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Requirement for a non-specific glycoprotein cytoplasmic domain sequence to drive efficient budding of vesicular stomatitis virus

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The cytoplasmic domains of viral glycoproteins are often involved in specific interactions with internal viral components. These interactions can concentrate glycoproteins at virus budding sites and drive efficient virus budding, or can determine virion morphology. To investigate the role of the vesicular stomatitis virus (VSV) glycoprotein (G) cytoplasmic and transmembrane domains in budding, we recovered recombinant VSVs expressing chimeric G proteins with the transmembrane and cytoplasmic domains derived from the human CD4 protein. These unrelated foreign sequences were capable of supporting efficient VSV budding. Further analysis of G protein cytoplasmic domain deletion mutants showed that a cytoplasmic domain of only 1 amino acid did not drive efficient budding, whereas 9 amino acids did. Additional studies in agreement with the CD4-chimera experiments indicated the requirement for a short cytoplasmic domain on VSV G without the requirement for a specific sequence in that domain. We propose a model for VSV budding in which a relatively non-specific interaction of a cytoplasmic domain with a pocket or groove in the viral nucleocapsid or matrix proteins generates a glycoprotein array that promotes viral budding.

Keywords: cytoplasmic domain/spike glycoprotein/vesicular stomatitis virus/virus assembly/virus budding

Introduction

Enveloped viruses are released from cells by budding from cellular membranes. During the budding process, viral spike glycoproteins can be preferentially incorporated into virions. This sorting process can be mediated by an interaction between the cytoplasmic domains of the glycoproteins and a component(s) of the viral core. Such interactions are also thought to drive the budding process itself in many cases (reviewed by Simons and Garoff, 1980; Stephens and Compans, 1988). For alphaviruses and hepadnaviruses, specific interactions between core and spike proteins are required for budding (Bruss and Ganem, 1991; Suomalainen *et al.*, 1992; Lopez *et al.*, 1993). For alphaviruses, such interaction was inferred from experiments in which even single amino acid changes within a tyrosine-based motif in the cytoplasmic domain of a virus envelope protein can abolish budding (Zhao *et al.*, 1994). More recently, the interactions between the cytoplasmic domains of alphavirus glycoproteins and their nucleocapsids have been analyzed at the molecular level (Lee *et al.*, 1996; Skoging *et al.*, 1996). On the other hand, retroviruses employ a different budding mechanism that is based on an interaction of core gag proteins with the plasma membrane, and the gag component alone is sufficient to drive a normal level of particle formation. In the case of human immunodeficiency virus type 1 (HIV-1), the specific incorporation of the env protein is driven by an interaction of the matrix protein and the cytoplasmic domain of env (Cosson, 1996).

For influenza virus, a segmented negative-strand RNA virus, deletion of the cytoplasmic domain of the hemagglutinin (HA) glycoprotein has only a small effect on virus budding and assembly of HA into the virion (Jin *et al.*, 1994). In contrast, deletion of the neuraminidase (NA) cytoplasmic domain reduces incorporation of NA into virions, reduces virus titers and results in production of filamentous particles (Mitnaul *et al.*, 1996). Deletion of the cytoplasmic domains of both the HA and NA proteins results in even more drastic effects on virus morphology (Jin *et al.*, 1997), probably because of the loss of specific interactions between the glycoproteins and matrix protein (Enami and Enami, 1996; Jin *et al.*, 1997).

The role of envelope proteins in budding of rhabdoviruses such as vesicular stomatitis virus (VSV) or rabies appears somewhat different. Initial studies with a temperature-sensitive mutant indicated that VSV particles lacking G protein could be produced, but only at very low levels (Knipe *et al.*, 1977; Schnitzer and Lodish, 1979). In addition, a VSV recombinant completely lacking G protein buds with at least 30-fold reduced efficiency (Schnell *et al.*, 1997). Similar results have been obtained in rabies virus where a deletion mutant lacking the glycoprotein was still able to bud from infected cells, but gave 30-fold reduced yields of particles (Mebatsion *et al.*, 1996). From these results it appears that efficient budding of rhabdoviruses requires the surface glycoprotein. However, the relative importance of the glycoprotein cytoplasmic domain is less clear. It was shown by a trans-complementation assay that at least the membrane-proximal 9 amino acids of the VSV G protein cytoplasmic domain (out of a total of 29) were required for efficient incorporation of G protein into virions of a temperature-sensitive mutant lacking G protein (Whitt *et al.*, 1989). Also, a recombinant rabies virus encoding a G protein with a deleted cytoplasmic domain was generated and found to produce ~5-fold fewer virus particles with reduced amounts of rabies G protein (Mebatsion *et al.*, 1996). These studies all suggested that the cytoplasmic domain was important in budding, but they did not identify a specific assembly

signal in this domain. Additional studies of incorporation of chimeric HIV-1 env/VSV G proteins also suggested that a specific assembly signal for incorporation resides in the cytoplasmic domain since HIV envelope protein was not incorporated into VSV or rabies virus unless the cytoplasmic domain of the HIV-1 envelope protein was substituted with that of G protein (Owens and Rose, 1993; Mebatsion and Conzelmann, 1996; Johnson *et al.*, 1997). These results did not, however, rule out the possibility that there was a negative signal preventing HIV-1 envelope protein localization at sites of VSV budding.

