JOURNAL OF VIROLOGY, Dec. 1992, p. 7049–7058
0022-538X/92/127049-10$02.00/0

Vol. 66, No. 12

Role of Ribosomes in Semliki Forest Virus Nucleocapsid Uncoating

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Received 8 July 1992/Accepted 8 September 1992

The mechanism by which Semliki Forest virus nucleocapsids are uncoated was analyzed in living cells and in vitro. In BHK-21 cells, uncoating occurred with virtually complete efficiency within 1 to 2 min after the nucleocapsids entered the cytoplasm. It was inhibited by monensin, which blocks nucleocapsid penetration from endosomes. As previously shown for Sindbis virus (G. Wengler and G. Wengler, Virology 134:435–442, 1984), the capsid proteins from incoming nucleocapsids became associated with ribosomes. The ribosome-bound capsid proteins were distributed throughout the cytoplasm, while the viral RNA remained associated with vacuolar membranes. Using purified nucleocapsids and ribosomes in vitro, we established that ribosomes alone were sufficient for uncoating. Their role was to release the capsid proteins from nucleocapsids and irreversibly sequester them, in a process independent of energy and translation. The process was stoichiometric rather than catalytic, with a maximum of three to six capsid proteins bound to each ribosome. More than 80% of the capsid proteins could thus be removed from the viral RNA, resulting in the formation of nucleocapsid remnants whose sedimentation coefficients progressively decreased from 140S to 80S as uncoating proceeded.

Within virus particles, the genome is present in a condensed and inactive form protected from the external environment. Upon entry into a host cell, it has to be released for replication and/or translation. The reactions that lead to such disassembly involve some of the least understood steps in the replication cycle of most viruses. In this report, we describe the mechanism of uncoating for nucleocapsids of Semliki Forest virus (SFV).

SFV is a member of the family *Togaviridae* (genus *Alphavirus*), a family of mosquito-borne enveloped viruses which can cause encephalitis and other diseases in mammals. Because of its structural simplicity and high infectivity, SFV has served as a useful model for studying viral entry into animal cells (18, 20, 21). Its single-stranded positive-sense RNA (~12 kb) is enclosed in an icosahedral nucleocapsid and surrounded by the viral envelope, which contains the spike glycoproteins E1, E2, and E3. The nucleocapsid is approximately 40 nm in diameter and has a sedimentation coefficient of about 150S (16, 31). It has been proposed to have a T=3 or T=4 icosahedral symmetry and contains either 180 or 240 copies of the viral capsid (C) protein (33 kDa) (3, 6, 7). X-ray crystallography of Sindbis virus, an alphavirus closely related to SFV, has revealed that the C protein differs from other known viral C proteins in having a structure similar to that of chymotrypsin (3). The shell formed by C proteins around the viral RNA has holes, so that the RNA is accessible to small molecules such as RNase (1, 16). However, these holes are insufficient for the viral RNA to be accessed and translated by ribosomes. The nucleocapsids must therefore be dissociated upon their entry into cells, and the viral RNA must be released.

SFV entry into cells begins by binding of the virus to receptors on the cell surface, which is followed by rapid internalization by receptor-mediated endocytosis (13). The low pH in early endosomes triggers a spike glycoprotein-mediated fusion event between the viral envelope and the endosomal membrane, releasing the nucleocapsid into the cytoplasm (18, 20). About 50% of cell-associated viruses deliver their nucleocapsids into the cytoplasm within the first 30 min following entry, while the rest fail to penetrate and are degraded in lysosomes (14). Immediately after fusion, the nucleocapsids can be detected by electron microscopy on the cytoplasmic side of the endosomal membrane, but they quickly disappear from view, presumably because of disassembly (11).

In their studies on Sindbis virus uncoating, Wengler and Wengler found that the incoming C proteins bind to ribosomes (38). In gradient centrifugation experiments on cell lysates, they found a large fraction of incoming C protein cosedimenting with 60S ribosomal subunits. The amount of C protein in the 60S peak was somewhat reduced in the presence of chloroquine (a drug that inhibits penetration of nucleocapsids into the cytoplasm), suggesting that the effect was dependent on nucleocapsid delivery into the cytoplasm. It was also observed that artificially assembled nucleocapsid-like particles disassemble when incubated in a reticulocyte lysate (39). A similar reaction was also mentioned, but not documented, for normal nucleocapsids (38). Previous studies by Ulmanen et al. had already demonstrated that newly synthesized C protein of SFV binds to the large subunit of the ribosome (34, 35). Together with the studies on incoming virus, these observations led Wengler and Wengler to propose that nucleocapsid uncoating is caused by C protein binding to ribosomes (37, 38). Alternative possibilities for togavirus uncoating, including a potential acid-induced destabilization of the nucleocapsid as the virus passes through endosomes, are also discussed in this report (25).

As an extension of our work on SFV entry into cells, we have analyzed nucleocapsid uncoating by using quantitative, kinetic, and morphological methods. Our experiments involved living cells as well as in vitro reactions with isolated

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components. These studies demonstrated that ribosomes are necessary and sufficient for efficient uncoating of nucleocapsids. We have also elucidated the mechanism by which the association of nucleocapsids with ribosomes leads to disassembly.

MATERIALS AND METHODS

Cells, virus, and infection. BHK-21 cells and SFV were grown as described previously (13, 15). Cells were plated 2 days before the initiation of infection. Virus was bound to cells at 4°C for 2 h in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) containing 0.2% bovine serum albumin and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 6.8). Cells were washed with cold RPMI to remove unbound virus. To initiate internalization of bound virus, RPMI at 37°C (supplemented with fetal bovine serum) was added, and the cells were shifted to a 37°C water bath. When monensin (Calbiochem, San Diego, Calif.) was used to inhibit viral penetration from endosomes, it was added to cells an hour before the binding of virus, at 25 μM, and was present during the binding and internalization stages.

