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Association of Vesicular Stomatitis Virus Proteins with HeLa Cell Membranes and Released Virus

LAWRENCE A. HUNT* AND DONALD F. SUMMERS

Department of Microbiology, University of Utah College of Medicine, Salt Lake City, Utah 84132

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The association of vesicular stomatitis virus proteins with intracellular and plasma membranes was examined by pulse and pulse-chase labeling of virus-infected HeLa cells with [^{35}S]methionine and separation of cell homogenates into three major membrane fractions in discontinuous sucrose gradients. The glycoprotein *G* was primarily associated with rough endoplasmic reticulum-like membranes after short radioactive pulses (2 to 4 min) but accumulated in the plasma membrane-enriched fraction and the smooth internal membrane fraction with longer pulse or chase periods. The nucleocapsid protein *N* and the matrix protein *M* accumulated in the rough endoplasmic reticulum and plasma membrane-like fractions but not in the smooth internal membrane fraction. Only a fraction (35 to 40%) of the viral protein synthesized during a short pulse in the mid-cycle of infection was apparently utilized in released virus. The newly synthesized virus proteins first appeared in released virus in the order: *M*, *N* and *L*, and *G*.

Vesicular stomatitis virus (VSV) is a rhabdovirus that matures by budding through host cell membranes (16, 27). The virion envelope contains two virus-specific proteins, the matrix protein *M* and the glycoprotein *G*, in association with cellular lipids and glycolipids (19, 26). Virus proteins *N*, *NS*, and *L* are components of the nucleocapsid core along with the viral RNA genome (26). VSV-infected cells have provided an excellent model for the maturation of membrane glycoproteins and the assembly of cellular plasma membranes on the basis of several criteria: (i) virus infection inhibits essentially all host protein synthesis shortly after infection (23); (ii) both envelope proteins become strongly associated with HeLa cell plasma membranes during infection (6, 8); (iii) these virus proteins associate with the plasma membranes with kinetics similar to those of host non-glycosylated membrane proteins and membrane glycoproteins (1, 2); and (iv) host glycosyl transferases are probably responsible for the glycosylation of the virus glycoprotein (4).

Previous studies of the association of virus-specific envelope proteins with plasma membranes were extended in this work to include the association of virus nucleocapsid and envelope polypeptides with both plasma and intracellular membranes in VSV-infected HeLa cells. In addition, the extent of utilization of intracellular VSV polypeptides in the assembly and release of virus and the order of appearance of newly synthesized virus polypeptides in released virions were examined.

MATERIALS AND METHODS

Cell, virus infection, and radioactive labeling. Suspension cultures of HeLa S3 cells were grown in Joklik-modified minimal essential medium (Flow Laboratories) supplemented with 2 mM glutamine plus 5% fetal calf serum (Flow Laboratories) at a concentration of 4×10^5 to 8×10^5 cells per ml. Stock preparations of VSV (Indiana serotype) were grown in HeLa cells, purified, and assayed as previously described (13, 23). For the short-term labeling experiments, cells were collected by centrifugation and resuspended in growth medium minus serum, supplemented with glutamine, at 10 times their previous density (4×10^6 to 8×10^6 cells per ml). Cells were infected with 10 PFU of VSV per cell, and at 1.5 h postinfection serum was added to 5% concentration. At times ranging from 3.5 to 4.0 h postinfection, when host protein synthesis was known to be fully inhibited and virus-specific macromolecular synthesis was maximal (23), cells were pulse-labeled with 10 to 20 μCi of L-[^{35}S]methionine (100 to 400 mCi/ml, New England Nuclear) per ml.

Processing of radioactively labeled cells. Cells (5- to 25-ml samples) were removed from cultures at designated intervals into centrifuge tubes containing 5 to 10 ml of Earle solution on ice. Cells were centrifuged for 2 min at 2,000 rpm at 4°C in an IEC PR-6000 centrifuge and resuspended in 3.0 ml of E-T buffer (10 mM Tris, pH 8.0-1 mM EDTA). The cells were allowed to swell for 5 min at 0°C and were then ruptured with 10 strokes of a stainless-steel Dounce homogenizer (clearance of 0.002 inch [ca. 0.005 cm]).

Nuclei were removed by centrifugation for 2 min at 1,500 rpm.