The recovery of infectious VSV from plasmid DNA (Lawson *et al.*, 1995; Whelan *et al.*, 1995) makes possible a directed molecular genetic analysis of the structure and function of VSV proteins. As we report here, a detailed analysis of VSV mutants expressing chimeric or deleted envelope proteins allows us to conclude that efficient budding requires the presence of a cytoplasmic domain on the VSV G protein, but that specific sequences are not required in that domain. Based on these results we propose a new model for rhabdovirus budding in which a critical spacing of G protein on the membrane is determined by non-specific interactions of the glycoprotein tail with a pocket or groove in the nucleocapsid/matrix structure beneath the viral membrane. We suggest that this spacing may help generate membrane curvature and enhance efficient envelopement of the viral cores composed of nucleocapsid and matrix proteins.

Results

To determine whether sequences in the VSV G protein cytoplasmic or transmembrane domains are important for efficient budding of VSV virions, we generated VSV recombinants in which the VSV glycoprotein gene was replaced with genes encoding VSV G–CD4 hybrid proteins. CD4 was chosen for these studies because it has a single hydrophobic membrane-spanning domain and a relatively short cytoplasmic domain, similar in length to the VSV G cytoplasmic domain. Neither the membrane-spanning nor the cytoplasmic domain are related in sequence to the corresponding sequences in VSV G. In addition, CD4 and certain other proteins can be incorporated into VSV virions, indicating that they are not excluded from regions where VSV buds (Schubert *et al.*, 1992; Schnell *et al.*, 1996b). In contrast, the HIV envelope protein and, presumably, other proteins are nearly completely excluded from VSV virions (Owens and Rose, 1993; Johnson *et al.*, 1997). To obtain a complete picture of the requirements of the transmembrane and/or cytoplasmic domain we used three different G–CD4 constructs designated GGC, GCC and GCG (Figure 1A). The first letter indicates that the ectodomain is from VSV G, the second letter indicates the origin of the transmembrane domain (G or CD4) and the third letter indicates the origin of the cytoplasmic domain. The three recombinant VSVs encoding each of the hybrid G proteins completely replacing wild-type (wt) G proteins were recovered using standard techniques (Lawson *et al.*, 1995).

Because of results obtained with the G–CD4 constructs, we also generated two VSV recombinants encoding G proteins with truncated cytoplasmic domains of 1 (CT1) or 9 (CT9) amino acids. These constructs were made by

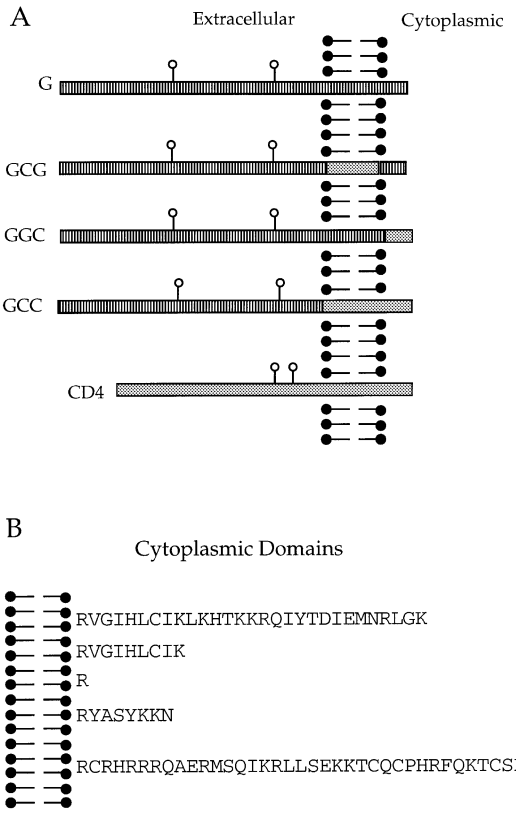


Fig. 1. Diagrams of chimeric G proteins and cytoplasmic domains. (**A**) Chimeric VSV G–CD4 protein constructs are illustrated. The first letter indicates that the ectodomain is from VSV G, the second letter indicates the origin of the transmembrane domain (VSV G = G, CD4 = C) and the third letter indicates the origin of the cytoplasmic domain. (**B**) Amino acid sequences of the cytoplasmic domains of VSV wt, VSV-CT9, VSV-CT1, VSV-CT1R and human CD4.

PCR using primers that introduced translation stop codons at the appropriate positions and deleted downstream coding and non-coding sequences from the G gene so that reversion could not occur by mutation of the stop codon. Although the mutant with a single amino acid in the cytoplasmic domain grew very poorly, it was still possible to recover it from plasmid DNA with our standard procedure.

Recombinants synthesize normal levels of mutant G and other VSV proteins

Because the different G protein constructs were expressed from the normal G gene position, we anticipated that the G proteins as well as the other viral proteins would all be synthesized at wt levels. To confirm this, we infected BHK cells with VSV wt, VSV-GCG, VSV-GGC, VSV-GCC, VSV-CT9, or VSV-CT1 for 4 h. Infected cells were then labeled with [^{35}S]methionine for 1 h and crude cell lysates were fractionated by SDS–PAGE. Because VSV infection shuts down host-cell protein synthesis, the viral proteins can be visualized without immunoprecipitation. The results in Figure 2 show that all mutant G proteins were expressed in similar amounts compared with VSV wt G protein, and that the synthesis of the other VSV proteins was not affected by the G protein mutations. The mobilities of the GGC, GCG and GCC proteins were consistent with the longer cytoplasmic domain of CD4 (38 amino acids) compared with the VSV G domain (29

