A Fusion-Defective Mutant of the Vesicular Stomatitis Virus Glycoprotein

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Vesicular stomatitis virus (VSV) is the prototype rhabdovirus and, like other members of this group, contains a single membrane-spanning spike glycoprotein (G) in its membrane envelope. Infection by many enveloped viruses such as VSV involves binding of the virus to a receptor on the cell surface, internalization by endocytosis, and fusion of the viral envelope with the endosomal membrane (13, 18). For VSV, the membrane fusion event is catalyzed by G protein (7, 22) and occurs in the endosome, where G protein undergoes a conformational change induced by low pH. Viral and endosomal membrane fusion releases the viral nucleocapsid into the cytoplasm and is thus required for infection. Although the amino acid sequences involved in receptor binding and membrane fusion have been determined for several enveloped viruses (see reference 28 for a review), these sequences have not been identified for VSV or other rhabdoviruses. Analysis of mutations in G protein affecting binding, fusion, or release of nucleocapsids should be useful in understanding the early events of infection.

We have recently described a system for identifying mutations affecting VSV G protein function. G protein expressed from cloned DNA is used to rescue a temperature-sensitive mutant of VSV (tsO45) which makes a defective G protein that is not transported to the plasma membrane at the nonpermissive temperature (31). This system provides a sensitive assay for examining the ability of mutant glycoproteins to be incorporated into particles or to function in the subsequent steps of virus binding and entry. Using this rescue assay, we were able to demonstrate a requirement for at least part of the normal cytoplasmic tail of VSV G protein in its assembly into virus particles (31). By using this system to analyze a variety of VSV G proteins carrying mutations in the extracellular domain, it should be possible to localize domains of the glycoprotein involved in later steps of virus infectivity.

We previously described the generation of point mutations that introduced new sites for asparagine-linked (N-linked) glycosylation in the VSV G protein. Although one of these proteins was misfolded and retained in the endoplasmic reticulum, two others appeared to fold correctly and were transported to the plasma membrane (16, 17). Here we show that one of the mutants (QN-1) that is transported normally was incorporated into virus particles but that it did not rescue infectivity, apparently because it lacked fusion activity. This mutant also formed heterotrimers with a hybrid G protein that has the wild-type G protein ectodomain and interfered with rescue by wild-type VSV G protein.

Although hydrophobic domains involved in membrane fusion have been identified in several viral fusion proteins (11, 28, 32), such a domain has not been identified in any rhabdovirus glycoprotein. Because the extra oligosaccharide at amino acid 117 in QN-1 immediately precedes a region consisting largely of uncharged amino acids (residues 118 to 136), we suggest that this domain may be directed involved in the membrane fusion activity of VSV G protein.

MATERIALS AND METHODS
Viruses, cell culture, plasmids, and rescue assay. Stocks of tsO45 and the recombinant vaccinia virus vTF7-3 were prepared as described previously (31). All cDNAs encoding G protein glycosylation mutants (see Fig. 1) were excised with BamHI from pJC119-based plasmids (16) and inserted into the unique BamHI site of pET-3 (25). Rescue of tsO45 was performed as described previously (31) except that all plasmid transfections were mediated by a modification of the lipofection procedure (6), using dimethylidioctadecyl ammonium bromide (Sigma Chemical Co.) as the cationic lipid (J. Rose, L. Buonocore, and M. Whitt, submitted for publication).

