Glycoprotein Cytoplasmic Domain Sequences Required for Rescue of a Vesicular Stomatitis Virus Glycoprotein Mutant

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We have used transient expression of the wild-type vesicular stomatitis virus (VSV) glycoprotein (G protein) from cloned cDNA to rescue a temperature-sensitive G protein mutant of VSV in cells at the nonpermissive temperature. Using cDNAs encoding G proteins with deletions in the normal 29-amino-acid cytoplasmic domain, we determined that the presence of either the membrane-proximal 9 amino acids or the membrane-distal 12 amino acids was sufficient for rescue of the temperature-sensitive mutant. G proteins with cytoplasmic domains derived from other cellular or viral G proteins did not rescue the mutant, nor did G proteins with one or three amino acids of the normal cytoplasmic domain. Rescue correlated directly with the ability of the G proteins to be incorporated into virus particles. This was shown by analysis of radiolabeled particles separated on sucrose gradients as well as by electron microscopy of rescued virus after immunogold labeling. Quantitation of surface expression showed that all of the mutated G proteins were expressed less efficiently on the cell surface than was wild-type G protein. However, we were able to correct for differences in rescue efficiency resulting from differences in the level of surface expression by reducing wild-type G protein expression to levels equivalent to those observed for the mutated G proteins. Our results provide evidence that at least a portion of the cytoplasmic domain is required for efficient assembly of the VSV G protein into virions during virus budding.

The assembly of enveloped viruses provides a simple and useful model for studying membrane sorting in animal cells (38). For many viruses, the final step in assembly involves budding from the plasma membrane. During the budding process, viral membrane proteins are preferentially incorporated into the nascent virion, whereas most host cell plasma membrane proteins are excluded from the assembly site. It has been suggested that this process requires specific interactions between the cytoplasmic domains of viral glycoproteins (G proteins) and maturing nucleocapsids on the cytoplasmic face of the plasma membrane (for reviews, see references 37 and 40). These interactions would facilitate the complete envelopment of the nucleocapsid by a patch of plasma membrane and ultimately result in the release of virus from the cell. For alphaviruses such as Semliki Forest virus and Sindbis virus, there is evidence for nucleocapsid-G protein interactions that would support such a mechanism (11, 14, 41), although there is no direct evidence that these interactions are required for assembly.

Viruses such as vesicular stomatitis virus (VSV) that contain matrix proteins present a more complicated picture than do alphaviruses. There is evidence that the VSV matrix protein assembles at the inner surface of the plasma membrane, and it is thought to form a bridge between the nucleocapsid and the 29-amino-acid cytoplasmic domain of the single viral transmembrane G protein (1, 5). Chemical cross-linking studies on mature virus particles have suggested interactions between the VSV G protein, the matrix protein, and the nucleocapsid protein (6, 21, 23). Other evidence has suggested that the matrix protein may be involved in the final condensation of nucleocapsids just before budding (25, 26).

The report that spikeless VSV particles lacking G protein could be produced at low levels in cells infected with a G protein temperature-sensitive mutant of VSV (*tsO45*) sug-

gested that G protein was not required for the budding process (36). However, a role for the G protein in VSV budding was resurrected when it was found that these particles, which are devoid of intact G protein, actually contain C-terminal fragments of G protein including the cytoplasmic domain (22). These fragments of G protein may provide an essential cytoplasmic scaffold for binding of matrix protein and nucleocapsid, resulting in budding of the spikeless particles, or they may be generated by proteolysis of intact G protein after virus budding (22).

In this study, we developed an assay system in which cells expressing the VSV G protein from cloned cDNA were used to rescue the *tsO45* mutant of VSV. Transient expression of wild-type VSV G protein in *tsO45*-infected cells resulted in the production of infectious *tsO45* particles at the nonpermissive temperature. We have used this system to demonstrate a requirement for sequences from the normal cytoplasmic domain of the VSV G protein in assembly.

MATERIALS AND METHODS

Viruses and cell culture. Stocks of *tsO45* were prepared by infecting BHK-21 monolayers with plaque-purified isolates at a multiplicity of infection (MOI) of 0.1. After adsorption for 30 min in serum-free Dulbecco modified Eagle medium (DMEM), the inoculum was removed and the culture was incubated at 34°C for 24 h in DMEM containing 5% fetal calf serum (FCS). Medium was collected, cleared by centrifugation at $1,200 \times g$ for 10 min, and titered on mouse L-cell monolayers at both 40 and 34°C. None of the plaque isolates formed plaques at the nonpermissive temperature. The plaque isolates were also tested for temperature-sensitive growth by inoculation of BHK-21 cells at an MOI of 3 and incubation of the cultures at 40°C for 18 h. The 40°C culture supernatants were then titered on L cells at 34°C. Stocks that did not produce infectious virus at 40°C were used for the rescue experiments.

Propagation and purification of the recombinant vaccinia

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virus vTF7-3 (10, 33) was performed essentially as described by Mackett et al. (20) except that the final step of sucrose gradient centrifugation was not performed.

Plasmids and oligonucleotide-directed mutagenesis. The gene encoding wild-type G protein was obtained from pSVGL1 (30) by digestion with *Bam*HI, and the fragment was inserted into the unique *Bam*HI site of pAR-2529 (10, 33). The resulting plasmid, pAR-G₁, in addition to being used for expression of wild-type G protein, was also used to generate a derivative of pAR-2529 that contains a *Xho*I site flanked by *Bam*HI sites (pAR-X). Insertion of all other G protein-related constructs was done by using the newly introduced *Xho*I site in pAR-X.

