Intracellular Distribution of Input Vesicular Stomatitis Virus Proteins after Uncoating

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We have examined the fate of input viral proteins following the uncoating of vesicular stomatitis virus (VSV) by immunofluorescence microscopy, immunoelectron microscopy, and cell fractionation. VSV was adsorbed to BHK cells and allowed to become internalized in the presence of 100 mM NH₄Cl; the NH₄Cl was then removed to initiate synchronized uncoating. The three major structural proteins of VSV, the matrix protein (M), the nucleocapsid protein (N), and the glycoprotein (G), were each distributed uniquely after uncoating. Immunofluorescence microscopy showed that both G and N proteins retained a punctate distribution, whereas M protein was diffusely distributed throughout the cytoplasm, suggesting that it had become soluble. Immunoelectron microscopy showed that N protein was found in clusters (presumably in intact nucleocapsids) associated with the cell cytoskeleton and in unfused virions in endosomes and lysosomes. M protein was found diffusely distributed throughout the cytoplasm and also in endosomes and lysosomes. G protein was found only in association with endosomes and lysosomes after uncoating. Electrophoretic analysis of the high-speed cytosol fraction from infected cells showed that it contained chiefly M protein. The amount of M protein in the cytosol increased continuously during 90 min of uncoating, confirming its solubilization during uncoating. M protein was not covalently modified by phosphorylation upon uncoating, as evidenced by its mobility on nonequilibrium pH gradient gel electrophoresis. We suggest that those nucleocapsids associating with the cytoskeleton after uncoating may represent the sites of primary viral transcription.

Vesicular stomatitis virus (VSV) is an enveloped RNA virus of negative polarity. Its 11-kb genome encodes five functional proteins (1). The three major structural proteins are the membrane-embedded glycoprotein spike (G), the nucleocapsid protein (N), which complexes with the viral RNA, forming a nucleocapsid, and the matrix protein (M), which is thought to bind the nucleocapsid to the external membrane during budding. The two additional proteins encoded by VSV are the L and NS proteins, which together constitute the viral RNA polymerase (2).

The pathway traversed by VSV as it enters the cell has been well documented (16, 19, 21, 22). After binding to the cell membrane, the virus is endocytosed by the clathrin-mediated pathway and passed to the endosomal-lysosomal compartments. The low pH (5.5 to 6.5) of the endosomes activates the fusogenic activity of the G protein on the viral surface, which catalyzes fusion between the viral envelope and the endosomal membrane, transferring the nucleocapsid and the M protein to the cytoplasmic side. Viral RNA transcription and replication occur exclusively in the cytoplasm, with no involvement of the nucleus. The cytoplasmic events following the fusion reaction and leading to the start of transcription, however, have not yet been defined.

In this study we have used an NH₄Cl-induced block to create a synchronized infection of BHK cells in order to investigate the distribution of the three major VSV proteins within 1 h after uncoating. Synchronization of uncoating of VSV and other viruses with lysosomotropic amines, which raise endosomal pH and thus reversibly inhibit virus-endosome fusion, has been used successfully in several previous studies (16, 19, 20, 22). Using immunofluorescence and immunoelectron microscopy as well as biochemical isolation,

we found that the M, G, and N proteins each had distinctly different locations after uncoating: the M protein became soluble in the cytoplasm, the G protein remained in the endosomal-lysosomal compartment, and the N protein apparently became associated, as intact nucleocapsids, with the cell cytoskeleton.

MATERIALS AND METHODS

Reagents. Monoclonal antibodies (MAbs) to the proteins of VSV were the generous gifts of Douglas S. Lyles, Bowman Gray School of Medicine (anti-G and anti-M [15, 18]) and of Leo Lefrancois, Upjohn Co., Kalamazoo, Mich. (anti-N). Anti-G and anti-M MAbs were used as culture supernatants. Anti-N MAb was contained in an ascites fluid and was diluted 1:1,000 in phosphate-buffered saline (PBS) before use. [³⁵S]methionine was purchased from ICN. Cell culture materials were purchased from GIBCO Laboratories. All other reagents were of the best grade available and were generally purchased from Sigma.

