

The Association of Virus-specified Proteins with Membrane Structures in Vero Cells Infected with Japanese Encephalitis or Murray Valley Encephalitis Virus

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Abstract: Vero cells infected with Japanese encephalitis (JE) or Murray Valley encephalitis (MVE) virus were fractionated into three extracts (E1, E2 and E3). Each extract was centrifuged through 25-60% discontinuous sucrose gradients. Three distinct bands were separated from Extract 1 (E1/F1, E1/F2, E1/F3) and Extract 2 (E2/F4, E2/F5, E2/F6) in contrast to a single band obtained from Extract 3 (E3/F7). In addition, pellets were also obtained from Extracts 1 and 2, and were termed P1 and P2 pellets respectively. Analyses of the labelled viral proteins and glycoproteins in the various fractions were carried out in conjunction with electron microscopy. MVE virus-specified proteins were present in virtually all fractions. In contrast, JE virus-specified proteins were observed almost exclusively in the smooth membranes of E1/F2, E1/F3 and E2/F4 fractions. Intense virus protein profiles were seen in the E1 pellet (P1) from both JE virus- and MVE virus-infected cells, and contained most of the virus-induced membranous structures and ribosomes. All the virus-specified glycoproteins (E, NS1 and PrM) were found in the P1 fraction but only E and NS1 were found in the E1/F2, E1/F3 and P2 fractions. In summary, the smooth membranes and membranes of the P1 fraction have strong association with the JE virus-specified proteins and probably have a role in their translation. However, the membranes very close to the nuclei had no accumulation of the JE viral proteins. The association of the MVE virus-specified proteins was found with almost all the membrane fractions including the membranes of the perinuclear region. Thus it seems that both smooth and rough membranes are important for the translation and accumulation of both the JE and viral proteins in Vero cells.

Key words: Japanese encephalitis virus, Murray Valley encephalitis virus, Virus-induced membranous structures, Proteins and glycoproteins.

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INTRODUCTION

Cell fractionation procedures have been employed for the isolation and separation of cytoplasmic membrane fractions from flavivirus-infected cells in discontinuous sucrose gradient centrifuged according to the method of Caligiuri and Tamm (1969, 1970) or with minor modifications (Takeda *et al.*, 1978; Kos *et al.*, 1975; Friedman *et al.*, 1972; Boulton and Westaway, 1976; Westaway and Ng, 1980). Polyacrylamide gel electrophoresis (PAGE) analysis on radioactively labelled, flavivirus-infected cell membranes showed the rough membranes to be mainly responsible for viral protein synthesis (Boulton and Westaway, 1976; Stohlman *et al.*, 1975; Westaway and Ng, 1980). Caligiuri and Tamm (1970) and Ter-shak (1984) also showed that most of the poliovirus proteins were preferentially associated with the rough endoplasmic reticulum (RER). However, Kos and co-workers (1975) found virus-specified proteins in both rough and smooth membrane fractions of JE virus-infected chick embryo fibroblast (CEF) cells. Reports on the actual sites of glycosylation were more varied. Boulton and Westaway (1976) excluded the smooth membranes while Stohlman and colleagues (1975) found radioactive glucose being actively incorporated by dengue-2 virus at the intermediate and smooth endoplasmic reticulum (SER). Kos and co-workers (1975) suggested that the enrichment of the glycoproteins E and GP19 (PrM) of JE virus in the rough membrane fractions might be due to their accumulation at the site of virus maturation. In this paper two flaviviruses, namely JE or MVE, were used to infect Vero cells. Then electron microscopic studies and PAGE analyses of the fractionated membranes involved with JE and MVE virus-specified proteins were studied.

MATERIALS AND METHODS

Cells and viruses: Vero cells were grown to confluency in Medium 199 containing 5% foetal calf serum and maintained after infection in Eagle's minimum essential medium containing 0.1% bovine serum albumin. The Nagayama strain of JE virus and MRM66 strain of MVE virus were obtained from Prof. E. G. Westaway, Monash University, Australia.

Labelling with radioactive substrates: Uninfected and infected cells were labelled *in vitro* from 40 to 43 hours post infection with ^{35}S -methionine (for 3 hours) or from 30-40 hours post infection with ^3H -glucosamine (for 30 hours) after 3 hours pretreatment with medium lacking the amino acid or carbohydrate of interest in the presence of 3 $\mu\text{g/ml}$ actinomycin D (a gift of Merck, Sharp and Dohme). Labelled compounds were obtained from the radiochemical Centre, Amersham and used at 5 $\mu\text{Ci/ml}$ for L-[^{35}S] methionine (1330 Ci/mmol) and 10 $\mu\text{Ci/ml}$ for D-[6- ^3H] glucosamine hydrochloride (33 Ci/mmol). Cells were harvested at about 48 hours post infection.