Degradation of viral proteins and SDS-polyacrylamide gel electrophoresis (PAGE). [^{35}S]methionine-labeled virus was bound and internalized into cells at a multiplicity of infection of 100 PFU per cell. After various times at 37°C, cells were shifted to ice, washed with ice cold 100 mM NaCl–50 mM Tris (pH 7.6) (TN buffer), and lysed in 0.5% Triton X-100 in TN buffer containing 10 μg each of chymostatin, leupeptin, antipain, and pepstatin (Sigma Chemical Co.) per ml. Lysates were scraped off the dishes, and electrophoresis sample buffer was added at a final concentration of 200 mM Tris–3% sodium dodecyl sulfate (SDS)–10% glycerol–1 mM EDTA (pH 6.8). Samples were immediately heated to 95°C for 5 min, sonicated to shear cellular DNA, and run on 10% polyacrylamide gels (19). Gels were stained with Coomassie blue, and fluorography was performed on salicylate-impregnated gels, using Kodak XAR-5 film. Fluorographs were scanned, and the integrated intensity of bands was determined by a digital scanner (Visage 2000; Bioimage, Ann Arbor, Mich.).

Antibodies and indirect immunofluorescence. Rabbit antiserum to detergent-free oligomers of spike glycoproteins was made by methods described elsewhere (2). Mouse monoclonal antibodies to C protein were a gift of Irene Greiser-Wilke, Hannover Veterinary School, Hannover, Germany (9).

Cells grown on glass coverslips were infected at a multiplicity of infection of 5,000 PFU per cell. This high multiplicity was required in order to visualize signal from incoming viral proteins. Virus was bound (at 4°C) and internalized (at 37°C) in the presence of 200 μM puromycin (Sigma) to inhibit protein synthesis and thus avoid confusion between incoming and newly synthesized viral proteins. After a given time at 37°C, cells were washed with cold phosphate-buffered saline, fixed with 3% paraformaldehyde, and processed for indirect immunofluorescence as described previously (24). Controls consisted of monensin treated-infected cells, uninfected cells, infected cells stained with non-SFV antibody, and infected cells stained with rabbit primary antibody and anti-mouse secondary antibody (and vice versa) to estimate cross-reactivity of the secondary antibodies.

Scanning laser confocal microscopy. To measure fluorescence intensity due to incoming C protein and spike glycoproteins during various early stages of virus entry, cells were processed for indirect immunofluorescence as described above and then viewed on a Bio-Rad MRC-600 confocal imaging system mounted on a Zeiss Axiovert 10 microscope. Images were collected by two methods. One method consisted of imaging cells with the confocal aperture set at maximum. In this way, images of 50 to 75 cells were collected for each time point of virus entry into cells. In the second method, the confocal aperture was set to the smallest size and z-series images were taken, by which optical sections were made every 1.8 μm from the top to the bottom of the cell. Eight to ten sections were collected for each cell, and images of five to ten cells were collected for each time point of virus entry. The two methods gave equivalent results in three separate experiments.

A 40× objective lens with a numerical aperture of 1.4 was used. Confocal settings were as follows: 0.3-mW laser power, photomultiplier gain at maximum, zoom of 1.0, Kalman filter, 1 s per scan, and 10 frames per image. The raster size was 512 by 512 pixels. Cells were labeled with Texas red or fluorescein isothiocyanate to label the C protein and the spike glycoproteins. The single-channel mode was used when only one label was used, and the double-channel mode was used in experiments with dual labeling. The two modes gave identical results.

The intensity of fluorescence in the whole cell or in optical sections through the cell was measured by using Bio-Rad AREA software. Cells were outlined by using a mouse. The AREA software displayed fluorescence intensity in the selected area in terms of integrated pixel values. The area over which the measurement was made was displayed in square millimeters.

Assay for penetration of nucleocapsids into the cytoplasm. Two methods were used to measure penetration of SFV nucleocapsids into the cytoplasm. One assay depended on the sensitivity of viral RNA to RNase as penetration occurred and is described elsewhere (14). The second depended on the sensitivity of penetrated C protein to digestion with proteinase K. Proteinase K cleaves C protein into two fragments (17). [^{35}S]methionine-labeled virus was bound to cells at 4°C and internalized at 37°C. At various times, cells were homogenized in TN buffer as described below. Proteinase K (Boehringer Mannheim) was added to the lysates at 100 μg/ml, and the digestion was allowed to proceed on ice for 15 min. Phenylmethanesulfonyl fluoride (2.5 mM), chymostatin, leupeptin, antipain, and pepstatin (each at 10 μg/ml), and bovine serum albumin (100 μg/ml) were added to inhibit proteolysis. Lysates were mixed with nonreducing electrophoresis buffer and subjected to SDS-PAGE, autoradiography, and densitometry. Samples not treated with proteinase K were used to determine the amount of total cell-associated C protein. Proteinase K-treated samples were used to determine the amount of C protein resistant to digestion and therefore still protected by the viral and endosomal membranes. Subtraction of the amount of resistant C protein from the total gave the amount of C protein that was proteinase K sensitive and had penetrated into the cytoplasm. In control samples in which the lysate was treated with Triton X-100 before the addition of proteinase K, all of the C protein was digested. In cells treated with monensin, which inhibits viral penetration, very small amounts of C protein became proteinase K sensitive.

Sedimentation assay for uncoating. (i) In cells. [^{35}S]methionine- and [^{3}H]uridine-labeled virus was bound and internalized into cells at a multiplicity of infection of 100 PFU per cell. After various times of internalization at 37°C, cells were

shifted to ice, washed with TN buffer, and scraped into TN buffer containing 10 μg each of chymostatin, leupeptin, antipain, and pepstatin per ml. Cells were homogenized by 30 passages through a no. 25 needle, resulting in over 95% breakage. Nuclei were separated by centrifuging the lysate at 1,200 × *g* for 5 min at 4°C. The postnuclear supernatants were layered on top of continuous gradients of 15 to 30% (wt/wt) sucrose in TN buffer with a 0.5-ml cushion of 66% (wt/wt) sucrose at the bottom. Gradients were centrifuged in a Beckman SW50.1 rotor at 50,000 rpm for 1 h at 4°C and fractionated from the top into 28 fractions. The sedimentation profile of cellular RNA was determined by measuring A_{260}. Half of each fraction was subjected to liquid scintillation counting. The rest of the fraction was pooled with the rest of one adjacent fraction and precipitated with trichloroacetic acid (TCA). Precipitated fractions were counted to measure the amount of intact viral RNA or protein, which could not be done by direct counting alone. Precipitated fractions were also analyzed by SDS-PAGE to determine the sedimentation profiles of individual viral proteins. Control cells were treated with 25 μM monensin to inhibit penetration of nucleocapsids into the cytoplasm. Others were treated with 200 μM puromycin to inhibit translation of viral RNA and determine its effect on nucleocapsid uncoating.