Cell culture and labeling. BHK-21F cells were grown in monolayer culture in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn calf serum at 37°C in a 5% CO₂ atmosphere. All cultures were supplemented with penicillin and streptomycin and used at subconfluent densities. VSV, Indiana serotype, was grown in BHK-21F cells on semiconfluent monolayers and purified as described previously (20). Viral proteins were labeled by the addition of [³⁵S]methionine to infected BHK cells grown in Eagle's minimal essential medium (MEM) that contained 5% of the usual amount of methionine.

Infection conditions. Cell monolayers in 100-mm plates were rinsed twice in PBS with 100 mM NH₄Cl, and VSV at a multiplicity of 1,000 was adsorbed for 90 min at 37°C in the presence of 100 mM NH₄Cl. After adsorption of the virus,

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the binding medium was washed away (time zero [t_0] of infection) and replaced by DMEM with or without cycloheximide (100 $\mu\text{g}/\text{ml}$). Control cells were continually blocked in virus-endosome fusion by incubation in DMEM plus 100 mM NH_4Cl . Unless otherwise indicated, cells were incubated for 1 h at 37°C (t_1).

Immunofluorescence microscopy. BHK-21F cells were grown to semiconfluence on coverslips and infected as described above. At t_1 cells were washed twice in PBS and fixed in methanol-acetone (1:1) for 5 min at 0°C. Nonspecific MAb binding was blocked by incubating the cells at room temperature in 10% calf serum-PBS. MAbs were added to the coverslips as indicated and incubated for 30 min at room temperature. After several washes in PBS, fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (IgG) heavy- and light-chain secondary MAb (Cappel Research Products, Durham, N.C.) was added, and the coverslips were incubated for an additional 30 min at room temperature. After another wash in PBS, the coverslips were mounted in glycerol or FITC-Guard (Test-tog, Chicago, Ill.). Experimental and control cells were observed and photographed with a Zeiss Photomicroscope III equipped for epifluorescence. Within an experiment, all specimens were photographed and all photographs were printed under identical conditions. The photographs were made with Kodak Tri-X film developed with Diafine developer. The following control experiments were performed in parallel with those localizing N, M, and G proteins: the primary MAb was omitted; the primary MAb was substituted with an irrelevant one; and uninfected BHK cells were labeled with the MAbs against M, N, and G proteins.

Electron microscopy. BHK-21F cells were grown to semiconfluency on three-well Teflon-coated slides and infected as described above. At t_1 cells were fixed in 6% paraformaldehyde-0.15% glutaraldehyde-0.1 M phosphate buffer (pH 7.4) for 3 h at 4°C. Following fixation, the cells were washed with PBS and then treated with NaBH_4 (50 mg/100 ml of PBS) for 20 min at 4°C to quench free aldehydes. Cells were again washed with PBS and then permeabilized with 1.0% Triton X-100 in 0.1 M phosphate buffer for 60 s at 4°C. After washing with PBS, nonspecific MAb binding was blocked by incubating the cells in 2% normal goat serum in PBS for 2 h at 4°C. The appropriate primary MAbs were diluted in PBS-1% bovine serum albumin-0.05% Tween 20 and incubated with the cells for 8 h at 4°C. After extensive washing in PBS-Tween with several changes over 1 h, the cells were incubated with 5-nm gold-conjugated secondary MAb, anti-mouse IgG (Janssen Pharmaceutical, Piscataway, N.J.), for 8 h at 4°C. Cells were washed again and then fixed in 2.5% glutaraldehyde in 0.1 M cacodylate (pH 7.3) for 15 to 30 min at room temperature. After being washed in this buffer, the cells were postfixed in 1% OsO_4 in the same buffer for 1 h at room temperature, washed with buffer, and dehydrated through 30 and 50% ethanol. The slides were then stained en bloc with uranyl acetate (2% in 50% ethanol) for 1 h at 4°C. Dehydration was continued through 70, 85, 95, and 100% ethanol and propylene oxide, and the cells were embedded in Epon-araldite. The embedded cells were mounted on epoxy cylinders and polymerized. The sections were popped off the slide by gently rocking the epoxy cylinder after placing the polymerized slide on a 100°C hot plate for 15 s. Sections with a pale gold interference color were cut with a diamond knife, picked up onto mesh grids, and stained with 2% aqueous uranyl acetate and 2% lead citrate. Sections were examined and photographed with a JEOL 1200EX transmission electron microscope. Control experiments were performed in

parallel, substituting an irrelevant MAb, against type II collagen, in place of those directed against N, M, or G viral proteins.

