Visualization of Intracellular Transport of Vesicular Stomatitis Virus Nucleocapsids in Living Cells

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Vesicular stomatitis virus (VSV) belongs to the family Rhabdoviridae in the order Mononegavirales. VSV is an enveloped virus with a negative-stranded RNA genome of 11,161 nucleotides. The genome encodes for five viral proteins, namely, nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and the RNA-dependent RNA polymerase (L) (1, 44). The viral genome is present within the virion as a ribonucleoprotein (RNP) being tightly encapsidated by the N protein and associated with the viral polymerase complex of L and P proteins (1, 44). The M protein is located underneath the viral envelope, whereas the G protein forms spikes on the viral envelope. The G protein binds to the cell surface receptor and is required for the entry of the virus into the cells. The virus enters susceptible cells by receptor-mediated endocytosis. Fusion of the viral envelope with the endosomal membrane in a pH-dependent process leads to the release of the viral nucleocapsids into the cytoplasm for viral gene expression. During VSV assembly, the progeny nucleocapsids are transported alone or in association with the viral M protein to the plasma membrane, where they are packaged into an envelope containing the viral G protein and are released from the infected cells. Although some of the fundamental steps in the VSV infection pathway have been deciphered, many questions concerning the entry mechanisms and transport of viral nucleocapsids to synthesis sites and from synthesis sites to assembly sites, as well as virus egress, remain poorly understood. Studies to provide an understanding of these processes have been hampered due to the inability to observe these events by live-cell imaging of infected cells.

In a recent report, it was demonstrated that the fusion of the viral envelope and the release of the nucleocapsid into the cytoplasm are two independent but successive steps in the endocytic pathway of VSV infection (29). These studies revealed that release of the viral nucleocapsid into the lumen of the endosomal vesicle occurs by the fusion of the viral envelope with the membranes of the endosomes but that the nucleocapsid release into the cytoplasm may require a back-fusion event in which the internal vesicles fuse with the membranes of the late endosome (29). Following synthesis in the cytoplasm, the progeny nucleocapsids must then be transported to the plasma membrane for viral assembly. The mechanism(s) by which the transport of such large RNP complexes is accomplished is unclear. Because of the high viscosity of the cytoplasm, movement of the RNP by diffusion is likely to be limited (32). Intracellular pathogens and their macromolecular components overcome this obstacle by hijacking cytoplasmic motors and utilizing the cellular cytoskeleton as a roadway for intracellular transport to reach their destination (23, 35, 49, 50).

An understanding of some of the mechanistic details of virus entry by endocytic pathway and nucleocapsid release into the cytoplasm of infected cells has been possible only with the use of VSV chemically labeled with lipophilic fluorescent dyes (29). The use of such labeled viruses is limited to studies involving tracking of the input virus (16, 26, 29, 47). Once the viral nucleocapsid is delivered into the cytoplasm, subsequent tracking of the viral nucleocapsids, particularly, the sites of synthesis and transport of the nucleocapsids to the sites of

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The region that links domain I and domain II is called the hinge region. The site of eGFP incorporation at aa position 196 is indicated by a vertical dotted line. (B) Expression of PeGFP fusion protein in transfected cells. Cells transfected with plasmids encoding P (lanes 2 and 5) or PeGFP (lanes 3 and 6) proteins or no plasmid (lanes 1 and 4) were radiolabeled with Expre35S-S35 label. The radiolabeled proteins were immunoprecipitated with antibodies as shown on the top (α-P, anti-P; α-eGFP, anti-eGFP), analyzed by SDS-PAGE, and detected by fluorography. Size markers in kDa are shown on the left. P and PeGFP proteins are identified on the right. (C) Replication and transcription activities of PeGFP protein relative to Pwt as determined by DI-particle replication or minigenome transcription assays (20, 40). The histograms represent the average data from three independent experiments, with standard deviations shown by error bars. Repln, replication; Txn, transcription.