(ii) **In vitro.** [^{3}H]uridine- and [^{35}S]methionine-labeled nucleocapsids were released from viral membranes by Triton X-100 and were used as such or were first completely separated from the spike glycoproteins as described below. Isolated ribosomes and nucleocapsids were mixed together in various ratios and incubated for different times at either 4 or 37°C. The mixture was analyzed by velocity sedimentation as detailed above for cell lysates.

Isolation of ribosomes. Ribosomes were isolated from untreated rabbit reticulocyte lysate (Promega, Madison, Wis.) essentially as described previously (32). Mitochondrion-free reticulocyte lysate containing 0.5 M KCl and 1 mM puromycin was incubated at 37°C for 15 min and layered over a cushion consisting of 500 mM sucrose, 20 mM Tris, 300 mM KCl, 3 mM $MgCl_2$, and 1 mM dithiothreitol. This material was centrifuged in a Beckman TL100.2 rotor at 55,000 rpm for 4 h at 4°C. Pellets were suspended in 20 mM Tris–100 mM KCl–3 mM $MgCl_2$–1 mM dithiothreitol and then recentrifuged as described above. Pellets were resuspended and stored in small aliquots in liquid nitrogen. The A_{260}/A_{235} ratio of the preparation was 1.49, and the A_{260}/A_{280} ratio was 2.6. Pure ribosomal preparations should have an A_{260}/A_{235} ratio of 1.45 or above and an A_{260}/A_{280} ratio of 1.85 or above. One microliter of isolated ribosomes contained 0.78 pmol of the 60S subunit.

Isolation of nucleocapsids. Nucleocapsids were isolated as described by Fuller (6a). Virus was lysed in 1% Triton X-100, layered over a 0.5-ml cushion of 10% (wt/wt) sucrose in TN buffer in a thick-walled polycarbonate tube, and centrifuged in a Beckman TLS-55 rotor at 55,000 rpm for 50 min at 4°C. The supernatant containing the spike glycoproteins was discarded, and the nucleocapsid pellet was suspended in TN buffer.

Metabolic labeling of cells and RNase treatment of cell lysates. To label cellular ribosomes, cells were treated with [^{3}H]uridine (50 μCi/2.5-cm dish of 5×10^5 cells; New England Nuclear Research Products, Boston, Mass.) for a period of 12 h before the start of viral infection. [^{35}S]methionine-labeled virus was internalized into these cells, and its uncoating was assayed by velocity sedimentation. Ribosomes were digested by treating cell lysates with 100 μg of RNase A per ml for 10 min on ice.

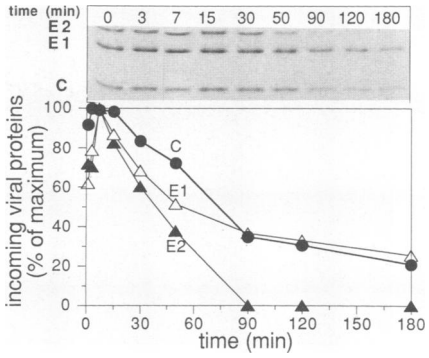

FIG. 1. Fate of incoming SFV proteins in BHK-21 cells. [^{35}S]methionine-labeled SFV was prebound to cells at 4°C and allowed to be internalized by shifting the temperature to 37°C. Cells were lysed at the indicated times, and the amount of incoming viral proteins that remained intact after various times at 37°C was determined by SDS-PAGE (top) and densitometric quantitation (bottom).

RESULTS

Fate of incoming C protein. To monitor the fate of incoming viral proteins, [^{35}S]methionine-labeled SFV was bound to BHK-21 cells at 4°C and allowed to internalize by shifting the temperature to 37°C. At different times after warming, the labeled proteins were analyzed by SDS-PAGE, autoradiography, and densitometry. It was found that all of the cell-associated C protein remained intact for up to 15 min. Most of it remained intact for up to 30 min, after which gradual degradation was observed (Fig. 1). Spike glycoprotein E1 was degraded with kinetics similar to those for C protein, whereas E2 was almost completely degraded within 90 min. As demonstrated below, more than 50% of the cell-associated virus penetrates into the cytoplasm and uncoats by 30 min (see Fig. 4). Thus, it is unlikely that proteolysis of incoming C protein is a mechanism for uncoating.

Double-label immunofluorescence was next used to visualize the C protein and the spike glycoproteins during entry (Fig. 2). Viruses bound to cells at 4°C were allowed to be internalized at 37°C. To ensure that only incoming viral proteins were seen, puromycin was added to inhibit synthesis of new proteins. As the virus entered cells, two major changes were observed: the C protein and the spike glycoproteins separated from each other, and there was an increase in the overall intensity of fluorescence. Initially, the C protein and the envelope glycoproteins colocalized on the cell surface (Fig. 2A and B) and in endocytic vacuoles (Fig. 2C and D). However, the vacuolar fluorescence pattern of C protein gradually changed, so that 30 min after warming, the C protein was distributed in a grainy cytoplasmic pattern and was excluded from the nucleus (Fig. 2E). The spike glycoproteins remained associated with vacuoles (Fig. 2F). When virus uptake was performed in the presence of monensin, which permits binding and internalization of the virus but inhibits penetration (22), the C protein and the spike glycoproteins remained vacuolar (Fig. 2G and H).

Along with the change in distribution of the viral proteins, there was a dramatic increase in the intensity of fluorescence due to both the C protein and the spike glycoproteins (Fig. 2). When quantitated by confocal microscopy and the Bio-Rad AREA software, the increase in fluorescence intensity was 30- to 50-fold as viral entry progressed (see Fig. 4A). This increase coincided with penetration and uncoating, as