SDS-PAGE. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), protein samples were electrophoresed on an SDS-PAGE system containing 12% acrylamide as described previously (14). After electrophoresis of [^{35}S]methionine-labeled proteins, the gels were stained with Coomassie blue, dried, and exposed to Kodak X-OMAT film at -80°C for autoradiography.

NEPHGE. For nonequilibrium pH gradient electrophoresis (NEPHGE), gels were made by a modification of the O'Farrell technique (26), modified as described previously (28). The conditions used for the running of the first dimension of an isoelectric focusing gel in a tube were adapted to a slab gel apparatus (12, 23). Samples were precipitated with cold 20% trichloroacetic acid, resuspended in lysis buffer (28), and run on NEPHGE gels.

Cytosol preparation. Cytosol was prepared by the method of Beckers et al. (3). Briefly, infected cells were harvested by scraping, pelleted, resuspended in 10 mM triethanolamine-150 mM KCl, pH 7.4, pelleted, washed in KCl-HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, and then homogenized with 20 strokes in a Dounce homogenizer. The homogenate was then subjected to high-speed centrifugation at $190,000 \times g$ for 75 min. The protein concentration of the resulting cytosol preparation was determined by the method of Bradford (5).

RESULTS

In the experiments to be described, VSV was adsorbed to BHK cells at high multiplicity (1,000 PFU/cell) in the presence of 100 mM NH_4Cl . This was determined by plaque assay to be the minimum concentration that completely blocked virus uncoating in these cells (data not shown). After a 90-min adsorption period, at t_0 the cells were washed and the culture medium was replaced. Cells were incubated, usually for an additional hour (until t_1), with medium containing cycloheximide (100 $\mu\text{g}/\text{ml}$). This treatment allowed normal uncoating by virus-endosome fusion to occur, while the cycloheximide ensured that no new viral proteins were synthesized. Control cells were incubated with DMEM containing 100 mM NH_4Cl (which continued to block virus fusion and uncoating) or with DMEM alone (which permitted normal uncoating and subsequent viral protein synthesis).

Phase micrographs of BHK cells at t_0 are shown in Fig. 1, row A. Some vacuolation is evident. This is a general response to incubation with lysosomotropic amines such as NH_4Cl (27). Vacuolation was somewhat more pronounced after incubation with cycloheximide for an additional hour, but no indications of virus-induced cytopathic effects were evident in any of the t_1 samples (not shown). At the end of the loading period, at t_0 , M, N, and G proteins were all distributed in a punctate pattern, consistent with their expected endosomal localization in intact virions before virus uncoating (Fig. 1, row B).

At t_1 of uncoating, differences in the distribution of the three viral proteins were seen. The most striking change was that M protein became diffusely distributed throughout the cytosol, while N and G proteins retained punctate distributions (Fig. 1, row C). This pattern of M protein staining suggested that it had become soluble in the cytoplasm. The cells at t_1 that were prevented from uncoating by continued

FIG. 1. Immunofluorescence microscopy showing the distribution of viral proteins in infected BHK cells. MAbs to VSV M, N, and G proteins were added to fixed cells following the experimental manipulations described. Rows A and B: Phase and immunofluorescence micrographs of the same cells at *t*0. At the end of the loading period, with virus uncoating blocked by NH_4Cl , all three MAbs stained the cells with a punctate pattern, indicating the expected endosomal distribution of intact virions. Row C: Cells incubated in DMEM with cycloheximide (100 $\mu\text{g}/\text{ml}$), at *t*1. Removal of the NH_4Cl block allowed uncoating to proceed. M protein was diffusely distributed throughout the cytoplasm, while N and G proteins retained a punctate distribution. Row D: Cells incubated in DMEM alone, at *t*1. Under these conditions, uncoating proceeds normally, as in row C, and is followed by viral protein synthesis. The fluorescent signal is more intense for all three proteins. M and N proteins are diffusely distributed throughout the cytoplasm, while G protein is localized in the endoplasmic reticulum and Golgi regions.