Fractionation of cell homogenates. Cellular membrane fractions were prepared by equilibrium centrifugation in discontinuous sucrose gradients using a modification of the techniques of Fleischer and Zambrano (10). Sucrose solutions were made in E-T buffer, and the percentage of sucrose is given as weight/weight solutions. The clarified cell homogenate (2.0 ml) was adjusted to a final concentration of 49% sucrose with the addition of 3 volumes of 65% sucrose and overlaid with 6 ml of 44% sucrose, 9 ml of 39% sucrose, 9 ml of 34% sucrose, and 6 ml of 29% sucrose. The discontinuous sucrose gradients were centrifuged for 16 h at 20,000 rpm at 4°C in a Beckman SW27 rotor. Fractions (1.1 ml) were collected from the bottom of the gradient with a LKB peristaltic pump and monitored continuously at 280 nm with a Gilford recording spectrophotometer. The localization of plasma membranes in the discontinuous gradient was determined with a lectin-binding assay (5) using wheat germ lectin (A grade, Calbiochem) that was labeled with [1-^{14}C]acetic anhydride (10 μCi/mmol; New England Nuclear) (21). Portions of the cell homogenate and each gradient fraction were assayed for trichloroacetic acid-insoluble radioactivity as previously described (1). Fractions corresponding to each of the peaks of radioactivity or optical density were pooled, diluted with E-T buffer, and pelleted by centrifugation for 90 min at 50,000 rpm at 4°C in a Beckman 65 or 50Ti rotors.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gel electrophoresis of ^{35}S-labeled protein from cell homogenates or cell membrane fractions was performed on 8 or 10% polyacrylamide slab gels using the Tris-glycine buffer system as previously described (11). Dried gels were subjected to autoradiography with Kodak RP Royal X-ray film. Densitometer tracings were made from the autoradiographs and integrated using an ORTEC 4310 densitometer with a digital printing integrator (Ortec Inc., Oak Ridge, Tenn.).

RESULTS

Distribution of pulse-labeled viral proteins in cellular membrane fractions. Cellular homogenates of [^{35}S]methionine pulse-labeled VSV-infected HeLa cells were separated into three major membrane fractions by equilibrium centrifugation in discontinous sucrose gradients (Fig. 1). The fractionation of total cellular membrane fragments (minus nuclei) is a relatively crude procedure that relies on differences in buoyant densities for the separation of the major classes of membranes (smooth endoplasmic reticulum [SER], rough endoplasmic reticulum [RER], and plasma membranes). For this reason, a clean separation between the membrane components was not possible and the identification of the three membrane fractions was an operational designation. The heavy-density fraction was assumed to be enriched for RER because rRNA was present in

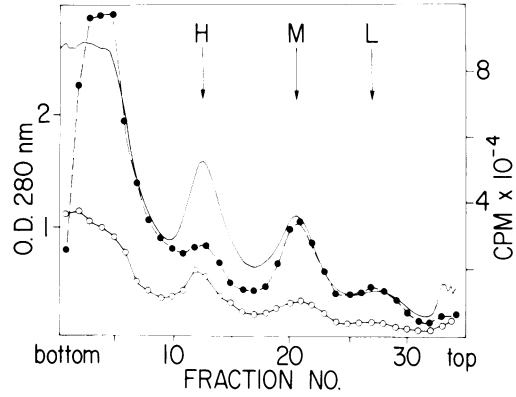

FIG. 1. *Fractionation of pulse-labeled VSV-infected HeLa cells by equilibrium centrifugation. VSV-infected cells (4 × 10^6 cells per ml) were pulse-labeled at 4 h postinfection with 15 μCi of [^{35}S]methionine per ml for 2, 4, 8, 12, 20, or 30 min. Cell homogenates were fractionated in 29 to 49% discontinuous sucrose gradients. An optical density profile* (——) *is presented with the positions of the three light-scattering bands indicated by arrows. H, M, and L correspond to the heavy, medium, and light density membranes, respectively. The heavy-density membranes were located at the 44 to 39% sucrose interface, the medium-density membranes were located at the 39 to 34% sucrose interface, and the light-density membranes were located at the 34 to 29% sucrose interface. Portions (0.1 ml) of each gradient fraction were assayed for acid-insoluble radioactivity. The profiles for the 4-min pulse sample* (○) *and the 30-min pulse sample* (●) *are presented.*

this fraction but was not detected in significant amounts in the other two membrane fractions. The medium density fraction was assumed to be enriched for plasma membranes, since approximately three-fourths of membrane-bound wheat germ agglutinin (^{14}C acetylated) co-sedimented with this fraction after incubation of intact HeLa cells with radioactive lectin. The light-density fraction was presumably enriched for intracellular membrane structures of lower density, commonly referred to as SER and "Golgi apparatus" (10). The recovery of these smooth internal membrane components in the light-density membrane fraction was expected to be the least quantitative, since the floating of membranes up from the bottom of the gradient was designed for the qualitative purification of SER and Golgi rather than the quantitative recovery of these membranes (10). Likewise, the heavy-density fraction was expected to be the most highly contaminated fraction (with non-RER membrane fragments, soluble protein, and heavy-density particulate structures such as VSV ribonucleocapsids).