Virus binding assays. The procedure used to determine the amount of virus binding was a modification of that used by Matlin et al. (19). Virus particles radioactively labeled with [35S]methionine were recovered from the medium by cen-
trifugation after rescue of tsO45 with the indicated G protein. A sample of the medium was titered by plaque assay on mouse L cells before centrifugation to determine the amount of infectious virus present. Virions were sedimented through a 20% sucrose cushion at 115,000 × g for 1 h and suspended in 100 µl of 10 mM Tris hydrochloride (pH 7.4), and the amount of radioactivity in the virus fraction was determined by scintillation counting. Confluent monolayers of mouse L cells were washed twice with ice-cold sodium bicarbonate-free Dulbecco modified Eagle medium (DMEM) containing 0.1% bovine serum albumin (BSA) and buffered to pH 6.4 to 6.6 with 2 mM 2-(N-morpholino)ethanesulfonic acid (MES) and 2 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (binding medium) and were then incubated on ice for an additional 10 min in binding medium. The monolayers were then maintained at 4°C during the binding assay. Approximately 3 × 10⁴ cpm of [³⁵S]methionine-labeled virus was adsorbed to the cells for 1.5 h in 200 µl of binding medium, and the plates were rocked every 15 min. All assays were performed in duplicate. After binding, the inoculum was removed and the monolayers were washed four times with ice-cold binding medium. The cells were then solubilized in phosphate-buffer saline (PBS) containing 1% Nonidet P-40 (Sigma) and scraped from the dish with a rubber policeman. Duplicate samples were suspended in Ecoscint scintillation fluid (National Diagnostics), and the amount of labeled virus bound was determined. To determine the amount of virus internalized, cells were removed from the plate with PBS containing 50 mM EDTA and then pelleted at 4,000 rpm for 1 min in a microcentrifuge. The cell pellet was resuspended in ice-cold binding medium (pH 7.4) without BSA but containing proteinase K (0.5 mg/ml; Sigma) and incubated on ice for 45 min to remove bound virus remaining on the cell surface. The protease-treated cells were then washed three times with ice-cold binding medium (pH 7.4) containing 0.1% BSA. Cell-associated radioactivity was determined after solubilizing as described above.

**Metabolic labeling, immunoprecipitation, and flow cytometry.** Baby hamster kidney (BHK-21) cells were infected with vTF7-3 and transfected with the appropriate plasmid(s). Cells transiently expressing G proteins were radioactively labeled with [³⁵S]methionine in serum-free, methionine-free DMEM at 3 to 4 h posttransfection, using Trans³⁵S Label (ICN Biomedicals, Inc.). Immunoprecipitation of single G proteins was performed as described previously (23). For coimmunoprecipitation of G protein heterotrimers, cells were solubilized in MNT buffer (pH 5.8) containing 1% Triton X-100 (4), nuclei and cytoskeleton were removed by centrifugation at 14,000 rpm for 2 min in a microcentrifuge (Eppendorf), and the cell extracts were precleared by incubation with an irrelevant antiserum for 6 h on ice, followed by incubation with Pansorbin (Calbiochem, Inc.) for an additional 6 h on ice. The supernatants were then divided into three portions and incubated with the appropriate antiserum for 6 h on ice. Immune complexes were recovered with Pansorbin and then washed with MNT buffer (pH 5.8) containing 1% Triton X-100. Labeled proteins were resolved on 10% polyacrylamide gels containing sodium dodecyl sulfate (SDS) (14) and visualized by fluorography (1). Quantitation of immunoprecipitated proteins was determined by scanning densitometry as described previously (15). Flow cytometry was performed as described previously (31) except that after fixation, the cells were washed once with PBS containing 10 mM glycine (PBS-glycine) and then pelleted in PBS-glycine for 30 min to block any residual fixative. The fixed cells were then incubated in PBS-glycine containing 0.2% BSA for an additional 30 min. All subsequent antibody incubations and washes were performed in PBS-glycine-BSA.

**RESULTS**

The wild-type VSV G protein contains two N-linked oligosaccharides at amino acids 179 and 336. Three mutant VSV G proteins were made in our laboratory previously with single amino acid changes introduced to generate additional sites for oligosaccharide attachment at amino acids 25, 49, and 117 (KT, HS, and QN-1, respectively) (16). The positions of the mutations are shown in Fig. 1. Two of the additional sites were glycosylated (KT and QN-1); however, the site designated HS was not glycosylated although a consensus site for N-linked sugar addition which is not glycosylated.

