The EMBO Journal vol.5 no.8 pp.1913-1920, 1986

The budding mechanism of spikeless vesicular stomatitis virus particles

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Communicated by K.Simons

Virus particles, lacking the spike G-glycoproteins, are produced during infection of Vero cells with the vesicular stomatitis virus mutant ts045 at the restrictive temperature 39.5°C. At this temperature the mutated G proteins are blocked in their intracellular transport in the endoplasmic reticulum. We have studied the role of the G proteins in the formation of these spikeless virus particles. The results showed that the spikeless particles contain a full complement of membrane anchors, derived from the carboxy-terminal end of the G protein. Our observations suggest that virus particles are formed at the restrictive temperature with G protein which is later cleaved to produce spikeless particles. We suggest that this is due to a leak of G protein to the cell surface at 39.5°C where budding then takes place, presumably driven by a G protein C-terminal tail-nucleocapsid interaction.

Key words: enveloped viruses/intracellular transport/membrane sorting/phenotypic mixing/virus budding

Introduction

One of the key issues in understanding membrane traffic in the animal cell is the mechanism of membrane sorting. The traffic between the cellular compartments is thought to be mediated by membrane vesicles which bud from one compartment and fuse to the next (Palade, 1975). These vesicles include proteins in transit to the next organelle, but exclude resident proteins. With the exception of clathrin-coated vesicles (Pearse and Bretscher, 1981) little is known about the carrier vesicles mediating traffic between cellular compartments.

One dramatic example of membrane sorting is the formation of an enveloped virus from the plasma membrane of an infected cell (Simons and Garoff, 1980; McCloskey and Poo, 1984). In this process the nucleocapsid binds to the cytoplasmic face of the plasma membrane and induces the formation of a membrane bud. This bud surrounds the nucleocapsid and as a result a complete virus particle is released into the extracellular fluid. Host proteins are excluded from the forming virus membrane, and viral glycoproteins pre-existing in the plasma membrane are concentrated in it. This membrane sorting is analogous to what happens during intracellular membrane sorting (Simons and Fuller, 1985). Only the direction of the budding process is reversed. The cytoplasmic face of the membrane is forced to assume a concave shape in the bud around a nucleocapsid whereas this face turns convex during the formation of an intracellular bud as, for example, when the clathrin polyhedral basket closes around an endocytic vesicle. At present, the virus model for membrane sorting is far easier to study than intracellular sorting. The genes and the proteins of several enveloped viruses are characterized in detail, whereas the essential components of intracellular carrier vesicles are yet to be defined.

The budding of virus has been postulated to be driven by transmembrane interactions between the virus membrane glycoprotein and the internal proteins of the virus (Garoff and Simons, 1974). No direct evidence for this simple mechanism is yet available. For alpha viruses such as Semliki Forest virus, which is composed of a nucleocapsid surrounded by a lipid envelope with transmembrane glycoprotein spikes, several observations support such a budding scheme (see Simons and Warren, 1984). However, for those enveloped viruses, e.g. vesicular somatitis virus (VSV), which contain an M protein, present data provide a confusing picture of the budding process. At the budding site, the nucleocapsid is thought to bind to M protein which condenses the nucleocapsid into its mature form; this structure functions as a scaffold for the budding process (Dubois-Dalcq *et al.*, 1984). During this process the viral surface glycoprotein (G protein) is selectively included into the bud, apparently by diffusion-mediated trapping (Reidler *et al.*, 1981), while host proteins are not.

Chemical crosslinks have been produced in the mature virus particles between G and internal virus proteins (Dubovi and Wagner, 1977; Zakowski and Wagner, 1980). Such crosslinks suggest transmembrane interactions between the G protein and the internal proteins that could drive VSV assembly. However, the formation of spikeless VSV particles during the infection of Vero cells with the VSV mutant ts045 at the non-permissive temperature does not fit into this scheme. Normal amounts of the mutant G protein are synthesized at 39°C but the protein is retained in the endoplasmic reticulum due to a transport block and no G protein has been found on the cell surface (Knipe *et al.*, 1977; Zilberstein *et al.*, 1980). Nevertheless, virions lacking spikes are formed at a relatively high yield, 5-10% of the amount of wild-type virus (Schnitzer *et al.*, 1979). These virions are completely devoid of G protein and they are not infectious. Also mutants of retroviruses are known to form particles lacking virus spike glycoproteins (Kawai and Hanafusa, 1973; de Giuli *et al.*, 1975). These observations have led to the conclusion that the virus glycoproteins do not have an essential role in budding. This role has been delegated to the M protein instead. Virus mutants with defective M protein do not form virus particles at the restrictive temperature (Knipe *et al.*, 1977). The VSV M protein is located in a strategic position immediately beneath the cytoplasmic face of the lipid bilayer (Pepinsky and Vogt, 1979) and has affinity for anionic lipids (Zakowski *et al.*, 1981). However, none of the known properties of the M protein explain the site of the normal budding process, and why the viral G proteins are included in the bud. The molecular mechanism underlying VSV assembly remains an enigma.

In this paper we have studied in detail the formation of spikeless VSV particles during infection of Vero cells with VSV ts045. Our studies have revealed that the spikeless VSV particles contain a full complement of membrane anchors, derived from the

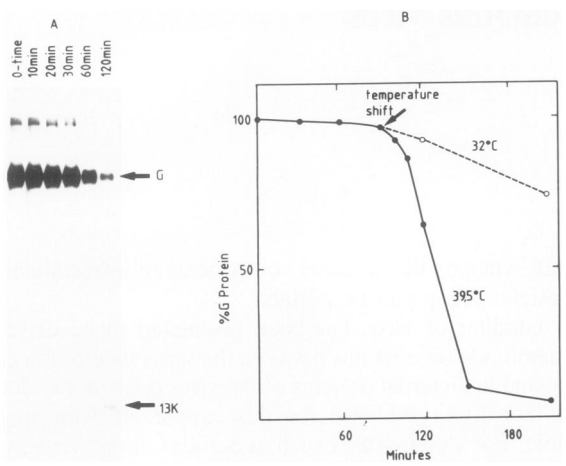

Fig. 1. The G protein of ts045 mutant is unstable on the cell surface at 39.5°C. Vero cells were infected with ts045 for 5 h and pulsed with [^{35}S]-methionine for 10 min at 32°C followed by a 90-min chase during which period labelled G protein moved to the cell surface. Temperature was then shifted to 39.5°C (0-time in A). The G protein associated with the cells was analysed at time intervals by immunoprecipitation with a monoclonal antibody against the cytoplasmic tail of G protein followed by SDS-gel electrophoresis. Along with the G protein decay at 39.5°C, a mol. wt 13 000 C-terminal fragment accumulated (**A**). In (**B**) normalized rates of the G protein disappearance during the chase period at 32°C and subsequent incubation at 39.5°C is shown (●——●). When temperature was left at 32°C after the 90-min chase period, G protein disappeared slowly (○ — — ○). Quantitation of the SDS-gel bands was performed by the method of Suissa (1983).

