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Proper Maturation of the Japanese Encephalitis Virus Envelope Glycoprotein Requires Cosynthesis with the Premembrane Protein

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The role of the Japanese encephalitis virus (JEV) premembrane (prM) protein in maturation of the envelope (E) glycoprotein was evaluated by using recombinant vaccinia viruses encoding E in the presence (vP829) or absence (vP658) of prM. Immunofluorescence analyses showed that E appeared to be localized in the endoplasmic reticulum of cells infected with JEV, vP829, or vP658. However, reactivity with monoclonal antibodies and behavior in Triton X-114 indicated that E produced in the absence of prM behaved abnormally. Furthermore, E produced in the presence of prM by recombinant vaccinia viruses could be incorporated into flavivirus pseudotypes, whereas E synthesized in the absence of prM could not. These results demonstrate that cosynthesis of prM is required for proper folding, membrane association, and assembly of the flavivirus E protein.

The flavivirus virion consists of a nucleocapsid surrounded by a lipid bilayer containing an envelope (E) glycoprotein and a nonglycosylated membrane (M) protein. The M protein is found in infected cells as a glycosylated precursor, prM, which is cleaved to form M in a late-stage cleavage event, presumably by a cellular protease located in the secretory pathway (reviewed in reference 3). The prM and E proteins are synthesized as a part of a polyprotein processed during translation through the endoplasmic reticulum (ER) membrane. These proteins appear inside the lumen of the ER with their N termini cleaved by signalase, and their C-terminal hydrophobic amino acid domains supply stop transfer sequences and membrane anchors. The E protein plays an important role in receptor binding and membrane fusion and contains most of the sites that react with neutralizing antibodies as well as many protective epitopes (reviewed in reference 7). The roles for prM in virion function and viral biology remain unclear, although prM is probably involved in virion maturation, since cleavage of prM to M is associated with changes in infectivity (14) and fusion activity (6) and since prM and E are found in cell-associated heterodimers (14).

We have developed recombinant vaccinia viruses encoding various combinations of Japanese encephalitis virus (JEV) structural and nonstructural (NS) proteins in order to define the roles of these proteins in inducing protective immunity (8, 13). Two of these viruses, vP555 and vP829, encode prM and E and induced the proper synthesis of both intracellular and extracellular forms of the prM/M and E proteins in infected cells (8, 13), in the presence (vP555) or absence (vP829) of NS1. Furthermore, we demonstrated that cells infected with vP829 released subviral particles of 20-nm diameter that were characterized as flavivirus RNA-free membrane vesicles with prM/M and E embedded in a lipid bilayer (9). Another recombinant, vP658, encodes E and NS1, and E produced by cells infected with this virus is not released into the extracellular fluid (13).

To determine whether E produced by vP658 was not released from cells because of its presence in a different subcellular compartment, we used indirect immunofluorescence microscopy. The results of these studies (Fig. 1) showed that E was distributed in a fine network in the cytoplasm of cells infected with either vP658 or vP829, suggesting that E produced in cells infected with either recombinant is present in the ER, consistent with previous N-linked glycosylation studies (8, 13).

To further probe the differences in the E protein produced by cells infected with these viruses, we compared the reactions of E prepared from cells infected with the Nakayama strain of JEV, vP829, or vP658 with a panel of monoclonal antibodies (MAbs). For these analyses, radioactive antigens prepared from cells infected with JEV, vP829, or vP658 were immunoprecipitated with MAbs and subjected to sodium dodecyl sulfate-containing polyacrylamide gel electrophoresis (SDS-PAGE), and the amount of E precipitated was determined by using a Molecular Dynamics PhosphorImager. Table 1 shows the results for three selected MAbs. Two of these, J3-11G5 and J3-14E6 (12), are shown since they exhibited the highest and lowest reactions with the authentic (JEV) E protein, and the third MAb, D1-4G2 (5), was selected since it reacts with a discontinuous epitope and its binding is dependent on correct disulfide bond formation within E (12). These results indicate that the vP658-encoded E was present in cell lysates in much lower amounts, probably as a result of degradation since identical vectors and promoters were used for construction of vP829 and vP658 (8, 13). Furthermore, the vP658 E is in an antigenically different conformation from E present in cells infected with JEV or vP829, since it reacted very poorly with D1-4G2.

