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Selective Association of Sindbis Virion Proteins with Different Membrane Fractions of Infected Cells

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Plasma and smooth membranes obtained from chicken embryo cells infected with Sindbis virus were solubilized and subjected to electrophoresis on acrylamide gels. The electrophoretic patterns showed that (i) the major proteins synthesized and associated with plasma membranes from infected cells are virion proteins and (ii) at 4 hr after infection virion proteins are not present at detectable levels in the smooth membranes of the cell.

At present, little is known about the biochemical events associated with morphogenesis of those enveloped animal viruses which acquire their outer membrane by budding from the surface of the infected cell (arboviruses, myxo- and paramyxoviruses, rhabdoviruses, and C-type tumor viruses). The events in viral morphogenesis have been determined primarily by electron microscopy examination of infected cells. Morphogenesis appears to involve three distinct steps: (i) assembly of the nucleocapsid, (ii) association of the nucleocapsid with the cell membrane, and (iii) budding and release of virions as enveloped nucleocapsids. Little is known about the nature and specificity of the association of virion proteins with cell membranes. Holland and Kiehn (13) demonstrated that the structural proteins of influenza virus associated with several different membrane classes in the infected cell. Similarly, Spear and co-workers (23) demonstrated that herpesvirus structural proteins were detected in both the internal and plasma membrane fractions obtained from infected cells. Morphological studies indicate that group A arboviruses preferentially mature at the plasma membrane (1). We were interested in determining if arbovirus virion proteins show differential affinities for different membrane classes. This paper reports the specific association of envelope protein with the plasma membrane of chicken embryo (CE) cells infected with Sindbis virus.

MATERIALS AND METHODS

Virus strains. The large-plaque variant of Sindbis virus was isolated from a stock culture of strain Ar339 by repeated single-plaque passages. Virus stocks were grown in 18-hr-old monolayer cultures of CE cells in Eagle medium (6) with twice the original amino acid concentration. Virus was quantitated in monolayer cultures of CE cells by a plaque assay.

Virus purification and nucleocapsid isolation. To prepare amino acid-labeled virus, CE cultures were treated for 2 hr with Eagle medium containing 0.025 the usual amino acid concentration and 5 μg of actinomycin D (Mann Research Laboratories) per ml. Cultures were infected at a multiplicity of 10, and virus was adsorbed for 1 hr and then overlaid with Eagle medium with 0.025 the normal, unlabeled amino-acid concentration containing 5 μCi of ^{14}C- or ^{3}H-labeled reconstituted protein hydrolysate per ml. Culture fluids containing virus were harvested 8 hr after infection. The virus was purified by differential centrifugation and zonal sedimentation through a 15 to 30% (w/w) linear sucrose gradient (26). Nucleocapsids were extracted from CE cells 4 hr after infection and purified as described by Sreevalsan and Allen (24).

Radioactive labeling of infected cell. Monolayer CE cultures were treated for 5 hr prior to labeling with 5 μg of actinomycin D (Mann Research Laboratories) per ml. The medium was removed and 2 ml of Eagle medium with 0.025 the usual amino acid concentration was added during the first hour of infection. A 4-ml amount of Eagle medium with 0.025 the normal, unlabeled amino acid concentration containing 3 μCi of ^{14}C-reconstituted protein hydrolysate (Schwarz) per ml was added. Cultures were incubated at 37 C for 3 additional hr. Samples containing radioactivity were precipitated in 5% trichloroacetic acid and collected by vacuum filtration through membrane filters (0.45-μm pore size). Each filter was washed twice with cold, 5% trichloroacetic acid and counted in an Ansitron liquid scintillation counter.

Cell fractionation. The medium was removed from chilled (4 C) CE monolayer cultures. The plates were washed with Eagle medium, and the cells were harvested with a rubber policeman. The cell suspension was centrifuged at 2,000 $\times$ g for 10 min at 4 C, and the cells were suspended in Eagle medium. After two washes with Eagle medium, the cells were suspended in 2.5 ml of 10^{-2} M tris(hydroxymethyl)aminomethane (Tris) and 10^{-3} M ethylenediaminetetraacetic acid (EDTA) and homogenized with 18 strokes of a

tight-fitting glass Dounce homogenizer held in an ice bath. The cell homogenate was centrifuged for 10 min at 4,000 × *g* to remove nuclei and unbroken cells. Approximately 15% of the 5′-nucleotidase activity was recovered in the pellet.