Synthetic oligonucleotides used for construction of genes encoding G proteins with truncated cytoplasmic tails consisted of 25-mers in which the codon for leucine (TTA) at amino 10 of the cytoplasmic domain was changed to TAG (CT-9; 5'-TTGCATTAATAGAAAGCACACCAAG-3') or tyrosine (TAT) at amino 19 was changed to TGA (CT-18; 5'-AAGACAGATTTGAACAGACATAGAG-3'). Oligonucleotides used in to generate the cytoplasmic tail internal deletion mutants were 5'-CTATTCTGGTTCTCCGAATT TATACAGACATAG-3' for CTΔ2-17 and 5'-CATCTTTGC ATTAATATATACAGACATAG-3' for CTΔ10-17. All procedures for oligonucleotide-directed mutagenesis of the G protein gene, using primer extension on single-stranded M13 templates, have been described previously (19, 29). Mutants were screened by differential hybridization, and the sequences were confirmed by dideoxynucleotide sequencing (35), using Sequenase (United States Biochemical Corp.). After confirmation by DNA sequence analysis, the mutant G protein genes were recovered from M13 replicative-form DNA and inserted into pAR-X.

Rescue assay. BHK-21 cells that were 75 to 80% confluent in 6-cm-diameter dishes were infected with tsO45 at an MOI of 3 in serum-free DMEM for 30 min at 37°C. The inoculum was removed, and the infected culture was incubated in DMEM containing 5% FCS at 40°C for 60 min. To maintain the temperature restriction, all subsequent manipulations were performed on an insulated incubation tray. After the 60-min incubation, the culture was superinfected with vTF7-3 at an MOI of 20. The recombinant vaccinia virus was adsorbed in serum-free DMEM, which was prewarmed to 40°C, for 30 min. Immediately after adsorption, the culture was transfected by using the lipid-mediated transfection reagent Lipofectin (Bethesda Research Laboratories Life Technologies, Inc.). Before the inoculum was removed, 15 µg of plasmid DNA and 30 µl of Lipofectin (1 mg/ml) were added to 2 ml of serum-free DMEM (40°C), and the mixture was kept in a 40°C water bath for 5 min. In some experiments we used a new cationic liposome transfection method (J. K. Rose and L. Buonocore, manuscript in preparation), which gave results equivalent to those obtained with Lipofectin. The mixture was added to the superinfected culture after removal of the vTF7-3 inoculum. At 3 h posttransfection, 2 ml of DMEM containing 10% FCS (40°C) was added to the superinfected, transfected culture, which was then incubated at 40°C. The medium was collected 18 to 21 h posttransfection, unless otherwise indicated, and assayed for infectious virus on mouse L cells at 34°C.

Flow cytometry. All culture manipulations were performed as described for the rescue assay except that the culture was not initially infected with tsO45. The cells were removed from the dish at 7 h posttransfection with phosphate-buffered saline (PBS) containing 50 mM EDTA, pelleted at 130 × g for 2 min, resuspended in 50 µl of PBS, and then fixed in

suspension by the addition of 2 ml of 3% paraformaldehyde (30). After fixation, the cells were washed twice in PBS containing 10 mM glycine (PBS-glycine) and then incubated with VSV G protein-specific mouse monoclonal antibody (MAb I1; 17) diluted 1:200 in PBS-glycine, followed by fluorescein-conjugated, affinity-purified goat anti-mouse antibody diluted 1:50 (Cooper Biomedical, Inc.) as described previously (30). Flow cytometry was performed on an EPICS Profile cell cytometer (EPICS Division, Coulter Corp.).

Surface immune precipitation and Western blot (immunoblot) analysis. BHK-21 cells transiently expressing G proteins at 40°C were removed from 35-mm-diameter dishes as described above and immediately placed on ice. All subsequent procedures were performed at 4°C. The cells were washed once in PBS containing 2% bovine serum albumin (BSA) (PBS-BSA) and then incubated in 100 µl of the same buffer containing 5 µl of polyclonal anti-VSV serum for 2.5 h. Excess antibody was removed by washing the cells three times in PBS-BSA, with incubation for 5 min between washes. The cells were then suspended in 50 µl of PBS and lysed as described previously (31). Nuclei and cell debris were removed by centrifugation for 2 min in an Eppendorf microcentrifuge, and the supernatant was made to 0.2% sodium dodecyl sulfate (SDS). G protein-antibody complexes were recovered by the addition of protein A-bearing *Staphylococcus* cells (Pansorbin; Calbiochem-Behring). Surface immunoprecipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE), and the proteins were transferred to nitrocellulose as described previously (43). VSV-specific proteins were detected after incubation with anti-VSV serum diluted 1:1,000 in serum incubation buffer (43) for 2 to 3 h and with ¹²⁵I-protein A (ICN Biomedicals, Inc.) in SIB for 1.5 h, followed by autoradiography at ambient temperature.