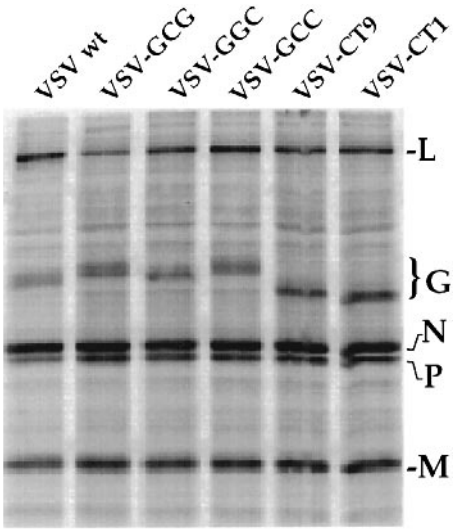
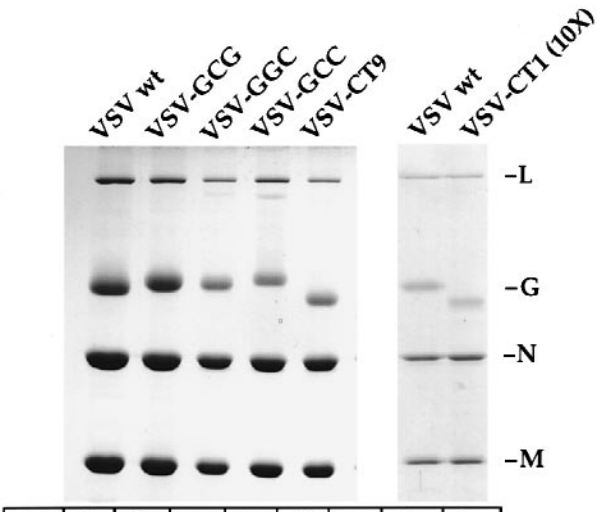


Fig. 2. Recombinant VSVs synthesize normal levels of mutant G and other VSV proteins. BHK cells were infected with VSV wt, VSV-GCG, VSV-GGC, VSV-GCC, VSV-CT9 or VSV-CT1 (m.o.i. = 10) for 4 h and then radiolabeled with [^{35}S]methionine for 1 h. Cell lysates were analyzed by SDS–PAGE. The positions of the VSV proteins are indicated.

amino acids), or the longer transmembrane domain of CD4 (35 amino acids) compared with VSV G (20 amino acids). The CT1 and CT9 proteins migrated as expected based on truncations of their cytoplasmic domains. We did not find any significant differences in the levels of the VSV proteins expressed by these recombinants.

VSV G–CD4 hybrids show nearly wild-type budding efficiency

To determine whether the chimeric G proteins affected virion production or viral titer, we infected 5×10^6 BHK cells with each recombinant and after 24 h, when virus production was complete and all cells were killed, we determined the viral titers in the medium. Virus was also purified from the medium by ultracentrifugation and 5% of each sample was analyzed by SDS–PAGE followed by staining with Coomassie Brilliant Blue. We found that the quantification of stained gels was highly reproducible and gave a linear response for each VSV protein over a wide range of protein concentrations. A photograph of the stained gel (Figure 3) along with quantification below the photo shows that wt VSV produced the largest amount of virions (based on N protein quantification). The mutants with the substituted transmembrane and/or cytoplasmic domains produced virus amounts from 49–80% of the wt levels. In addition, the titers of these viruses were consistent with the yields of virus proteins, indicating that the specific infectivity of the particles was not significantly altered in these constructs. These results suggest that the substitution in VSV G protein of foreign transmembrane, cytoplasmic, or both domains has a relatively small effect on VSV budding from BHK cells. We conclude that there cannot be strong and specific budding or assembly signals in the transmembrane or cytoplasmic domains of VSV G protein.



	1	2	3	4	5		6	7
N*	100	82	49	68	51		100	11.1
titer	3.1×10^9	2.2×10^9	9.3×10^8	9.2×10^8	1.3×10^9		1.6×10^9	1.1×10^8
N/M	1.1	1.1	1.2	1.1	1.1		1.1	1.3
G/M	0.53	0.61	0.42	0.31	0.39		0.55	0.31

Fig. 3. Incorporation of mutant G proteins into VSV virions. Purified virions derived from cells infected with VSV wt, VSV-GCG, VSV-GGC, VSV-GCC, VSV-CT9 (lanes 1–5), or VSV wt and VSV-CT1 (lanes 6 and 7) were separated by SDS–PAGE and the proteins visualized by Coomassie Blue staining. The stained gels were scanned to obtain the protein quantitation given below the gel. For lanes 1–5 the amount of N protein in virions obtained from 10^5 cells infected with wt virus is set at a value of 100, and the values for the other mutants are expressed relative to this. Lanes 6 and 7 show a comparison of wt and CT1, where the CT1 virus was obtained from 10 times more infected cells than the wt virus. The ratios of N:M protein or G:M protein for each recombinant are also given, as are the titers of the viral stocks from each recombinant.

Complete truncation of the G cytoplasmic domain severely reduces virus production

A previous study showed that at least 9 amino acids of the cytoplasmic domain of VSV G are necessary for phenotypic rescue of a VSV G temperature-sensitive mutant (tsO45) at non-permissive temperature (Whitt *et al.*, 1989). In that study, because the rescue by complementation was relatively inefficient even with wt G protein, it was not possible to measure an increase in particle production over the background of non-infectious particle production by the mutant. However, there was a 5–6 log increase in viral titer when complementing with wt G and no detectable increase when complementing with a G-CT1 construct. In contrast a G-CT9 construct did complement, suggesting that a G incorporation signal resided in the membrane-proximal 9 amino acids of the cytoplasmic domain.