VSV nucleocapsids are multiprotein-RNA complexes composed of viral RNA that is tightly wrapped with the N protein and is associated with P and L proteins. P protein is a multifunctional protein that is an essential subunit of the viral RNA-dependent RNA polymerase. In addition to its role in polymerase functions, it binds to the L protein and stabilizes it from proteolytic degradation (4, 13); it acts as a chaperone for the N protein, which then specifically encapsidates the viral RNA (9, 34, 41); and it interacts with terminal sequences of viral genome for viral RNA synthesis (21, 24). Our previous studies showed that the protein is organized in a modular fashion relative to its function (Fig. 1A) (6, 8, 19, 39). While phosphorylation of specific amino acid residues at the aminoterminal domain I (amino acid residues 1 to 150) is responsible for transcription activity (39), phosphorylation of specific amino acid residues at the carboxy-terminal domain II (amino acid residues 210 to 244) is important for optimal replication activity (19) of P protein. Phosphorylation of these residues at both domain I and domain II is indispensable for virus growth (6). Domain III, which comprises 21 to 25 residues at the extreme carboxy-terminal region, is important for mediating the binding of P protein to the N RNA template (8). The region that links domain I and domain II is called the hyper-variable hinge region (approximately spanning amino acid residues 150 to 210). We recently studied the role of this hinge region and found that it plays an important role in VSV RNA synthesis and assembly of infectious particles (7). In that study, we also demonstrated that insertion of 19 amino acids (aa) within the hinge region of the protein (Fig. 1A) has no significant adverse effects on virus replication (7). In the present study, we show that a fusion protein (PeGFP), in which full-length enhanced green fluorescent protein (eGFP) was inserted in the hinge region of the P protein, is functional in viral genome transcription and replication. A recombinant VSV encoding the PeGFP protein in place of P protein (VSV-PeGFP) was recovered. Using VSV-PeGFP, we have examined the intracellular sites of viral RNA synthesis. By live-cell imaging of VSV-PeGFP-infected cells, we have found that the movement of newly synthesized viral nucleocapsids toward the cell periphery is mediated by microtubules (MTs). In addition, our studies indicate that mitochondria may play a role in intracellular transport of the viral nucleocapsids.
RESULTS

PeGFP fusion protein supports transcription and replication of VSV. Our recent observation (7) that insertion of 19 aa at position 196 in the hinge region of P protein had no significant adverse effect on transcription and replication prompted us to insert full-length eGFP coding sequence at this position (Fig. 1A). To determine if the insertion resulted in a stable protein, we examined the level of expression and the size of the PeGFP fusion protein in transfected cells. Accordingly, BHK-21 cells were transiently transfected with plasmids encoding either the wt or fusion proteins following vTF7-3 infection, and the proteins were radiolabeled and examined by immunoprecipitation with anti-P and/or anti-eGFP antibody and SDS-PAGE. As can be seen in Fig. 1B, the fusion protein PeGFP was stably expressed, could be immunoprecipitated with both anti-P and anti-eGFP antibodies, and possessed a predicted molecular mass of ~73 kDa.

In order to examine the effect of eGFP insertion on P protein function in viral RNA synthesis, we used DI particles to determine replication activity and a minigenome template (p10BN) (20) to assess the transcription activity of PeGFP fusion protein. The radiolabeled DI RNAs and minigenome RNA were analyzed on urea-agarose gels and quantitated by densitometry. Results from three independent experiments show that the PeGFP protein is ~55% active in replication, whereas it is ~22% active in transcription compared to the wt P protein (Fig. 1C). These results suggest that the PeGFP fusion protein is functional, albeit with reduced activity.

Recovery of infectious VSV encoding PeGFP fusion protein. To test whether infectious virus could be recovered from VSV genome plasmids encoding P protein into which eGFP was inserted in frame in the hinge region, a recombinant VSV genome plasmid (pVSV-PeGFP) encoding PeGFP in place of wt P protein was constructed (Fig. 2A). We also constructed another recombinant VSV genome plasmid (pVSV-eGFP), containing eGFP coding sequence as an extra gene between the G and L gene junctions (Fig. 2A). Both of these genomic constructs led to recovery of recombinant VSV from transfected cells. In order to examine if the eGFP insertion into the P open reading frame has any effect on the growth of the virus, we examined single-cycle growth kinetics of the wt and mutant viruses. As can be seen from Fig. 2B, VSV-eGFP grew to titers similar to those of the wt VSV, whereas VSV-PeGFP grew to titers that were on average 8- to 10-fold less than those of the wt VSV. To determine if the viral growth correlated with the extent of viral macromolecular synthesis in infected cells, we examined RNA and viral protein synthesis in cells infected with these viruses. Our results show that overall synthesis of viral RNAs (Fig. 2C) and viral proteins (Fig. 2D) was not significantly different from results obtained with cells infected with wt VSV. These results indicate that in these mutant viruses the extent of viral protein and RNA synthesis did not correlate with the viral growth rate. The sizes of the viral mRNAs were as predicted, with the PeGFP mRNA migrating more slowly than mRNA for wt P protein or eGFP (Fig. 2C,
compare lane 2 with lanes 1, 3, and 3'). As expected, six specific proteins (N, P, M, G, eGFP, and L) were detected in cells infected with VSV-eGFP (Fig. 2D, lane 4), whereas five proteins (N, PeGFP, M, G, and L) were detected in cells infected with VSV-PeGFP (Fig. 2D, lane 3). Immunoprecipitation of the proteins with anti-P (Fig. 2D, lanes 5 to 8) or anti-eGFP (Fig. 2D, lanes 9 to 12) antibody resulted in detection of P, PeGFP, and eGFP proteins of expected sizes from cells infected with the appropriate recombinant viruses. The N and L proteins and to some extent the M protein were also immunoprecipitated with these antibodies. This is not surprising since the P protein interacts with N and L proteins and is also associated with viral nucleocapsids that may contain M protein.