The hydrophobic properties of E produced by JEV-, vP829-, and vP658-infected cells were compared by using Triton X-114 phase separation experiments. As shown in Fig. 2, most of the radioactive E obtained from JEV- and

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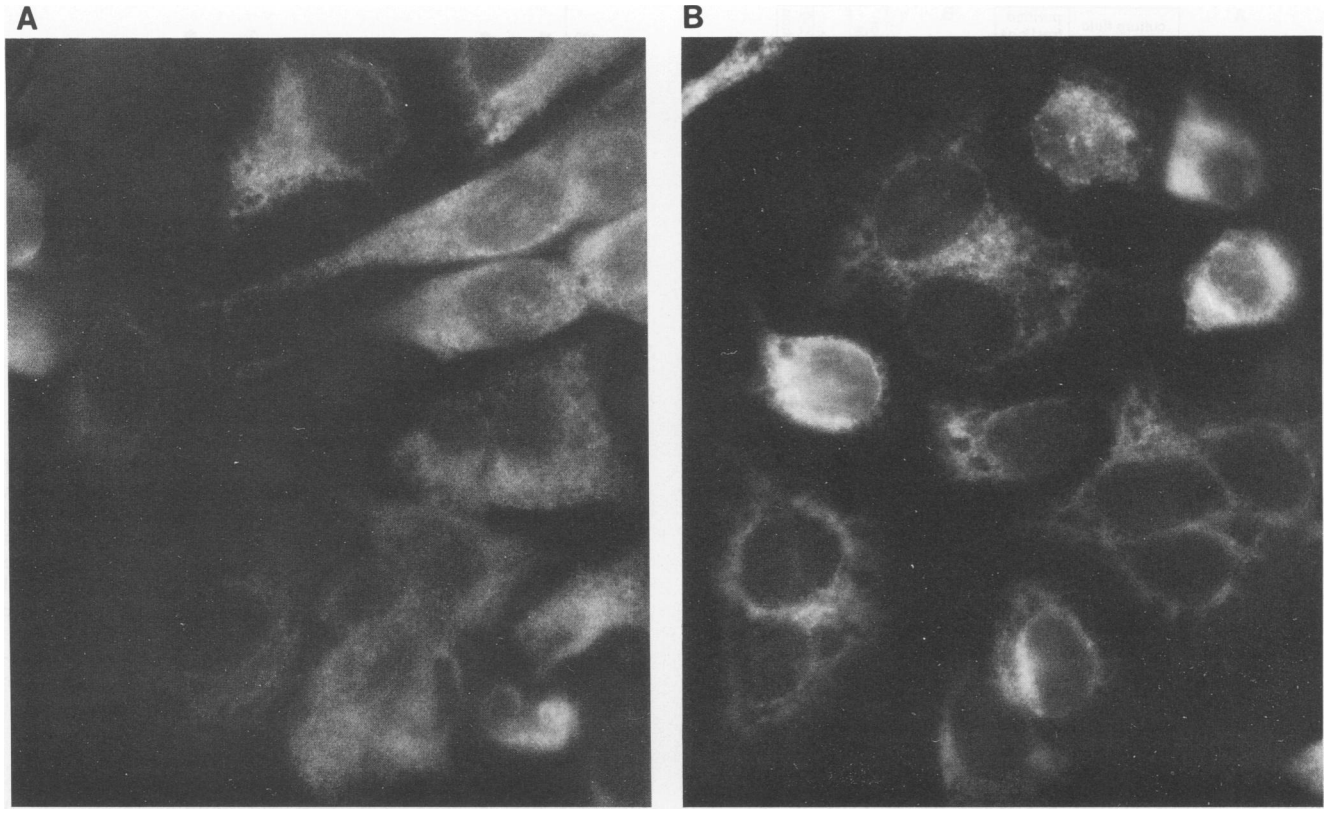