Here we recovered infectious VSV with the G-CT1 or G-CT9 genes replacing the wt G gene, making it possible to quantitate directly the effects of cytoplasmic tail truncations on virus production. Figure 3 (lanes 5 and 7) illustrates the virus yield from each recombinant, determined by staining of the virion proteins separated by SDS–PAGE. VSV G-CT9 produced ~2-fold less virus protein and infectivity per infected cell, while virus production by G-CT1 was much more reduced. To obtain sufficient G-CT1 viral proteins compared with the other

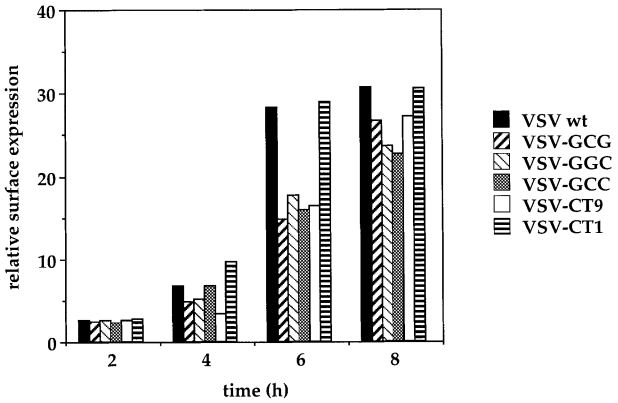


Fig. 4. Relative surface expression of chimeric VSV G proteins. BHK cells were infected with recombinant VSVs at an m.o.i. of 10 and removed from the dish at the indicated time points. Relative surface expression of VSV G protein was determined by flow cytometry after first staining with a G-protein-specific monoclonal antibody, followed by an FITC-conjugated secondary antibody.

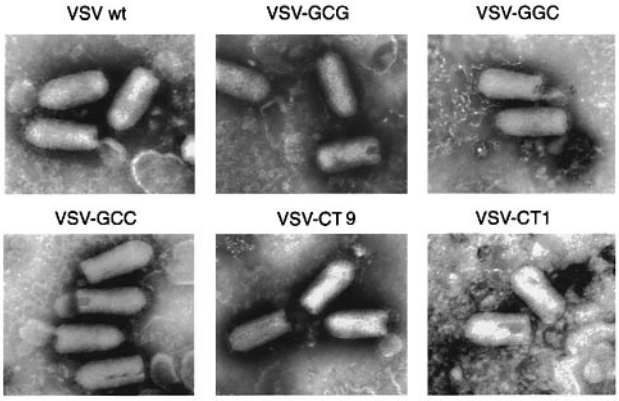


Fig. 5. Electron microscopy of purified virions. Purified VSV wt, VSV-GGC, VSV-GCG, VSV-GCC, VSV-CT1 and VSV-CT9 particles were adsorbed onto carbon-coated grids and negatively stained with phosphotungstic acid. All particles show the typical bullet shape, indicating that the exchange or deletion of the VSV G cytoplasmic tail does not affect virus morphology.

mutants, it was necessary to load virus derived from 10 times more infected cells than used for the other mutants. Quantitation revealed that virus production by VSV-GCT1 was reduced 10-fold compared with wt, and that titer was reduced similarly (Figure 3). We also noted that in all virus mutants analyzed, the ratio of N to M proteins in the virions remained nearly constant, while ratio of G protein to M varied over a 2-fold range.

High-level surface expression of G proteins

One possible explanation for the reduced budding of CT1 or other mutants is slower transport and lower level accumulation of the glycoproteins at the cell surface. Previous studies in fact noted that CT1 and CT9 G mutants acquired complex oligosaccharides ~6-fold more slowly than wt G protein, indicating a reduced rate of transport from the endoplasmic reticulum to the Golgi apparatus and presumably to the cell surface (Whitt *et al.*, 1989). Because the critical issue in particle production is the amount of each protein on the cell surface at the time of virus budding, we measured the relative surface expression of each G protein directly by flow cytometry. BHK cells were infected with each virus, and G protein on the surface of the cells was quantified at different times after infection (Figure 4). G proteins were detectable on the surface as early as 2 h after infection. By 4 or 6 h the expression of G-CT1 protein on the cell surface was actually greater than wt G, while all of the other mutants were reduced by 30–50% compared with wt. At 8 h, when the majority of VSV budding had already occurred, the surface expression was very similar for wt and all mutants. Although the absolute values varied from experiment to experiment, in three independent experiments like the one shown here, G-CT1 was always expressed on the surface at higher levels than wt G. Therefore, the reduced budding of virus from cells infected with this mutant cannot be explained by reduced surface expression of the G protein. Although transport of the G-CT1 protein is slower than wt, it apparently accumulates to higher levels than wt G on the cell surface because it does not support budding as well as wt or other G proteins, and is therefore not being released into budding virions.

Analysis of particles by electron microscopy

Recent studies of influenza virus have shown that deletion of the cytoplasmic domains of the glycoproteins can cause changes in particle morphology, often resulting in long filamentous structures (Mitnaul *et al.*, 1996; Jin *et al.*, 1997). To determine whether the changes in the transmembrane or cytoplasmic domains of G proteins altered the normally bullet-shaped morphology of VSV, we examined the particles from all mutants by electron microscopy (Figure 5). They all had the typical bullet shape and we did not observe any filamentous particles even for the complete cytoplasmic deletion mutant, CT1. In addition, for all virions except VSV-GCT1, we could often see a typical array of G protein spikes protruding from the virion surface, especially at the rounded end. The ability to see this array depends greatly on the staining in any particular area of the grid, but despite inspection of >100 particles of VSV-GCT1, we could not visualize the spikes. The lack of organized spikes may be related to the reduced budding of this mutant.