A 2-ml amount of the 4,000 × *g* supernatant fluids containing 85% of the 5′-nucleotidase activity was mixed with sucrose to bring the final concentration to 45% (w/w). This preparation was layered on top of a 2-ml, 60% sucrose cushion, and a discontinuous sucrose gradient was prepared as described by Bosmann (2). The gradient was centrifuged for 16 hr at 70,000 × *g* in a SW25.1 rotor of a Spinco model L ultracentrifuge. Fractions were collected with a syringe beginning at the top of the tube: fraction 1 of 3.5 ml, 2 of 5.5 ml, 3 of 6 ml, 4 of 4 ml, 5 of 4 ml, 6 of 6 ml, and 7 of 2 ml.

Samples were assayed for enzyme activity (5′-nucleotidase and glucose 6-phosphatase) and radioactivity. The plasma membrane- and smooth membrane-containing fractions were diluted in Tris-EDTA buffer and centrifuged at 70,000 × *g* for 2 hr. The pellet was suspended in 2 ml of Tris buffer with a pipette and centrifuged in a second discontinuous Bosmann gradient. All gradient fractions were held at 4 C in an ice bath to avoid inactivation of enzymes. The membrane fractions from the second gradient were pelleted at 70,000 × *g* for 2 hr and suspended in Tris buffer.

Enzyme assays. The enzyme 5′-nucleotidase was assayed by the method of Heppel and Hilmoe (12). Glucose 6-phosphatase was assayed by the method of Weaver and Boyle (29). Protein was assayed by the method of Lowry et al. (14). Specific activities are expressed as milligrams of inorganic orthophosphate (P_i) per hour per microgram of protein.

Polyacrylamide gel electrophoresis. Membrane fractions were solubilized by the procedure of Summers et al. (27). Sodium dodecyl sulfate (SDS) was added to the membrane fractions to a final concentration of 1 to 2%; the extracts then were acidified by the addition of 0.1 volume of glacial acetic acid. Urea was added to a final concentration of 0.5 M. After incubation at 45 C for 1 to 2 hr, the samples were dialyzed for 48 to 72 hr against 8 liters of 0.01 M sodium phosphate buffer, *p*H 7.1, containing 0.1% SDS, 0.5 M urea, and 0.1% 2-mercaptoethanol. After dialysis the samples were stored at −70 C.

Polyacrylamide gels (10%) were prepared by the technique of Ornstein (16) and Davis (4) as modified by Hay et al. (11). Electrophoresis was performed in glass tubes (0.6 by 12.5 cm) on vertical 10-cm separating columns with 0.5-ml (3.5%) stacking gels. Samples were electrophoresed with bromophenol blue marker at 8 ma/gel for 7.5 to 8 hr. After electrophoresis the gels were frozen at −70 C and sliced into 1-mm discs with a transverse gel slicer (Diversified Scientific Instruments, Inc.). Gel slices were dissolved by incubation at 37 C in 0.1 to 0.2 ml of 30% hydrogen peroxide for 24 to 36 hr. Ten milliliters of Cab-O-Sil scintillation fluid and 0.5 ml of 1 N NaOH were added to each sample, and the radioactivity was determined.

Envelope (molecular weight = 55,000 daltons) and nucleocapsid (molecular weight = 30,000 daltons) protein markers (22) were prepared by digestion of sucrose gradient-purified, ^{3}H-amino acid-labeled Sindbis virus. These were coelectrophoresed with the various membrane preparations.

