NS1 protein(s) in an extracellular particle during infection of a vertebrate host could help to account for the strong immunological response to this protein (Russell *et al.*, 1980) and the synthesis of a similar particle may be important for the production of an effective NS1-based vaccine.

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