Infectivity of the recombinants with the CD4 cytoplasmic domain is unstable

We noticed after storage at 4°C for 3–5 days that the titers for VSV-GGC and VSV-GCC dropped drastically compared with those for the other recombinant viruses. To analyze stability, we measured virus titers before and after incubation at 37° C for 8 h. The results showed that for VSV wt, VSV-GCG, VSV-CT9 and VSV-CT1, the titers were reduced ~10-fold, whereas for both viruses with a CD4 cytoplasmic domain (VSV-GGC and VSV-GCC) the titer was reduced at least 1000-fold. A more detailed time course of inactivation for VSV wt, VSV-GCG, VSV-GGC and VSV-GCC showed that the titers for VSV wt and VSV-GCG dropped 10- or 40-fold within the first 8 h, whereas VSV-GGC and VSV-GCC showed 2000- or 3400-fold titer reductions (data not shown). When inactivated virus particles were isolated by centrifugation, we observed no physical loss of VSV G protein and electron microscopic study did not show any change of the viral particle structure of VSV-GGC or VSV-GCC compared with the other viruses. These results suggest

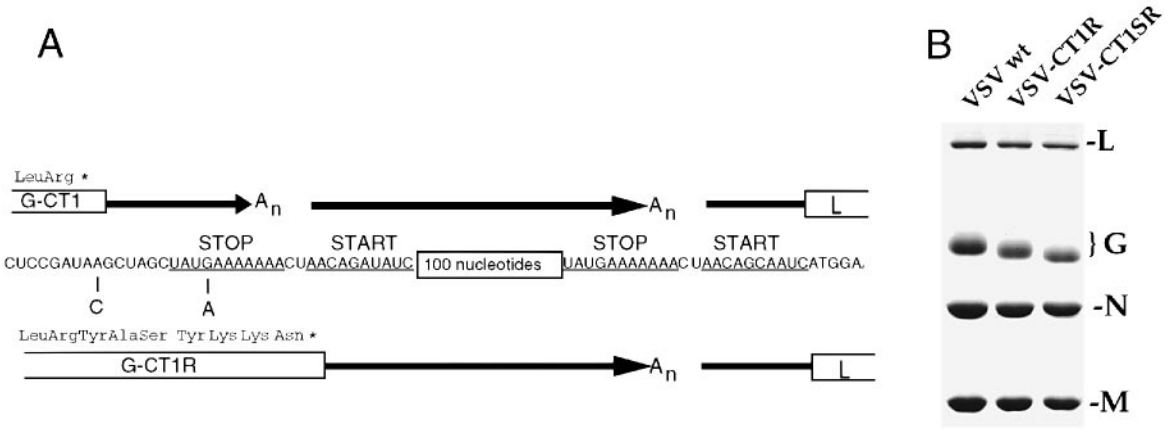


Fig. 6. Analysis of a VSV CT1 revertant. (**A**) The two mutations indicated below the VSV-CT1 sequence (A–C, G–A) were found after 20 passages of the virus. One mutation changed the original G CT1 translation stop codon to a tyrosine codon. The other eliminated a downstream transcription stop-start signal. The two mutations resulted in the gain of an 8 amino acid tail (G-CT1R). (**B**) Purified virions of VSV wt, VSV-CT1R or VSV-CT1SR, derived from the same number of infected cells, were analyzed by SDS–PAGE and the proteins visualized by Coomassie Blue staining. VSV-CT1SR has the 8 amino acid cytoplasmic domain of CT1R substituted for the wt cytoplasmic domain in the background of otherwise wt VSV G protein. The stained gels were scanned and the viral proteins quantified. Virus protein yields and titers were similar for each virus.

that the foreign cytoplasmic domain from CD4 interferes in some way with virus stability.

Selection of a VSV-CT1 revertant

In the construction of the VSV-CT1 mutant, the coding and non-coding sequences downstream of the introduced stop codon were deleted to prevent reversion through mutation of the stop codon. Because the CT1 virus grew so poorly and caused a greatly reduced cytopathic effect (CPE), we thought that it might be possible to obtain revertants, perhaps through mutation in other VSV genes, after passaging and selection of viruses that assembled more efficiently. We therefore carried out extensive low multiplicity passaging of the mutant. After passage 19, we detected a stronger and faster CPE more like that of wt virus. This stock produced both large plaques (typical of wt virus) and small plaques (typical of the CT1 mutant). Stocks of the large plaque virus, the original CT1 mutant and wt virus were then prepared in parallel and analyzed by SDS–PAGE and Coomassie Blue staining. The result in Figure 6B shows that the large plaque revertant (CT1R) released nearly as much virus as wt VSV. In addition, its VSV G protein appeared larger than the CT1 mutant. To determine the sequence changes responsible for the reversion, we sequenced the G protein mRNAs from the VSV-CT1 mutant and the revertant after RT–PCR of mRNA from infected cells. The results verified the expected sequence as shown for CT1 whereas VSV-CT1R contained two mutations that resulted in the gain of an 8 amino acid cytoplasmic domain (Figure 6A). First the stop codon UAA was mutated to UAC (Tyr). In addition, the conserved transcription stop/polyadenylation signal $UAUGA_7$ was mutated to $UAUA_8$ immediately after this. Under normal circumstances, elimination of the G transcription stop would prevent virus replication by eliminating expression of the downstream L mRNA. In this construct, however, there was a 100 nucleotide transcription unit placed between L and G. Elimination of the G transcription stop would be expected to generate an mRNA 150 nucleotides longer, terminating at the stop-start signal preceding the L gene (Figure 6A). This longer RNA would then encode a VSV G protein with the 8 amino acid cytoplasmic domain illustrated in Figure 6A. Complete elimination of the transcription stop was verified by Northern blot analysis showing a longer G mRNA produced by the CT1R mutant (not shown).