Cell Fractionation: Cell fractionation was carried out according to the method of Westaway and Ng (1980) with some modifications. The cell monolayer (10^8 cells) was washed twice with cold phosphate buffered saline (PBS) followed by three rinses with Buffer A (0.1 M NaCl, 0.01 M Tris-HCl, pH 7.4 and 1.5 mM MgCl_2). The cells were allowed to swell for

about 5 minutes and then disrupted with 20 strokes in tight fitting Dounce homogenizer (Jensons, England). The disrupted cells were then passed 20 times through a 21-Gauge needle attached to a 5 ml syringe. The disrupted cell suspension was centrifuged (3,000 rpm) for one minute in a bench centrifuge. The supernatant was then carefully aspirated, and designated as Extract 1. The remaining pellet was resuspended in an appropriate amount of Buffer A, then passed vigorously 20 times through a 26G needle to mechanically strip the attaching membranes off the nuclei. The suspension was again centrifuged for 1 minute using a bench centrifuge. The supernatant was retained and labelled as Extract 2. The pellet (nuclei and tightly associated membranes) was resuspended in Buffer B (identical to Buffer A, except the pH is adjusted to 8.5) and treated with 1% sodium deoxycholate (DOC) and 1.5% Tween 80 (final concentrations). The detergent extract was retained after centrifugation and designated as Extract 3. All procedures, from initial washings to final extraction steps were carried out at the temperature of melting ice. Extracts 1, 2 and 3 were layered separately onto a 25 to 60% discontinuous sucrose gradient and spun for 4 hrs at 40,000 rpm using SW41 rotor. At the end of the spin, the portion of visible bands were collected and pelleted at 50,000 rpm for 1hr. The membrane pellets obtained were either dissolved in 2% SDS for PAGE or fixed in fresh glutaraldehyde for electron microscopy.

Polyacrylamide gel electrophoresis (PAGE): Before loading onto 11% SDS-discontinuous polyacrylamide gels (Laemmli, 1970), the samples (25-30 μ l) were mixed with bromophenol blue dye mixture and boiled for 1 minute. Constant current of 7-8 mA/hr was administered for each gel in overnight electrophoresis (approximately 18 hours). The slab gels were then dried at 80°C under vacuum and exposed to X-ray film (Dupont X-ray films) for the appropriate time.

Electronmicroscopy: The cells or membrane fractions were processed for electron microscopy as described by Ho and co-workers (1987).

RESULTS

Infected whole cells

The ultrastructural changes induced by Japanese encephalitis (JE) and Murray Valley encephalitis (MVE) viruses in Vero cells have been described by Ho and coworkers (1987). Briefly, in infected cells the rough endoplasmic reticulum (RER) was distended, elongated and often contained virus particles. In addition, clusters of vesicles usually with thread-like enclosures or dense cores were seen in close vicinity to the smooth convoluted membranes (CM) and RER.

Cell fractionation

A total of seven membrane and two pellet fractions were obtained after ultracentrifugation of the three extracts through discontinuous sucrose gradients (Fig. 1). Fractions 1 (E1/F1), 4 (E2/F4) and 7 (E3/F7) banded at the 0-25% sucrose interface, fractions 2 (E1/F2) and 5 (E2/F5) were found of the 30-40% sucrose interface while fractions 3

