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A tyrosine-based motif in the cytoplasmic domain of the alphavirus envelope protein is essential for budding

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The budding of enveloped viruses from cellular membranes is believed to be dependent on the specific interaction between transmembrane spike proteins and cytoplasmic core components of the virus. We found that the cytoplasmic domain of the E2 transmembrane spike glycoprotein of Semliki Forest virus contains two essential determinants which are absolutely needed for budding. The first constitutes a single tyrosine residue in the context of a direct pentapeptide repeat. The tyrosine could only partially be substituted for other residues with aromatic or bulky hydrophobic side chains, although these immediately reverted to the original genotype. The second determinant involves palmitylated cysteine residues flanking the tyrosine repeat motif. The function of these is probably to anchor the tail against the inner surface of the membrane so that the tyrosine-containing motif is properly presented to the nucleocapsid. This is the first example where a membrane virus employs a tyrosine signal for the selective incorporation of spike proteins into budding structures.

Key words: alphavirus/budding/membrane protein/tyrosine motif/virus assembly

Introduction

Enveloped viruses are released from infected cells by budding at cellular membranes. During this process, the viral core protein complex becomes enwrapped within a lipid bilayer containing viral transmembrane spike proteins. According to the prevailing hypothesis, the incorporation of a full spike complement and the simultaneous exclusion of host proteins are dependent on direct interactions between the cytoplasmic domains of spike proteins and core components. Such interactions are also thought to drive the budding process itself. In the cases of hepadnaviruses (Bruss and Ganem, 1991) and alphaviruses (Suomalainen *et al.*, 1992) it has been conclusively shown that both core and spike are absolutely required for budding. For all other viruses (except the retroviruses, see below) this situation remains unclear. The retroviruses seem to employ a different mechanism for budding since the core (gag) component alone is sufficient for particle formation (Delchambre *et al.*, 1989; Gheysen *et al.*, 1989; Rhee *et al.*, 1990), but even here the effective inclusion of spikes probably depends on interactions with the core component. However, for no viruses have direct spike–nucleocapsid (NC) protein–protein interactions been shown, although some suggestive evidence has been obtained for vesicular stomatitis virus (Whitt *et al.*, 1989; Owens and Rose, 1993), influenza virus (Collier *et al.*, 1991; Bilsel *et al.*, 1993) and Semliki Forest virus (SFV; Helenius and Kartenbeck, 1980; Metsikkö and Garoff, 1990). In particular, the exact molecular mechanisms that drive budding and guide the inclusion of spike proteins into the mature virus particle remain unsolved.

We are studying the assembly of SFV. SFV encodes three transmembrane proteins which are made from the same polyprotein precursor. Alternating signal peptide and transmembrane anchor sequences direct the translocation and integration of this precursor into the endoplasmic reticulum (ER) membrane where signal peptidase cleavage events generate the individual proteins p62, 6K and E1 (Garoff *et al.*, 1990; Liljeström and Garoff, 1991a). These proteins form heterotrimeric complexes in the ER for transport to the cell surface (Wahlberg *et al.*, 1989; Lusa *et al.*, 1991). At a late stage of transport the p62 protein is cleaved to its mature form, E2, by a host endoprotease (de Curtis and Simmons, 1988). At the cell surface, 6K dissociates from the complex and only E1–E2 heterodimers are included in mature virions forming the viral spike complexes (Ziemiecki and Garoff, 1978; Liljeström *et al.*, 1991).

Since all three membrane proteins carry cytoplasmic domains, any or all of them might be interacting with the NC and thus play an important role in budding. The construction of a full-length cDNA clone of SFV (Liljeström *et al.*, 1991) now allows the use of reverse genetics as a means to define these interactions. We showed that deletion of the 6K gene still allowed virus budding, although the release of virus was slowed (Liljeström *et al.*, 1991). Removal of the E1 cytoplasmic domain had no major effect on budding (Barth *et al.*, 1992). Therefore, the only possible portion of the spike that could interact with the NC is the cytoplasmic tail of E2 protein. This part consists of 31 COOH-terminal residues of the protein (Figure 1). In this study we have analyzed a series of E2 tail mutants for their effect on virus budding. Our results show conclusively that a single, highly conserved, tyrosine residue within a direct repeat motif is absolutely required for efficient budding. In this respect, the formation of viral buds mimics the mechanism used for the formation of vesicles for intracellular transport of transmembrane proteins.