Incorporation of reduced levels of PeGFP and L proteins into virions. Since the overall size of PeGFP protein increased by almost twofold (524 aa for PeGFP as opposed to 265 aa for wt P protein), we were curious to examine what effect this would have on the efficiency of incorporation of PeGFP into VSV-PeGFP particles relative to other viral proteins as well as relative to wt P protein in VSV. To test the efficiency of incorporation of the PeGFP fusion protein into VSV-PeGFP particles, we radiolabeled the virus-infected cells with Expre35S35S, purified the extracellular virions, and analyzed the proteins incorporated into virions by SDS-PAGE analysis. Interestingly, we observed that PeGFP and L proteins were incorporated consistently at levels below that seen for wt VSV (Fig. 3). In these experiments, we examined viral proteins from all three viruses (VSVwt, VSV-PeGFP, and VSV-eGFP) that correspond to nearly equal amounts of N, M, and G proteins, the three major structural proteins within the virions. Unequivocally, the data shown in Fig. 3 and other similar independent experiments suggested that PeGFP and L proteins were incorporated less efficiently into VSV-PeGFP virions than into VSVwt and VSV-eGFP virions. Taking into account the number of methionine and cysteine residues in P and PeGFP proteins, we estimated from three independent experiments that the amounts of PeGFP and L proteins incorporated into the VSV-PeGFP virions were on average about 50% of the amounts of P and L proteins in VSVwt. In the control virus expressing eGFP as an extra gene (VSV-eGFP), the incorporation of L and P proteins was similar to that of VSVwt. These results indicate that the larger PeGFP protein may have influenced incorpo-
ration of L-PeGFP protein complexes into the viral nucleocapsids that are packaged in the virions.

Examination of intracellular sites of viral RNA synthesis. Subcellular localizations of eGFP were noticeably different in cells infected with VSV-eGFP and VSV-PeGFP. While VSV-eGFP-infected cells showed typical eGFP distribution evenly throughout the cytoplasm as well as in the nucleus (Fig. 4A), the fluorescence pattern in cells infected with VSV-PeGFP appeared granular and the granules were distributed throughout the cytoplasm but not in the nucleus (Fig. 4B). The differential distribution patterns of PeGFP and eGFP likely reflect the fact that PeGFP is associated with the viral nucleocapsids and is intimately involved in viral genome replication and transcription, whereas eGFP is not.

To determine if the locations of the granular fluorescence seen in cells infected with VSV-PeGFP represent the sites of synthesis of viral RNA, we examined the sites of de novo synthesis of viral RNA. Accordingly, cells infected with VSV-PeGFP were treated with actinomycin D followed by BrUTP to label de novo-synthesized RNA and examined by immunofluorescence using anti-BrdU antibody. Newly synthesized RNA labeled with BrUTP was detected throughout the cytoplasm (Fig. 4D), and PeGFP protein (Fig. 4C) colocalized to these sites (Fig. 4E). The viral N and L proteins also colocalized with the PeGFP protein (Fig. 4F to K) in a manner similar to viral RNA. These data suggest that synthesis of viral RNA occurs at these sites. The sites of viral RNA synthesis appear to be distributed throughout the cytoplasm. The staining observed near the cell periphery may represent the viral nucleocapsids in transit to the cell surface for assembly.

Association of viral nucleocapsids with mitochondria. Live-cell imaging of VSV-PeGFP-infected cells revealed that many green fluorescent dots were detectable as early as 30 min after infection and became numerous by 1 to 2 hpi (data not shown). A size estimation of several individual green dots suggested that they represent individual viral nucleocapsids. Most of these fluorescent nucleocapsids were mobile within the cytoplasm with time, moving toward the cell periphery away from the nucleus. High-magnification DIC images of infected cells showed that many of these nucleocapsids (Fig. 5A) appeared to be moving along or in close association with mitochondria or mitochondrion-like structures in a nonlinear fashion toward the cell periphery (Fig. 5A0 to A210). Tracking of several such nucleocapsids (n = 12) over time indicated that they moved with an average speed of approximately 30 nm/s.