Electron microscopy and immunogold labeling. Virus particles from rescue assays performed in 10-cm-diameter dishes were recovered from culture medium by first pelleting cell debris at 1,500 × g for 10 min. The virus fraction was then concentrated by centrifugation through a 20% sucrose cushion (20% [wt/vol] sucrose in 10 mM Tris hydrochloride [pH 7.4]) at 115,000 × g for 60 min in an SW41 rotor (Beckman Instruments, Inc.), and the pellet was suspended in 30 µl of 10 mM Tris hydrochloride (pH 7.4) on ice. Virus samples were adsorbed to a carbon film that had been vaporized onto mica supports, using an Edwards evaporator. The carbon layer was then adsorbed to 400-mesh copper grids for 2 min. The grids were then either negative stained with 1% phosphotungstic acid (pH 7.1) or processed for immunogold labeling of virus particles. Immunogold labeling was performed essentially as described by Murti and Webster (24). After virus-carbon adsorption, the grid was rinsed briefly with Dulbecco PBS for 5 s, and excess PBS was removed by blotting. The surface of the grid to which the virus-carbon layer had been adsorbed was then blocked with 2% BSA in PBS for 15 min at 20°C. The grid was rinsed briefly in PBS and then placed on a 100-µl drop of MAb I1 (17) diluted 1:50 in PBS for 45 min. Excess antibody was removed by sequentially by placing the grid onto four 100-µl drops of PBS for 1 min each time. The grid was then placed onto a solution of 5-nm colloidal gold particles coated with rabbit anti-mouse immunoglobulin for 45 min. Preparation of 5-nm colloidal gold and its adsorption to immunoglobulin G were performed as described by Slot and Geuze (39). Unbound gold conjugates were removed by four sequential 5-min incubations in PBS. The virus-immunogold complexes were then negative stained by incubating the grids for 1 min

each on two 50- μ l drops of 1% phosphotungstic acid (pH 7.1). Excess stain was removed by a brief rinse in deionized water, and the grids were air dried after excess water was blotted from the grid. Images of negative-stained and immunogold-labeled virus preparations were obtained with a JEOL JEM-100 CX II electron microscope at 80 keV.

Metabolic labeling, immunoprecipitation, and sucrose gradient centrifugation. Labeling with [35 S]methionine was achieved by using Trans 35 S label (ICN Biomedicals, Inc.) in methionine-free, serum-free DMEM unless indicated otherwise. Immunoprecipitation of G protein and digestion with endo-*N*-acetylglucosaminidase H (endo H) were performed as described previously (31). Labeled proteins were resolved on 10% polyacrylamide gels containing SDS as described elsewhere (16) and visualized by fluorography (2). Scanning densitometry was performed with a Hoefer GS 300 densitometer as described previously (19). For immunoprecipitation of VSV proteins from crude virus pellets or from gradient fractions, virus particles were first disrupted with detergent solution (31); the suspension was made to 0.2% SDS and then incubated with rabbit anti-VSV antiserum. Linear sucrose gradients were prepared in Beckman SW41 tubes from stocks of 20 and 45% sucrose dissolved in 10 mM Tris hydrochloride (pH 7.4). Virus particles sedimented to the middle of the gradient after centrifugation at $180,000 \times g$ for 45 min.

RESULTS

To determine whether VSV G protein expressed from cloned cDNA could be incorporated into VSV particles, we used a temperature-sensitive mutant (*tsO45*) of VSV. Cells infected with *tsO45* and incubated at 40°C produce greatly reduced levels of infectious virus (8) as well as a small quantity of noninfectious particles lacking G protein spikes (36). A single amino acid substitution in the ectodomain (13) causes misfolding and aggregation of *tsO45* G protein at 40°C (3), which results in retention of the protein within the endoplasmic reticulum. We reasoned that high-level expression of wild-type G protein should rescue infectious virus particles from *tsO45*-infected cells. To obtain high-level transient expression, we used the system of Fuerst et al. (10), which is based on a recombinant vaccinia virus (vTF7-3) encoding bacteriophage T7 RNA polymerase. Cells infected with vTF7-3 produce T7 RNA polymerase, which then drives expression of genes under control of a bacteriophage T7 promoter carried on a plasmid transfected into the infected cells. Cells infected with vTF7-3 and transfected with a plasmid containing a G protein cDNA express large amounts of G protein. A similar approach was used by Li et al. (18) to rescue a VSV M-protein temperature-sensitive mutant by using a cDNA clone encoding wild-type M protein.

Rescue of *tsO45* by VSV G proteins from two serotypes. To obtain plasmids encoding VSV G proteins under control of the bacteriophage T7 promoter, we cloned cDNAs encoding G proteins from both Indiana (32) and New Jersey (12) serotypes into the vector pAR-2529 (10), also referred to as pET-3 (33). (The Indiana and New Jersey serotypes are hereafter indicated by subscripts I and NJ, respectively.) These plasmids are designated pAR-G_I and pAR-G_{NJ}. Transfection of these DNAs into BHK-21 cells infected with the vaccinia virus T7 polymerase vector vTF7-3 resulted in production of approximately 3×10^6 molecules of G protein per cell in the first 6 h after transfection (data not shown).