RESULTS

Separation of internal and plasma membrane fractions from CE cells. The density flotation technique developed by Bosmann (2) for HeLa cells was employed to obtain preparations enriched for plasma and smooth membranes from CE cells. Plasma and smooth membranes were identified by enzyme markers and phospholipid-to-protein ratios. Figure 1A shows the distribution of glucose 6-phosphatase, an enzyme associated with the endoplasmic reticulum (2) on the first Bosmann gradient. The greatest amount of enzyme activity was concentrated in fraction 1 which is consistent with Bosmann's results with HeLa cells (2). The activity of 5′-nucleotidase, a plasma membrane enzyme marker in HeLa (20, 29) and in CE cells (18), was highest in fraction 7 (Fig. 1B). The plasma membrane fraction then was pelleted and subjected to a second gradient centrifugation. After the second centrifugation, the 5′-nucleotidase activity was detected in the same position and glucose 6-phosphatase activity was not detected in fraction 7. Consistent with Bosmann's results with HeLa cells, the lipid-protein ratios in these two membrane classes also were different. The ratio of lipid-to-protein in the plasma membrane fraction (S7) was one-eighth of the ratio found in the smooth membrane fraction (S1). Although this membrane fractionation procedure does not yield pure membrane preparations, it does yield fractions enriched in one membrane type while depleted in the other. This is evidenced by the observed lipid-to-protein enrichment and by the depletion of marker enzymes associated with the two membrane species.

The removal of extracellular virus from the membrane fractions obtained from infected cells during the purification procedure is shown in Table 1. At 4 hr after infection, the culture fluid contained 10^7 plaque-forming units/ml and the intracellular virus concentration was less than 1 plaque-forming unit/cell. In four experiments, between 30 to 90% of the infectious virus, together with nuclei and unbroken cells, was removed from the cell homogenate by centrifugation at 4,000 × *g*. Because only 15% of the 5′-nucleotidase activity pelleted at 4,000 × *g*, the removal of infectious virus was not thought to be the result of attachment of virions to plasma membrane vesicles. The removal of infectious virus from the cell homogenate by centrifugation at 4,000 × *g* may reflect inactivation of virus in

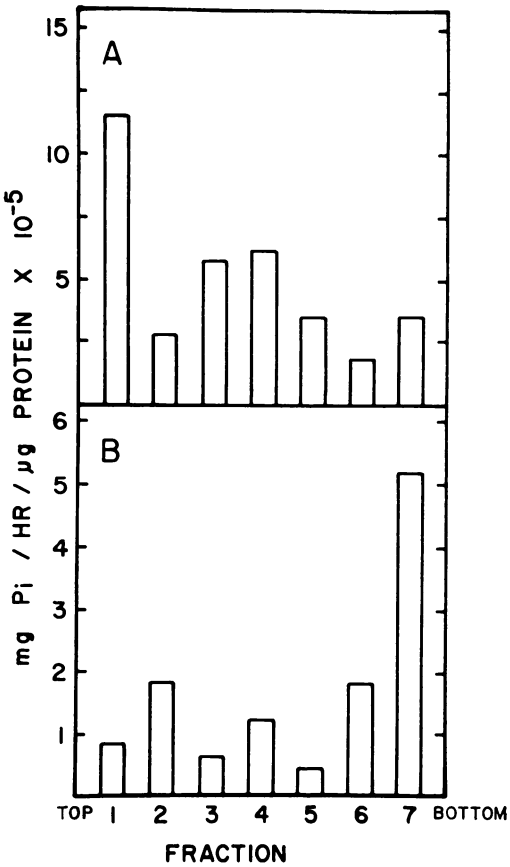

FIG. 1. *Separation of plasma and smooth membranes from uninfected chick embryo cells on the first Bosmann sucrose gradient. Cells were suspended in 10^{-2} M tris(hydroxymethyl)aminomethane and 10^{-3} M ethylenediaminetetraacetic acid and homogenized with a glass Dounce homogenizer. The cell homogenate was centrifuged for 10 min at 4,000 × g to remove nuclei. The supernatant fluids containing the cellular membranes were mixed with sucrose to bring the final concentration to 45% (w/w). This preparation was placed in the bottom of cellulose nitrate tubes and a discontinuous sucrose gradient was prepared as described by Bosmann. The gradients were centrifuged for 16 hr at 70,000 × g in a Spinco SW25.1 rotor. Sedimentation was from left to right. Fractions of unequal volume were collected and enzyme activities were measured in each fraction. (A) Distribution of glucose 6-phosphatase activity, an internal membrane marker. (B) Distribution of 5'-nucleotidase activity, an enzyme associated with the plasma membrane.*

the cell homogenate and physical entrapment. The plasma membrane fraction used in the electrophoresis studies contained less than 0.1% of the initial virus. The removal of free viral nucleocapsids from the membrane preparation also was monitored. A purified, ^{3}H-uridine-labeled nucleocapsid preparation was mixed with unlabeled, infected cell homogenates (4000 × *g* supernatant fluid). As indicated in Table 1, the plasma membrane fraction contained less than 1% of the added, labeled nucleocapsid preparation.