FIG. 1. Immunofluorescence micrographs of HeLa cells infected with vP829 (A) and vP658 (B). Cell monolayers were infected with recombinant vaccinia viruses (multiplicity of infection of 2); 24 h later, the monolayers were fixed with cold ethanol and stained with a MAb against E (J3-11B9) and a goat anti-mouse fluorescein isothiocyanate-conjugated antibody.

vP829-infected cells partitioned into the detergent phase, whereas more than half of the E protein molecules synthesized in vP658-infected cells were found in the aqueous phase. Furthermore, essentially 100% of E found in culture fluids of JEV- and vP829-infected cells remained in the detergent phase during extraction, and no E was released from vP658-infected cells, confirming earlier studies (13). These results suggest that E synthesized in vP658-infected cells was improperly folded, preventing it from displaying the amphipathic properties of E produced by JEV- and vP829-infected cells. The hydrophilic forms of E found in vP658-infected cells are probably similar to misfolded disulfide-linked aggregates of newly synthesized viral glycoproteins recently identified by Marquardt and Helenius (10). The E protein produced by vP658 is present in similar types of aggregates, since most of the E protein found in vP658-infected cells does not enter the separatory gel in SDS-PAGE in the absence of reduction (data not shown).

Production of pseudotype viruses by using vector-encoded proteins in cells coinfected with related viruses has recently been used to assay oligomerization of the rhabdovirus E glycoprotein (15). We have used a similar approach to determine whether E produced by our recombinant vaccinia viruses could be incorporated into flavivirus pseudotypes.

TABLE 1. Reaction of E produced by JEV-, vP829-, and vP658-infected HeLa cells with MAbs[a]

Virus	Relative amt[b] after immunoprecipitation with MAb:		
	J3-11G5	J3-14E6	D1-4G2
JEV	57	35	39
vP829	95	60	55
vP658	8.8	5.6	1.1

[a] HeLa cell monolayers were infected with recombinant vaccinia viruses (multiplicity of infection of 2) or JEV (multiplicity of infection of 5) and then were pulse-labeled for 2 h with [^{35}S]methionine and chased for 6 h in the presence of excess unlabeled methionine (13). Radiolabeled cell lysates and culture fluids were immunoprecipitated with MAbs against E, J3-11G5, J3-14E6 (12), and D1-4G2 (5), and then resolved by SDS-PAGE.

[b] Relative amounts of immunoprecipitated E protein present in SDS-polyacrylamide gels determined with a PhosphorImager.

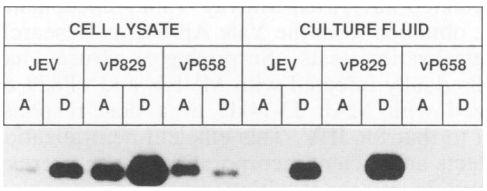

FIG. 2. Radioimmunoprecipitates of Triton X-114-extracted lysates of HeLa cells infected with JEV, vP829, or vP658. Cell monolayers and culture fluids obtained from [^{35}S]Met-labeled, infected cells were subjected to phase separation by using 2% Triton X-114 prepared in 50 mM Tris-HCl (pH 7.5) containing protease inhibitors as previously described (2, 11); E was immunoprecipitated from equal portions of the aqueous (A) and washed detergent (D) phases by using MAb J3-11B9, subjected to SDS-PAGE, and autoradiographed.

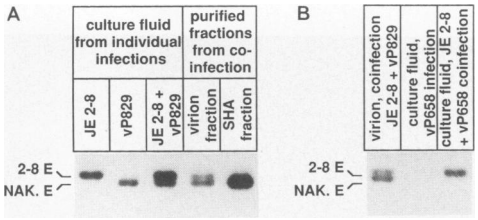

FIG. 3. Results of assays using a pseudotype virus containing E proteins of two different molecular weights that was produced by HeLa cells dually infected with the 2-8 strain of JEV and a recombinant vaccinia virus, vP829 (both at a multiplicity of infection of 5). (A) Immunoprecipitates from culture fluids obtained from cells infected singly with the 2-8 strain of JEV or vP829 electrophoresed either separately or together as a mixture, as indicated. The two lanes on the right show the immunoprecipitates prepared from the sucrose density gradient-purified virion and slowly sedimenting hemagglutinin (SHA) fractions obtained from culture fluids of cells coinfected with 2-8 and vP829. (B) Immunoprecipitates prepared from the virion fraction obtained from cells coinfected with 2-8 and vP829, from the culture fluid of cells infected with vP658 alone, or from the culture fluid from cells coinfected with 2-8 and vP658, as indicated. In all cases, immunoprecipitates were digested with 0.4 U of glycopeptidase F prior to SDS-PAGE (1).