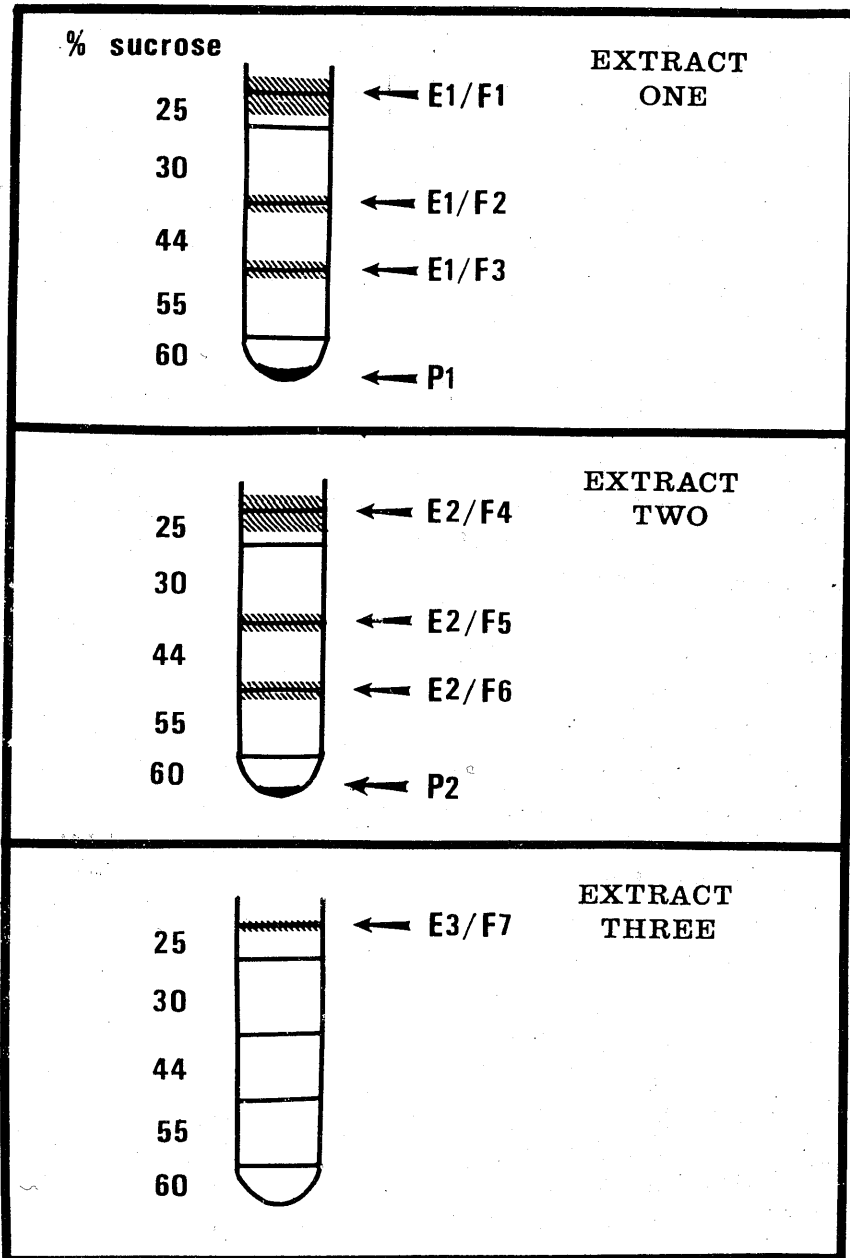


Fig. 1. Diagrammatic representation of the positions of the membrane fractions after ultracentrifugation.

The three extracts were spun at 40,000 rpm through a 25-60% discontinuous sucrose gradient for 4 hours. The fractions are numbered sequentially from 1 to 7 to avoid confusion. Fractions 1 (E1/F1), 4 (E2/F4) and 7 (E3/F7) banded at the 0-25% sucrose interface, fractions 2 (E1/F2) and 5 (E2/F5) were found at the 30-40% sucrose interface and fractions 3 (E1/F3) and 6 (E2/F6) were observed at the 44-55% sucrose interface. Two pellet fractions (P1 and P2) were also obtained, namely P1 and P2 from Extracts 1 and 2 respectively.

(E1/F3) and 6 (E2/F6) were observed at the 44-55% sucrose interface. The P1 and P2 fractions from the infected Extracts 1 and 2 respectively were much more pronounced than those from uninfected cells especially the E1 pellet which was very compact and sticky. Flocculant aggregates were visible in the E2/F4 and E2/F5 fractions while small particulate material was seen in the E3/F7 fraction. The other bands were homogeneous and opaque but varied in thickness and intensity.

Electron microscopy of membrane structures from fractionated cells

The first three fractions (E1/F1, E1/F2 and E1/F3) of Extract 1 from the uninfected Vero cells consisted mainly of ribosomes and small amounts of membranes (Fig. 2A). The membranes of the first two fractions of both JE or MVE virus-infected cells were predominantly smooth. Structures resembling the smooth endoplasmic reticulum (SER) were observed (arrow - Fig. 2B & 2C). Majority of the membranes in E1/F3 fraction from the JE virus-infected cells were smooth (Fig. 2C). For the MVE virus-infected cells fraction E1/F3 consisted of a mixture of rough and smooth membranes (Fig. 2D).