To determine whether this transient expression of G protein could rescue *tsO45*, cells that had been infected with

TABLE 1. Titers and neutralization of *tsO45* rescued with wild-type G protein

Plasmid	Titer (PFU/ml)	Neutralization ^a	
		Anti-VSV _I	Anti-VSV _{NJ}
pAR-2529	3×10^1	ND	ND
pAR-G _I	1.4×10^5	<10 ¹	10 ⁵
pAR-G _{NJ}	1.1×10^5	10 ⁵	<10 ¹

^a Neutralization assays were performed by incubating culture supernatants for 30 min at 37°C with a heat-inactivated (56°C, 30 min) polyclonal rabbit anti-VSV serum diluted 1:1,000. The supernatants were then titrated on mouse L cells at 34°C. ND, Not determined.

tsO45 were then superinfected with vTF7-3, transfected with pAR-G_I or pAR-G_{NJ}, and incubated at 40°C. Culture medium was collected after 18 to 21 h, and virus titers were determined on mouse L-cell monolayers at the permissive temperature (34°C). The titers from cultures transfected with pAR-G_I or pAR-G_{NJ} were greater than 10⁵ PFU/ml, whereas those from mock- or vector-transfected cells were 0 to 50 PFU/ml (Table 1). No plaques were observed when the culture medium was assayed at 40°C, indicating that the rescued virus was still temperature sensitive and showing that rescue of *tsO45* did not result from recombination or reversion of the temperature-sensitive phenotype (data not shown).

Infectivity results from the expressed G protein. G protein from the New Jersey serotype (G_{NJ}) was almost as efficient in rescuing *tsO45* as was G protein from the Indiana serotype (G_I) (Table 1). To determine whether the increase in infectivity after rescue was due to the expressed G protein, we examined whether antisera specific for VSV_{NJ} could neutralize the pAR-G_{NJ}-rescued virus. Neutralizing antisera are directed exclusively to the G protein (15). Because the G proteins from the two serotypes have only 50% homology, there is no cross-neutralization by antisera made against the individual serotypes. Anti-VSV_{NJ} serum neutralized virus rescued by G_{NJ} but not G_I. This result demonstrates that rescue resulted from the expressed VSV_{NJ} G protein. Similarly, anti-VSV_I serum neutralized only virus from cultures expressing G_I. Since *tsO45* belongs to the Indiana serotype of VSV, neutralization of pAR-G_{NJ} rescued virus by anti-VSV_{NJ} but not by anti-VSV_I antiserum indicates that expression of the VSV_{NJ} G protein does not stimulate *tsO45* G-protein transport.

Expression of G proteins with cytoplasmic tail deletions. To determine whether the 29-amino-acid cytoplasmic domain of VSV G protein was required for rescue, we first examined a series of G proteins with deletions in the cytoplasmic C terminus. Two precise deletions were described previously. These proteins retained one or three of the membrane-proximal cytoplasmic amino acids (4, 28). We also generated four additional deletion mutants for this study. These retained 9 or 18 of the membrane-proximal amino acids or had internal deletions (Table 3, Fig. 1). The cytoplasmic deletion

FIG. 1. Internal deletions of deletion mutants CTΔ2-17 and CTΔ10-17.

FIG. 2. Transport kinetics of G protein mutants. BHK-21 cells (3×10^5 cells in 35-mm-diameter dishes) were infected with vTF7-3 and transfected with the plasmids indicated. At 3 h posttransfection, the medium was replaced with methionine-free, serum-free DMEM and the cultures were incubated for 15 min at 37°C. The cultures were then pulse-labeled for 10 min in 1 ml of methionine-free DMEM without serum containing 50 μ Ci of [35 S]methionine. Cells were lysed immediately or chased with DMEM containing excess (2 mM) unlabeled methionine and 5% FCS for the times indicated. G protein was immunoprecipitated with anti-VSV₁ antiserum, and half of the immune complexes were incubated with or without endo H for 18 h at 37°C, followed by SDS-PAGE. The fraction of G protein having endo H-resistant oligosaccharides was determined by scanning densitometry of autoradiograms.

mutants are designated by the number of amino acids remaining in the cytoplasmic tail (CT-1, CT-3, CT-9, and CT-18) or by the residues deleted (CTΔ2-17 and CTΔ10-17).

In previous studies, CT-3 was observed to be transported about sixfold more slowly than wild-type G protein between the endoplasmic reticulum and Golgi compartments, as measured by the rate of acquisition of endo H-resistant oligosaccharides (28). Endo H resistance indicates transport to the medial Golgi compartment (7). To determine the transport rates of the other CT deletion mutants, the kinetics of acquisition of endo H-resistant oligosaccharides were examined. Each of the G proteins having partially deleted cytoplasmic tails was transported more slowly than the wild-type G protein, acquiring endo H-resistant sugars with half-times of approximately 1.5 to 2 h (Fig. 2). In contrast, the wild-type protein acquired endo H-resistant sugars with a half-time of 15 to 20 min. Because of the differences in transport, we felt it essential to establish the level of each protein on the cell surface.