C-terminal portion of the VSV G protein, and reaffirm an essential role for the G protein in the budding process.

Results

Vero cells degrade surface-bound or surface-associated G protein at 39.5°C

One possible explanation for the production of spikeless particles of VSV ts045 at the restrictive temperature would be leakage of G protein to the surface and subsequent cleavage of the G protein spikes from the virus particles during or after budding. To test this hypothesis we analysed the stability of the mutant G protein on the cell surface at the restrictive temperature, 39.5°C. Vero cells were infected with ts045 at 32°C. Five hours after infection, the cells were given a 10-min pulse of [^{35}S]methionine followed by a 90-min chase at 32°C. During this period, the mutant G protein became fully glycosylated as revealed by a mobility change on an SDS-gel. The protein should thus have reached a late Golgi compartment or the cell surface (Zilberstein *et al.*, 1980). The temperature was then shifted to 39.5°C and the cells were harvested at different times. Cells were lysed and the G protein was extracted, immunoprecipitated with antibody against a peptide consisting of the 15 C-terminal amino acids of the G protein (anti-G protein tail antibody) followed by analysis by SDS electrophoresis on polyacrylamide gel (Figure 1). Quantitation of the data showed that the labelled G protein disappeared rapidly ($t_{½}$ 40 min) after the temperature shift. After 120 min at 39.5°C, only traces of labelled G protein were found in the cells. For comparison, a similar experiment was done without a temperature shift from 32 to 39.5°C. At the permissive temperature G protein disappeared with a $t_{½}$ of >2.5 h.

Concomitantly with the disappearance of G protein at 39.5°C, a fragment showing an apparent mol. wt of 13 000 accumulated in the cells. This fragment represented the C-terminal portion

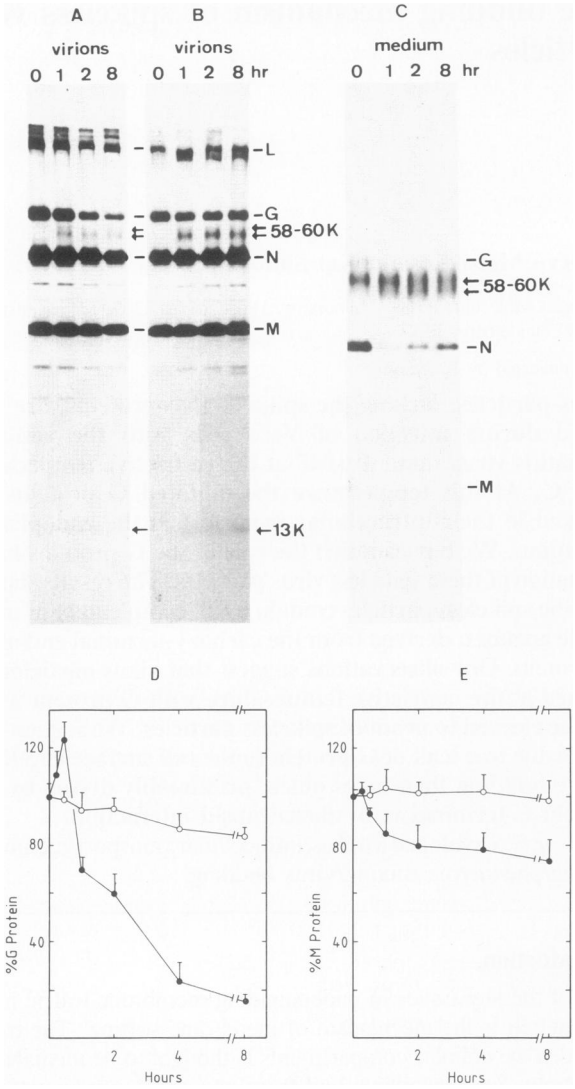

Fig. 2. Disappearance of G protein from ts045 virus particles produced at 32°C after a shift to 39.5°C. Vero cells were pulsed for 10 min with [^{35}S]-methionine after a 5-h infection with ts045 at 32°C, and then chased for 120 min at 32°C to allow viruses to bud into the medium. Temperature was then shifted to 39.5°C and incubation was continued for various periods with cells on the culture dishes, or in the medium alone which was removed from the dishes and incubated separately from the cells in plastic tubes. Viruses were pelleted from clarified medium after each incubation period (at 39.5°C) and analysed by SDS-gel electrophoresis on a 13% polyacrylamide gel. Incubation with the cells is shown in **A** and without cells in **B**. In (**C**) the corresponding media incubated without cells were immunoprecipitated with anti-G protein spike antibodies after pelleting virions and analysed on a 10% polyacrylamide gel. Normalized rates of the G and M protein disappearance from virions in the presence (●———●) and absence (○——○) of cells are shown in (**D**) and (**E**), respectively. Duplicate 3.3-cm diameter dishes were used for each time point, and quantitation was performed by the method of Suissa (1983). Variation between duplicates is indicated by vertical bars. At zero-time point the M protein band on the film was 2.3 times darker than the G protein band.

of the G protein since it was recognized by the anti-G protein tail antibody (Figure 1A). These results suggest that cell surface-associated G protein is cleaved at 39.5°C leaving behind a mol. wt 13 000 peptide in the membrane.