The contents of P1 which was found as a pellet at the base of the tube after ultracentrifugation of Extract 1 was found to be most interesting. Most of the virus-induced membranous structures were sedimented here (Fig. 3A & 3B). Mixtures of smooth and rough membranes were observed in close association with the virus-induced vesicles. In the MVE virus-infected E1 pellet, convoluted membranous (CM) structures were also seen in association with these vesicles (Fig. 3B). The uninfected E1 pellet contained only plasma membranes and some amorphous components (Fig. 3C). The percentage of non-lipid content of the membranes in the E1 pellet from the uninfected Vero cells was found to be very close to that of Hela cells plasma membranes as reported by Bosmann and colleagues (1968, 1969).

Structural characteristics of the membranes in the three fractions from Extract 2 (E2/F4, E2/F5 and E2/F6) were again predominantly smooth. Fractions from both the uninfected and infected cells consisted of smooth membranes of different densities. Mitochondria were present in large numbers in fraction E2/F6 (Fig. not shown). The uninfected cell pellet from extract 2, P2, was also found to be heterogeneous with mixtures of RER, ribosomes and smooth membrane (Fig. 4A). Occasionally, swollen and distorted mitochondria were seen. In addition to this same set of membrane structures seen in the uninfected cell fraction, remnants of the endoplasmic reticulum with virus particles still within the lumen were seen in the virus-infected P2 fractions (Fig. 4B and inset). Some clusters of fairly intact convoluted membranes were also seen among the RER components in the MVE virus-infected pellet (not shown). Residual amount of mitochondria were also observed.

Ribosomes and some free nuclear material seemed to be the components of the only visible band from Extract 3 (E3/F7 - Fig. 4C) for both the uninfected and infected cell fractions. The nuclear pellet after detergent treatment is shown in Fig. 4D. Some of the nuclei were disrupted, but most were stripped of their closely associated membranes.

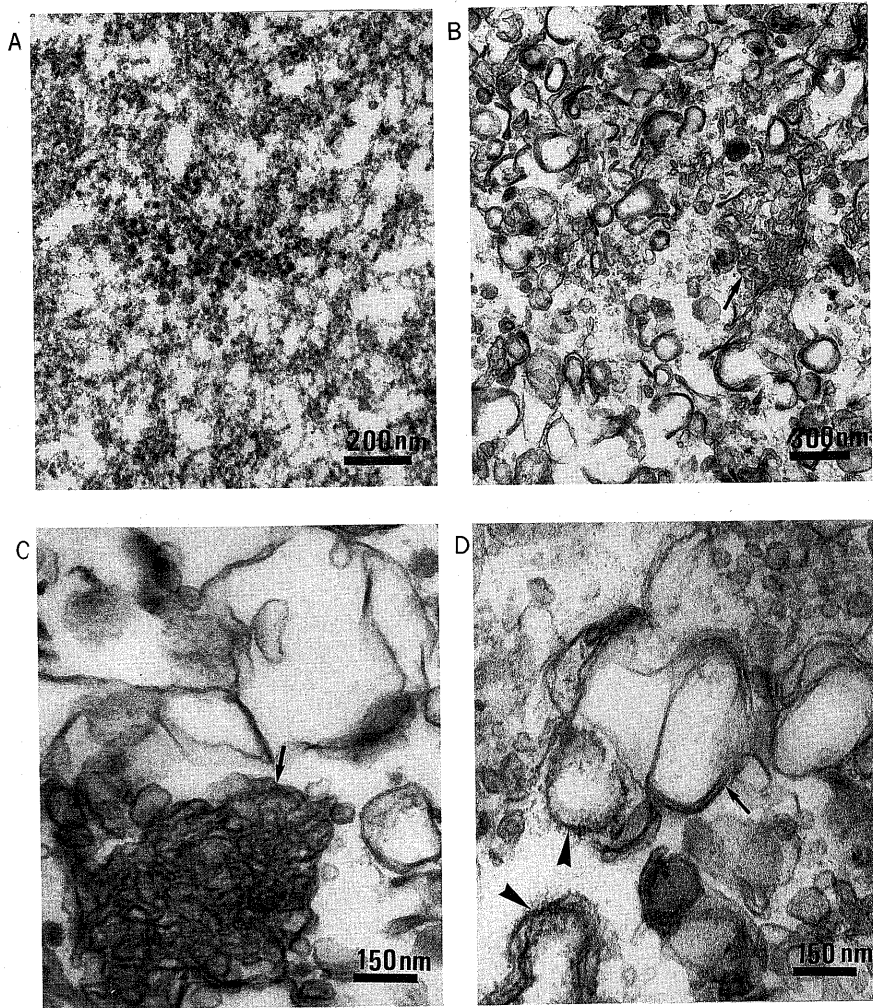


Fig. 2. Ultrastructures of membrane fractions.