Biochemical evidence for JEV pseudotype formation was obtained by evaluation of E in virion fractions obtained from cells coinfected with the recombinant vaccinia viruses and the 2-8 strain of JEV (4; obtained from the Yale Arbovirus Research Unit), which produces an E protein with an apparent higher molecular weight. Figure 3A shows that sucrose density gradient-purified virions produced by dual infection with the 2-8 strain and vP829 contained E proteins corresponding in size to E produced by the 2-8 strain and by vP829. This result indicates that E encoded by vP829 had been incorporated into the 2-8 virion, demonstrating pseudotype formation in cells coinfected with vP829. In contrast, culture fluids of cells dually infected with the 2-8 strain and vP658 showed only a single band corresponding to E encoded by the 2-8 strain (Fig. 3B), indicating that virions produced by coinfection with vP658 did not possess the E protein encoded by vP658; even overexposure of the gel failed to show any evidence for the Nakayama-sized E protein in this sample. Furthermore, the absence of any vaccinia virus-derived E in the culture fluid indicates that the prM protein synthesized by 2-8 could not rescue the vaccinia virus-encoded E protein.

The production of flavivirus pseudotypes by coinfection with recombinant vaccinia viruses was further examined by using a related flavivirus, Murray Valley encephalitis virus (MVEV; obtained from the Yale Arbovirus Research Unit), and neutralization tests. Surprisingly, viruses harvested from cells dually infected with MVEV and vP829 could be neutralized with MAb J3-14H5 at a dose-response level identical to that for JEV. This efficient neutralization probably reflects an efficient incorporation of the overexpressed JEV E protein into the resulting viruses and may also reflect the ability of this MAb to neutralize virus by binding to only a small number of sites on the virion, consistent with its high neutralization titer (12). No detectable neutralization with this JEV-specific MAb was noted in the virus harvested from cells dually infected with MVEV and vP658 (Fig. 4). These results show that JEV E cosynthesized with prM was capable of being incorporated into the MVEV virion, whereas JEV E produced in the absence of prM was not.

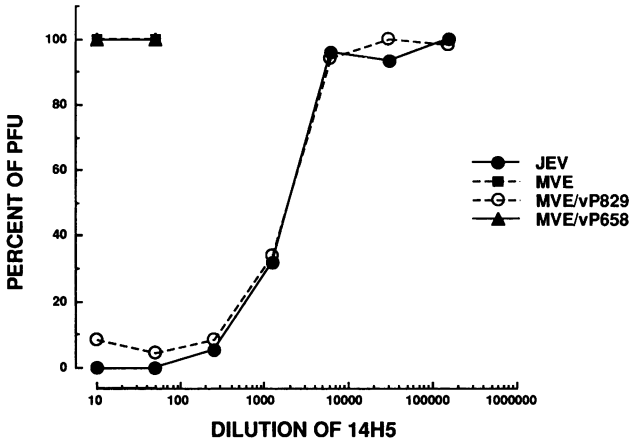

FIG. 4. Neutralization of virus produced by dual infection of HeLa cells with MVEV and recombinant vaccinia viruses. Cells were infected with MVEV (multiplicity of infection of 2), incubated for 24 h, and superinfected with vP829 or vP658 (multiplicity of infection of 5). The medium was renewed 16 h later, and the sucrose density gradient-purified virion fractions prepared from culture fluid harvested 8 h later were tested for neutralization by using the JEV-specific MAb J3-14H5 (12), in the presence of mouse anti-wild-type vaccinia virus (added to inhibit vaccinia virus plaque formation). Data are expressed as percentage of PFU obtained in the absence of the MAb.