Not all of the cell-associated G protein is degraded at 39.5°C. A fraction of the G protein disappearing from the cell surface is assembled into new virus particles. We next studied the stability of the mutant G protein incorporated into the virus. After a 2-h chase at 32°C the temperature was shifted to 39.5°C. Virions

were isolated from the medium after varying time periods at 39.5°C and analysed by SDS-gel electrophoresis (Figure 2). Quantitation of the G protein revealed an initial increase during the first 30 min at 39.5°C, followed by a decrease. The initial increase must have been due to viruses budding into the medium. When the G protein began to disappear we observed the appearance of a faint mol. wt 13 000 peptide associated with the virus (Figure 2A and B). This peptide was recognized by the anti-G protein tail antibody (not shown) and was probably identical to the mol. wt 13 000 fragment in Figure 1. However, this band did not accumulate with time, suggesting its further processing.

To determine whether the cleavage of the mutant G protein in the virus was dependent on cells, we separated the medium from the cells before shifting the temperature to 39.5°C. After incubating the medium containing the viruses for different time periods at 39.5°C, the virions were pelleted and analysed for G protein. This experiment showed that although the G protein was not stable in the medium separated from cells, the degradation rate was significantly faster in the presence of the cells (Figure 2D).

In addition to the mol. wt 13 000 band, fragments with slightly lower molecular weights than the intact G protein were found in the pelleted virions especially when cells were not present (Figure 2B). We analysed the corresponding media, after pelleting the virus particles, by immunoprecipitation with anti-G protein spike antibodies and detected the same fragments (Figure 2C). These bands had apparent mol. wts between 58 000 and 60 000. During the incubation at 39.5°C, the amount of these fragments in the medium increased somewhat, but they did not account for the disappearance of G protein. The fragments soluble in the medium were not recognized by the anti-G protein tail antibody (not shown). They thus lack the C-terminal part of the G protein and probably correspond to the soluble G_s protein described by Little and Huang (1978). The fact that part of the G_s remained attached to the virus particles would be explained by the oligomeric nature of the G protein spike (Kreis and Lodish, 1986). All the subunits of a spike have to be cleaved before G_s is released.

These results suggest that the Vero cells possess a surface protease which is able to cleave the mutant G protein at 39.5°C. A structural change in the mutant G protein may make it more susceptible to the attack of the protease. Slow degradation of the G protein appears to occur in the medium either due to a soluble or virus-associated protease. Presumably, most of the spike protein is degraded to smaller fragments not detected by our antibodies.

Spikeless virions contain G protein membrane anchors

The degradation of the mutant G protein at 39.5°C left behind a C-terminal peptide of apparent mol. wt 13 000 (Figures 1 and 2). We examined whether similar peptides were present in the spikeless ts045 particles produced at 39.5°C. First, we tried to immunoprecipitate [^{35}S]methionine-labelled proteins from solubilized virions using anti-G protein tail antibody. This approach verified the earlier observation that the ts045 particles harvested 16 h post-adsorption at 39.5°C are devoid of G protein (Schnitzer *et al.*, 1979) but we did not detect any fragments precipitating with the tail antibody either. As a control, we showed that the G protein membrane anchors produced by proteolytic shaving of [^{35}S]methionine-labelled wild-type VSV (Mudd, 1974) were recovered quantitatively with the same procedure. We then shaved the spikeless ts045 particles and subjected them to immunoprecipitation but we did not observe any anchors. Second, we searched for G protein membrane anchors by Western blotting on nitrocellulose but this approach gave a negative answer. Third, analysis of [^{35}S]methionine-labelled ts045 particles after phase partitioning into Triton X-114 (Bordier, 1981) did not reveal any anchors. Neither did chloroform–methanol extraction (Utermann and Simons, 1974) of the spikeless virus. However, immunoblotting of the polyacrylamide gel after electrophoresing an unreduced sample of spikeless particles revealed a surprising result. When we stained the gel slab with ^{125}I-labelled anti-G protein tail antibody, an intensive reaction was observed on top of the stacking gel where the spikeless ts045 virus was applied (Figure 3, lane 1). Incubation of the antibody with a synthetic peptide corresponding to the sequence of the G protein C-terminal tail abolished the reaction (Figure 3, lane 3). The unexpected position of the G protein tail peptides in the SDS-gel suggests that they were aggregated and did not penetrate the gel. Besides this aggregation, another odd feature with the G protein fragments was that they were not detectable after reduction of the sample before electrophoresis. This anomalous behavior explains our failure to detect the G protein fragments by commonly used methods.

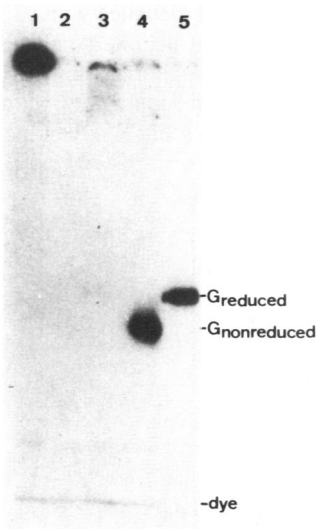

Fig. 3. Immunoblotting of G protein C-terminal fragments in spikeless ts045 virions. Spikeless ts045 was produced in Vero cells at the restrictive temperature and purified. The viral polypeptides were separated by SDS-polyacrylamide gel electrophoresis using a 5% stacking gel and an 8% running gel. The gel slab was fixed and then washed thoroughly with PBS, followed by agitation with ^{125}I-labelled anti-G protein tail antibody. After washing off unbound antibody, the gel was dried and autoradiography was performed. The spikeless ts045 virus shows a reaction on top of the stacking gel when not reduced (**lane 1**) while upon reduction the reaction disappears (**lane 2**). In the non-reduced sample the reaction was abolished when the ^{125}I-labelled antibody was pre-incubated for 1 h with 5 μM peptide whose amino acid sequence corresponded to 11 amino acids of the G protein C-terminus (**lane 3**; this was blotted separately from the rest of the gel). Wild-type VSV grown in BHK cells at 37°C shows a reaction with ^{125}I-labelled anti-tail antibody at the position of G protein only, whether reduced (**lane 5**) or not (**lane 4**). 2 μg of viral protein were applied onto each lane.