![FIG. 1. Schematic representation of wild-type (wt) G protein and G-protein glycosylation mutants. The three glycosylation mutants HS, KT, and QN-1 were generated previously (16). The locations of N-linked oligosaccharides are indicated (Δ), and the amino acid residues to which the additional glycans are attached are given in parentheses. Amino acid numbering corresponds to the protein before signal sequence cleavage (24). The amino acid residues that are glycosylated in the wild-type G protein and all of the mutants are indicated above the wild-type sequence. The HS mutant contains a consensus site for N-linked sugar addition which is not glycosylated.](http://jvi.asm.org/fig/fig1.jpg)

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To examine the effects of these mutations on the abilities of the altered G proteins to assemble into and function in virus particles, we used a system in which transient expression of wild-type G protein from cloned cDNA results in the rescue of the temperature-sensitive mutant of VSV (tsO45) at the permissive temperature (31). Cells were infected with tsO45, and the indicated G protein was expressed by using the transient system of Fuerst et al. (8) at 40.5°C. The rescue of tsO45 by each of the G-protein mutants was then determined by titration at 34°C of virus produced. We found that neither KT nor QN-1 rescued tsO45 and that the titer obtained after rescue with HS was significantly lower than that obtained with wild-type G protein (Table 1).

To determine whether the mutant proteins were expressed on the cell surface at the high temperature required for the rescue assay (40.5°C), we examined the expression of each of the mutant G proteins at both 37 and 40.5°C by flow cytometry. At 37°C, the relative surface expression of the three mutant G proteins was equivalent to that of wild-type G protein. At 40.5°C, QN-1 showed the same level of surface expression as did wild-type G protein; however, cells ex-
pressing KT showed only 5% and HS showed approximately 50% of the surface expression levels of wild-type G protein at the higher temperature (Table 1). Pulse-chase experiments showed that KT was degraded at 40.5°C with a half-life of approximately 2 h, whereas HS had a half-life of approximately 3.5 to 4 h. In contrast, both QN-1 and wild-type G protein were relatively stable at 40.5°C, with half-lives of greater than 5 h (data not shown). The reduction in rescue efficiency observed with HS could therefore be explained by a shorter half-life resulting in a lower level of surface expression than for wild-type G protein. Similarly, the rapid degradation of KT and low levels of surface expression could explain the inability of KT to rescue tsO45. These results also suggested that the single point mutation in HS and the mutation or the extra oligosaccharide on KT interfered with protein folding at 40.5°C. In contrast, the additional carbohydrate on QN-1 had no discernible effect on protein stability or transport at the elevated temperature.

**QN-1 is efficiently incorporated into tsO45 virions.** Although expression of QN-1 did not result in the production of infectious particles in the rescue assay, the protein was present on the cell surface at a level comparable to that of wild-type G protein. This result suggested that the extra oligosaccharide was either blocking incorporation of QN-1 into particles or affecting some early step in infection. To determine whether QN-1 was incorporated into virions, we examined the protein composition of [35S]methionine-labeled particles recovered from the medium of tsO45-infect ed cells expressing QN-1. Radioactively labeled virus particles were collected by centrifugation and purified by sedimentation in a sucrose gradient. Fractions were immunoprecipitated and then analyzed by SDS-polyacrylamide gel electrophoresis. A peak of QN-1 was found in fractions that contained intact virions (31), indicating that QN-1 was incorporated into VSV particles (Fig. 2). Quantitation of QN-1 present in fractions 6 and 7, relative to the amount of N or M in the same fractions, showed that the amount of QN-1 incorporated was similar to that found for particles rescued with wild-type G protein (31), indicating that the lack of infectivity observed after rescue of tsO45 with QN-1 was not due to the inability of QN-1 to assemble into virus particles.

**Viruses containing QN-1 bind to cells and are endocytosed.** To determine whether the failure of particles containing QN-1 to infect cells was due to a defect in the binding of these particles to cells, we compared the binding properties of particles containing either QN-1 or wild-type G protein. Rescued particles labeled with [35S]methionine were concentrated from the medium by centrifugation and adsorbed to confluent monolayers of L cells at 4°C. After adsorption, the cells were washed extensively, the monolayers were solubilized, and cell-associated radioactivity was determined.Particles rescued with wild-type G protein or QN-1 bound equally well to the monolayers; however, only 10 to 17% of the input radioactivity remained cell associated after washing. A similar low level of virus binding has been shown previously for wild-type VSV virions (19).