To determine the relative amount of surface expression of each of the G proteins, cultures were transfected with plasmids encoding the different G proteins, and the cells were examined for surface fluorescence by flow cytometry. All of the CT mutants were expressed on the cell surface at 40°C, although at levels approximately two- to sixfold below that of wild-type G protein 7 h after transfection (Table 2). The amount of G protein on the cell surface was also examined by surface immunoprecipitation for several of the

TABLE 2. Surface expression of G proteins

Source of DNA	Mean (SD)		Relative surface expression ^c
	% of cells positive ^a	MFI ^b	
Wild type			
G ₁	71.9 (5.9)	81.2 (17.2)	1.0
G ₁ (10) ^d	49.7 (10.4)	64.4 (17.0)	0.55
G ₁ (50) ^d	32.3 (5.1)	31.1 (1.5)	0.17
CT-1	41.6 (2.4)	24.1 (2.7)	0.17
CT-3	42.3 (1.6)	26.8 (1.25)	0.19
CT-9	54.4 (6.5)	37.6 (5.6)	0.38
CT-18	52.5 (8.1)	43.0 (8.0)	0.38
CTΔ2-17	36.0 (8.7)	27.8 (3.7)	0.17
CTΔ10-17	53.7 (9.0)	43.6 (8.8)	0.40
G _μ	39.9 (8.7)	27.9 (3.0)	0.19
GHA	44.1 (9.7)	28.4 (3.4)	0.20
G23	38.9 (7.5)	28.2 (4.1)	0.19

^a From three experiments.

^b MFI, Mean fluorescence intensity.

^c Relative amount (compared with the value for wild-type G protein) of total surface expression in the culture for each G protein, determined from the percentage of cells positive $\times$ MFI for each of the G protein mutants divided by the same value for wild-type G₁.

^d Fold reduction in DNA used for transfection.

mutated G proteins. The results of cell surface immunoprecipitations showed reductions in surface expression similar to those found by flow cytometric analysis. For example, CT-9 and CT-1 were reduced approximately three- and fourfold, respectively, compared with the value for wild-type G protein.

Both membrane-proximal and membrane-distal cytoplasmic residues are sufficient for rescue. To determine whether the cytoplasmic domain of G protein was required for rescue, all of the CT mutants were tested in the rescue assay. Both CT-9 and CT-18 rescued *tsO45* at levels about three- to fivefold below that of the wild-type G protein (Table 3). In contrast, CT-1 and CT-3 gave only a marginal increase in titer over the value for the mock-transfected control. Reasonably efficient rescue of *tsO45* with CT-9 but not CT-3 suggested that amino acids in this membrane proximal portion of the cytoplasmic tail might be critical for rescue. Alternatively, the twofold reduction of surface expression obtained with CT-1 and CT-3 in comparison with CT-9 might have precluded the rescue of *tsO45*. To determine whether there was a minimum amount of surface expression required for rescue, we reduced the amount of wild-type G protein expression by transfecting limiting amounts of pAR-G₁ in the rescue assay. These experiments showed that a level of wild-type G protein surface expression equal to or less than that found for CT-3 gave rescue approximately 10- to 20-fold greater than that obtained with CT-1 or CT-3 (Tables 2 and 3). These results indicate that the failure of CT-1 and CT-3 to rescue *tsO45* was not due to an insufficient level of surface expression.

To assess the importance of the membrane-proximal residues, we examined two more mutants with internal deletions of residues 2 to 17 (CTΔ2-17) and 10 to 17 (CTΔ10-17) of the cytoplasmic tail. Rescue of *tsO45* with CTΔ2-17 gave titers equivalent to those of wild-type G protein when similar levels of surface expression were compared. Likewise, CTΔ10-17 rescued *tsO45* to near-wild-type levels (Table 3). Thus, G proteins with either the membrane-proximal 9 or membrane-distal 12 amino acids of the cytoplasmic domain could rescue *tsO45* as efficiently as wild-type G protein when expressed at similar levels. In contrast, G proteins with only

TABLE 3. Rescue of *tsO45* with G protein cytoplasmic tail deletion mutants

Source of DNA	Cytoplasmic tail	Titer (PFU/ml) ^a
Wild type		
G ₁	RVGIHLCKLKHHTKKRQIYTDIEMNRLGK	1.1–1.9 × 10 ⁵
G ₁ (10) ^b		5.2–5.7 × 10 ⁴
G ₁ (50) ^b		5.5–5.8 × 10 ³
CT-1	R	1.6–4.2 × 10 ²
CT-3	RVG	2.3–2.5 × 10 ²
CT-9	RVGIHLCK	2.5–3.5 × 10 ⁴
CT-18	RVGIHLCKLKHHTKKRQI	3.3–6.2 × 10 ⁴
CTΔ2-17	RIYTDIEMNRLGK	4.0–6.0 × 10 ³
CTΔ10-17	RVGIHLCKIYTDIEMNRLGK	5.4–7.7 × 10 ⁴
None		0–5 × 10 ¹

^a Range determined after rescue with each G protein. Titers were determined from at least three separate rescue experiments except in the case of G₁ (10) and G₁ (50), for which values from two rescue assays are presented.

^b Fold reduction in the amount of pAR-G₁ used for transfection.

one or three amino acid cytoplasmic domains did not rescue *tsO45* efficiently.