To show that this G protein C-terminal peptide was associated with the membranes of the spikeless virions, we used alkaline treatment. Mudd (1974) has shown that treatment of VSV or proteolytically shaved VSV with 0.1 M NaOH releases the membrane envelopes from the internal virus components. The glycoprotein or the membrane anchors left in the shaved virions remain attached to the membrane. The envelopes can then be isolated by sucrose gradient centrifugation. When this procedure was applied to the spikeless ts045 virions, ~1% of the radioactivity

of whole virions metabolically labelled with [^{35}S]methionine was recovered in the envelope fraction. Analysis of the stripped envelopes by SDS-gel electrophoresis failed to reveal either G protein or G fragments in the running gel, although traces of M and N proteins of VSV were seen. We then incubated the spikeless envelopes with ^{125}I-labelled anti-G protein tail antibody and separated the unbound antibody in a sucrose gradient. By this method specific binding of the ^{125}I-labelled tail antibody to the envelopes of spikeless ts045 virus was detected (Figure 4). The ts045 envelopes bound ~1% of the ^{125}I-labelled antibody while a corresponding amount of wild-type VSV envelopes bound ~2%. We presume that only a small fraction of the envelopes were inside-out, explaining the low binding of the ^{125}I-labelled antibody.

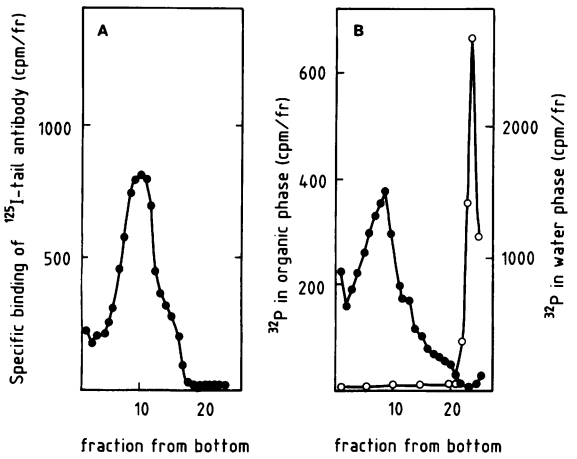

Fig. 4. Demonstration of G protein membrane anchors in the envelopes of spikeless ts045. (**A**) Purified ts045 virus (20 μg) produced at 39.5°C in Vero cells was treated with 0.1 M NaOH (0°C, 5 min) and centrifuged into a stepwise 10–55% (w/w) sucrose gradient in a Beckman SW50.1 rotor (40 000 r.p.m., 60 min, 4°C). The envelopes were collected from the gradient interface and incubated with ^{125}I-labelled monoclonal anti-G protein tail antibody (5 × 10^5 c.p.m., 1 h at 20°C) in the absence and presence of the G protein C-terminal peptide (5 μM). Incubation volume was 150 μl. Unbound antibody was separated from the envelopes by centrifugation in a linear 5–20% (w/w) sucrose gradient containing 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA, in a Beckman SW40 rotor (90 min, 40 000 r.p.m.). Fractions were collected and counted for ^{125}I radioactivity. Specific binding of the antibody to the envelopes was obtained from the difference of counts in the absence and presence of the peptide. When the peptide was present, no ^{125}I-radioactivity sedimented in the position of the envelopes. (**B**) ^{32}P-Labelled envelopes were used to indicate the position of the envelopes in the gradient. The mutant ts045 was metabolically labelled with [^{32}P]phosphate at 39.5°C, purified and treated with 0.1 M NaOH. After centrifugation in an identical linear sucrose gradient as in (**A**) fractions were collected and extracted with chloroform–methanol (2:1, v/v). The position of the alkali-treated envelopes ([^{32}P]phospholipids, ●——●) in the gradient corresponds to that of the specific binding of the ^{125}I-labelled antibody in (**A**). [^{32}P]RNA remains on the surface of the gradient (○——○).

Next, we quantitated the amount of the G protein membrane anchors directly by SDS-gel electrophoresis since we now knew that the anchors aggregated in the stacking gel when not reduced (Figure 3). Mutant virus proteins were labelled with [^{35}S]-cysteine at 39.5°C. There is a single cysteine residue in the cytoplasmic tail of the mutant G protein 23 amino acids from the C terminus, while the next cysteine residue is on the amino-terminal side of the second glycosylation site of the G protein (Gallione and Rose, 1985). From the fact that the spikeless ts045 virions contain no mannose residues (unpublished) we conclude that the G protein membrane anchors must be cleaved in such a way that only one cysteine residue remains. We found that the amount of ^{35}S radioactivity in the G protein anchors in the stacking gel was 6.9% of the total ^{35}S radioactivity of the viral proteins. Wild-type VSV grown in BHK cells was used as a reference. In the wild-type 48% of the [^{35}S]cysteine label of the total viral proteins was in the G protein. Using the known number of cysteine residues in the intact G protein (Rose and Gallione, 1981) we calculated that a ts045 virus particle contains approximately the same number of G protein membrane anchors as there are intact G proteins in a wild-type virion (Table I).

G protein is present in newly formed ts045 virions at 39.5°C

Our data so far show that the membrane anchors of the G protein are found in the spikeless ts045 virions. They do not, however, show whether these anchors are incorporated as such into the virions or rather as intact G protein molecules which are cleaved later to remove the spikes. To address this question we focused on the time point when the newly formed ts045 particles are released into the medium at the restrictive temperature.

We consistently saw a faint band of G protein appearing in the ts045 virions harvested during early chase periods after a pulse of [^{35}S]methionine at 39.5°C (Figure 5). The G protein band was

Table I. The number of G protein membrane anchors in the spikeless ts045 particles

	Band in the stacking gel (d.p.m.)	Intact G protein (d.p.m.)	Cytoplasmic tail (d.p.m.)	N, NS, M and L[c] proteins (d.p.m.)	% Membrane[d] anchors
ts045, not reduced	329 ± 24	–	259 ± 31[a]	3527 ± 184	109 ± 20
ts045, reduced	70 ± 7	–	–	3535 ± 121	–
Wild-type VSV, not-reduced	61 ± 8	2418 ± 84	186 ± 6[b]	2780 ± 123	100

The ts045 was metabolically labelled in Vero cells at 39.5°C with [^{35}S]cysteine. Wild-type VSV was labelled in the same way at 37°C in BHK cells and was used as a reference. The viruses were purified by sucrose gradient centrifugation and the viral proteins (either reduced or non-reduced) were separated by SDS-gel electrophoresis. The gel slab was dried and the viral proteins localized by autoradiography, and subsequently cut out of the running gel; the G-protein anchor band was cut out from the stacking gel. The gel pieces were solubilized and counted for ^{35}S radioactivity. The means and standard deviations (n = 4) are shown.