Because of the extensive passaging required to obtain the revertant, we were concerned that the tail change might not be the only explanation for the reversion. Indeed, the G protein of the revertant contained three single-base changes that changed 3 additional amino acids in the G ectodomain, and additional changes may have occurred in other VSV proteins. Therefore, to determine if the new cytoplasmic tail was sufficient to give a wt phenotype, we generated this tail sequence on G protein in an otherwise wt VSV background (VSV-CT1SR). The yields of this virus were nearly equivalent to wt or the CT1R mutant (Figure 6B) indicating that the new tail was sufficient to explain the reversion of CT1. Thus, a second cytoplasmic domain, unrelated to the original sequence can, like the CD4 domain, promote nearly wt VSV budding efficiency. The small difference in the mobilities of CT1R and CT1SR proteins (Figure 6B) must result from one or more of the 3 amino acid changes found in the extracellular domain of CT1R.

Discussion

Based on determinations of virus protein yields from BHK cells infected with VSV, we calculate that each infected cell buds ~100 000 virus particles. From virus titers we calculate that up to 10% of these particles can be infectious. The specific interactions among the VSV proteins that are responsible for this prodigious and specific particle production are only partially understood.

VSV virions are composed almost entirely of the five VSV structural proteins, although traces of cellular membrane proteins are present as well (reviewed by Wagner and Rose, 1996). VSV nucleocapsids containing the genomic RNA and the N, P and L proteins assemble in the cytoplasm. The VSV matrix (M) protein interacts with both the cytoplasmic side of the plasma membrane

(Chong and Rose, 1993) and with the helical nucleocapsids, and is critical to viral budding (Knipe *et al.*, 1977; Wagner and Rose, 1996). Electron microscopy indicates that at least some M protein is inside the helical nucleocapsids (Barge *et al.*, 1993) suggesting that M protein may interact with the membrane at the ends of the nucleocapsid.

Although VSV can bud particles lacking G protein, the efficiency of budding without G is reduced at least 20- to 30-fold (Knipe *et al.*, 1977; Schnitzer *et al.*, 1979; Schnell *et al.*, 1997). Similarly, rabies virus, a distantly related rhabdovirus, buds virus particles in the absence of G protein, but with 30-fold reduced efficiency (Mebatsion *et al.*, 1996). These results illustrate the importance of G protein in budding, but do not define the mechanism through which budding is enhanced by G protein.

In the studies reported here we focused on sequences in the cytoplasmic and transmembrane domains of the VSV G protein to determine if specific interaction signals important to VSV budding were present in either, or both, domains. Surprisingly, we found that the G sequences in the cytoplasmic and transmembrane domains could be completely replaced by the corresponding, unrelated, sequences from the human CD4 protein. Recombinant VSVs containing these chimeric G proteins were recovered and were found to bud virus with an efficiency at least half that of wt VSV. These experiments illustrate that highly specific interactions in these domains do not play a major role in virus budding from BHK cells. Because these conclusions from the VSV–CD4 chimeras were different from those obtained in earlier complementation studies on deletion mutants in the VSV G tail (Whitt *et al.*, 1989), we repeated the deletion studies by constructing and recovering recombinant VSVs with only the membrane-proximal 1 (CT1) or 9 (CT9) amino acids of the cytoplasmic domain. In agreement with the earlier complementation studies, the experiments with these mutants showed that 9 amino acids of the normal tail were sufficient to drive nearly normal budding efficiency, but that G protein with only 1 cytoplasmic amino acid showed only 10% of normal budding efficiency. In addition, the G–CT1 mutant produced low virus titers and small plaques. Our results were similar to those obtained with a rabies G-tail deletion which reduced rabies budding 6-fold.

After extensive passaging of the VSV-CT1 mutant, we were able to select a revertant that had acquired a new cytoplasmic domain through two point mutations that allowed transcription and translation of normally untranscribed and untranslated sequences. This revertant encoded an 8 amino acid cytoplasmic domain unrelated in sequence to the normal G tail. Further studies showed that this short tail on G (and not some other mutation in the revertant) was sufficient to promote normal VSV budding.

How can one explain the strong requirement for a G cytoplasmic domain in promoting virus budding, but the lack of a specific sequence requirement in that domain? We considered first that the normal tail might affect G protein expression on the cell surface. However, quantitation of G protein expression on the cell surface by flow cytometry showed that this was not the case. The only feature that the functional tail sequences have in common is the presence of at least 2 basic amino acids, but the positions of these residues are different. Thus, there might be weak charge interactions with an acidic pocket in the VSV nucleocapsid or matrix proteins. Alternatively, it may be important to have a tail present to fit non-specifically into a groove or pocket and thus promote a specific arrangement of VSV G trimers on the cell surface. A specific array of G proteins might then promote specific interactions between the extracellular domains of G trimers that cause membrane curvature and promote virus budding. Our inability to observe the normal pattern of G protein spikes on the CT1 mutant would be consistent with the lack of an ordered array of trimers.