To confirm that the viral nucleocapsids were moving along and/or associated with mitochondria, we examined the infected cells treated with MitoTracker Red, which specifically stains only mitochondria. By live-cell imaging, the green fluorescent nucleocapsids were seen closely associated with the red-stained mitochondria (Fig. 5B) and were moving along mitochondria.
with time (Fig. 5B0 to B50). Tracking of two such nucleocapsids (Fig. 5B0 to B50) indicates that they are clearly seen very close to, if not attached to, mitochondria. These results suggest that mitochondria may be involved in transport of viral nucleocapsids from the sites of synthesis to the cell periphery.

Involvement of MTs in transport of progeny viral nucleocapsids. Since it is known that movement and intracellular distribution of mitochondria are dependent on cytoskeletal components, especially the MTs (18, 54), we investigated whether MTs are involved in the observed movement of the viral nucleocapsids. Initially, we examined the distribution of fluorescent viral nucleocapsids in infected cells in the absence or presence of NOC, a drug known to inhibit microtubule polymerization. Cells not treated with the drug and infected with VSV-PE-GFP showed typical MT distribution in the cytoplasm (Fig. 6A). This was representative of most infected cells. In addition, high-magnification images revealed that many of these nucleocapsids were found to be closely associated with MTs. Nucleocapsids were also seen distributed throughout the cytoplasm (Fig. 6A). The effect of the drugs on virus yield was not due to
reduced levels of viral protein synthesis or genome replication (data not shown).

A time course experiment of untreated and NOC-treated cells infected with VSV-PeGFP was performed to examine, by live-cell recording, the synthesis of progeny nucleocapsids for up to 4 h. Different patterns of fluorescence distribution were observed in cells without and with NOC treatment. At the beginning of infection, green fluorescence was barely detectable, but at 1 hpi, fluorescence was readily detectable in untreated and drug-treated cells and gradually increased in intensity with time. By 2 and 4 hpi, numerous nucleocapsids were visible in the cytoplasm of untreated cells (Fig. 7A and B). In contrast, nucleocapsids were seen in aggregates at 2 hpi (Fig. 7C) and became larger, brighter, and more numerous by 4 hpi (Fig. 7D).

Immunostaining of infected cells with antitubulin antibody in the presence of MitoTracker Red and with or without NOC treatment further confirmed the involvement of MTs in the transport of nucleocapsids. In the absence of NOC (Fig. 7E), the nucleocapsids were dispersed throughout the cytoplasm and largely remained as individual nucleocapsids, the majority of which were associated with or in close proximity to MTs as well as mitochondria (Fig. 7F and G). In cells treated with NOC (Fig. 7H to J), aggregates of fluorescent nucleocapsids were detected. Thus, the loss of MTs in NOC-treated cells led to an accumulation of progeny viral nucleocapsids in the cytoplasm, resulting in the formation of these aggregates.

DISCUSSION

In recent years, the use of fluorescent molecules as protein tags, combined with advanced imaging techniques, has been instrumental in providing unprecedented opportunities for visualizing many dynamic processes in living cells (12, 27, 31). By use of viruses genetically tagged with fluorescent proteins, several aspects of virus biology and virus-cell interactions in vitro and in vivo have been examined. In particular, fluorescent-tagged viruses were used to study intracellular transport of human immunodeficiency virus capsids (35), capsid assembly (36), and genome recombination (30, 43); herpes virus entry, capsid transport, and egress (33, 45, 48); rhabdovirus gene expression (14) and immune response (25); and morbillivirus tissue tropism (53). In this report, we generated a recombinant VSV encoding P protein fused in frame with eGFP (VSV-PeGFP), which allowed us to track the movement of viral nucleocapsids in infected cells by real-time imaging. Our results suggest that the nucleocapsids are synthesized at sites distributed throughout the cytoplasm and that the progeny nucleocapsids are transported to the cell periphery by an MT-mediated process. Our observation that nucleocapsids are in close proximity to mitochondria suggests that mitochondria may also be involved in the nucleocapsid transport process.