[a]This value was obtained by subtracting the d.p.m. of the reduced sample in the position of the stacking gel from that corresponding to the position of the band in the non-reduced sample (see text).

[b]The d.p.m. in the G protein were divided by 13, the number of cysteine residues in the whole G protein (Rose and Gallione, 1981).

[c]The d.p.m. in the slices containing the N+NS, M and L protein were added together.

[d]The percent of membrane anchors found in the spikeless particles was calculated by fixing the amount of G proteins in wild-type VSV as 100.

maximal at 1−2 h after the pulse, and then it disappeared with time. Some G protein fragments with a mol. wt 58 000−60 000 also appeared in the virions along with the G protein. Twelve hours after the [^{35}S]methionine pulse G protein or G fragments were no longer detected in the virions. These data are consistent with the idea that the ts045 buds with at least some G protein which is then cleaved. They also suggest that there is a leak of G protein to the cell surface at 39.5°C.

A significant increase was observed in the yield of [^{35}S]-methionine-labelled ts045 virions during the chase period when unlabelled VSV was added to the chase medium. With 50 μg/ml of VSV in the medium, a 2-fold increase was obtained while 100 μg/ml of VSV increased the yield of labelled virus 6-fold after a 90-min chase period. G protein was clearly seen in the labelled virus collected between 1 and 2 h after the pulse in the presence of VSV (Figure 6). Quantitation of the viral bands showed that the G protein accounted for ~20% of the amount of the G protein present in the wild-type VSV. Our interpretation of this finding is that the newly formed virions bind to a surface receptor immediately after budding and this binding can be blocked by excess unlabelled VSV. This would suggest that most virions possess enough G protein to mediate the attachment. Later, these G protein spikes are removed.

To exclude the possibility that receptor-bound virions were taken into the cell by endocytosis and then returned to the medium with the spikes cleaved, we tested the effect of inhibiting lysosomal enzymes with chloroquine (100 μM). This treatment did not increase the yield of the G protein in [^{35}S]-methionine-labelled ts045 virus either in the absence or presence of unlabelled VSV (data not shown). Neither did it affect the amount of labelled N and M proteins incorporated into ts045 virus.

The phospholipid composition of spikeless ts405 particles is identical to that of wild-type VSV

It can be argued that the ts045 does not bud from the plasma membrane at 39.5°C but instead in the endoplasmic reticulum where the G protein accumulates (Knipe *et al.*, 1977). The spikes could then be cleaved during (and after) transport of the virions to the cell surface. To test whether this is the case or not, we studied the phospholipid composition of the spikeless virus. The phospholipid composition of VSV budding from the plasma membrane reflects the host cell plasma membrane lipid composition (Patzer *et al.*, 1979). As expected, it was found that the phospholipid composition of the spikeless ts045 was very similar to that of the wild-type VSV (Table II) which is known to bud from the plasma membrane. In contrast, viruses budding from the endoplasmic reticulum have a similar composition to that of the total cellular lipid (Pike and Garwes, 1977). We conclude that ts045 does not bud from the endoplasmic reticulum at 39.5°C.

Two cellular polypeptides become incorporated into ts045 spikeless particles

Both wild-type VSV and a temperature-sensitive G protein mutant of VSV (tsL513) incorporate selectively two surface-labelled cellular proteins of mol. wts 20 000 and 110 000 at 39.5°C (Lodish and Porter, 1980). Since no G protein was found in the tsL513 virions grown at this temperature, the possibility was raised that the cellular surface proteins could direct VSV budding. We checked whether or not non-VSV coded proteins became incorporated into the spikeless ts045 virions at the restrictive

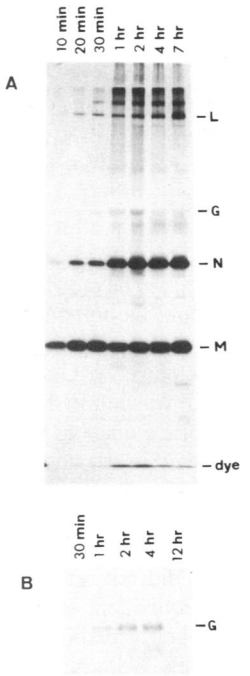

Fig. 5. G protein is transiently detectable in ts045 particles produced at 39.5°C in a pulse-chase experiment. Vero cells infected for 5 h at 39.5°C were pulse-labelled and chased for indicated periods at 39.5°C. The cumulative virus yield at each time point was pelleted from clarified medium. The viral pellets were subjected to electrophoresis in SDS-gels, followed by fluorography. A faint band of G protein gradually appears with time and begins to decay slowly after a 2-h chase (**A**). In (**B**), the viral pellets were solubilized in 1% deoxycholate−1% Triton X-100 in PBS and immunoprecipitated with anti-G protein tail antibody before electrophoresis. By 12 h after the pulse all G protein has disappeared.

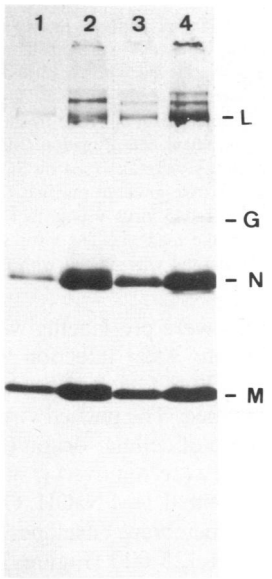

Fig. 6. Excess unlabelled VSV in the medium increases the yield of ts045 particles produced at 39.5°C. Confluent monolayers of Vero cells were infected with ts045 for 5 h at 39.5°C and given a 10-min pulse of [^{35}S]-methionine. Chase was performed in the absence and presence of VSV (100 μg/ml) in the medium. Virus was harvested from the chase medium 1 h after the pulse (**lane 1** without VSV, and **lane 2** with VSV). The medium was replaced and the new medium was collected between 1 and 2 h after the pulse (**lane 3**, without VSV; **lane 4**, with VSV). The presence of unlabelled VSV increases the yield of the labelled mutant virus. Between 1 and 2 h after the pulse G protein is visible in the virus when VSV was added to the medium.