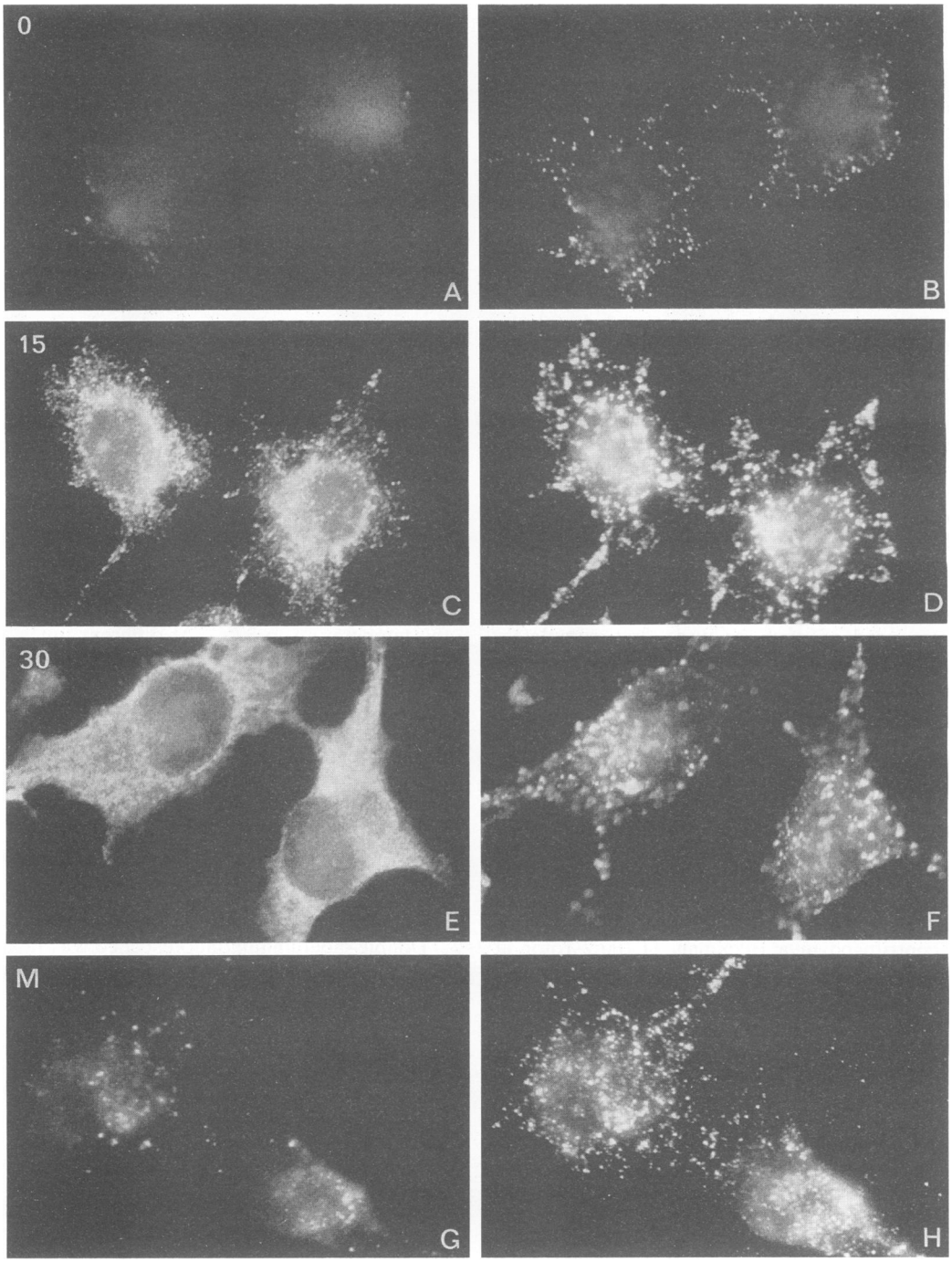

FIG. 2. Cellular distribution of incoming SFV proteins determined by double-label immunofluorescence. SFV was bound to cells at 4°C and internalized for various times at 37°C. Cells were fixed with paraformaldehyde and stained with a pool of mouse monoclonal antibodies to C protein (A, C, E, and G) and rabbit polyclonal antibodies to spike glycoproteins (B, D, F, and H). Cells in panels A and B remained at 4°C and showed colocalization of spike glycoproteins and C protein on the cell surface. Cells in panels C and D were shifted to 37°C for 15 min. Much of the C protein colocalized with the vacuolar distribution of spike glycoproteins. At 30 min after the shift, spike glycoproteins remained in vacuoles, whereas C protein showed a grainy cytoplasmic staining pattern (E and F). In the presence of monensin, C protein and spike glycoproteins remained in vacuoles 90 min after the temperature shift (G and H). Exposure times for all photographs were identical.

assayed biochemically (see Fig. 4 and the following set of experiments). The most likely reason for the increase in fluorescence intensity was that viral proteins, which are packed densely in the intact virus, become dispersed as the viral envelope dissociates and the nucleocapsids disassemble, thus providing greater access to primary and secondary antibodies. In the presence of monensin, the increase influorescence intensity was much reduced. A similar increase in

fluorescence signal has been previously demonstrated for influenza virus antigens during entry and disassembly of virus (23). These findings for influenza virus and SFV suggest that measuring changes in the intensity of the fluorescence signal from incoming viral proteins could provide a general method for monitoring disassembly of viruses.

Uncoating of nucleocapsids in the cytoplasm. When released from the viral envelope by a nonionic detergent, intact nucleocapsids have a sedimentation coefficient of about 140S in sucrose gradients (16). Upon entry of viruses into cells and the subsequent uncoating of nucleocapsids, we expected to see a change in sedimentation, as described by Wengler and Wengler for Sindbis virus (38). [^{35}S]methionine- and [^{3}H]uridine-labeled viruses were allowed to be internalized into cells for 30 min. The cells were then homogenized in the absence of detergent, and the postnuclear supernatants were analyzed on sucrose velocity gradients. No labeled material sedimented at the position expected of intact nucleocapsids (Fig. 3A). The spike glycoproteins E1 and E2, the viral RNA, and about half of the total C protein were found on the 66% sucrose cushion placed at the bottom of the gradient to retain membranous organelles. A peak sedimenting at 60S contained the other half of the C protein. Since this peak was devoid of viral RNA, we concluded that it represented the C protein that had dissociated from the viral RNA; i.e., it was a product of the uncoating reaction.

Additional experiments confirmed that the 60S peak corresponded to C protein released during nucleocapsid uncoating. No C protein sedimented at 60S when virus was simply bound to cells at 4°C and not allowed to internalize (not shown), nor was there a 60S peak when virus was internalized in the presence of 25 μM monensin, which blocks the low-pH-induced fusion event in endosomes (Fig. 3B). Moreover, when penetration of nucleocapsids from endosomes into the cytoplasm was measured by using a novel protease accessibility assay, penetration was found to closely parallel the increase of C protein at 60S, both in kinetics and in efficiency (Fig. 4B and C; see Materials and Methods for details of the penetration assay). Within 1 to 2 min after penetration, the amount of C protein in 60S complexes increased rapidly, reaching a maximum of about 50% of the total within 30 min of virus entry. Uncoating, as measured by this velocity sedimentation assay, paralleled the increase in fluorescence intensity observed by confocal microscopy (Fig. 4A and B).