Insertion of eGFP into the hinge region of the P protein led to PeGFP fusion protein, which was active in viral genome transcription and replication, indicating that the hinge region can accommodate large insertions without much adverse effect on protein function. However, incorporation of PeGFP as well as the L protein into virions was affected (Fig. 3). It is possible that the large size of the fusion protein might have sterically hindered the association of P-L complexes with the viral N RNA template, resulting in reduced incorporation of PeGFP and L proteins in the virions. Although the levels of viral macromolecular synthesis in cells infected with VSV, VSV-PeGFP, or VSV-GFP were not significantly different from each other, VSV-PeGFP grew to titers that were almost 10-fold less than those of the other viruses. Whether the reduced levels of incorporation of L and P proteins in VSV-PeGFP account for its retarded growth phenotype remains to be examined. We have recently demonstrated that the P protein of VSV plays a major role in assembly of infectious particles (7). It is therefore possible that insertion of eGFP into P protein
may have affected the assembly functions of the protein to some extent, resulting in retarded growth of VSV-PeGFP. It should be noted that a recombinant rabies virus encoding the P protein with eGFP fused in frame at its amino terminus possessed a significantly retarded growth phenotype (14). In addition, eGFP incorporation into the L protein of measles virus (11) led to a reduced growth phenotype of the recombinant virus. However, a recombinant rinderpest virus with insertion of eGFP at a similar location in the L protein grew like the wt virus in vitro but was attenuated for growth and virulence in its natural animal host, cattle (2). Clearly, these data indicate that growth and virulence properties can be altered by insertion of eGFP into the viral polymerase. It will be interesting to examine whether VSV-PeGFP with insertion of eGFP in the P protein of the viral polymerase complex possesses altered growth and virulence phenotypes in vivo.

In cells infected with VSV-PeGFP, the intracellular distribution of fluorescence was punctate. The majority of these puncta were seen distributed throughout the cytoplasm, and their locations represented the sites of viral replication since the de novo-synthesized RNA as well as other components of viral replication machinery, namely, the N and the L proteins, colocalized to these sites. The interpretation that viral RNA synthesis occurs throughout the cytoplasm but in proximity to the cell nucleus was strengthened by the observation that in cells treated with NOC or Colcemid, which inhibit MT polymerization, leading to a disruption of nucleocapsid transport from the sites of synthesis to the cell periphery, the nucleocapsids were seen as aggregates around the nucleus (Fig. 6 and 7). VSV contains approximately 450 copies of P molecules per nucleocapsid (51). Although VSV-PeGFP contains almost half as many PeGFP molecules as there are P molecules in wt VSV (Fig. 3), it still has more than the 120 copies of eGFP necessary to detect individual rotavirus particles (5). Clearly, by live-cell imaging of infected cells at high magnification, we were able to detect individual nucleocapsids as tiny green fluorescent structures (Fig. 5 and 6). Many of these structures were not seen in cells expressing PeGFP alone or in combination with N and/or L proteins (data not shown). Many of these fluorescent nucleocapsids appeared to be associated with mitochondria and moved in parallel with the longitudinal axis of mitochondria toward the cell periphery. The significance of the association of the nucleocapsid with mitochondria is not clear at this time, but it is possible that cytoskeletal structures, like MTs, that are used by mitochondria for their intracellular distribution (18, 54) might be involved in nucleocapsid transport. The...
association of nucleocapsids with mitochondria may thus be transient and possibly serve as bridges linking MTs during transport of the nucleocapsids. Alternatively, it is possible that the association of nucleocapsids with mitochondria may be just random. Further work will be necessary to provide any functional significance to the association of nucleocapsids with mitochondria.

Although the effect of NOC on nucleocapsid distribution was dramatic (Fig. 6B), virus yield was reduced to about 20% of that from untreated control cells (Fig. 6C). It is possible that nucleocapsid transport may occur by additional mechanisms that are independent of MT. In this regard, it is of note that for DNA viruses whose capsids are specifically transported by MTs toward the cell periphery, a 20 to 25% reduction in virus yield in the presence of NOC has been considered to be significant (3, 37). In the light of these observations, our data are consistent with the interpretation that MTs are involved in the transport of viral nucleocapsids by an anterograde movement toward the cell periphery. Although the nucleocapsid transport toward the cell periphery was affected by NOC or Colcemid treatment, virus entry and uncoating remained unaffected, as seen by synthesis and accumulation of viral nucleocapsids with time in NOC-treated cells (Fig. 7C and D). This is consistent with the recent findings that NOC has no significant adverse effect on VSV entry, uncoating, or viral macromolecular synthesis (29).

How might the MTs be involved in transport of viral nucleocapsids toward the cell periphery? Since the MTs form tracks on which cellular cargos are transported by intracellular kinesin and dynein motors (10, 52), it is possible to envision a platform of the NS phosphoprotein of vesicular stomatitis virus. Proc. Natl. Acad. Sci. USA 75:8653–8659.