Proteins incorporated into the plasma membrane of Sindbis virus-infected cells. Actinomycin D-treated CE cells were infected at a multiplicity of 50 plaque-forming units, and the cells were labeled with ^{14}C-amino acids from 1 through 4 hr after infection. The cells were harvested, and the plasma membrane was partially purified by two discontinuous sucrose gradients. In Figure 2 the polypeptide composition of the plasma membrane from Sindbis-infected, actinomycin D-treated cells is compared with the polypeptide composition of membrane from uninfected actinomycin D-treated cells. The major peaks in the plasma membrane obtained from virus-infected cells corresponded to the Sindbis envelope and nucleocapsid marker proteins. The ratio of radioactivity in the envelope peak to that in the nucleocapsid peak was 3:1. The other major peak of membrane-associated protein electrophoresed more slowly than either viral structural protein. This pattern is similar to that seen in extracts of whole-cell extracts of Sindbis-infected CE cells (22, 25). In agreement with the results of Strauss and coworkers (25) with whole-cell extracts, we found that actinomycin D greatly reduces the appearance of radioactively labeled proteins in uninfected cells. The major proteins in the plasma membrane fraction of Sindbis-infected cells do not correspond to significant protein peaks from the uninfected plasma membrane fraction.

Because the ratio of envelope-to-nucleocapsid protein in the virus-infected plasma membrane fraction was the same as in the virion (26), we had to consider the possibility that the protein was contained in newly synthesized virions attached to the plasma membrane fragments. To demonstrate that the viral structural proteins in the membrane fraction were not due to contaminating intact virions, mixing experiments were performed. Uninfected, unlabeled CE cells were mixed with purified ^{14}C-amino acid-labeled virus and homogenized, and the plasma membrane fractions were purified. In parallel, plasma membrane fractions from infected cells labeled with ^{14}C-amino acids were purified. The viral infectivity associated with the membrane fractions in the major steps of the purification procedure is shown in Table 2. The specific activity and amount of infectious virus in these parallel studies were comparable. A comparison of the electrophoretic profiles of each plasma membrane preparation (Fig. 3) showed that only 17% of the total radioactivity found in the envelope peak of the

TABLE 1. *Removal of free virion and nucleocapsids from the plasma membrane fraction during the course of membrane purification*

Fraction	PFU/fraction[a]	Per cent initial PFU	Labeled[b] nucleocapsid fraction	Per cent initial radioactivity
Cell homogenate	$2,300 \times 10^5$	100		
$4,000 \times g$ Supernatant fluids	210×10^5	10	80×10^3	100
First gradient				
1	1.7×10^5	0.074	3.5×10^3	4.4
2	5.5×10^5	0.24	4.9×10^3	6.1
3	12.0×10^5	0.57	9.0×10^3	11.2
4	14.0×10^5	0.61	4.0×10^3	5.0
5	12.0×10^5	0.57	7.2×10^3	9.0
6	16.0×10^5	0.70	10.2×10^3	12.8
7	13.0×10^5	0.56	4.6×10^3	5.8
Second gradient (fraction 7)				
1	0.4×10^5	0.017	0.7×10^3	0.88
2	2.7×10^5	0.12	0.6×10^3	0.75
3	3.1×10^5	0.13	0.6×10^3	0.75
4	2.1×10^5	0.09	0.6×10^3	0.75
5	4.1×10^5	0.18	0.8×10^3	1.0
6	3.0×10^5	0.13	1.0×10^3	1.2
7	2.4×10^5	0.10	0.4×10^3	0.5

[a] PFU = plaque-forming units.