- (A) Fractions E1/F1, E1/F2 and E1/F3 from uninfected Vero cells mainly consisted of ribosomes.
- (B) The E1/F1 and E1/F2 fractions of JE and MVE virus-infected cells comprise of smooth membranes. Arrow indicates membrane structures resembling the smooth endoplasmic reticulum.
- (C) In E1/F3 fraction from JE-virus-infected cells again smooth membranes are the predominant component. The arrow indicates possible clump of smooth endoplasmic reticulum.
- (D) Unlike the JE virus infection, the E1/F3 fraction of MVE virus infected cell has a mixture of smooth (arrow) and rough (arrowheads) membranes.

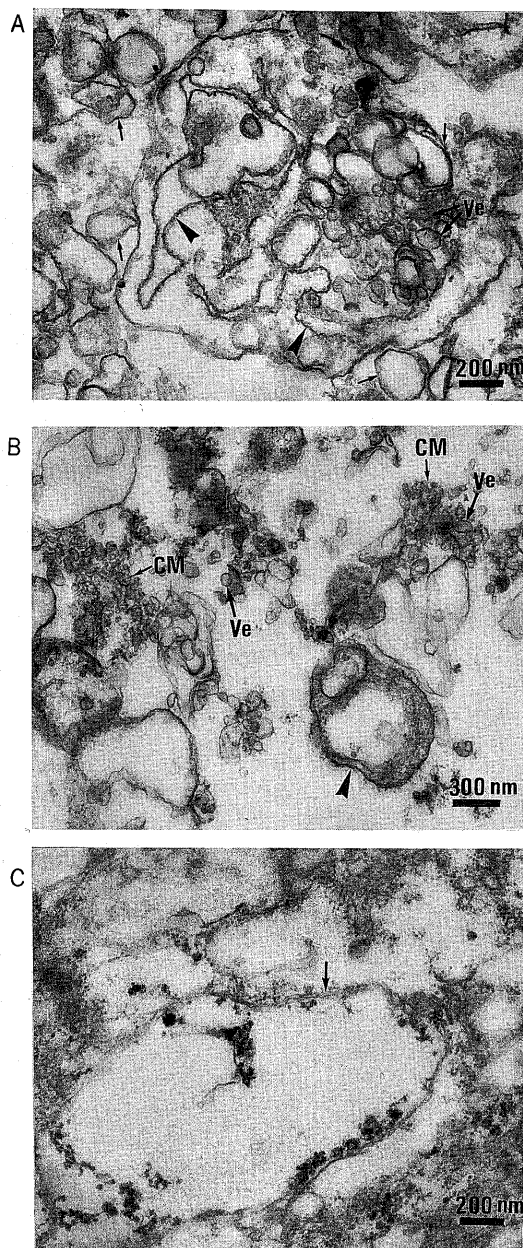


Fig. 3. Ultrastructures of the P1 pellet.

(A) The P1 fraction of JE virus-infected cells has most of the virus-induced vesicles (Ve). There are also a mixture of smooth (small arrows) and rough (arrowheads) membranes.

(B) With the P1 fraction from MVE virus-infected cells, virus-induced vesicles (Ve) are also present. Membrane structure similar to the convoluted membranes (CM) is seen together with the rough (arrowhead) membranes.

(C) In the P1 fraction of the uninfected Vero cells only the plasma membrane (arrow) and some undefined amorphous components are seen.