exposure to 100 mM NH_4Cl continued to demonstrate a punctate staining pattern for all viral proteins, emphasizing the dependence of virus uncoating on the acidic pH of the endosomes (data not shown). Cells rapidly recover their normal, acidic endosomal pH after removal of NH_4Cl (27).

When, after adsorption, fresh DMEM lacking NH_4Cl or cycloheximide was added to the cells, uncoating and subsequent viral transcription and translation proceeded normally; newly synthesized M and N proteins were distributed throughout the cytoplasm, whereas newly synthesized G

FIG. 2. Electron microscopy of infected BHK cells. (A) Cells were fixed with standard fixative after loading with VSV, at t_0 . Several endosomes (E) are seen, in which numerous VSV particles are visible. Magnification, $\times 20,000$. (B) Cells were lightly fixed at t_0 for immunoelectron microscopy and stained with anti-collagen type II MAb. Endosomally localized VSV is evident, with no colloidal gold labeling following treatment with this irrelevant MAb. Magnification $\times 20,000$. (C) Cells were lightly fixed at t_0 and stained with anti-N MAb. Note the endosomal localization of the colloidal gold label (arrows). Magnification, $\times 60,000$. Abbreviations: M, mitochondria; E, endosome; G, Golgi apparatus; RER, rough endoplasmic reticulum; PM, plasma membrane; N, nucleus.

protein became localized to the rough endoplasmic reticulum and the Golgi apparatus (Fig. 1, row D).

Two controls were done. The primary MAb was substituted with an irrelevant one (anti-type II collagen) or with no MAb; no staining was observed in either case. Treatment of uninfected cells with MAbs directed against the N, M, or G protein followed by the usual treatment with the secondary MAb likewise gave negative results (data not shown).

The localization of viral proteins after uncoating was examined further by electron microscopy. Cells were examined after VSV loading, at t_0 . Numerous endosomes were seen to be loaded with VSV; other cellular organelles, such as mitochondria, the Golgi apparatus, and the rough endoplasmic reticulum, were also easily discerned (Fig. 2A). Some vacuolation of the cytoplasm was seen, consistent with that observed by phase microscopy (Fig. 1, row A).

Immunoelectron microscopy of these cells required that they be lightly fixed with paraformaldehyde in order to retain antigenicity of viral epitopes. Although cellular architecture was not ideally preserved under these fixation conditions, reasonable conclusions as to the cellular localization of viral proteins could be made.

As was previously seen with immunofluorescence microscopy, all three viral proteins were found in endosomes at the end of the loading period, at t_0 . Viral particles labeled with anti-N antibody are shown in Fig. 2C. Similar observations were made with anti-M and anti-G antibodies (data not shown). On the other hand, when infected cells were stained at t_0 with an irrelevant MAb (anti-collagen type II), virtually no labeling was seen (Fig. 2B).

One hour after the initiation of fusion (t_1), differences in distribution of the three viral proteins were observed. G protein was still associated with the endosomes, as expected from its disposition in the viral membrane and from its role in facilitating virus-endosome membrane fusion. N protein, on the other hand, was distributed between two cellular sites: most remained associated with the endosome in intact, unfused viral particles, but a substantial fraction was present outside of endosomes, in large clusters. These presumably correspond to intact nucleocapsids, apparently associated with cytoskeletal structures (Fig. 3A and B). The nonendosomal portion of M protein was also distributed throughout the cytoplasm of the cell, but it was not clustered and did not seem to be associated with any particular cellular structure

FIG. 3. Immunoelectron microscopy of BHK cells at t_1 of uncoating stained with anti-N (A and B) or anti-M (C) MAbs. (A and B) Anti-N labeling showed gold particles present in large clusters (large arrows) throughout the cytoplasm, usually associated with cytoskeletal filaments (small arrows). (C) Anti-M labeling showed gold particles distributed singly or in small groups throughout the cytoplasm, not associated with cytoskeletal structures. N, Nucleus; PM, plasma membrane. Magnification: (A) $\times 40,000$; (B) $\times 40,000$; (C) $\times 57,000$.