Table II. Phospholipid compositions of ts045 and wild-type VSV produced at 39.5°C

	% of phospholipid				
	Phosphatidyl-choline	Phosphatidyl-inositol	Phosphatidyl-serine	Phosphatidyl-ethanolamine	Sphingomyelin
ts045	37.2 ± 0.7	6.1 ± 0.0	14.3 ± 0.2	37.7 ± 0.6	4.9 ± 0.1
wtVSV	34.4 ± 0.5	5.0 ± 0.2	17.3 ± 0.2	36.7 ± 0.2	6.6 ± 0.2
Vero cells	59.2 ± 0.3	6.7 ± 0.2	6.0 ± 0.1	24.6 ± 0.2	3.6 ± 0.1

Vero cells were grown in the presence of [^{32}P]phosphate (0.1 mCi/ml) for 24 h and then infected with ts045 or wild-type VSV and grown further at 39.5°C for 15 h. Virions were purified from the culture medium by sucrose and tartrate gradient centrifugation. Lipids were extracted from the virions and the [^{32}P]-phospholipids were separated by two-dimensional h.p.t.l.c. according to van Meer and Simons (1982). After localization of the phospholipid spots by autoradiography, they were scraped and counted for ^{32}P radioactivity in a scintillation counter. The mean of two determinations is given, and is followed by the deviation from the mean.

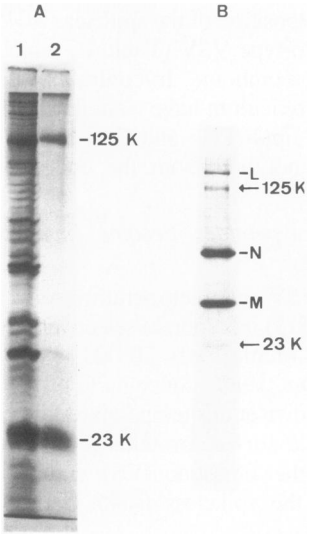

Fig. 7. Incorporation of cellular polypeptides into ts045 particles produced at 39.5°C. (**A**) Subconfluent Vero cells were pre-labelled for 24 h at 37°C with [^{35}S]methionine and then infected with ts045 and grown further at 39.5°C for 15 h. Virions were purified from the culture medium. Membrane envelopes were obtained by alkaline treatment of the purified virus, followed by sucrose gradient centrifugation (Mudd, 1974). The purified virus and the envelopes were analysed by SDS-gel electrophoresis and fluorography. **Lane 1**, tartrate-gradient purified ts045 virions; **lane 2**, alkali-derived envelopes. (**B**) Ts045 virus was grown in Vero cells for 15 h at 39.5°C and purified from the medium. The virus was subjected to electrophoresis in SDS and the gel was stained with Coomassie Brilliant blue.

temperature. Vero cells were pre-labelled with [^{35}S]methionine for 12 h before infection. After infection with ts045 the temperature was shifted to 39.5°C for 16 h whereafter the virions were harvested and purified. The purfied virus contained several polypeptides which were of cellular origin (Figure 7A, lane 1). However, most of these were removed from the envelope after the virions were treated with 0.1 M NaOH. Only two major polypeptides were left in the membrane envelopes, exhibiting apparent mol. wts of 23 000 and 125 000 (Figure 7A, lane 2). These polypeptides are probably identical to those detected by Lodish and Porter (1980). The same bands were also visible by Coomassie blue-staining in SDS-gel electropherograms of spikeless virus particles isolated by potassium tartrate gradient centrifugation (Figure 7B).

Discussion

This study demonstrates that the spikeless VSV particles formed at the restrictive temperature in Vero cells infected with ts045 VSV contain membrane anchors derived from the G protein. These anchors were difficult to detect due to their tendency to aggregate. After trying many different procedures with little success, the membrane anchors were finally detected in the stacking gel after SDS-polyacrylamide gel electrophoresis of unreduced samples. Our findings readily explain why these membrane anchors were not observed previously in ts045 spikeless particles. Quantitation using [^{35}S]cysteine labelling showed that the number of anchors in the ts045 particles corresponded closely to the full complement of G proteins in wild-type particles (Table I).

How the spikeless particles are formed is not yet settled. Our working hypthesis is that these arise at the cell surface of the infected cells. First, the phospholipid composition of the envelopes of the spikeless particles is similar to that of the wild-type VSV (Table II). If the particles had been formed in the endoplasmic reticulum, where the G protein accumulates at the restrictive temperature, their phospholipid composition should resemble that of the total cell (Pike and Garwes, 1977). Second, pulse-chase studies showed that some G protein is incorporated into the ts045 particles formed at 39.5°C (Figure 5). If unlabelled VSV was added during the chase to saturate the cell surface receptors for VSV a 6-fold higher yield of particles was observed in the extracellular medium. These virions contain appreciable amounts of G protein (Figure 6). However, this G protein was degraded at 39.5°C, so that 16 h after infection the particles in the medium lack spikes. Third, if pulse-labelled G protein was allowed to move to the cell surface or into virus particles at the permissive temperature, and the temperature was subsequently raised to 39.5°C, a rapid degradation of the cell and virus-associated G protein took place. A C-terminal fragment of the G protein (mol. wt 13 000) was left in the membrane. This probably represents the membrane anchors which later turn into the aggregating species, detected in the stacking gel during SDS-gel electrophoresis of spikeless particles (Figure 3). Some soluble G protein was released into the extracellular medium but the amount formed did not account for the loss of membrane-bound G. The soluble G protein did not react with anti-G protein tail antibody and is probably equivalent to the G_s protein (Little and Huang, 1978) reported to be formed intracellularly during wild-type VSV infection (Garreis-Wabnitz and Kruppa, 1984; Chen and Huang, 1986). Also under our experimental conditions, most of the G_s was formed and secreted before the shift to 39.5°C (Figure 2C).