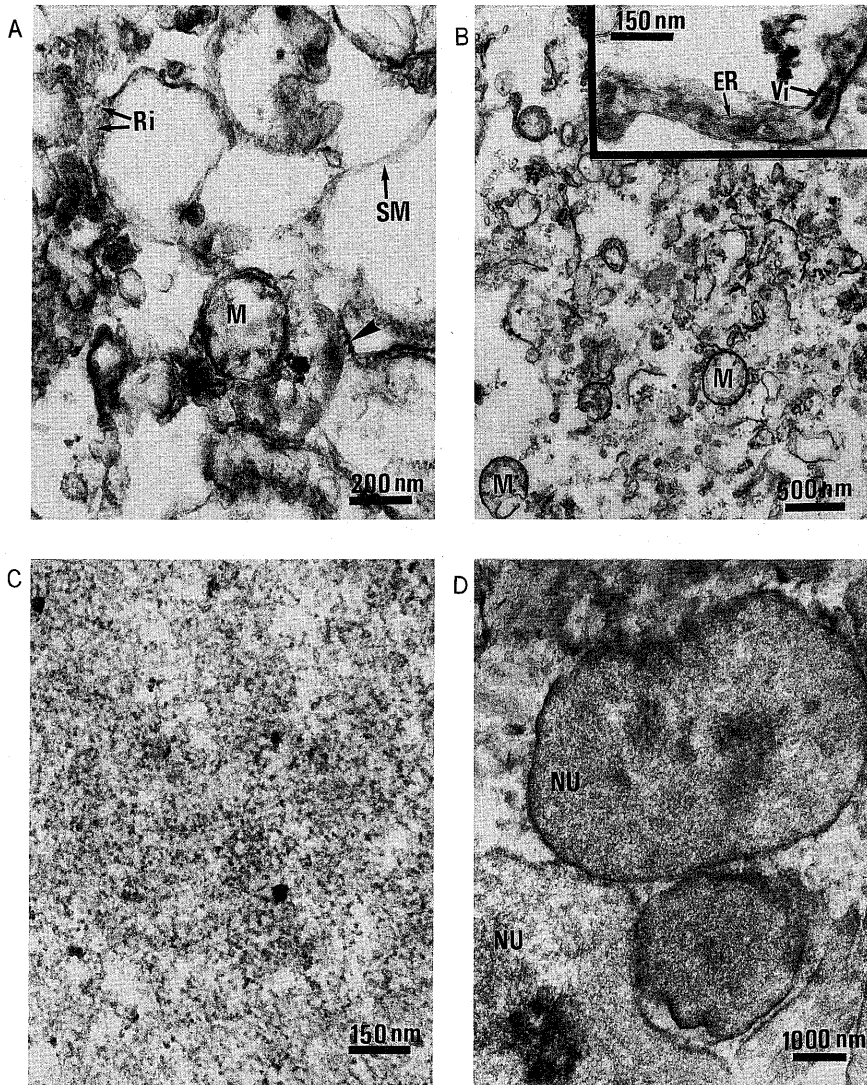


Fig. 4. Ultrastructures of the P2, E3/F7 and nuclear.

(A) The P2 fraction of the uninfected Vero cells consisted of a mixture of RER (arrowhead), ribosomes (Ri) and smooth membrane (SM). Distorted and swollen mitochondria (M) are seen scattered among these membrane structures.

(B) The membranous structures present in the P2 fraction of the infected cells were similar to the uninfected fraction. Mixture of smooth and rough membranes together with swollen mitochondria (M) are observed. In addition, remnants of endoplasmic reticulum (ER) consisting of virus (Vi) particles are found (inset).

(C) The only band from Extract 3 (E3/F7), is comprised of mostly ribosomes for the uninfected as well as the infected cells.

(D) After the detergent treatment, most of the nuclei (Nu) are seen to have lost their closely associated membranes.

Distribution of Virus-specified Proteins in the Various Membrane Fractions.

Autoradiography of viral protein bands after PAGE revealed several interesting differences between the two viruses (Fig. 5A & 5B). Three main viral proteins of JE virus, namely P92 (NS5), P72 (NS3) and GP 53 (E) were found in fractions 2, 3 and at very low level in fraction 4 (E1/F2, E1/F3 and E2/F4 respectively) but not in fractions 5, 6 and 7 (E2/F5, E2/F6 and E2/F7 respectively). The E1 pellet (P1) showed strong virus-protein profiles, with decreasing intensity in the E2 pellet (P2). Only residual amounts of these virus-specified proteins were observed in the nuclear pellet after the detergent and deoxycholate treatment. In contrast, most of the different fractions from the MVE virus-infected cells contained three main virus-specified proteins, namely, P98 (NS5), P67 (NS3) and GP 56 (E), although in varying quantities judging from their intensities (Fig. 5B). Both E1 and E2 pellets (P1 and P2) contained substantial amounts of virus-specified proteins. The nuclear pellet (NP) also contained some residual virus-specified proteins. Of all the virus-specified proteins, NS5 appeared in higher concentration in the smooth membranes of E1/F2 and E1/F3 fractions for both JE and MVE virus-infected specimens (Fig. 5A & 5B).