The finding that dual infection with vP658 failed to produce pseudotypes is consistent with the conclusion presented above that E produced in the absence of prM accumulates in an aggregated, antigenically altered form in the ER. Thus, proper folding, maturation, and assembly of E require cosynthesis with prM.

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REFERENCES

1. **Bangs, J. D., N. W. Andrews, G. W. Hart, and P. T. Englund.** 1986. Posttranslational modification and intracellular transport of a trypanosome variant surface glycoprotein. J. Cell. Biol. **103**:255–263.
2. **Bordier, C.** 1981. Phase separation of integral membrane proteins in Triton X-114 solution. J. Biol. Chem. **256**:1604–1607.
3. **Chambers, T. J., C. S. Hahn, R. Galler, and C. M. Rice.** 1990. Flavivirus genome organization, expression, and replication. Annu. Rev. Microbiol. **44**:649–688.
4. **Chen, B. Q., and B. J. Beaty.** 1982. Japanese encephalitis vaccine (2-8 strain) and parent (SA 14 strain) viruses in *Culex tritaeniorhynchus* mosquitoes. Am. J. Trop. Med. Hyg. **31**:403–407.
5. **Gentry, M. K., E. A. Henchal, J. M. McCown, W. E. Brandt, and J. M. Dalrymple.** 1982. Identification of distinct antigenic determinants on dengue-2 virus using monoclonal antibodies. Am. J. Trop. Med. Hyg. **31**:548–555.
6. **Guirakhoo, F., F. X. Heinz, C. W. Mandl, H. Holzmann, and C. Kunz.** 1991. Fusion activity of flaviviruses: comparison of mature and immature (prM-containing) tick-borne encephalitis virions. J. Gen. Virol. **72**:1323–1329.
7. **Heinz, F. X.** 1986. Epitope mapping of flavivirus glycoproteins.

Adv. Virus Res. **31:**103–168.

8. **Konishi, E., S. Pincus, B. A. L. Fonseca, R. E. Shope, E. Paoletti, and P. W. Mason.** 1991. Comparison of protective immunity elicited by recombinant vaccinia viruses that synthesize E or NS1 of Japanese encephalitis virus. Virology **185:**401–410.
9. **Konishi, E., S. Pincus, E. Paoletti, R. E. Shope, T. Burrage, and P. W. Mason.** 1992. Mice immunized with a sub-viral particle containing the Japanese encephalitis virus prM/M and E proteins are protected from lethal JEV infection. Virology **188:**714–720.
10. **Marquardt, T., and A. Helenius.** 1992. Misfolding and aggregation of newly synthesized proteins in the endoplasmic reticulum. J. Cell Biol. **117:**505–513.
11. **Mason, P. W.** 1989. Maturation of Japanese encephalitis virus glycoproteins produced by infected mammalian and mosquito cells. Virology **169:**354–364.
12. **Mason, P. W., J. M. Dalrymple, M. K. Gentry, J. M. McCown, C. H. Hoke, D. S. Burke, M. J. Fournier, and T. L. Mason.** 1989. Molecular characterization of a neutralizing domain of the Japanese encephalitis virus structural glycoprotein. J. Gen. Virol. **70:**2037–2049.
13. **Mason, P. W., S. Pincus, M. J. Fournier, T. L. Mason, R. E. Shope, and E. Paoletti.** 1991. Japanese encephalitis virus-vaccinia recombinants produce particulate forms of the structural membrane proteins and induce high levels of protection against lethal JEV infection. Virology **180:**294–305.
14. **Wengler, G., and G. Wengler.** 1989. Cell-associated West Nile flavivirus is covered with E + pre-M protein heterodimers which are destroyed and reorganized by proteolytic cleavage during virus release. J. Virol. **63:**2521–2526.
15. **Whitt, M. A., L. Buonocore, C. Prehaud, and J. K. Rose.** 1991. Membrane fusion activity, oligomerization, and assembly of the rabies virus glycoprotein. Virology **185:**681–688.