To explain that the spikeless particles are formed at the cell surface, we assume that there is a leak of G protein to the plasma membrane at the non-permissive temperature. Budding would be driven by G protein binding to condensed nucleocapsids. The mutant G protein is then degraded during budding and/or in the virus particles bound to cell surface receptors or free in the

medium. Our data make it very likely that the spike glycoprotein plays an important role in budding. Several features of the budding process would be easier to comprehend if the G protein, instead of the M protein, were to direct virus assembly at the cell surface. The specificity of the budding process would be ensured by the presence of binding sites for the G protein cytoplasmic tails in the M protein-coated nucleocapsid. To explain why budding occurs at the cell surface during VSV infection and not intracellularly one could simply postulate that the G protein on the cell surface undergoes a conformational change caused by conditions in the extracellular environment being different from those prevailing in the lumen of the Golgi complex and the endoplasmic reticulum. This conformational change would have to be transmitted to the cytosolic side to facilitate the interaction with the nucleocapsid. One possibility would be the formation of G protein clusters in the membrane plane favoring binding. Similar interactions have been postulated to take place during surface receptor−ligand binding to trigger events ocurring on the cytoplasmic side of the plasma membrane (McCloskey and Poo, 1984).

One most intriguing aspect of the membrane sorting occurring during the budding of VSV is the production of mixed phenotypes during double infection of a cell (Zavada, 1982). Under these conditions, virus particles are formed which contain the nucleocapsid and the M protein of one virus and spike glycoproteins from other viruses used to infect the cell. Thus some basic element of specificity in the assembly process is still shared among the enveloped viruses. VSV can include into its envelope the surface glycoproteins of several enveloped RNA viruses and also of enveloped DNA viruses. On the other hand, host cell proteins are, with some exceptions, excluded from the virus particle. The Thy-1 protein has been reported to be included into VSV particles from murine leukaemia cells (Calafat *et al.*, 1983). Moreover, Lodish and Porter (1980) have shown that two surface polypeptides (mol. wts. 20 000 and 110 000) are incorporated from Vero cells into wild-type VSV particles as well as into a G protein-affected mutant (tsL513) under conditions where spikeless particles are produced. We have confirmed this latter result for ts045. These spikeless particles contain, in addition to G protein membrane anchors, two cellular polypeptides of mol. wts. 23 000 and 125 000 (Figure 7). These two polypeptides may have structural homology to virus glycoproteins, and they could represent *env* gene products deriving from retroviral genomes (Little *et al.*, 1983; Russ *et al.*, 1983). The mechanism of their inclusion into VSV particles would be similar to that of phenotypic mixing.

On the basis of results obtained with spikeless VSV particles formed during double infection with retroviruses and G protein mutants of VSV, it has been postulated that no G protein is needed to incorporate foreign glycoproteins into VSV particles (Lodish and Weiss, 1979; Weiss and Bennett, 1980). The pure pseudotypes observed in earlier studies should be re-examined in the light of our finding that spikeless ts045 particles contain G protein membrane anchors. Our studies support the conclusion of Witte and Baltimore (1977) who reported that some G protein had to be included to form mixed VSV particles during double infection with Moloney murine leukaemia virus in 3T3 fibroblasts. They found no particles containing VSV nucleocapsid and M protein with retrovirus glycoproteins, but lacking G protein. Another finding which supports the role of the homologous spike proteins in directing virus assembly is the inhibition of mixed phenotype formation in polarized epithelial cells. Mixed phenotypes were not formed between influenza virus and VSV early in double infections (Roth and Compans, 1981) because influenza virus buds from the apical membrane domain and VSV from the basolateral membrane of polarized MDCK cells. Formation of mixed viruses was observed only after cytopathic effects had led to loss of surface polarity and to mixing of the basolateral VSV G protein and the apical influenza surface glycoprotein over the whole cell surface. One would thus assume that phenotypic mixing involves transmembrane interactions between the G proteins and the underlying nucleocapsid. Foreign viral spike proteins could be incorporated into the budding virion either by direct interactions with the nucleocapsid or by lateral interactions with G protein. The cellular Thy-1 protein which has been found to be included into VSV (Calafat *et al.*, 1983) is not a transmembrane protein (Low and Kincade, 1985; Tse *et al.*, 1985). It is attached to the outer aspect of the plasma membrane by a covalently bound glycophospholipid tail. In this case, interactions with the VSV proteins would only be possible on the extracytoplasmic side. A closer look at this process may reveal interesting facets of phenotypic mixing and of the mechanism of the budding process.

The heavy reliance on the normal functions of the host cell has justified the use of viruses as tools to study processes such as exocytosis and endocytosis in the animal cell. Enveloped virus budding may seem at first sight a purely virus-related phenomenon. However, the basic mechanism of budding may only be a variation of a general theme recurring in membrane traffic in the cell (Simons and Fuller, 1986). Perhaps the first virus which invented the budding mechanism modified cellular sorting proteins for its own purposes. Seen from this viewpoint an understanding of viral budding is not only essential for pathogenesis of virus disease, but might also lead to insights into the mechanism of intracellular membrane sorting.

Materials and methods

Cells and viruses

Monkey Vero cells were grown as monolayers in Eagle's MEM (Gibco) supplemented with 5% fetal calf serum (Boehringer) and non-essential amino acids (Gibco). Mutant VSV ts045 (Griffiths *et al.*, 1985) was produced in Vero cells. For infection, ts045 was adsorbed for 1 h at 32°C using 40 p.f.u./cell. For restrictive growth conditions, the temperature was then shifted to 39.5°C. The ts045 virions were purified according to Matlin *et al.* (1983). Wild-type VSV was of Indiana serotype and it was produced in BHK 21 cells as described by Matlin *et al.* (1983).