Distribution of Virus-specified Glycoproteins in the Various Membrane Fractions

There are three recognisable glycoproteins in the flavivirus-infected Vero cells. They are E, NS1 and PrM. The glycoproteins E and NS1 (GP53 and GP47 respectively for JE virus; GP56 and GP48 respectively for MVE virus) appeared in fractions 2 (E1/F2) and 3 (E1/F3) and the E1 and E2 pellets (Fig. 5c & 5D). Surprisingly, the third glycoprotein PrM (GP19), which is the precursor of the M protein in extracellular virus, was seen only in the E1 pellet and unfractionated cells (lanes J or M) when labelled with either ^3H -glucosamine or ^{35}S -methionine.

DISCUSSION

Cell fractionation technique was used as a means to correlate the different membrane structures with the presence of the virus proteins. It was found that JE virus-specified proteins (Fig. 5A) were observed to be associated with the membrane structures from fractions E1/F2, E1/F3, E2/F4 and E1 pellet (P1). These fractions consisted predominantly of smooth membranes (Fig. 2B & 2C) except for the E1 pellet (Fig. 3A). This pellet is made up of a mixture of the RER, SER, ribosomes and virus-induced vesicles. The observation was consistent with the study done by Kos and co-workers (1975). They reported that JE virus-specified proteins were found in both smooth and rough membrane fractions from infected chick embryo fibroblast cells. However other studies by several workers (Stohman *et al.*, 1975; Boulton and Westaway, 1976; Westaway and Ng, 1980) have shown that the synthesis of flavivirus-specified proteins was preferentially associated with the RER.

The largest protein, NS5 (P92) was consistently found to be in the highest concentration in the smooth membrane fractions (E1/F2, E1/F3 and E2/F4). Its possible role in RNA polymerase activity has been implicated by Rice and co-workers (1985) and Chu and Westaway (1985 & 1987).

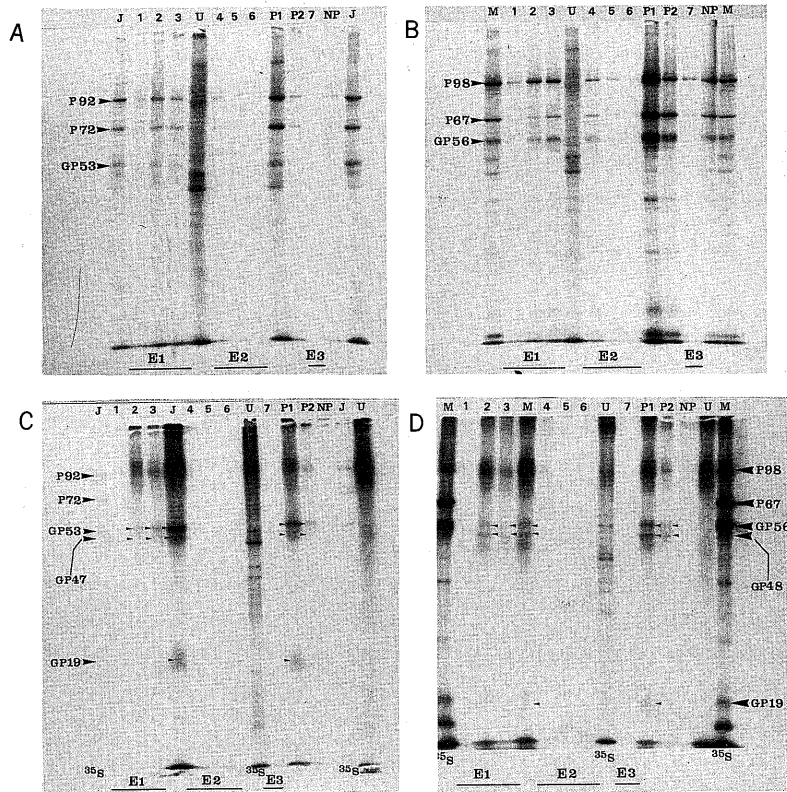


Fig. 5. Distribution of virus-specified proteins and glycoproteins in the membrane fractions.

The various membrane fractions isolated after ultracentrifugation were analysed by polyacrylamide gel electrophoresis. The virus-specified proteins and glycoproteins were labelled by ^{35}S -methionine (A and B) and by ^{35}S -glucosamine (C and D) and were separated in 11% polyacrylamide gels.

(A) From the fractions obtained in the JE virus-infected cells, three prominent virus-specified proteins NS5 (P92), NS3 (P72) and E (GP53) are found in fractions E1/F2 (Lane 2), E1/F3 (Lane 3), P1 and faintly in P2.