Inclusion of the protein synthesis inhibitor puromycin (200 μM), both before and during virus entry, did not affect the formation of the 60S peak. This finding indicated that the uncoating process was independent of translation of the viral RNA.

Taken together, the results demonstrated that within cells, nucleocapsid uncoating was a rapid and efficient process. Evidently, all of the nucleocapsids that were delivered to the cytoplasm were uncoated within 1 to 2 min. The released C proteins were recovered in 60S complexes distributed throughout the cytoplasm, whereas the uncoated viral RNA, along with unpenetrated virus particles, remained associated with cellular membranes and with membrane-bound organelles. Being extremely rapid and unaffected by puromycin, uncoating was clearly independent of translation of viral RNA. It was thus different from the mechanism reported for tobacco mosaic virus, whereby the majority of coat proteins are removed during translation (28).

It is important to mention that entirely different results were obtained if cells were lysed with a nonionic detergent instead of by mechanical disruption. In Triton X-100 lysates, the 60S peak containing the C protein was seen even when the virus was only bound to the cell surface and not internalized or when fusion in endosomes was blocked by monensin. Disassembly could, in fact, be observed when virus particles were added to a Triton X-100 lysate of BHK-21 cells. As will be shown below, the reason for such disassembly was that the envelope of unpenetrated viruses was solubilized by the detergent, thus exposing the nucleocapsids to the disassembly factors present in the cell lysate. In detergent-free lysates, the nucleocapsids of unpenetrated viruses remained protected by the viral envelope and the endosomal membrane.

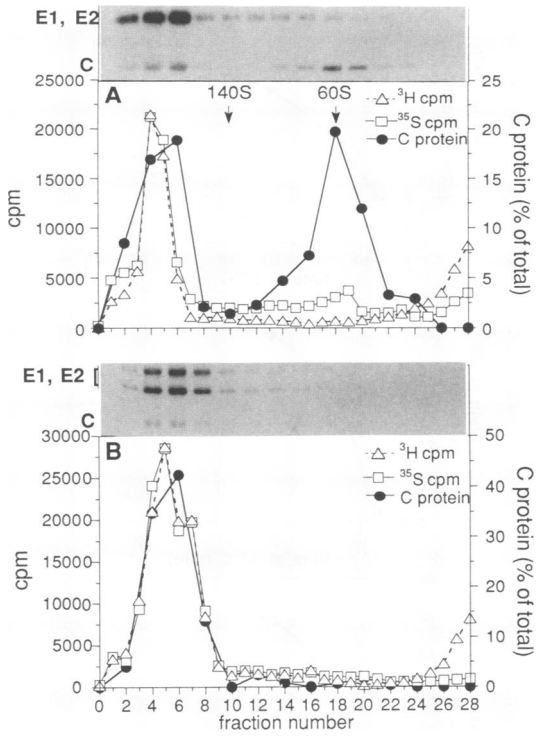

FIG. 3. Uncoating in BHK-21 cells. (A) [^{35}S]methionine- and [^{3}H]uridine-labeled SFV was prebound to cells at 4°C and internalized at 37°C for 30 min. (B) Binding and internalization were carried out in the presence of 25 μM monensin. In both panel A and panel B, cells were homogenized and the lysates were centrifuged on continuous 15 to 30% sucrose gradients with a 66% sucrose cushion at the bottom in an SW50.1 rotor at 50,000 rpm for 1 h at 4°C. Gradient fractions were subjected to liquid scintillation counting in order to determine sedimentation profiles of viral proteins (□) and viral RNA (△). Adjacent pairs of fractions were pooled, TCA precipitated, and subjected to SDS-PAGE and fluorography. Fluorographs above the gradient profiles show the sedimentation patterns of the viral proteins. In panel A, the gel was run in reducing conditions, which results in glycoproteins E1 and E2 migrating as a single band. In panel B, nonreducing conditions were used. C protein was quantitated from the fluorographs by using densitometry. The amount of C protein in each fraction is expressed as a fraction of the total C protein in the gradient (●). The top of the gradients is on the right. The small peak of ^{35}S and ^{3}H radioactivity observed near the top of the gradient corresponded to TCA-soluble protein and RNA.

Uncoating depends on ribosomes. The sedimentation of the dissociated C protein at 60S could mean either that the C protein was in the form of multimers or that it was com-

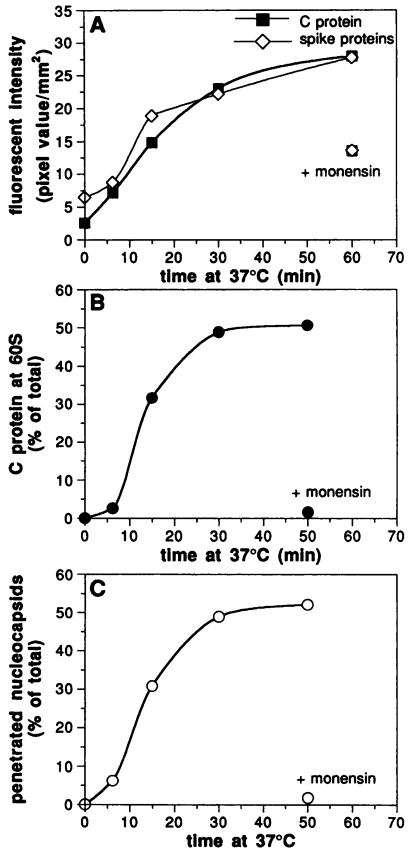

FIG. 4. Kinetics of penetration and uncoating, determined by confocal microscopy and by biochemical assays. (A) Confocal microscopy. Virus was bound to cells on coverslips at 4°C. Control cells were treated with 25 μM monensin. Cells were warmed to 37°C for various times, fixed, and processed for indirect immunofluorescence by using anti-C and anti-spike glycoprotein antibodies. The intensity of fluorescence in cells was measured by using a confocal microscope and the Bio-Rad AREA software as described in Materials and Methods. (B and C) Biochemical assays for uncoating and penetration. [^{35}S]methionine-labeled SFV was bound to cells at 4°C and internalized at 37°C for various times. Control cells were treated with 25 μM monensin. (B) To determine the extent of uncoating (●) at each time point, cells were lysed and subjected to centrifugation and analysis as described in the legend to Fig. 3. The fraction of total cell-associated C protein that sedimented in the 60S peak was determined by TCA precipitation, fluorography, and densitometry as described in the legend to Fig. 3. (C) Viral penetration (○) was assayed by determining amount of total C protein in the cell homogenate that was sensitive to proteinase K (see Materials and Methods).