The distribution of radioactive VSV-specific

proteins in the sucrose gradients (Fig. 1) indicated that after a short pulse (4 min) with [^{35}S]methionine these polypeptides were associated with the three membrane fractions in approximately the same proportion as the optical density at 280 nm. Membrane-bound radioactivity apparently accumulated in the medium-density fraction during a longer pulse-labeling period (30 min). The majority of radioactive protein remained at the bottom of the gradients in the position of soluble protein and higher-density particulate structures.

The distribution of individual virus proteins in the different cellular membrane fractions was determined by electrophoresis in polyacrylamide slab gels (Fig. 2). The analysis showed that the different species of viral protein associated differentially with the heavy-, medium-, and light-density membranes. After 2- to 4-min pulses the glycoprotein (*G*) was the major species associated with membranes, primarily with the heavy fraction and to a lesser extent with the medium fraction. *G* accumulated rapidly in the heavy and medium fraction and more slowly in the light fraction during longer pulse-labeling periods. The glycoprotein was the only virus protein that associated in large amounts with the light-density membranes. *N* and *M* accumulated in large amounts in both the heavy- and medium-density fractions during the 2- to 30-min pulse-labeling periods. Slightly more *N* was detected in the heavy fraction and more *M* was detected in the medium fraction after the 4- to 12-min pulse-labeling periods. *L* and *NS* were present in membrane fractions in relatively low amounts, associated primarily with the heavy- and medium-density membranes. The virus or host origins of the minor proteins gp140 and gp62 are presently under investigation. These proteins were designated as glycoproteins on the basis of their co-electrophoresis with glucosamine- and mannose-labeled proteins from VSV-infected HeLa cells (17). A glycoprotein similar in size to gp140 has been observed in small amounts in VSV grown in BHK-21 cells (9) and in large amounts in BHK-21 cells infected with a mutant of VSV obtained from *Drosophila melanogaster* cells persistently infected with VSV (22).

The bottom of the sucrose gradients (49 and 44% sucrose layers), which contained soluble protein and higher-density particulate structures (free nucleocapsids), was greatly enriched for *N* protein after labeling periods up to 30 min (data not shown). Small amounts of *M*, but almost no *G*, were found in this fraction. The gradient pellets contained variable amounts of protein and included small amounts of all the major viral proteins (data not shown).

Distribution of intracellular viral proteins after pulse-chase labeling. Figure 3 shows the distribution of [^{35}S]methionine-labeled polypeptides in cell homogenates and the three membrane fractions of VSV-infected cells after a 3-min pulse and 0- to 30-min chase periods. The "chase" with unlabeled methionine was effective, as demonstrated by the similarity of the gel profiles of the cell homogenates from the 0-, 10-, 15-, and 30-min chase periods.

The major ^{35}S-labeled virus component associated with cell membranes at the end of the 3-min pulse was *G*, and the majority of the glycoprotein was in the heavy fraction. ^{35}S-labeled *G* was lost from the heavy fraction during the chase period and accumulated in both the medium and light fractions. In the light-density membranes, this accumulation was most prominent during the 5- to 15-min chase period, with no significant increase observed after 20- and 30-min chase periods. In contrast, *G* continued to accumulate in the medium fraction during the 20- to 30-min chase. The minor glycoprotein species, gp140, also accumulated in small amounts in both the medium- and light-density membrane fractions and was apparently lost from the heavy-density membrane fraction.

As previously noted with the pulse-labeling studies, *M* and *N* did not associate with the light-density membranes in large amounts relative to *G*. The VSV *M* and *N* proteins accumulated in both heavy- and medium-density fractions. There was a short delay (5 to 10 min) in the association of *N* with the plasma membrane-enriched fraction (medium density) relative to *M*. Small amounts of ^{35}S-labeled *L* protein were observed in the heavy- and medium-density membranes after chase times of 10 to 30 min.

In a similar pulse-chase experiment (data not shown), the distribution of ^{35}S-labeled viral proteins after 60- or 90-min chase periods was not dramatically changed from the distribution observed after 30 min in Fig. 3: (i) pulse-labeled *G* could not be completely "chased" out of the heavy-density fraction or significantly decreased in the light-density fraction; (ii) the majority of the pulse-labeled *N* protein remained at the bottom of the discontinuous sucrose gradients; and (iii) membrane-associated *N* and *M* proteins were found predominantly in the heavy and medium fractions.