(B) With the fractions from MVE virus-infected cells, the NS5 (P98), NS3 (P67) and E (GP56) proteins seemed to be present in most of the fractions albeit at varying quantities.

(C) The JE virus glycoproteins, E (GP53), NS1 (GP47) and PrM (GP19) are observed in the unfractionated infected cells (Lane J) and in the P1 fraction (Extract 1 pellet). In E1/F2 (Lane 2), E1/F3 (Lane 3) and P2 (Extract 2 pellet) fractions only E (GP 52) and NS1 (GP47) are found.

(D) The observation in the MVE virus-infected cells is similar to (C). The glycoproteins E (GP56), NS1 (GP48) were found in P1, P2 and fractions 2 and 3. In contrast PrM (GP19) is seen only in the P1 fraction and the unfractionated cells (Lane M), and not in fractions E1/F2 (Lane 2), E1/F3 (Lane 3) and P2.

Lanes 1 to 7 represent the different fractions obtained after ultracentrifugation. P1 and P2 indicate pellets obtained from Extract 1 and 2 respectively. NP represents nuclear pellet after detergent treatment. U denotes unfractionated whole cell (unfractionated). J and M represent JE virus- and MVE virus-infected cells respectively (unfractionated). The ^{35}S -methionine labelled samples as protein controls (in C and D) are depicted at the bottom of the respective lanes.

Analysis of the P1 fraction from JE virus-infected Vero cells indicated that the three major viral proteins were present in similar quantities (judging from the intensities of the autoradiogram - Fig. 5A). The barely visible band of the envelope protein, E, seen in the protein profile of the E2 pellet (P2) fraction was somewhat surprising considering the presence of rows of virus still enclosed within the RER (Fig. 4B inset). Unlike the P2 fraction from JE virus-infected cells, the P2 fraction of MVE virus-infected cells had strong association with the viral proteins. The association of the MVE viral proteins with the membrane around the perinuclear region (E3/F7) was also not surprising. Fluorescent antibody studies conducted by Ng and colleagues (1983) have shown the involvement of these membranes in Kunjin virus replication. For both JE and MVE virus infections, the viral glycoproteins were observed to be associated with the E1/F2, E1/F3 as well as the P1 and P2 pellets (Fig. 5C & D). In conclusion from this study, it appears that both smooth and rough membranes are important for the translation and accumulation of the flaviviruses JE and MVE viral proteins in Vero cells.

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日本脳炎ウイルスまたはマレー-溪谷脳炎ウイルスに感染した Vero 細胞の膜構造に存在するウイルス特異的タンパク

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日本脳炎ウイルス (JE) またはマレー-溪谷脳炎ウイルス (MEV) に感染した Vero 細胞から 3 つの分画 (E1, E2, E3) を抽出し、それぞれを不連続蔗糖密度勾配超速心法で分画したところ、E1 から 3 つのバンド (E1/F1, E1/F2, E1/F3), E2 から 3 つのバンド (E2/F4, E2/F5, E2/F6) が得られたが、E3 からは 1 つのバンド (E3/F7) だけが得られた。超速心の後、E1 と E2 から管底に得られた pellet を各々 P1 と P2 となづけた。各分画について放射線性同位元素で標識したウイルス特異的タンパクと糖タンパクを解析し電子顕微鏡による観察結果と比較した。MVE 特異的タンパクは殆ど全ての分画に存在したが、JE 特異的タンパクは平滑膜を含む E1/F2, E1/F3, E2/F4 分画に存在した。JE 及び MVC 両者共、感染細胞の E1 分画から得られた pellet (P1) に多量のウイルス特異的タンパクが存在し、ウイルス感染によって生じた膜構造とリボソームが観察された。P1 分画にはウイルス特異的タンパクの全ての種類 (E, ES1, PrM) が存在したが、E1/F2, E1/F3 及び P2 分画には E と NS1 のみ認められた。すなわち、平滑膜及び P1 分画の膜構造が JE 特異的タンパクと強く関係しており多分それらの翻訳過程に関すると推測されるが、核に極めて近接した膜構造に JE 特異的なタンパクは集積していなかった。これに対して、MBE 特異的タンパクは核周辺膜を含む殆ど全ての膜構造に存在した。従って、平滑膜、及び粗面膜共に JE 及び MVE に特異的なタンパクの翻訳と集積に重要であるとみられる。