At least 40% of the binding observed with particles containing QN-1 appeared to be specific because this fraction could be competed with increasing concentrations of wild-type VSV virions (Fig. 3A). Furthermore, particles containing QN-1 were internalized with kinetics similar to those of rescued virions containing wild-type G protein (Fig. 3B). This amount of specific binding correlated well with our previous results showing that only 30% of the virions have detectable amounts of G protein after rescue of tsO45 with wild-type G protein (31).

**QN-1 is defective in cell-cell fusion activity.** We next determined whether QN-1 had the membrane fusion activity characteristic of wild-type VSVG protein. When cells expressing wild-type G protein are incubated briefly at a pH of 6.0 or lower, the acid-induced fusogenic activity of VSVG protein causes cell-cell fusion (7, 22). Cells expressing either wild-type G protein or QN-1 were incubated for 1 min at pH 5.5 and then monitored for cell-cell fusion after incubation at 37°C in normal growth medium. Parallel cultures were also

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Table 1. Surface expression and rescue with G protein glycosylation mutants

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<thead>
<tr>
<th>DNA</th>
<th>Relative surface expression*</th>
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<td></td>
<td>37C</td>
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<td>Wild-type G1</td>
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<td>HS</td>
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<tr>
<td>KT</td>
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<td>0.05</td>
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<tr>
<td>QN-1</td>
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* Determined by flow cytometry for cells expressing G proteins at the indicated temperatures in the absence of tsO45. Values represent the amount of surface G protein in each culture relative to the amount wild-type G protein.

* Values from at least three separate rescue experiments with each G protein.
examined by flow cytometry to determine the relative level of surface expression for each protein. After exposure to pH 5.5, cells expressing wild-type G protein formed extensive syncytia, but the cells expressing QN-1 showed no syncytium formation (Fig. 4). The lack of fusion by QN-1 was not explained by reduced surface expression because the level of expression observed by flow cytometry was slightly higher than in the wild-type G control. To determine whether QN-1 had a different pH requirement for fusion than wild-type G protein, we examined the fusion activity of either QN-1 or wild-type G protein after exposure to pHs from 4.0 to 6.5, in increments of 0.5 pH unit. Cells expressing wild-type G protein formed syncytia after incubation at pH 6.0 or lower, but under no circumstances did cells expressing QN-1 form syncytia (data not shown). These results indicate that QN-1 is not able to induce membrane fusion and suggest that the lack of infectivity of particles containing QN-1 is due to a fusion defect.

QN-1 interferes with rescue by wild-type G protein. The inability of particles containing QN-1 to infect cells and the failure of cells expressing QN-1 to form syncytia after exposure to low pH suggested that the additional oligosaccharide on G protein was preventing fusion of the viral envelope with the endosomal membrane. To determine whether QN-1 might interfere with the function of wild-type G protein (perhaps through the formation of hetero-oligomers), we performed a rescue by expressing wild-type G protein and QN-1 simultaneously. Cells infected with tsO45 and vTF7-3 were cotransfected with plasmids encoding QN-1 and wild-type G protein of the Indiana serotype (G1), and the medium was assayed for infectious virus after incubation at 40.5°C. To control for any inhibitory effect resulting from the coexpression of two proteins in the rescue assay, we also examined the titers obtained after coexpressing G and an unrelated protein, the alpha subunit of human chorionic gonadotropin (hCG-α) (12). Coexpression of QN-1 and wild-type G protein, at a ratio of 1:1.5, respectively, resulted in a 32-fold reduction in the titer of rescued virus compared with the titers found after rescue by coexpression of wild-type G and hCG-α (Table 2). Increasing the amount of QN-1 expression 2-fold over that of wild-type G protein decreased infectivity greater than 180-fold.