Extracellular appearance of viral proteins. A culture of VSV-infected Hela cells was pulse-labeled with [^{35}S]methionine for 10 min, incubated in the presence of excess unlabeled methionine for additional time periods of between 0 and 4 h, and separated into cellular and released-virus fractions by differential centrifu-

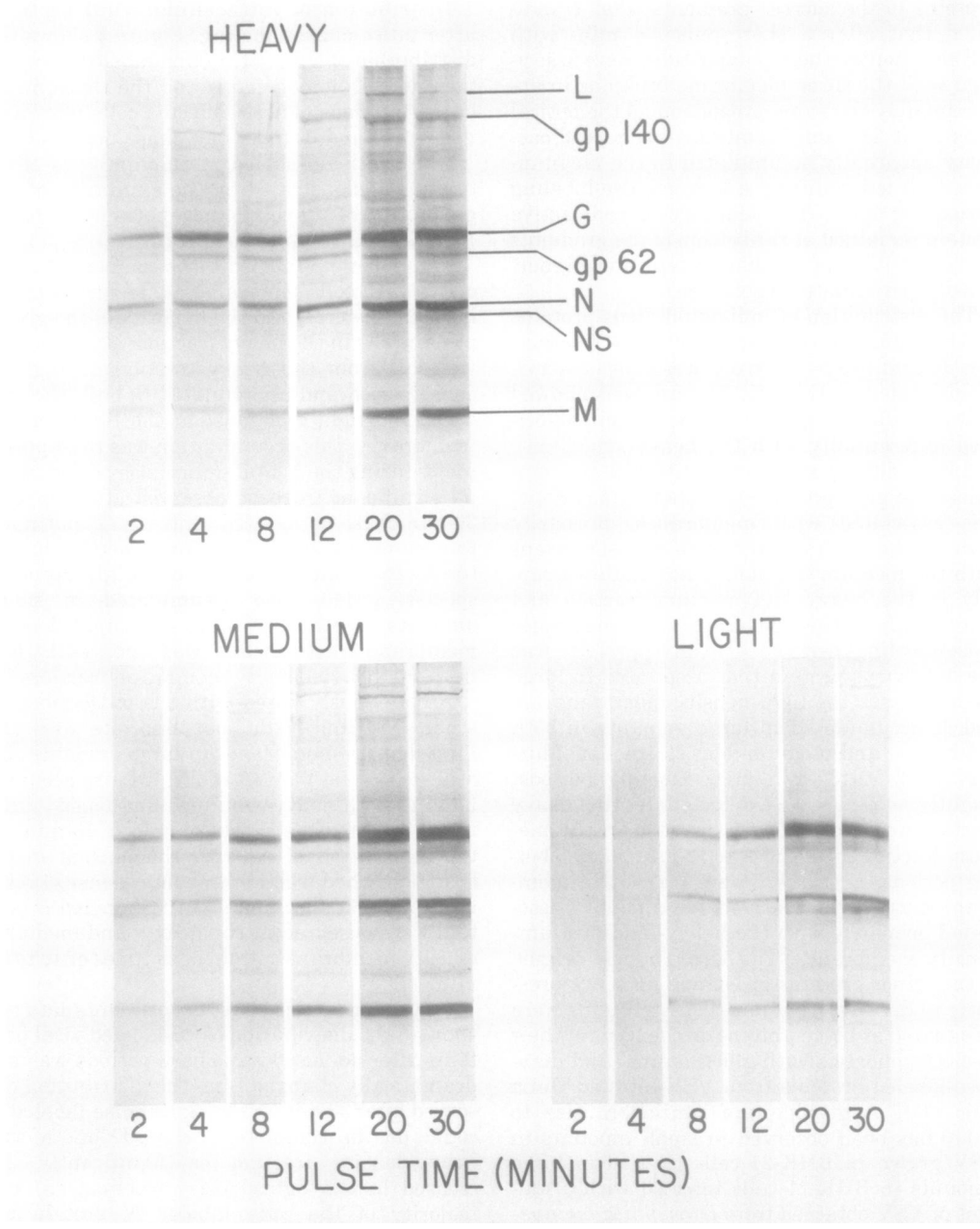

FIG. 2. *Polypeptides associated with cellular membrane fractions from pulse-labeled VSV-infected HeLa cells. Pooled fractions from the heavy-, medium-, and light-density membrane peaks in the experiment described in Fig. 1 were subjected to electrophoresis in SDS-polyacrylamide slab gels. Autoradiographs of the gels are presented. L, N, and NS are virus nucleocapsid polypeptides (26); G and M are components of the virus envelope (26). The two minor cell-associated glycoproteins were designated by gp62 and gp140 on the basis of their approximate molecular weight* ($\times 10^3$).

gation. The results (Fig. 4) indicated that the total radioactive protein (sum of released and cell-associated) remaining approximately constant during the 4-h chase period. Only 35 to 40% of the virus protein that was labeled during the 10-min pulse appeared in released virus. Most of the ^{35}S-labeled protein that appeared in released virus accumulated between