Metabolic labelling of viruses

Labelling of wild-type VSV with [^{35}S]methionine (Amersham, >800 Ci/mmol) or [^{35}S]cysteine (Amersham, >600 Ci/mmol) was performed in BHK 21 cells (Matlin *et al.*, 1982). Labelling of ts045 was performed in Vero cells at 39.5°C as described by Schnitzer *et al.* (1979). Briefly, the label (40 μCi/ml) was applied at 4 h post-adsorption and the medium was harvested at 16 h post-adsorption. After clarification of the medium from cellular debris (8000 g_{av}, 20 min, 4°C) the virions were banded on a 55% (w/w) sucrose cushion containing 0.5 mM EDTA (Matlin *et al.*, 1983). The virus band was applied on top of a 5−40% (w/w) potassium tartrate gradient and centrifuged at 30 000 r.p.m. for 90 min at 4°C in a Beckman SW40 rotor. The virus band was collected and the potassium tartrate was removed by pelleting the virions in a Beckman 75Ti rotor provided with adapters for Eppendorf tubes (40 000 r.p.m., 60 min, 4°C).

Pre-labelling of Vero cells with [^{35}S]methionine (20 μCi/ml) was performed at 37°C in growth medium containing 1/15 of normal methionine concentration. The pre-labelling was continued for 12 h, then the labelling medium was removed and the cells were infected with ts045 virus as before and grown at 39.5°C. Virions were harvested 16 h post-adsorption and purified as described above. The position of the virus in the tartrate gradient was determined after fractionation by performing SDS-polyacrylamide gel electrophoresis from each fraction followed by staining of the gel with silver (Nielsen and Brown, 1984).

For labelling of ts045 and wild-type VSV virions with ^{32}P, subconfluent Vero cells were grown for 24 h at 37°C in the presence of [^{32}P]orthophosphate (NEN) (100 μCi/ml). After removal of the label the cells were infected (40 p.f.u./cell) and grown further at 39.5°C for 16 h. Virions were purified from the medium as before.

Pulse-chase experiments

Vero cells just reaching confluency were infected with ts045 on 3.3-cm diameter Falcon dishes. Four hours after virus adsorption the cell monolayers were washed and pulse labelled by incubation in normal growth medium with a reduced bicarbonate concentration (4 mM), containing 10 mM Hepes, pH 7.25 and 20 μCi/ml of [^{35}S]methionine. After a 10-min pulse, the labelling medium was replaced with chase medium (normal growth medium containing 10× methionine and 10 mM Hepes, pH 7.25, with reduced bicarbonate concentration). All labellings and chases shorter than 2 h were done in a water bath and the solutions were pre-warmed. Longer incubations were performed in a CO_2-incubator to prevent excessive evaporation, using normal bicarbonate concentration (26 mM). After each chase period, the medium was collected and clarified from cell debris by low-speed centrifugation (8000 g_{av}, 20 min at 4°C). The virions were pelleted from the clear medium in a Backman 75 Ti rotor using the adapters for Eppendorf tubes (40 000 r.p.m., 60 min at 4°C). The viral pellet was analysed by SDS-polyacrylamide gel electrophoresis or solubilized in 1% Triton X-100/1% deoxycholate in phosphate-buffered saline (PBS) for immunoprecipitation before SDS-gel electrophoresis. The cells were scraped in 1% Triton X-100/1% deoxycholate in PBS, the nuclei were pelleted and the supernatant clarified by high-speed centrifugation (Beckman 75Ti rotor equipped with Eppendorf-adapters, 40 000 r.p.m., 60 min, 4°C).

Immunoprecipitation of cell extracts and solubilized virus was performed using affinity-purified monoclonal antibody against the G protein cytoplasmic tail (anti-tail antibody). This was a kind gift from Thomas Kreis, EMBL, who produced the antibody using a peptide containing the 15 C-terminal amino acids as the immunogen (Kreis, 1986). A pre-titred amount of affinity-purified rabbit anti-mouse IgG (Cooper Biomedical) was added and the formed complex was precipitated wtih Pansorbin (Calbiochem), using the procedure of Kessler (1975). Affinity-purified polyclonal rabbit anti-G protein IgG (anti-spike antibody, Matlin *et al.*, 1983) was used to immunoprecipitate soluble G protein fragments from the medium (Kessler, 1975). The antigens were dissociated from the adsorbent–IgG complex by boiling for 2 min in electrophoresis sample buffer under reducing conditions.

Immunoblotting

The affinity-purified monoclonal antibody against the cytoplasmic tail of G protein was labelled with Na^{125}I (Amersham) using Iodo-beads (Pierce). The antibody was diluted in PBS (25 μg/50 μl) and 1.25 mCi of Na^{125}I was added. Radioiodine left in the reaction mixture was quenched with excess tyrosine (5 mM), and the product was separated from byproducts on a column of Sephadex G-25 (Pharmacia) using 0.1% bovine serum albumin in PBS as eluant.

Viral proteins were separated by SDS-gel electrophoresis on a 1-mm thick gel using 5% stacking gel and 8% running gel. After fixing and washing with water three times for 2 h, the gel was agitated with ^{125}I-labelled antibody solution (5×10^5 c.p.m./ml of PBS containing 0.1% BSA) for 20 h at 4°C. A synthetic peptide possessing the sequence of eleven C-terminal amino acids of the G protein (mol. wt 1493) was used to block specific binding (Kreis, 1986). The gel was then washed twice for 10 h in 100 ml of PBS and dried. Autoradiography was carried out using Kodak XAR-5 film with an intensification screen.

Analytical

SDS-polyacrylamide gel eletrophoresis was performed using the buffer system of Laemmli (1970). ^{14}C-methylated protein markers (Amersham) were used as standards for apparent mol. wt. The gels were fixed with 45% methanol/7% acetic acid. For fluorography, the gels were impregnated with Enhance (NEN), dried and exposed on Kodak XAR-5 film at −70°C. Radioactivity in individual bands was quantitated either on the film by the method of Suissa (1983) as modified by Pfeiffer *et al.* (1985), or by cutting out the band from the gel after autoradiography. The bands were rehydrated and solubilized (Walter *et al.*, 1979) followed by scintillation counting. Phospholipids were analysed as described by van Meer and Simons (1982).

Acknowledgements

We wish to acknowledge Hilkka Virta for excellent technical assistance, Thomas Kreis for the gift of the anti-tail antibody and Gerrit van Meer for performing the phospholipid analyses. We thank Henrik Garoff, Stephen Fuller, Thomas Kreis, Gerrit van Meer and John Tooze for a critical reading of the manuscript and for helpful suggestions. K.M. was supported by a long-term EMBO fellowship.

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Received on 24 April 1986; revised on 27 May 1986