QN-1 and wild-type G protein can form heterotrimers when coexpressed. The strong inhibition of rescued virus infectivity resulting from the coexpression of QN-1 and wild-type G protein suggested that QN-1 might be preventing wild-type G protein from inducing fusion of the viral envelope with the endosomal membrane, thereby preventing infection. One mechanism by which QN-1 could interfere strongly with the activity of wild-type G protein would be if QN-1 and wild-type G protein formed mixed oligomers that were then fusion defective. Recently we have shown that wild-type and some mutant forms of G protein can form heterotrimers efficiently when coexpressed (P. Zagouras, A. Ruusala, and J. Rose, unpublished data). To determine whether QN-1 could form heterotrimers with other G proteins, we used a coimmunoprecipitation assay. This assay relies on the use of two different antibodies that can distinguish between the two proteins involved in heterotrimer formation. Because all of our antibodies recognize both QN-1 and wild-type G protein equally well, we examined whether QN-1 could form heterotrimers with a hybrid G protein (G23) which contains the extracellular and transmembrane domains of wild-type VSV G protein and the cytoplasmic domain of the infectious bronchitis virus p23 membrane protein (21). Immunoprecipitation of cell lysates with an antibody specific for the cytoplasmic tail of infectious bronchitis virus p23 resulted in the coprecipitation of G23 and QN-1. Likewise, antiserum specific for the C-terminal portion of the VSV G protein cytoplasmic domain precipitated both proteins (Fig. 5A). Sucrose gradient analysis of lysates from cells coexpressing QN-1 and G23 followed by coimmunoprecipitation of the trimer peak with the cytoplasmic tail antibodies confirmed that QN-1 and G23 were in heterotrimers (data not shown). Because the determinants for trimer formation are in the
extracellular domain (3), it is therefore very likely that wild-type G protein and QN-1 also form heterotrimers.

If the strong inhibition of rescue caused by the coexpression of QN-1 with wild-type G protein were due to heterotrimer formation, then QN-1 should not interfere as strongly with rescue by a glycoprotein with which it does not form heterotrimers. To test this hypothesis, we performed a rescue assay by coexpressing QN-1 and the wild-type G protein of the New Jersey serotype (G_NJ). We have shown previously that G_NJ is efficiently incorporated into tsO45 particles and produces infectious virions (31); however, QN-1 (an Indiana serotype G protein) and G_NJ did not form heterotrimers, apparently because of extensive divergence of the Indiana serotype and New Jersey serotype G protein ectodomain sequences (Fig. 5B). The effect of QN-1 on rescue by G_NJ was, in fact, much smaller than that seen for the homologous G_I protein (Table 2); however, there was still significant interference by QN-1.

DISCUSSION

In this report, we have described the incorporation into virions of a mutated VSV G protein (QN-1) that has an extra N-linked glycan at position 117 in the extracellular domain. Particles formed with the mutant glycoprotein are noninfectious, apparently because the QN-1 protein lacks all membrane fusion activity. We postulate a mechanism involving the failure of QN-1 to effect membrane fusion because cells expressing QN-1 did not form syncitia after exposure to acidic pH. This mutation (a single amino acid change) does not appear to have major effects on protein folding because it does not interfere with G protein oligomerization (5),

![FIG. 4. Acid-induced syncytium formation in cells expressing QN-1 and wild-type G protein. HeLa cells were infected with vTF7-3 and transfected with plasmids containing the cDNAs for either wild-type G_I protein or QN-1. The cultures were rinsed once with PBS and then incubated for 1 h in fusion medium (PBS containing 10 mM MES and 10 mM HEPES, pH 5.5) at 7 h posttransfection. The fusion medium was removed, and the cultures were incubated in DME containing 5% fetal calf serum for 1 h to visualize cell-cell fusion. To ensure that the cultures were expressing equivalent amounts of QN-1 and wild-type G protein, surface expression was determined by flow cytometry from parallel cultures expressing QN-1 and wild-type G protein. (A) Mock-transfected; (B) wild-type G protein transfected; (C) QN-1 transfected.](http://jvi.asm.org/)

![FIG. 5. Coimmunoprecipitation of QN-1-G protein heterotrimers. Cells transiently expressing the indicated G proteins were pulse-labeled with [35S]methionine for 30 min, chased with excess unlabeled methionine for 1 h, and then solubilized as described in Materials and Methods. (A) G proteins were immunoprecipitated from the detergent lysates with anti-G_I cytoplasmic tail antisera (T; lanes 1, 4, and 7), anti-infectious bronchitis virus p23 cytoplasmic tail antisera (23; lanes 2, 5, and 8), or anti-VSV_I antisera recognizing the ectodomain of G_I protein (I; lanes 3, 6, and 9). (B) G proteins were immunoprecipitated with anti-G_I cytoplasmic tail antisera (T; lanes 1, 4, and 7), anti-VSV_NJ antisera (N; lanes 2, 5, and 8), or anti-VSV_I antisera (I; lanes 3, 6, and 9). G proteins in lane 9 were immunoprecipitated with an admixture of anti-VSV_NJ and anti-VSV_I antisera.](http://jvi.asm.org/)
transport to the cell surface, incorporation into virus particles, or subsequent binding and endocytosis of virus particles.