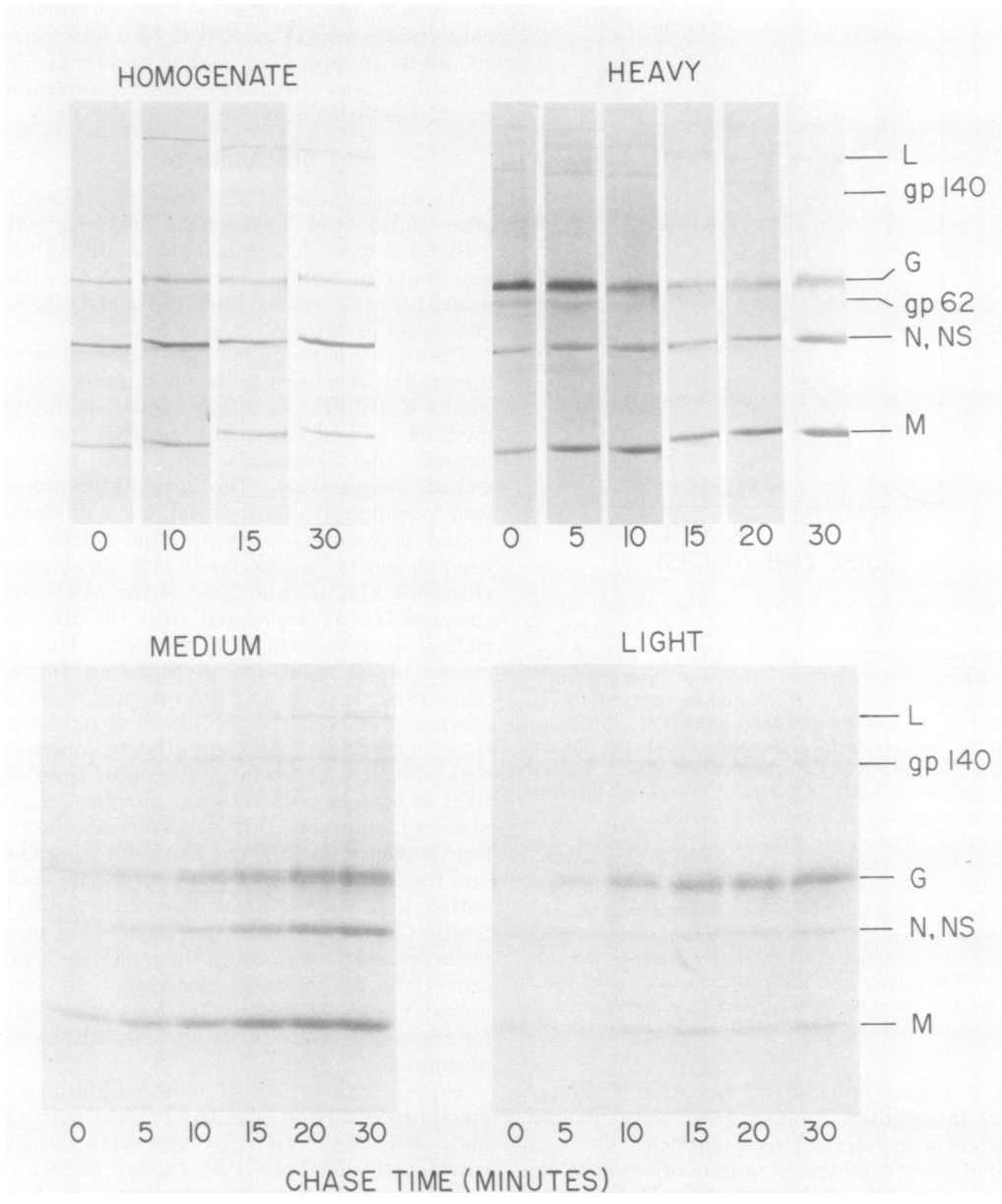

FIG. 3. *Polypeptides associated with cellular membrane fractions after pulse-chase labeling of VSV-infected HeLa cells. VSV-infected cells (8 × 10⁶ cells per ml) were pulse-labeled at 4 h postinfection with 20 μCi of [³⁵S]methionine per ml for 3 min. The cells were then incubated for an additional 0, 5, 10, 15, 20, or 30 min in the presence of excess unlabeled methionine (2 mM added to 0.1 mM in labeling medium). Heavy-, medium-, and light-density membrane fractions were obtained from the optical density peaks of discontinuous sucrose gradients. Portions of the cell homogenates and membrane fractions were subjected to electrophoresis on SDS-polyacrylamide slab gels, and the gels were subjected to autoradiography.*

1 and 2 h after the beginning of the chase period. The apparent end of the accumulation that was observed after a 3-h chase period was not an experimental artifact, since the total protein that appeared in released virus (stained in polyacrylamide gels with Coomaissie brilliant blue) steadily increased during the entire 4-h chase period (data not shown).