(Fig. 3C). Infected cells stained with anti-collagen II MAb as a control showed little or no labeling (data not shown).

To obtain further information about M protein distribution, the labeled viral proteins present in the high-speed supernatant after uncoating of [35 S]methionine-labeled VSV were analyzed by SDS-PAGE. By t_1 , the amount of M and L proteins in this fraction had specifically increased (Fig. 4,

FIG. 4. Autoradiography of SDS-polyacrylamide gels of cytosolic proteins obtained from BHK cells infected with [35 S]methionine-labeled VSV. Lane 1, Purified VSV; lane 2, cytosol, t_0 ; lane 3, cytosol, t_1 of incubation with cycloheximide (uncoating).

lane 3) compared with the amounts present at t_0 (Fig. 4, lane 2). In contrast, the small amount of N protein seen at t_0 was not increased by t_1 , as shown by scanning densitometry (Fig. 5).

In order to determine the rate of appearance of M protein in the cytosol, the amount of [35 S]methionine in high-speed supernatants was determined at different times after the start of uncoating (Fig. 5). The amount of cytosolic M increased up to 90 min, when it reached a value corresponding to ca.

FIG. 5. Rate of appearance of M and N protein in the cytoplasm. [35 S]methionine-labeled VSV was adsorbed to cells and allowed to uncoat for different times, and the amount of cytosolic M was determined by scintillation counting of cytosol prepared at the indicated times (□). The amount of N was determined by scanning densitometer tracings of SDS-PAGE gels (●). Error bars represent the standard deviation of the mean from four experiments.

FIG. 6. Radioautographs of NEPHGE gels of VSV M protein. (A) Purified VSV. Due to its basic character, M protein is the only viral protein to migrate in NEPHGE. Lane 1, [35 S]methionine-labeled VSV, untreated. Lane 2, Unlabeled VSV, phosphorylated with [γ - 32 P]ATP by incubation for 1 h at 32°C in 0.1% Nonidet P-40, 50 mM Tris-acetate, 8 mM magnesium acetate, 25 μ M ATP (4). Lane 3, [35 S]methionine-labeled VSV after phosphatase treatment. VSV was incubated for 1 h at 32°C with 30 U of calf intestinal phosphatase (Promega) in 200 μ l of 0.1% Triton X-100–10 mM Tris-HCl–1 mM MgCl₂–0.1 mM ZnCl₂–50 mM KCl, pH 8.0. (B) Experiment showing that VSV M protein is not phosphorylated upon uncoating. [35 S]methionine-labeled VSV was used throughout. Lane 1, Input VSV. Lane 2, Cell extract prepared after loading VSV into cells in the presence of NH₄Cl, at t0. Lane 3, Cell extract prepared after uncoating in DMEM plus cycloheximide, at t1. Lane 4, Cell extract prepared after uncoating in DMEM alone, at t1.

13% of total cell-associated M protein, while the amount of N protein in the cytosol did not increase.

We investigated the possibility that the solubilization of M protein after uncoating could arise from a covalent modification that would alter its intrinsic solubility properties. In order to analyze the phosphorylation state of uncoated cytoplasmic M protein, NEPHGE gels were used. M protein, because of its basic isoelectric point, is the only VSV protein that penetrates this basic gel, and M protein variants differing in a single charge can be clearly separated on NEPHGE gels (12, 23). Accordingly, NEPHGE gels were run on cellular extracts obtained from cells that had been infected with [35 S]methionine-labeled VSV. The M protein present in these extracts (Fig. 6B, lanes 2 and 4) migrated identically to M protein in unfused virions (Fig. 6B, lane 1). A more slowly migrating band, corresponding to M protein phosphorylated by virus-associated kinases *in vitro*, became evident after incubation of detergent-disrupted virions with [γ - 32 P]ATP (Fig. 6A, lane 2). In contrast, a band migrating identically with untreated VSV was obtained after detergent-disrupted VSV was treated with phosphatase (Fig. 6A, lane 3). We conclude, therefore, that M protein was not phosphorylated upon uncoating, nor did it undergo any other covalent modification that would change its net charge.