[b] Sucrose gradient-purified ^{3}H-uridine-labeled Sindbis nucleocapsids were mixed with unlabeled, infected-cell 4,000 × *g* supernatant fluids.

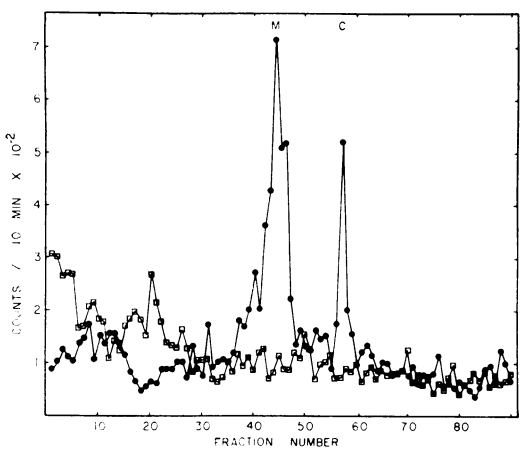

FIG. 2. *Electrophoretic profiles of the proteins of the plasma membrane fraction obtained from uninfected* (□) *and infected* (●) *actinomycin D-treated cells. In both studies cell cultures were pretreated with actinomycin D (5 μg/ml) for 5 hr before labeling for 3 hr with ^{14}C-amino acids. Actinomycin D-treated cells were infected at a multiplicity of 50 plaque-forming units and the cells were labeled with ^{14}C-amino acids from one through four hr after infection. Nucleocapsid and envelope proteins were identified by coelectrophoresis with ^{3}H-amino acid-labeled markers.*

infected plasma membrane profile could be accounted for by contaminating mature virions.

A reciprocal mixing experiment also was conducted by adding 3.2×10^5 counts/min of ^{14}C-amino acid-labeled Sindbis virus to infected, unlabeled cells. As indicated in Table 3, membranes from infected cells failed to adsorb the virus irreversibly.

Infection of CE cells by Semliki Forest virus or Sindbis virus leads to the formation of unique cytoplasmic vacuoles early in the logarithmic phase of virus replication (9). These vacuoles are involved in viral synthesis and maturation. These cytoplasmic vacuoles were concentrated in fraction 5 in the first Bosmann gradient. Because these cytoplasmic vacuoles are sites for viral ribonucleic acid synthesis (9), their position in the discontinuous sucrose gradient was identified in membrane fractions obtained from cells pulsed for 15 min with ^{3}H-uridine. This fraction was devoid of 5′-nucleotidase activity but did contain virion structural proteins. To demonstrate that the plasma membrane fractions were not contaminated with these virus-specific cytoplasmic vacuoles, an alternate membrane separation procedure was employed (20). Infected cells were homogenized in isotonic sucrose containing 1 mM EDTA and centrifuged at 800 × *g* for 15 min to remove whole cells and nuclei. The supernatant fluids then were centrifuged at 2,000 × *g* for 15 min. Although 75% of the 5′-nucleotidase activity pellets at this speed, the small cytoplasmic vacuoles remain in the supernatant fluids (7). Mature virions and free nucleocapsids also remain in the

TABLE 2. *Removal of free virus from the membrane fraction during the purification procedure in the mixing experiment*

Source of membrane fraction	4,000 × *g* Supernatant fluids (PFU/ml)[a]	Plasma membrane fraction, 1st gradient (PFU/ml)	Plasma membrane fraction, 2nd gradient (PFU/ml)	Specific activity of virus (counts per min per PFU)
Infected cells	1.35×10^8	2.7×10^6	1.96×10^5	1.59×10^{-2}
Uninfected cells with added labeled virus	4.2×10^7	5.0×10^5	2.9×10^5	1.02×10^{-2}

[a] PFU = plaque-forming units.