As shown by the polyacrylamide gel electrophoresis of the cell-associated polypeptides in

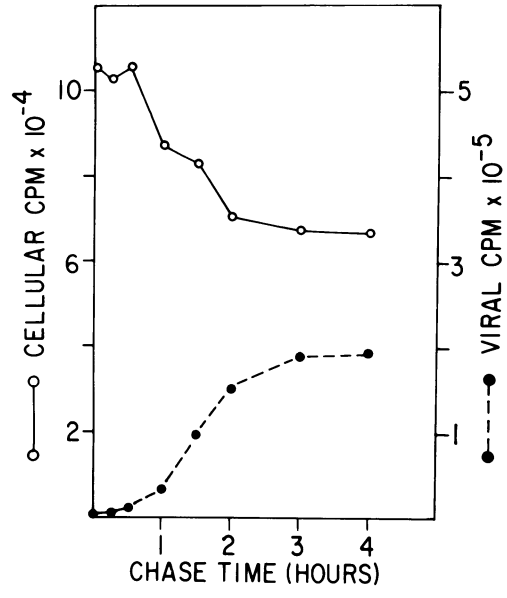

FIG. 4. *Movement of radioactive protein from VSV-infected HeLa cells to released virus during a pulse-chase experiment. VSV-infected cells (7.5 × 10⁶ cells per ml) were pulse-labeled at 3.5 h postinfection with 10 μCi of [³⁵S]methionine per ml for 10 min. The cell culture was then diluted to 5 times the original volume, and cells were incubated for an additional 0 to 4 h in the presence of excess unlabeled methionine (2 mM added to 0.1 mM in labeling medium). At the indicated chase periods (0, 1/4, 1/2, 1, 1 1/2, 2, 3, and 4 h), portions of the culture were removed and separated into cell and supernatant fractions. The homogenates and resuspended high-speed virus pellets (obtained by centrifuging the media supernate for 90 min at 40,000 rpm in a Beckman 50Ti rotor) were assayed for acid-insoluble radioactivity. The scales for the cell-associated and viral counts per minute were adjusted to correspond to equivalent fractions of the total sample.*

Fig. 5 (right), none of the species of VSV-specific, intracellular polypeptides were utilized efficiently in released virus particles. At least half of the pulse-labeled protein of each species remained associated with the HeLa cells. Newly synthesized protein of each species appeared in released virus at different times after the pulse-labeling (Fig. 5, left): (i) *M* was present in released virus at the end of the 10-min pulse; (ii) *L* and *N* were detected in small amounts by the end of the 15-min chase period; and (iii) *G* was not detected in significant amounts until the end of the 60-min chase period. The major species of protein accumulated in released virus at a nearly linear rate for approximately 2 h after different delay times (Fig. 6). The approximate chase period before the "linear" incorporation of the pulse-labeled polypeptides was estimated to be 0 min for *M*, 20 min for *N* (plus *NS*), and 45 min for *G*. The value for *L* was difficult to estimate accurately but may have been similar to the value for *N*.

DISCUSSION

The fractionation of VSV-infected HeLa cells after pulse and pulse-chase labeling with [³⁵S]methionine has indicated a differential membrane association between the VSV glycoprotein *G*, the matrix protein *M*, and the nucleocapsid proteins *N*, *L*, and *NS*.

The results of the pulse-chase labeling experiment (Fig. 3) confirmed the operational assignments of the three membrane fractions (heavy, medium, and light density) as RER-enriched, plasma membrane-enriched, and SER-enriched, respectively. The virus glycoprotein was presumably synthesized on membrane-bound polysomes, since specific mRNA has been shown to be associated with this class of ribosomes (11, 12) and most of the newly synthesized *G* was associated with the RER-enriched, heavy-density membranes. The decrease in pulse-labeled glycoprotein in this membrane fraction and the increase in pulse-labeled *G* in both the medium and light fractions during the chase period were consistent with the flow of virus glycoprotein from the RER to both smooth internal membranes and plasma membranes. The experimental distinction between the plasma membrane-enriched and the SER-enriched fractions was the observation that only *G* associated with the light fraction in large amounts relative to the other virus proteins, whereas all the major virus proteins (*N*, *M*, and *G*) associated in large amounts with the medium fraction (as expected for the assembly of virus particles at the cell plasma membrane).

The enrichment for *G* in the light-density membrane fraction was compatible with the maturation of the VSV glycoprotein in glycosyltransferase-enriched (3, 10, 14, 24), smooth internal membranes prior to the insertion of mature *G* into HeLa cell plasma membranes. Similar pulse-labeling studies with radioactive sugar precursors suggested that the addition of the distal sugar residues to the carbohydrate side chain of the VSV glycoprotein occurred primarily in the membranes of the light-density fraction (17). Wagner et al. (25) and Lafay (20) have also observed the relative enrichment for *G* protein versus the other virus protein species in light-density membrane fractions after labeling VSV-infected mouse L cells or VSV-infected chicken embryo fibroblast cells for 20 or 30 min.