Although both of the VSV recombinants containing the CD4 tail on VSV G budded virus with nearly normal efficiency, we noted that the infectivity of the virus particles was extremely unstable when compared with wt VSV. These experiments suggest that a foreign tail sequence, despite promoting normal budding, can have disruptive effects on virus function. We did attempt to discern changes in virion morphology after heat inactivation of these virions, but we could not observe any differences by electron microscopy. The foreign tails may interfere with internal structure of the virions.

There is also a conclusion from our studies that is unrelated to virus budding. In the selection of the revertant of CT1, two mutations occurred, one of which eliminated the consensus VSV transcription termination/poly(A) signal, $UAUGA_7$, by changing only the G to A. Because this mutation eliminated all transcription termination, we can conclude that this G residue is a critical part of the transcription termination signal.

Materials and methods

Plasmid constructions

Construction of additional VSV expression vectors from a full length infectious clone was performed as previously described (Schnell *et al.*, 1996a). The single *Nhe*I site in the 3′ non-coding region of the G gene was used for introduction of a linker containing a minimal VSV polymerase stop-start signal and an *Xma*I site followed by a *Not*I site. The linker was prepared from the following two complementary oligonucleotides: 5′-CTAGCTATGAAAAAAACTAACAGATATCACGCCCGGGAGCTAGTTGCGGCCGC-3′ and 5′-CTAGGCGGCCGCAACTAGCTCCCGGGCGTGATATCTGTTAGTTTTTTTCATAG-3′ which were annealed and cloned into the unique *Nhe*I site of pVSVFL-2. The resulting plasmid was designated pVSV-XMN, and the presence of the correct sequence was confirmed. For the construction of genes encoding G proteins with truncated cytoplasmic domains, PCRs were performed with VENT polymerase (New England BioLabs, Inc.) to minimize introduction of sequence errors. The VSV G gene containing a cytoplasmic tail of 1 (CT1) or 9 (CT9) amino acids (see Figure 1) was amplified by PCR from the plasmid pVSVFl-2 (Schnell *et al.*, 1996a). The forward primer was 5′-CGAACAACTAATATCCTGTC-3′ for both PCR constructs and the reverse primer was 5′-CGCGGGATCCGCTAGCTTATCGGAGAACCAAGAATAGTCC-3′ for CT1 or 5′-CGCGGGATCCGCTAGCTTATTTAATGCAAAGATGGATACCAAC-3′ for CT9. The reverse primers contained the indicated *Nhe*I site (underlined). The PCR products were cloned into the *Mlu*I and *Nhe*I sites of pVSV-XMN that had been digested with *Mlu*I and *Nhe*I. The resulting plasmids were designated pVSV-CT1 and pVSV-CT9.

The gene encoding the extracellular and transmembrane domains linked precisely to the human CD4 cytoplasmic domain was obtained from G CD4T (Buonocore *et al.*, 1994) by digestion with *Swa*I and *Xho*I, and the fragment was inserted into the unique *Swa*I and *Nhe*I (which is compatible with *Xho*I) of pVSVFl-2 (Schnell *et al.*, 1996a). The resulting plasmid was called VSV-GGC. Both constructs containing the G transmembrane and cytoplasmic domain of CD4 fused to the ectodomain of VSV G, or a VSV G gene with a substitution of the original G transmembrane domain for that of CD4 (see Figure1), were obtained from pXMGA6 (Li *et al.*, 1993; Odell *et al.*, 1997). First, a

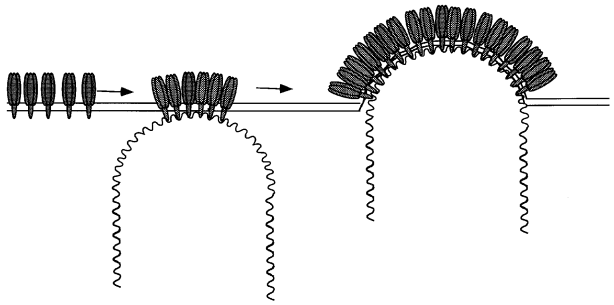


Fig. 7. Budding model for VSV. The model illustrates how VSV glycoprotein tails might fit non-specifically into a groove or slot formed by the nucleocapsid or matrix proteins.

*Bss*HII site was introduced at position 1473 of pXMGA6, which already contained an *Xho*I site at nucleotide position 1416. The plasmid was called pXMGA6-*Bss*HII. Using primers 5′-TTACGCGCGCGAAGA-AGATGCCTAGCCCAA-3′, 5′-CTCGAGCTCGAGCGCCCTGATTG-TGCTGGGG-3′ and 5′-GCGCGCGCGCGCGGGAGGCTGCAAGTG-GAA-3′, the transmembrane, or transmembrane and cytoplasmic domain sequences, of CD4 were amplified by PCR from the human CD4 clone pcDNA–CD4 (Raja *et al.*, 1993; Schnell *et al.*, 1996a) to replace wt G. The resulting plasmids were called VSV-GCG and pVSV-GCC.