The events that bring about fusion of the viral envelope with the endosomal membrane are not understood in precise molecular detail for any virus fusogen. In the case of influenza virus hemagglutinin and several other viral glycoproteins, proteolytic cleavage is required before acid activation in order to expose a critical hydrophobic domain which is thought to insert into the opposing membrane and facilitate redistribution of the two bilayers (28). For VSV G protein, no posttranslational cleavage occurs and there are no obvious hydrophobic regions that could participate in the fusion reaction. It has been suggested that a domain at the N terminus of G protein may be the fusion peptide (26); however, a G protein having a mutation in the critical region retained fusogenic activity equivalent to that of wild-type G protein (33).

How does the QN-1 mutation interfere with the fusion activity? The VSV G protein, like other fusogenic proteins that act at low pH (10, 13, 18, 27), is thought to undergo a conformational change induced by low pH (2, 4). This change may expose a hydrophobic domain that inserts into a target membrane (11, 30). It is conceivable that the extra glycan or the mutation itself might prevent such a conformational change. The only assay available for this conformational change is the stabilization of the G protein trimer at pHs below 6.0 (4). Because trimers formed by the QN-1 mutant are stable at low pH (5), the mutant is probably undergoing a conformational change, although there might well be differences in the conformation from wild-type G protein that prevent fusogenic activity.

An alternative explanation is that the mutation has a more direct effect and falls in or near the fusion domain itself. The hydrophilic glycan would presumably be a strong inhibitor of any membrane binding or insertion. Inspection of the G protein amino acid sequence reveals that the mutation immediately precedes a sequence of 19 uncharged amino acids (residues 118 to 136). Previously, Ohnishi suggested that residues 116 to 148 (residue numbering was changed to correspond to VSV G protein containing the signal sequence) may be involved in the fusion activity of VSV G protein because this sequence is highly conserved between the two VSV serotypes, although it contains several polar residues and is not strongly hydrophobic (20). Similarly, it has been proposed that a sequence of uncharged amino acids in the ectodomain of Semliki Forest virus E1 glycoprotein may be involved in membrane fusion (9). The sequence around the QN-1 mutation clearly deserves further investigation, since a fusion domain has not yet been identified in any rhabdovirus glycoprotein.

The other interesting aspect of the fusion-defective mutant was that it interfered with infection when it was incorporated into VSV particles with the wild-type glycoprotein. When the mutant protein was expressed at levels only 2-fold greater than those of wild-type G protein, the virus titers were reduced 180-fold. Such strong interference might occur through the formation of fusion-inactive heterotrimers of QN-1 and wild-type G protein. Although we did not have antibodies that could distinguish between QN-1 and wild-type G protein, which prevented us from detecting QN-1 wild-type G protein heterotrimer formation directly, we did find that QN-1 is capable of forming heterotrimers with another mutant G protein containing a wild-type extracellular domain. The strong interference of infectivity observed after QN-1 and wild-type G protein coexpression suggests that the presence of mutant subunits might well interfere with wild-type G protein fusion activity. Consistent with such a model, we found that when QN-1 was incorporated into virions with the VSV NJ glycoprotein (with which it does not form heterotrimers), there was much less inhibition of infectivity by QN-1.

The expression of mutant subunits of oligomeric viral proteins to confer dominant negative effects on virus assembly and growth has been proposed as a strategy for controlling viral diseases such as acquired immunodeficiency syndrome (29). A dominant negative mutation that allowed assembly of the etiological agent and rendered it noninfectious could be more effective than a condition blocking virus replication, since the host immune system might then respond more effectively to the particles produced. The results presented here suggest that a fusion-defective viral glycoprotein subunit might be very useful in preventing production of infectious virus.

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LITERATURE CITED

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