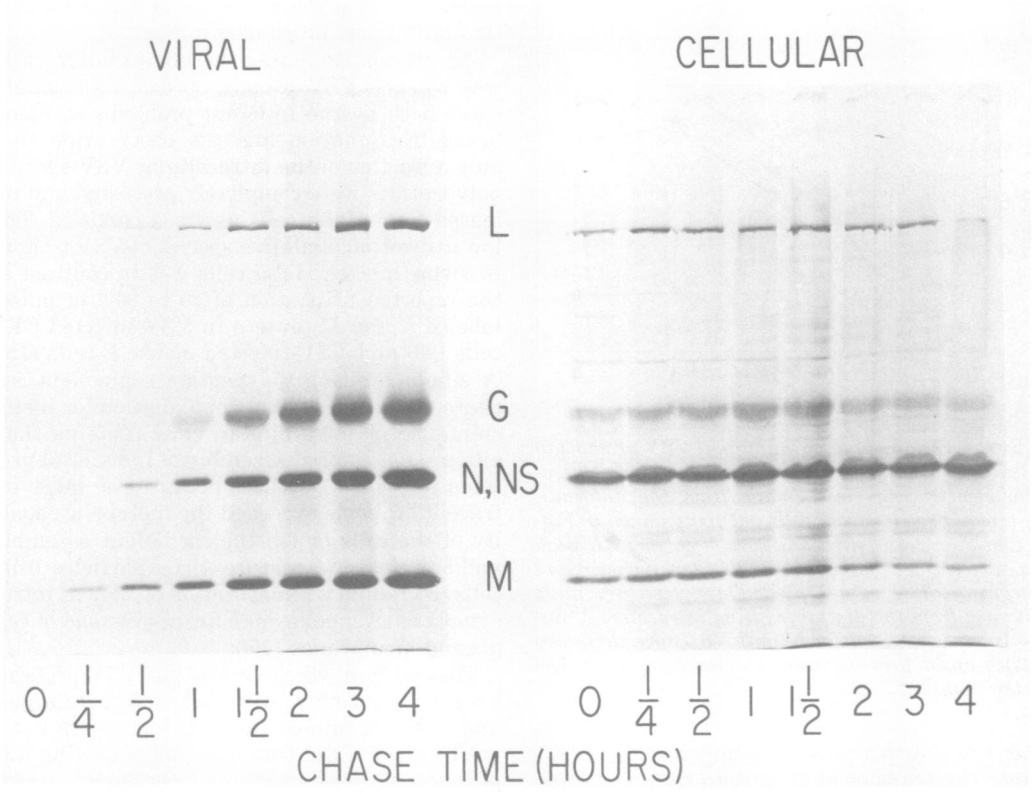

FIG. 5. *Polypeptides associated with VSV-infected HeLa cells and released virus during a pulse-chase experiment. Portions of the ^{35}S-labeled cell homogenates and resuspended virus pellets from the experiment described in the legend to Fig. 4 were subjected to electrophoresis in SDS-polyacrylamide gels. The amounts of cell-associated versus virus material do not correspond to equivalent fractions of the total sample, so that the viral and cellular slab gel autoradiographs cannot be directly compared. The apparent decrease in cell-associated L protein in the 4-h chase sample was an artifact of the gel electrophoresis (induced by the profile of Coomassie brilliant blue-stained proteins).*

However, with the chicken embryo fibroblast cells no more than one-tenth of the total cell-associated *G* protein was even detected in the lightest membrane fraction during the pulse-chase labeling period (20). The results with both of these systems could not be directly compared with our results, because their procedures apparently failed to adequately separate plasma membranes from RER and smooth internal membrane fractions.

Similar studies with influenza virus-infected chicken embryo fibroblast cells (14) suggested that the virus-specific glycoproteins were synthesized in close association with the RER and were transferred via the SER to the plasma membrane. Our results did not prove that this was the major pathway for the VSV glycoprotein in VSV-infected HeLa cells. Some of the pulse-labeled *G* protein associated immediately with the plasma membrane-enriched fraction. In addition, much of the *G* protein that associated with the light-density membranes was apparently not transferred to the plasma membrane-enriched fraction. Previous studies (2) indicated that pulse-labeled VSV glycoprotein accumulated at a slow rate in plasma membranes with no apparent delay, but accumulation occurred at a much higher rate after a 20-min lag time. These studies also indicated that the initial accumulation of *G* could have represented nonspecific association.