DISCUSSION

It is now well established that transfer of the transcriptional complex of VSV into the cytoplasm of a newly infected cell (uncoating) occurs by fusion between a viral envelope and an endosomal membrane. The fusogenic capacity of the virion is activated by the low pH of the endosome interior. In the presence of amines such as NH₄Cl, VSV is normally internalized but is unable to fuse with the endosomal membrane because of the increase in endosomal pH mediated by the amine (16, 19, 22). Subse-

quent removal of the amine results in the prompt recovery of the normal, acidic interior of the endosome, which in turn causes a synchronized burst of virus uncoating. In this study we have examined the redistribution of VSV proteins that occurs immediately following the fusion of VSV with the endosomes of BHK-21F cells. Two novel observations were made. First, M protein became soluble in the cytoplasm of the infected cell after uncoating. Second, uncoated N protein appeared in large clusters, presumably representing intact nucleocapsids, in association with the cytoskeleton of the infected cell.

The amount of soluble cytosolic M protein continued to increase for about 90 min after the initiation of synchronized uncoating. This finding clarifies what has appeared as a problem in VSV uncoating: since M protein binds to nucleocapsids during viral assembly (24), and since M also inhibits transcription by isolated nucleocapsids (6, 11, 29), how does primary transcription begin during infection? The immunolocalization studies presented in this article show that M protein was dissociated from nucleocapsids after uncoating. It became soluble in the cytoplasm, while the nucleocapsids became associated with cytoskeletal structures.

How might this solubilization of M protein occur? We considered the possibility that its solubility might be enhanced by covalent modification upon entering the cytoplasm. Phosphorylation attaches charged, hydrophilic groups to proteins, and M protein has been shown to undergo phosphorylation under a variety of conditions (4, 9, 10). Furthermore, phosphorylation of M protein has been suggested to play a role during VSV uncoating (31). Analysis on NEPHGE gels, however, showed that the cytosolic M protein after uncoating had a mobility identical to that in the infecting virus. Since NEPHGE gels resolve M proteins differing from each other by a single charge (12, 23) (Fig. 5), this finding rules out the existence of a significant amount of postuncoating phosphorylation or of any other modification that would change the protein's net charge. The tendency of M protein to become soluble would therefore appear to be intrinsic to the unmodified protein.

We have recently characterized the interaction between M protein and the viral lipid bilayer with the hydrophobic photoreactive probe 3-(trifluoromethyl)-3-(*m*-[125 I]iodophenyl)-3H-diazirine ([125 I]TID) (17). Reaction of [125 I]TID with intact virions resulted in labeling of both G and M proteins, in the ratio of 1:0.4, indicating a substantial penetration of M protein into the bilayer of the viral envelope. This label was largely localized in the extreme N-terminal 5 to 10 amino acid residues of the protein, a region rich in both lysine and leucine residues. It was proposed that this region could form two to three turns of an amphipathic helix. If such a short structure is really all that holds M protein to the membrane, the binding energy would be expected to be minimal, and dissociation from the membrane after uncoating might occur by simple mass action (30).

The second notable observation reported here is that uncoated nucleocapsids appear to be associated with the cell cytoskeleton. Penman et al. have reported that VSV messages were translated only when associated with the cytoskeleton (7, 8). Our observations suggest that the cytoskeleton may also play a role in viral transcription. Interestingly, tubulin and/or microtubule-associated proteins have previously been shown to greatly enhance (or perhaps be required for) transcription from nucleocapsids prepared from purified virions (13, 24). The relationship between cytoskeletal association and primary viral transcription will be investigated in future experiments.

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ADDENDUM IN PROOF

In more recent experiments, no hsp70 or other chaperone-like protein was found associated with input cytosolic M protein upon immunoprecipitation of M.

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