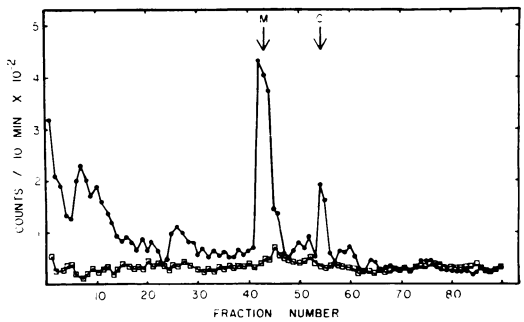

FIG. 3. *Electrophoretic profiles of the plasma membrane fraction of infected, actinomycin D-treated cells (●) and uninfected, unlabeled chick embryo plasma membrane fraction mixed with labeled Sindbis virus (□). Uninfected, unlabeled chick embryo cells were mixed with sucrose gradient-purified ^{14}C-amino acid-labeled virus, homogenized, and the plasma membrane fraction separated from the homogenate by the Bosmann procedure. In parallel, plasma membrane fractions obtained from infected cells labeled from 1 to 4 hr after infection were purified. Both membrane preparations were digested and coelectrophoresed with Sindbis membrane and nucleocapsid protein markers.*

TABLE 3. *Exclusion of added labeled Sindbis from preinfected cells*[a]

Fraction	Counts per min of acid-insoluble ^{14}C-labeled virus	Per cent
Inoculum of superinfecting virus to preinfected cells	3.2×10^5	100
Cell-associated virus after two consecutive low-speed centrifugations	1.2×10^4	3.7
4,000 × *g* Supernatant fluid	8.5×10^3	2.6
First Bosmann gradient		
Fraction 1	1.9×10^3	0.59
2	1.3×10^3	0.41
3	0.7×10^3	0.22
4	1.0×10^3	0.31
5	0.7×10^3	0.22
6	0.5×10^3	0.16
7	0.1×10^3	0.03
Second gradient		
Fraction 7	0	0

[a] Sucrose gradient-purified, amino acid-labeled Sindbis virus was mixed with unlabeled cells infected with Sindbis virus for 4 hr.

supernatant fluids. The pelleted membrane fraction was suspended and purified by two consecutive Bosmann gradients. The ratio of envelope-to-nucleocapsid protein in the membrane fractions obtained by this alternate purification procedure was also 3:1. This provided further evidence that the virion protein associated with the membranes of infected cells is not due to contaminating free nucleocapsids or virions.

Proteins of the internal membrane in Sindbis-infected cells. To determine whether the membrane protein of Sindbis virus is associated with the internal membrane fraction (fraction 1) obtained from infected cells, the polypeptide composition of internal membranes from Sindbis-infected, actinomycin D-treated, and uninfected actinomycin D-treated cells was analyzed. As indicated in Fig. 4, viral structural protein could not be detected in the internal cell membranes 4 hr after virus infection. Although virion protein was the major protein isolated with the plasma membrane of infected cells, envelope protein could not be detected in the internal membrane of the same cells. The absence of Sindbis virion proteins in the internal membranes is further evidence that a valid separation has been achieved. Further, the absence of virion proteins in the internal membranes suggests specificity in the association of virion protein to different classes of cell membranes.

DISCUSSION

The lipoprotein envelope of arboviruses is acquired as the nucleocapsid transverses cellular membranes. During maturation the nucleocapsid must recognize those regions of the infected cell membrane that contain viral envelope protein because host cell proteins are not present in the mature virion (19).

Arboviruses belonging to the different antigenic groups are thought to differ in the intracellular

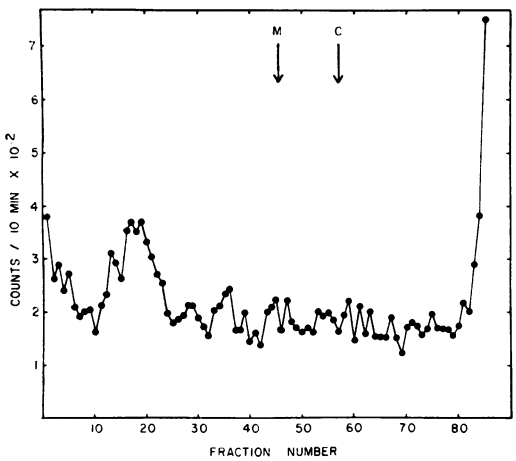

FIG. 4. *Electrophoretic profiles of the proteins of the smooth membrane fraction obtained from infected, actinomycin D-treated chick embryo cells. The smooth membrane fractions from cells infected with Sindbis virus at 4 hr after infection were digested and coelectrophoresed with membrane and nucleocapsid marker protein.*

site at which mature virions are produced (4, 15). Electron microscopy examination of porcine kidney or mosquito cells infected with Murray Valley or Japanese equine encephalitis viruses indicates that the final maturation of group B viruses takes place on the internal membranes of the cell (6). Electron microscopy studies of Semliki Forest virus, a group A arbovirus, suggest that the final stage of assembly occurs primarily at the plasma membrane (1).