In contrast to the synthesis of *G* on membrane-bound polysomes, the matrix protein *M* and the nucleocapsid proteins *N*, *L*, and *NS* were apparently synthesized on free cytoplasmic polysomes (11, 12) and subsequently became associated with cellular membranes. The VSV *M* protein began to associate with the plasma membrane-enriched fraction and also with the RER-enriched fraction immediately

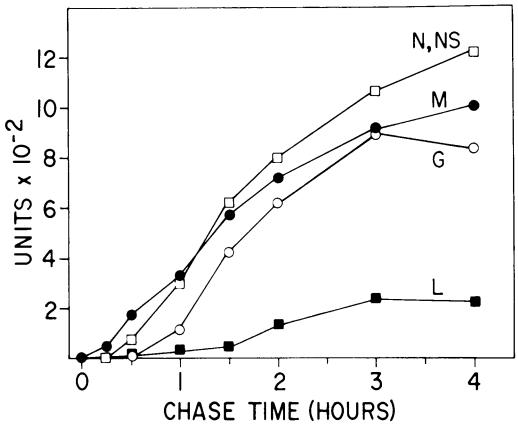

FIG. 6. *Appearance of individual VSV proteins in released virus during a pulse-chase experiment. Densitometer tracings were made from the autoradiograph of proteins from released virus shown in Fig. 5, and the relative area under each polypeptide peak was determined with an ORTEC densitometer and integrator. The values plotted are arbitrary units obtained from the digital-printing integrator. A single value was given for N and NS, since these two nucleocapsid proteins were not resolved in the gel autoradiograph.*

after the shortest pulse-labeling periods (2 or 3 min). The transfer of *M* protein from the soluble cytoplasmic fraction to the heavy-density membrane fraction was not compatible with the model of virus assembly at the plasma membranes but might have been partially caused by the contamination of the heavy fraction by plasma membrane fragments. Two other explanations are possible: (i) the affinity of *M* protein for all cell membranes either in vivo or during the homogenization process (6, 7), and (ii) the maturation of some VSV particles at cytoplasmic vacuolar membranes of HeLa cells (27).

The nucleocapsid proteins (*N*, *L*, and *NS*) also associated with both the RER and plasma membrane-enriched fractions and, in much smaller amounts, with the light-density membrane fraction. These proteins exhibited an apparent lag time of 5 to 10 min in plasma membrane association relative to the *M* protein. Cohen et al. (6) previously noted a delay in the association of *N* protein with plasma membranes, and this delay could have represented that time necessary for the nucleocapsid proteins to preassemble with the viral genome in the cytoplasm and travel to the plasma membrane. Nonspecific contamination of the heavy fraction with VSV nucleocapsids might have been responsible for the relatively high amounts of *N* protein associated with this fraction after short pulse-labeling periods.

An attempt to assess the intracellular pathway of each VSV protein species was complicated both by the inherent problems in membrane fractionation and the observation that only a fraction of the intracellular VSV-specific polypeptides were completely processed and released from HeLa cells as virus particles. The low utilization of all five species of VSV protein in virus-infected HeLa cells was in contrast to the reported utilization of 80 to 90% of pulse-labeled *L* and *M* protein in VSV-infected CEF cells (20) and VSV-infected mouse *L* cells (18). A single limiting polypeptide component appeared to be a less likely explanation for inefficient use of intracellular viral proteins than either: (i) a limited assembly of individual proteins into virus particles because the large intracellular pools exceeded the metabolic capacity of the cells or (ii) the inefficient assembly and/or release of mature virus particles from cells (including the maturation of VSV at intracytoplasmicvacuolar membranes instead of cell plasma membranes [25]).

The order in which each of the VSV proteins became associated with plasma membranes and were assembled into virus particles could not be determined from the pulse-labeling and pulse-chase experiments because large amounts of each species of virus protein were being continuously synthesized and processed in the virus-infected cells. What could be derived from these results was the minimal processing time for each VSV protein species between its biosynthesis and its appearance in free virus particles. The delay in the accumulation of newly synthesized *M*, *N*, and *L* proteins into released virus corresponded roughly to the delay in the accumulation of these VSV proteins in the plasma membrane-enriched cellular fraction. This supported an earlier suggestion (2) that the processing time between the association of VSV proteins with the plasma membrane and their appearance in released virus was minimal. The relatively long delay in the appearance of pulse-labeled glycoprotein in released virus suggested that: (i) the ^{35}S-labeled *G* that rapidly associated with the plasma membrane-like fraction was not incorporated into virus particles, and (ii) *G* may have been processed through the light-density internal membranes before it was assembled into virus particles at the plasma membranes and released from the cells. These results supported the general conclusion that the three major classes of VSV protein (glycoprotein, matrix protein, and nucleocapsid protein) were assem-

bled into mature virus particles at the host plasma membranes by separate pathways.

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