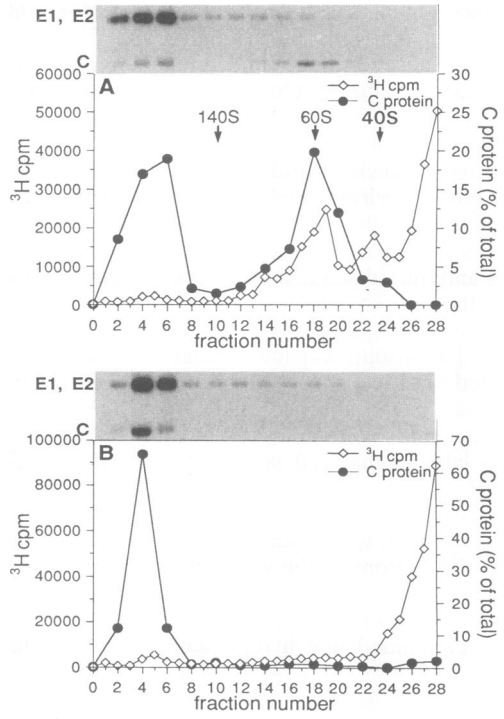

FIG. 5. Binding of C protein to the large subunit of the ribosome. Cellular RNA was labeled by treating cells with [^{3}H]uridine. [^{35}S]methionine-labeled SFV (200,000 cpm) was bound to these cells and internalized for 30 min. Cells were homogenized and centrifuged on a sucrose gradient as described in the legend to Fig. 3 (A), or the homogenate was treated with RNase and then centrifuged (B). Gradient fractions were subjected to liquid scintillation counting to determine sedimentation profiles of [^{3}H]uridine-labeled ribosomes (◇). Adjacent pairs of fractions were pooled, TCA precipitated, and subjected to SDS-PAGE and fluorography to determine sedimentation patterns of the viral proteins. C protein was measured by densitometry from the fluorographs (●). The top of the gradients is on the right.

plexed with cellular material. In support of the latter possibility, when the peak of C protein at 60S was isolated and rerun on a cesium chloride gradient, it had a density of 1.47 g/cm^3, indicating that it did not consist of protein alone but also contained nucleic acid. In agreement with the observation of Wengler and Wengler, the 60S peak coincided with the main peak of A_{260} corresponding to ribosomes (data not shown) (38). When [^{35}S]methionine-labeled virus was allowed to enter cells in which the ribosomes were labeled with [^{3}H]uridine, the C protein sedimented with the labeled peak corresponding to 60S ribosomal subunits (Fig. 5A). When these cell lysates were subsequently treated with RNase to digest the rRNA, the 60S peak was lost. After RNase treatment, the C protein sedimented in aggregated form close to the bottom of the gradient (Fig. 5B). Had the 60S peak consisted of multimers of C protein alone, digestion of rRNA would not have affected it. When cell lysates were made with buffer containing Mg^{2+}, to keep polysomes intact, the C protein sedimented to the bottom of the gradient along with the polysomes. Thus, the data showed that C protein from uncoated nucleocapsids quantitatively associated with ribosomes, confirming the conclusions that Wengler and Wengler (38) made for Sindbis virus.

Ribosome-induced uncoating in vitro. To determine whether ribosomes actively disassemble nucleocapsids or whether they simply sequester the dissociated C protein after uncoating has occurred, we analyzed uncoating in vitro, using purified ribosomes and nucleocapsids. The starting point for these in vitro experiments was the observation, mentioned above, that nucleocapsids from detergent-solubilized viruses dissociate when added to cell lysates. Ribosomes were isolated from reticulocyte lysates. Nucleocapsids were obtained from virus in one of two different ways: (i) viral envelopes were solubilized by Triton X-100, and the released nucleocapsids were isolated by centrifugation through a detergent free-sucrose cushion and resuspended in a detergent-free buffer, or (ii) Triton X-100-solubilized virus was added directly to the assay mixture.

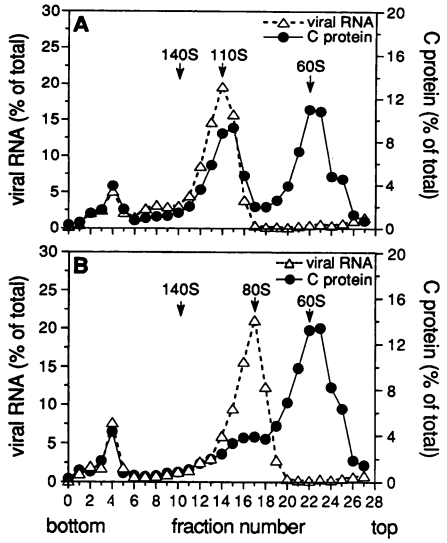

FIG. 6. Uncoating of isolated nucleocapsids in vitro. Isolated [^{35}S]methionine- and [^{3}H]uridine-labeled SFV nucleocapsids (100,000 and 200,000 cpm, respectively; 0.6 μg of viral protein per incubation) were incubated with purified ribosomes (4.2 pmol) at 37°C. After various times of incubation, the samples were centrifuged on sucrose gradients and analyzed as described for Fig. 3. (A) After 1 min of incubation, none of the viral RNA or C protein sedimented at 140S, the sedimentation coefficient of intact nucleocapsids. About half of the C protein sedimented at 60S. The rest of the C protein remained associated with the viral RNA, which now sedimented between 140S and 80S. Some aggregates of nucleocapsids arose in the process of isolating nucleocapsids from spike glycoprotein, and these sedimented to the cushion at the bottom of the gradient. (B) After 20 min of incubation, most of the C protein sedimented at 60S or 80S. All of the viral RNA sedimented at 80S.