G proteins with foreign cytoplasmic tails do not rescue *tsO45*. The results obtained with CT-9 and CTΔ2-17 suggested that only a certain length of cytoplasmic domain might be required for rescue of *tsO45*. To test this possibility, we examined the ability of G protein with foreign cytoplasmic domains to rescue *tsO45*. Several G proteins in which the cytoplasmic domain of G protein was replaced with the cytoplasmic domain of a heterologous protein were constructed previously (28). These G proteins, containing the cytoplasmic domains of influenza virus hemagglutinin (GHA; 10-amino-acid cytoplasmic domain), infectious bronchitis virus glycoprotein p23 (G23; 128 amino acids), and the membrane-bound form of immunoglobulin heavy chain (G_μ; 3 amino acids) were examined in the rescue assay. None of the G proteins with foreign cytoplasmic tails gave significant virus titers (Table 4). All of the G proteins with foreign tails were transported as fast as or faster than both CT-9 or CT-18, and all were expressed on the cell surface at levels approximately fivefold below that of wild-type G protein at 40°C (Table 2). Thus, the failure of these mutants to rescue was not the result of a transport defect. These results also show that heterologous cytoplasmic domains do not substitute for the G protein cytoplasmic domain and demonstrate that it is not simply a minimum length of amino acid sequence that is required for rescue.

Correlation of rescue with incorporation of the expressed G protein into virions. To determine whether the rescued virions were morphologically similar to wild-type VSV particles, we examined particles by electron microscopy after sedimentation from the medium. Virions produced in *tsO45*-infected cells grown at the permissive temperature were examined first. The particles were intact and had the typical array of G protein spikes protruding from the virion surface

(Fig. 3A). In contrast, only spikeless particles were produced in *tsO45*-infected cells at the nonpermissive temperature (not shown). The spikeless particles were approximately 1% of the total number of virions observed at the permissive temperature. Although virions rescued by G protein had clear areas of G protein spikes, they never appeared to have a full complement of spikes on the viral envelope (Fig. 3B). Importantly, only those virions obtained from culture medium containing infectious virus had discernible spikes on the virion surface. In addition, we did not observe a significant increase in particle number even after rescue with wild-type G protein. The absence of a complete complement of G protein on the virion surface was not a function of propagation at 40°C, since wild-type VSV particles grown at 40°C in the presence of vaccinia virus contained a full complement of spikes (data not shown). Particles obtained from cells in which rescue was attempted with CT-1, CT-3, GHA, G23, or G_μ were spikeless. An example from an attempted rescue with CT-1 is shown in Fig. 3C.

To make certain that the spikes observed on the rescued particles were truly G protein, the rescued virions were also examined by electron microscopy after immunogold labeling. Figure 3D and E show typical examples of virions rescued by wild-type G₁ and CT-9, and Fig. 3F shows a typical nonrescued particle. Quantitation of immunogold-labeled virions rescued by wild-type G₁ protein and CT-9 revealed that 25 to 35% of virus particles had enough G protein to be detected by our immunogold-labeling procedure (Table 5). In contrast, only 4 to 7% of the particles from mock- or CT-1-transfected cultures showed any gold labeling.

Absence of *tsO45* G protein in rescued virions. As described earlier, *tsO45* virions rescued by G_{NJ} were neutralized only by antisera to VSV_{NJ}. This suggested that the particles did not contain the *tsO45* G protein. To examine this question

TABLE 4. Sequence, transport, and rescue of G proteins with foreign cytoplasmic tails

Source of G protein	Cytoplasmic tail	Endo H ^a t _{1/2} ^a	Titer (PFU/ml) ^b
Wild-type G ₁	RVGIHLCKLKHHTKKRQIYTDIEMNRLGK	20 min	1.2–1.4 × 10 ⁵
GHA	RGNIRCNICI	1 h	4–8 × 10 ¹
G _μ	KVK	1.75 h	8–9 × 10 ¹
G23	<u>RV</u> GRLFKRCR...(n = 128) ^c	25 min	0–2 × 10 ¹
None			0–3 × 10 ¹

^a Half-times for acquisition of Endo H-resistant sugars; determined by Puddington et al. (28).

^b Ranges from two experiments.

^c The underlined amino acids are VSV G protein cytoplasmic tail sequences present in this construct.

FIG. 3. Electron microscopy of rescued particles after negative staining or immunogold labeling. Virus particles recovered from medium were examined after negative staining (A to C) or after labeling with mouse anti-G-protein monoclonal antibody and rabbit anti-mouse immunoglobulin G-coated colloidal gold, followed by negative staining (D to F). (A) *tsO45* particles grown at 34°C; (B) G_1 -rescued particles (arrow indicates surface projections); (C) spikeless particle from a CT-1-transfected culture; (D to F) immunogold-labeled particles from G_1 -, CT-9-, and CT-1-transfected cultures, respectively.

directly, particles obtained from G_{NJ} -rescued cultures were examined by immunogold electron microscopy, using either G_{NJ} -specific or G_1 -specific monoclonal antibody. Virions rescued by expression of G_{NJ} were recognized only by the G_{NJ} -specific antibody. As was determined for G_1 -rescued

virions, the G_{NJ} -rescued particles were approximately 30% of the total particles observed. Immunogold analysis of the same virus preparation by using a G_1 -specific monoclonal antibody gave only background levels of labeled virions (5.7% of the total). This result confirms the neutralization data suggesting the absence of significant amounts of *tsO45* G protein in rescued particles.