The VSV G gene encoding the wt ectodomain, transmembrane domain and the 8 amino acid domain found in the VSV-CT1 revertant (CT1R, see Figure 7) was amplified by PCR from the plasmid pVSVFl-2 using the forward primer 5′-CGAACAACTAATATCCTGTC-3′ and the reverse primer 5′-CCGCTAGCTTATTAGTTCTTCTTATAGCTGGCGTATCG-GAGAACCAAGAATAGTCC-3′. The PCR product was digested with *Nhe*I (underlined) and *Mlu*I (which is located downstream of the forward primer within G) and ligated to pVSVFL-2 that had been cleaved with *Nhe*I and *Mlu*I.

Recovery of VSV recombinants

Baby hamster kidney cells (BHK-21, ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum. Cells on 10 cm dishes (~80% confluent) were infected at a multiplicity of ten with vTF7-3 (Fuerst *et al.*, 1986). After 1 h, plasmids encoding the N, P and L proteins and the appropriate recombinant antigenomic RNA were transfected into the cells using a transfection reagent composed of dimethyldioctadecyl ammonium bromide and phosphatidylethanolamine (Rose *et al.*, 1991), as previously described (Schnell *et al.*, 1996a). Plasmid amounts were 10 μg of the respective full length plasmid (VSV wt, VSV-GCG, VSV-GGC, VSV-GCC, VSV-CT9, VSV-CT1), 3 μg pBS-N, 5 μg pBS-P and 1 μg pBS-L. Subsequent steps were performed as described (Schnell *et al.*, 1996a).

Preparation and analysis of protein from recombinant VSV

For metabolic labelling of the VSV proteins, BHK cells on a 3.5 cm plate (~80% confluent) were infected with recombinant or wt VSV at an m.o.i. of ~20. After 4 h, cells were washed with methionine-free DMEM and incubated for 1 h at 37°C in 1 ml of methionine-free DMEM with 100 mCi of [^{35}S]methionine. Cell extracts (2% of the total) were analyzed by 10% SDS–PAGE and detected with a PhosphorImager (Molecular Dynamics).

Preparation and analysis of VSV virions

For isolation of VSV wt and recombinant VSV virions, a monolayer of BHK cells (~80% confluent) on a 10 cm dish in 10 ml DMEM plus 5% fetal calf serum was infected with virus at an m.o.i. of 5. After 24 h, cell debris and nuclei were removed by centrifugation at 1250 *g* for 5 min. Virus was then pelleted from the medium through 20% sucrose at 36 000 r.p.m. in a Beckman SW41 rotor for 1 h. Virus pellets were resuspended in 500 ml of 10 mM Tris–HCl, pH 7.4, and spun again through 20% sucrose. Virus pellets were resuspended in 50 μl of 10 mM Tris–HCl, pH 7.4, and 2.5–25 μl were analyzed by 10% SDS–PAGE. Gels were stained with Commassie Blue (Wilson, 1984) and scanning densitometry was performed with a densitometer.

Flow cytometry

BHK-21 cells were infected with recombinant VSVs at an m.o.i. of 10 and removed from the dish at different time points (as indicated, see Figure 4) 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 addition of 2 ml of 3% paraformaldehyde. After fixation, the cells were washed twice in PBS containing 10 mM glycine and 1% BSA (PBS–glycine–BSA), then incubated with VSV G protein-specific mouse monoclonal antibody I1 (Lefrancois and Lyles, 1982) diluted in 1:200 PBS–glycine–BSA, followed by fluorescein-conjugated, affinity-purified goat anti-mouse antibody diluted 1:50 (Jackson Research, Inc.). Flow cytometry was performed on a EPICS® profile analyzer.

Electron microscopy

Virus particles were recovered from cultures by first pelleting cell debris at 1500 *g* for 10 min. The virus was then concentrated and purified twice by centrifugation through 20% sucrose [20% sucrose (w/v) in 1× TE pH 7.5] at 120 000 *g* for 60 min. Virus samples were adsorbed to carbon-coated grids for 5 min and then blocked with 1% BSA in PBS for 10 min at room temperature. The viruses were then negatively stained by incubating the grids for 1 min on 50 μl drops of 2% phosphotungstic acid, pH 7.5. Excess stain was removed and the grids were air dried. Images of viruses were obtained with a Zeiss EM910 electron microscope.

RNA isolation, RT–PCR and sequencing

For RNA isolation, BHK cells on 6 cm plates were infected with the respective recombinant VSV at an m.o.i. of 20. At 4 h after infection, cellular RNA was isolated using RNeasy Total RNA kit (Qiagen Inc.) as recommended by the manufacturer. For RT–PCR, the first-strand DNA synthesis reaction was carried out with 1 μg total RNA, and 0.75 mM RT1 5′-GAGGGAATAATCCAAGATCC-3′ or RT2 5′-GGT-CTCAAAATCGTGGACTTCCAT-3′ as positive-strand G or L primer, and SuperScript[a]II reverse transcriptase (Life Technologies), as indicated by the manufacturer. PCR was carried out using 10% of the reverse transcriptase reaction in 1× Vent buffer, 0.5 U Vent polymerase (New England Biolabs, Inc.), 200 μM each of dNTP and 0.4 μM RT1, or RT2. The second PCR primer was 3′-CGAACAACTAATATCCTGTC-5′ for RT1, or 5′-CCTGAATGCCCAGAAGGGTC-3′ for RT2. The reactions were subjected to 25 thermal cycles: 94°C, 0.45 min; 50°C, 0.45 min; 72°C, 1.0 min. The resulting PCR products were gel purified, and sequences were determined by the Yale oligonucleotide sequence facility, using primers RT1, RT2, 5′-AATAAACTCTCATCATCAGG-3′ and 5′-CAATCACTGCTTCGGCATCC-3′.

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