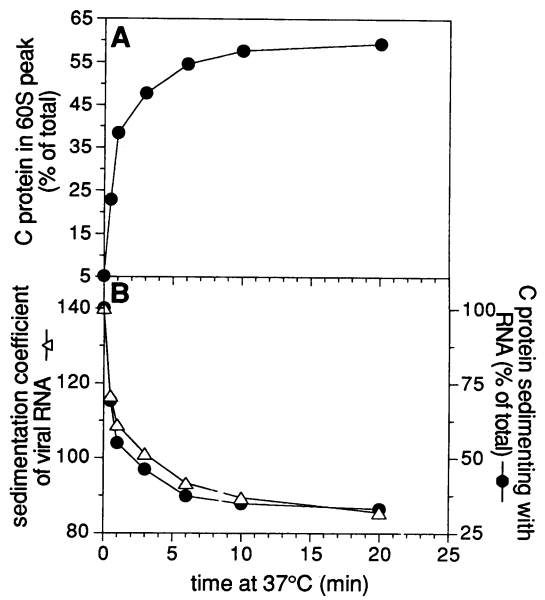

FIG. 7. Kinetics of uncoating and sedimentation properties of the disassembling nucleocapsid. Isolated [^{35}S]methionine- and [^{3}H]uridine-labeled nucleocapsids were incubated with ribosomes for various times, and uncoating was assayed on sucrose gradients. (A) Kinetics of uncoating in vitro. The fraction of total C protein that sedimented at 60S is shown. (B) Sedimentation properties of the intermediates of uncoating. Labeled viral RNA (△) was used as a marker for the disassembling nucleocapsids. The sedimentation coefficient of these disassembling nucleocapsids was calculated by using 140S as the value for intact nucleocapsids. The fraction of the total C protein (●) that remained associated with the viral RNA in the various uncoating intermediates is also shown.

When nucleocapsids were incubated with ribosomes and then analyzed on sucrose gradients, it was found that the C protein moved from 140S to 60S, as it did in living cells (Fig. 6). Uncoating could therefore be reproduced in vitro. The process did not need any source of metabolic energy, such as ATP or any other additives. The extent of uncoating at a given ribosome concentration depended on the period of incubation (Fig. 6). When incubated for long enough and at sufficient ribosome concentrations, 80% of the C protein dissociated from the viral RNA and sedimented at 60S (Fig. 6B). Like the C protein-containing 60S peak in cells, the density of the 60S peak produced in vitro was also 1.47 g/cm^3 in isopycnic CsCl gradients. The time course of the in vitro uncoating reaction is shown in Fig. 7A. At 37°C, the half time of uncoating was approximately 1 min, consistent with the rapid uncoating seen in cells. The reaction could also occur at 4°C, though at a slower rate (not shown).

In living cells, the viral RNA from uncoated nucleocapsids remained associated with cellular membranes (Fig. 3A) and was therefore difficult to analyze. The absence of membranes in the in vitro experiments allowed us to characterize what was left of the nucleocapsids after uncoating. We found that as progressively greater uncoating occurred, the sedimentation coefficient of the nucleocapsid remnant (containing the viral RNA and decreasing amounts of C protein) decreased steadily from 140S to 80S (Fig. 7B). We did not observe any remnants that sedimented slower than 80S, suggesting that the 80S species was the final product of uncoating. Also, at no point did we observe more than a single population of remnants, suggesting that the removal of C proteins proceeded without the formation stable intermediates.

The fraction of C protein transferred from the nucleocapsids to the 60S peak was directly proportional to the ribosome/nucleocapsid ratio in the sample (Fig. 8). In the presence of excess ribosomes (>800 ribosomes per nucleocapsid), 80% of the C protein was removed from the nucleocapsid peak and transferred to the 60S peak (Fig. 6B). It is unclear at present whether the remaining C proteins can be removed by ribosomes in vitro. We estimated that three to six C protein molecules could bind to each 60S ribosomal subunit.

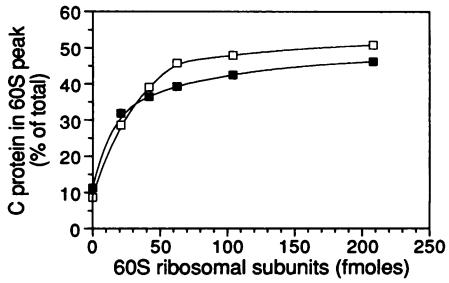

FIG. 8. Effects of ribosome concentration and duration of incubation on disassembly in vitro. Fixed amounts of [^{35}S]methionine-labeled SFV (100,000 cpm; 1.5 μg of protein) were mixed with various amounts of ribosomes and incubated at 37°C for either 5 min (□) or 30 min (■). The fraction of total C protein that sedimented at 60S was then determined as described for Fig. 3.

When constant amounts of nucleocapsids were incubated with variable amounts of ribosomes, the extent of uncoating at any given nucleocapsid/ribosome ratio did not increase with time (Fig. 8). Hence, ribosomes were unlikely to be playing a catalytic role in the process of uncoating. Also, when increasing amounts of unlabeled nucleocapsids were mixed with a fixed amount of labeled nucleocapsids and then incubated with ribosomes, a progressive decrease in the uncoating of the labeled nucleocapsids was observed (data not shown). The binding of C protein to ribosomes was, therefore, stable and saturable.

Taken together, the results demonstrated that ribosomes were responsible for rapidly removing the majority of C proteins from nucleocapsids. They were not acting catalytically but rather as saturable binding sites for C protein. Uncoating occurred through binding of C proteins directly from the nucleocapsids to the large ribosomal subunit, thereby stripping the viral RNA of its protective protein coat.

Uncoating with lysates from infected cells. Since newly synthesized C protein has been shown to bind to ribosomes (34, 35), it has been suggested that ribosomes in infected cells are saturated with C protein and that this prevents uncoating of newly assembled nucleocapsids (37, 39). We tried to test this hypothesis by examining whether ribosomes from infected cells were capable of uncoating nucleocapsids in vitro. We found that lysates from cells infected with SFV for 4 to 8 h were just as efficient in uncoating isolated [^{35}S]methionine-labeled nucleocapsids as were lysates from uninfected cells (data not shown). A number of different methods were used to make the lysates, with the objective of keeping the C protein-ribosome association intact. We used nonionic detergents to make lysates, homogenized cells in the absence of any detergent, used low-salt buffers for homogenization, added unlabeled nucleocapsids to the infected cells during homogenization, etc. Regardless of the method used, the uncoating capacity of ribosomes from infected cells was equal to that of ribosomes from noninfected cells. Saturation of ribosomes with newly synthesized C proteins is, therefore, an unlikely mechanism for mediating the switch between disassembly and assembly in infected cells.