Analysis of rescued virions by SDS-PAGE. To examine the composition of rescued virions, we recovered [^{35}S]methionine-labeled, rescued particles from the medium centrifugation and purified the virions in the pellet by sedimentation in sucrose gradients. To determine the protein composition of the crude virus pellet before sedimentation, a fraction was immunoprecipitated directly and the remainder was loaded onto a sucrose gradient. Gradient fractions were immunoprecipitated and analyzed by SDS-PAGE. Samples of the gradient fractions were also examined for infectivity by plaque assay. The peak of infectious virus was located in fractions 6 and 7 (not shown). Virions obtained after rescue with G_1 contained detectable amounts of the wild-type G protein (Fig. 4A). This protein was distinguishable from the *tsO45* G protein because of its faster mobility. The truncated

TABLE 5. Quantitation of rescued virions containing G protein^a

Source of G protein	Gold-labeled virions/total virions	% of particles containing G protein
Wild-type G_1	132/424	31
CT-9	179/665	27
CT-1	15/228	7
None	7/175	4

^a Virus particles were recovered from culture supernatants by centrifugation at $115,000 \times g$ for 60 min through a 20% sucrose cushion. The particulate fraction was suspended in 10 mM Tris hydrochloride (pH 7.4) and processed for electron microscopy after immunogold labeling. Fields of the grid were photographed at a magnification of $\times 10,000$, and virions were counted after the photographs were enlarged threefold. Virions that were decorated with five or more gold particles were considered positive for G protein. The total number of virions counted was obtained from 12 or more random fields.

FIG. 4. Analysis of rescued virus particles by sedimentation in sucrose gradients. Rescue assays were performed as described in Materials and Methods, with the following modifications. Transfections were carried out in 2 ml of DMEM containing 20% (0.04 mM) of the normal amount of methionine and no FCS. At 3 h posttransfection, the cultures were pulse-labeled with 100 μ Ci of [35 S]methionine per ml for 90 min. After the pulse, an equal volume of DMEM containing 10% FCS was added and the cultures were incubated for 18 to 21 h at 40°C. Virus particles were recovered from the medium by centrifugation at $115,000 \times g$ and suspended in 100 μ l of 10 mM Tris hydrochloride (pH 7.4), and 80 μ l was layered onto the top of linear 20 to 45% sucrose gradients in Beckman SW41 tubes. The gradients were centrifuged at 38,000 rpm for 45 min, and then 1-ml fractions were collected by bottom puncture. The fractions, in addition to the remaining 20 μ l of the crude virus pellet, were immunoprecipitated with rabbit anti-VSV₁ antiserum, and the immunoprecipitates were analyzed by SDS-PAGE. Gradient fractions from the bottom (fraction 1) to the top (fraction 12) are shown. Lane M, Wild-type VSV₁ marker proteins; lane P, crude virus pellet. Virus particles were from G₁-transfected (A), CT-18-transfected (B), mock-transfected (C), and GHA-transfected (D) cultures.

G protein, CT-18, was also incorporated into virions (Fig. 4B, fraction 6), although the amount of CT-18 in virions appeared to be less than the amount of wild-type G protein found in the G₁-rescued virus. In contrast, particles from mock-transfected cultures or cultures in which rescue was attempted with GHA contained very little G protein (Fig. 4C and D). Some *tsO45* G protein, however, could be detected in particles from mock-transfected cultures after longer exposures.

In all cases, regardless of whether rescue was obtained, the crude virus pellet fraction contained the expressed G protein. This can be seen clearly in Fig. 4D with the GHA protein (lane P). Although GHA was detected in the crude virus pellet, it was not incorporated into particles (fractions 6 and 7) and probably represents G protein in membrane vesicles released from the infected culture at 40°C. In fact, vesicles containing G or mutant G proteins were always observed in the crude virus fraction by electron microscopy after immunogold labeling.

DISCUSSION

Models of enveloped virus assembly have generally proposed that interactions between nucleocapsid components and the cytoplasmic domains of viral membrane G proteins drive the virus assembly process and may result in the exclusion of host proteins from the viral envelope (reviewed in reference 37). Although such models are attractive, they have been difficult to test. The elegant work of Vaux et al. (41) demonstrated an interaction between the cytoplasmic tail of Semliki Forest virus E2 envelope protein and the nucleocapsid, but it was not shown that this interaction is essential for virus assembly.

We described here an assay in which the requirements for G protein assembly into virions were tested directly. In this assay, transient expression of wild-type VSV G protein from a cDNA clone resulted in the rescue of infectious virus particles from cells infected with a temperature-sensitive mutant of VSV (*tsO45*) encoding a G protein that is not transported to the cell surface. Rescue of *tsO45* was also obtained with the G protein from a different serotype of VSV, and the infectious particles were neutralized only with antisera directed against the foreign G protein. This critical result shows that rescue must occur by incorporation into the mutant virus particles of the G protein encoded by the cDNA. Further experiments analyzing rescue by using G proteins with deleted cytoplasmic tails demonstrated that either the membrane-proximal or membrane-distal C-terminal portion of the normal cytoplasmic domain was sufficient for incorporation of G protein into virions with near-wild-type efficiency when corrected for expression levels. In contrast, G proteins with only one or three of the membrane-proximal amino acids or G proteins with foreign cytoplasmic domains of 3, 10, or 128 amino acids gave very low levels of rescue and were not detected in virus particles. The low level of rescue obtained with some of the mutated G proteins may reflect random, nonspecific incorporation into virus particles. The effects of cytoplasmic domain alterations on the rescue of *tsO45* did not appear to result from indirect effects on folding elsewhere in the G protein molecule because mutant G proteins having as little as one cytoplasmic amino acid (CT-1) form trimers and apparently have correctly folded extracellular domains (4).