These earlier reports led us to examine whether virion proteins of Sindbis virus selectively associate with different membrane classes in the infected cell. To test this hypothesis, we isolated fractions enriched in either plasma membrane or smooth internal membrane from infected CE cells. We examined these two membrane fractions because they could be distinguished by enzyme markers and lipid-protein ratios and because Sindbis is known to mature at the plasma membrane. Although the membrane separation procedure did not yield pure membrane preparations, it did yield fractions enriched in one membrane type but depleted in the other type.

The envelope protein of the Sindbis virion was the major newly synthesized protein associated with the plasma membrane of CE cells 4 hr after infection. The presence of envelope protein in the plasma membrane fractions obtained from infected cells suggests that the envelope protein binds to the plasma membrane, modifying the membrane prior to viral envelopment. The nature of the association of virion protein with the plasma membrane is not known. Viral structural proteins were not detected in the smooth cell membranes obtained from the same cells at 4 hr after infection. The absence of virion proteins in the smooth membranes suggests specificity in the association of virion protein with different membrane types. Although envelope protein was not detected 4 hr after infection, smooth membrane fractions obtained from cells late in infection may contain detectable levels of envelope protein. Scheele and Pfefferkorn (21) reported that envelope protein synthesized early during Sindbis infection has a greater probability of being incorporated into virions than envelope protein synthesized later in the infectious cycle. They postulated that much of the envelope protein synthesized later becomes associated with intracellular membranes not destined for viral maturation.

In contrast to our studies, Holland and Kiehn (13) reported that the structural proteins of influenza virus were associated with plasma, microsomal, and mitochondrial membrane fractions. Spear and co-workers (23) also reported that herpesvirus structural proteins could be detected in the smooth and plasma membranes obtained from herpesvirus-infected cells. Cohen and coworkers (3) recently reported that structural proteins of vesicular stomatitis virus, obtained from soluble extracts, associated in vitro at 0 C with the plasma membrane fraction obtained from uninfected HeLa cells. They concluded that recovery of virion proteins in cell membranes or sedimentable fractions does not constitute evidence that virus proteins are inserted into cellular membranes in vivo. Soluble virion proteins may adsorb to membranes during cell fractionation and membrane isolation. In the case of arboviruses, virion proteins are not present in detectable levels in soluble cell extracts (8).

The plasma membrane fractions obtained from infected cells also had nucleocapsid protein associated with them. Mixing experiments indicated that the presence of nucleocapsid protein cannot be explained on the basis of contamination of the membrane fraction with free nucleocapsids or mature virions. The inability to obtain plasma membrane fractions free of nucleocapsid protein suggests that the nucleocapsid binds firmly to those regions on the membrane that contain virion protein. At 4 hr after infection, virions may be budding out at such a rate that the ratio of envelope-to-nucleocapsid protein associated with the membrane was similar to that found in mature virions. Recent X-ray diffraction studies revealing the organization of protein and lipid in the Sindbis virus indicate that the nucleocapsid protein may be directly associated with the lipid bilayer (10).

The nucleocapsid protein therefore may be considered a membrane protein. If the nucleocapsid is directly associated with the lipid bilayer in the virion, then firm attachment of nucleocapsids to cellular membranes at the site of maturation would be expected. Waite and Pfefferkorn (28) have shown that, in Sindbis-infected cells where maturation is inhibited by low-ionic-strength medium, the nucleocapsids are attached so firmly to the membranes that the association remains after sonic or freeze-thaw disruption. Viral maturation takes place after the ionic strength is raised, indicating that a firm association of nucleocapsid with membrane occurs prior to budding.

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