DISCUSSION

The overall entry pathway for SFV is now one of the best understood among animal viruses. After receptor-mediated endocytosis in coated-vesicles, about 50% of the incoming viruses release their nucleocapsids into the cytoplasm by low-pH-activated fusion between the viral envelope and the limiting membranes of early endosomes (for a review, see reference 18). Once in the cytoplasm, these nucleocapsids are efficiently and rapidly uncoated. Uncoating depends on cellular uncoating factors identified as the 60S ribosomal subunits. These factors not only sequester the dissociated C proteins but actively bring about the uncoating process. Bound to ribosomes, the C proteins distribute throughout the cytoplasm, while the viral RNA remains associated with cellular membranes. Replication and translation occur in complexes localized to modified endosomes and lysosomes (4, 10).

We evaluated other potential mechanisms for uncoating. One possibility was that proteolysis of C protein could lead to nucleocapsid disassembly. The observation that the C protein of the incoming nucleocapsids remained undegraded while uncoating occurred (Fig. 1) indicated that proteolytic digestion was not involved. In rubella virus, which is also a member of the togavirus family, the C protein becomes hydrophobic when exposed to low pH (25). This finding has been interpreted as indicating that the C protein is exposed to low pH as the virus passes through the endosomes and that this exposure primes it to disassemble once it reaches the cytoplasm. It is unlikely that this is the case with alphaviruses, whose C proteins undergo a similar hydrophobic change in vitro (41). Although isolated SFV nucleocapsids undergo low-pH-induced changes in morphology and sedimentation properties (31), these changes do not take place when intact viruses are treated with low pH or when viruses are fused at low pH with liposomes in vitro (40), indicating that the viral envelope, in carefully made virus preparations, is impermeable to protons. This conclusion is consistent with recent X-ray solution scattering data demonstrating that exposure of Sindbis virus to pH 5 does not alter the structure of the nucleocapsid enclosed within the viral envelope (33). Furthermore, nucleocapsids isolated from virus by using nonionic detergents at neutral pH are fully infectious when microinjected into cells (5), suggesting that passage through acidic endosomes is not required for uncoating and infectivity. Although crucial for inducing the fusion reaction in endosomes, low pH is not needed for priming nucleocapsid uncoating.

We estimated that each ribosome can bind three to six C proteins. Whether there are multiple binding sites on each ribosome, or whether the C proteins are removed from the nucleocapsid and bound to the ribosome as oligomers, is unclear. The C protein does not affect the hydrodynamic properties of the 60S subunit sufficiently to produce a detectable change in sedimentation on sucrose gradients. The binding is essentially irreversible, judging from our failure to elute detectable amounts of labeled C protein after dilution or after prolonged incubation or to exchange the bound unlabeled C protein with labeled C protein. It will be interesting to determine whether the bound C proteins induce functional changes in the ribosomes.

Preliminary results suggest that the C protein binding sites on ribosomes contain exposed rRNA. Isolated 28S rRNA causes complete dissociation of the C protein from the viral RNA in vitro (30). It is interesting that the mammalian 28S rRNA contains a sequence of 400 nucleotides that has 54% identity to the encapsidation sequence proposed by Weiss et al. (36) for Sindbis viral RNA. It is possible that the C protein binds preferentially to this sequence in the 28S rRNA during uncoating. It has been shown that newly synthesized C protein can be UV cross-linked to rRNA in infected cells (27a). The amino-terminal portion of the C protein, with its numerous positively charged residues and prolines, has been proposed to be the segment that interacts with the viral RNA in the nucleocapsid (8). While this segment of the protein is too disordered to be completely visible in the X-ray structure, it is apparent that a part of it is exposed on the surface of the nucleocapsid (3). The accessibility of the amino-terminal segment may allow it to mediate the initial interaction of the C protein in the nucleocapsid with the rRNA.

In summary, our studies and those of Wengler and Wengler (38) indicate that alphavirus uncoating depends on the presence of a cellular uncoating factor, the ribosome, which competes for the C protein and thereby dissociates incoming nucleocapsids. How general is this strategy for uncoating among viruses? Very little is known about the early cytoplasmic events during entry of most animal viruses into cells (reviewed in reference 29). Given the overall similarities within the togavirus family, it is likely that its members rely

on ribosome binding. Whether flaviviruses and other, more distantly related viruses do so remains to be seen. Some viruses, however, seem to utilize different strategies. For instance, influenza virus has a monovalent cation channel in its envelope, which allows protons to enter the virus as it passes through the acidic endosomes, thus priming the incoming nucleocapsids to disassemble when they reach the cytoplasm (12, 23, 27). The nucleoproteins of some negative-stranded viruses, such as influenza virus and vesicular stomatitis virus, may not dissociate from the viral RNA for replication to occur (2a). Yet another strategy, involving a two-step uncoating process, is utilized by poxviruses and herpesviruses. In the case of poxviruses, the mechanism of the first step is not understood, but it results in partial uncoating and the synthesis of a virus-coded uncoating protein (26) that brings about the second step of complete nucleocapsid disassembly. Further studies on the molecular basis of uncoating in different virus systems will provide insights into some of the most crucial interactions between viral pathogens and their host cells.

Wengler and Wengler have suggested that the saturation of ribosomes with newly synthesized C protein may be responsible for the stability of newly assembled nucleocapsids in infected cells (37, 38). More studies are clearly needed to evaluate this hypothesis. That lysates from infected cells could uncoat nucleocapsids in vitro with the same efficiency as do lysates from uninfected cells suggests that infected cells have uncoating-competent ribosomes. Ulmanen et al. have shown that even after 8 h of infection, only 20% of total cellular ribosomes are associated with the newly synthesized C protein (35). The remaining ribosomes should in principle affect the stability of newly assembled or superinfecting nucleocapsids. Together, these findings suggest that the processes of maintaining stability of newly assembled nucleocapsids and of superinfection exclusion might be more complex than that proposed by the ribosome saturation hypothesis.

ACKNOWLEDGMENTS

We thank Rebekka Zirbel for initiating the experiments which led to the in vitro uncoating assay. We are grateful to Irene Greiser-Wilke for monoclonal antibodies to SFV C protein. We thank Robert Doms, Kelsey Martin, and Ira Mellman for critical reading of manuscript, Henry Tan for expert and generous help with the photography, Philippe Male for guidance in confocal microscopy, and members of the Helenius-Mellman laboratory for helpful advice and criticism.

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