One factor that could obviously affect the ability of the mutated G proteins to rescue *tsO45* was the amount of each on the cell surface at 40°C. In general, G proteins that did not

rescue, or gave marginal rescue of, *tsO45* were expressed on the cell surface at levels approximately five- to sixfold below that found for wild-type G protein, whereas the CT mutants that rescued *tsO45* efficiently showed only two- to threefold reductions in surface expression. By varying wild-type G protein expression, we showed that expression level is probably responsible for the lower titers obtained with mutants having nine or more of the normal cytoplasmic amino acids. However, the reduction in surface expression did not account for the failure of the short-tail (CT-1 and CT-3) or foreign-tail mutants to rescue. We have considered an alternative explanation for our data on the lack of incorporation of mutant G proteins into virus particles. Those with short or foreign cytoplasmic domains might be incorporated well but subsequently released from the virion envelope after budding. Although it is impossible to rule out this possibility, it seems unlikely since none of the mutant G proteins are found to be released from the plasma membrane in transfected cells.

It was surprising that two different regions of the normal cytoplasmic domain, the first 9 amino and the last 12 residues, appeared sufficient for the incorporation of G protein into virions. It is possible that two regions of the cytoplasmic domain have evolved to interact with nucleocapsids during virus assembly. Such a redundancy would ensure the efficient incorporation of VSV G protein into virions; however, further analysis is required to examine this possibility. The fact that all of the deletion mutants are transported to the Golgi apparatus more slowly and all are expressed at lower levels on the cell surface than is wild-type G protein suggests that the intact cytoplasmic domain not only is important for assembly of G protein into virus particles but also contributes to the rapid intracellular transport and high-level surface expression found for wild-type G protein. Both of these factors would be important for rapid maturation of the virus.

Our results contrast somewhat with those of Perez et al. (27), who reported that the cytoplasmic domain of the Rous sarcoma virus envelope G protein complex gp85/gp37 is not required for efficient production of infectious Rous sarcoma virus particles. This difference may reflect fundamental differences in the budding processes of retroviruses and other enveloped viruses such as VSV. Alternatively, the difference may simply reflect differences in the nature or sensitivity of the assays used.

The release of spikeless particles from *tsO45*-infected cells (36) prevents us from determining whether G protein is required to initiate virus budding. These particles contain a C-terminal fragment of G protein (22), which may be required for budding of the spikeless particles. In our experiments, rescue with wild-type G protein gave a 10⁴- to 10⁵-fold increase in titer, but we did not see a significant increase in virus particle number by electron microscopy or with metabolically labeled virions purified on sucrose gradients. This is not surprising, since only about 30% of the particles contain patches of G protein on their surfaces, and a 30% increase in particle number would not easily be detected. Thus, the rescued virions may result in part from the incorporation of the expressed G protein into particles already containing some C-terminal G protein fragments.

Our studies raise one point relevant to the mechanism of virus pseudotype production. These particles are formed after mixed infections with several enveloped viruses (reviewed in reference 45) and contain the nucleocapsid of one virus surrounded by an envelope containing G protein spikes from another virus. It was proposed that VSV pseudotype

production might result from a few G protein molecules that nucleate VSV nucleocapsid budding in regions of the plasma membrane containing a high density of the other viral G protein (34, 44). Alternatively, the formation of pseudotypes might result from the inability of VSV nucleocapsids to discriminate between regions of the plasma membrane containing heterologous viral G proteins. Our results show that a G protein having the cytoplasmic domain of an influenza virus hemagglutinin (GHA) is not incorporated into VSV virions. This finding suggests that the cytoplasmic domain of hemagglutinin does not interact with VSV nucleocapsids and that formation of VSV pseudotypes containing influenza virus hemagglutinin(46) probably results from fortuitous trapping of hemagglutinin molecules in virus particles already containing VSV G protein. Alternatively, these pseudotypes might contain some internal influenza virus components.

Growth of *tsO45* at the nonpermissive temperature in cells producing avian leukosis virus (ALV) results in titers of infectious VSV that are 10^4 -fold higher than in control cells, whereas cells releasing murine leukemia virus or avian reticuloendotheliosis virus rescue *tsO45* much less efficiently (42). The particles rescued by ALV contain ALV envelope protein but no detectable G protein, suggesting that the ALV envelope protein might contain a sequence that interacts well with VSV nucleocapsids or matrix protein. This model should be testable in our system.

Although we had previously attempted rescue of the *tsO45* mutant by using simian virus 40-based vectors expressing G protein (30), the results were poor and irreproducible, probably because of interference between simian virus 40 replication and VSV replication (unpublished results). Also, cell lines expressing G protein from a bovine papillomavirus vector (9) gave only marginal rescue, perhaps because of low rates of G protein synthesis (C. J. Gallione and J. K. Rose, unpublished results). The success of the vaccinia virus-T7 polymerase system probably derives from the high rate and early onset of G protein synthesis and from a lack of interference between vaccinia virus and VSV at early times after vaccinia virus infection. The system we have described here should be adaptable to other viruses and may allow incorporation of foreign proteins into VSV particles.

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