Results

Characterization of E2 tail mutants

The objective of this study was to determine the role of the E2 cytoplasmic tail (Figure 1) in SFV budding and to identify domains or residues within this region that were essential for the process. Our reverse genetics strategy was guided by a number of earlier observations. The distal half of the tail is known to function as a signal peptide for the translocation of the 6K polypeptide (Liljeström and Garroff, 1991a). However, this region has been suggested to flip back into the cytoplasm after signal peptidase cleavage (Liu and Brown, 1993), and therefore could play some important role in budding. This role could involve the two cysteine ($C_{24}C_{25}$) residues both of which are fully conserved (Figure 1). They are believed to be palmitylated (Gaedigk-Nitschko and Schlesinger, 1991; Ivanova and Schlesinger, 1993), and it is possible that this could serve to anchor the tail against the inner surface of the membrane. The same argument holds for the fully conserved C_4 residue at the NH_2-terminal end, which also becomes palmitylated (Gaedigk-Nitschko and Schlesinger, 1991). Accordingly, all three C residues were changed to alanines (A). The proximal half of the tail is highly conserved, especially the region spanning residues L_5-A_{14}. This stretch forms the direct repeat LTPYA/LTPGA, suggesting some underlying structural requirement. Within this motif, the completely conserved tyrosine (Y) would appear to be of special interest. Accordingly, this was substituted by a number of other residues (Figure 1).

To study the budding process specifically, it was imperative that the E2 protein mutants would not be defective in synthesis or intracellular transport to the cell surface. This was studied by pulse–chase analysis for which mutant RNAs were first packaged into SFV particles by cotransfecting the RNAs into cells together with a helper RNA species encoding the wild type proteins of the virus (Liljeström and Garoff, 1991b). The resulting virus stocks, now carrying the mutant genomic RNAs, were subsequently used to infect cells which then were pulse-labelled and chased for different times, after which the state and localization of the spike proteins was analyzed. Immunoprecipitation with monoclonal antibodies directed against both envelope proteins showed that the p62/E2 and E1 proteins of all mutants were correctly produced in amounts comparable to the wild type control (Figure 2, lane a). Formation of p62-E1 heterodimers occurs immediately after completed translocation of the polypeptide chains into the ER (Wahlberg *et al.*, 1989). This heterodimerization is conveniently assayed by immunoprecipitation using an E1 monoclonal antibody and measuring coprecipitation of p62. We found that the formation of p62–E1 heterodimers in all of the mutants occurred with the same efficiency as in the wild type where the p62 protein quantitatively coprecipitated with E1 (Figure 2, lane b).

A measure for heterodimer transport out of the ER is the cleavage of p62 to give E2, which occurs in the *trans*-Golgi compartment. This was analyzed by comparing the amounts of p62 and E2 in the 20 and 60 min chase samples. All mutants showed wild type kinetics of p62 cleavage (Figure 2, lanes a, b, d and e). Finally, to determine whether the heterodimers efficiently reached

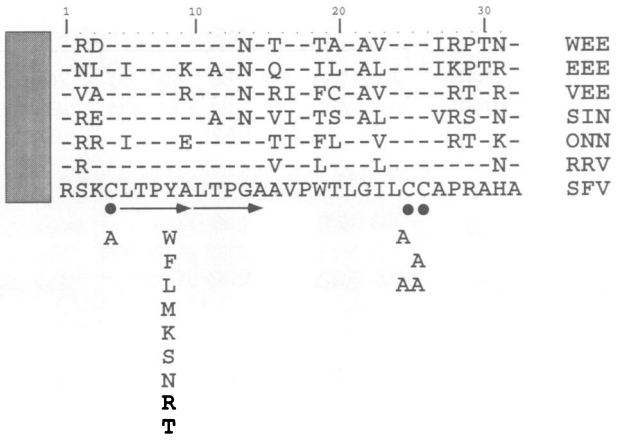

Fig. 1. Amino acid sequence of the cytoplasmic domains of SFV and six other alphaviruses. The amino acid sequences of western equine encephalitis virus (WEE) (Hahn *et al.*, 1988), eastern equine encephalitis virus (EEE) (Chang and Trent, 1987), Venezuelan equine encephalitis virus (VEE) (Kinney *et al.*, 1986), Sindbis virus (SIN) (Rice and Strauss, 1981), O'Nyong-Nyong (ONN) (Levinson *et al.*, 1990), Rose River virus (RRV) (Dalgarno *et al.*, 1983) and Semliki Forest virus (SFV) (Garoff *et al.*, 1980) are shown in single-letter codes. The SFV sequence is shown in its entirety, while for the other viruses, only those residues that differ from the SFV residues are indicated. Identical residues are shown with a dash. The hatched box denotes the membrane. The three conserved cysteine residues are indicated by dots, and the highly conserved double repeat stretch with the tyrosine residue is shown with two arrows. *In vitro*-made substitution mutations are shown below the SFV sequence at the position of replacement. All mutations represent single changes except the double mutant AA (boxed) of the COOH-terminal CC residues. Residues 15–31 constitute the signal peptide for the 6K protein.

the plasma membrane (PM) for virus budding, the pulse-labelled cells were biotinylated under conditions where only surface proteins were labelled. The results showed that no viral membrane proteins had reached the cell surface after a 20 min chase, whereas a major portion of them had done so after 60 min (Figure 2, lanes c and f). Thus, the efficiency of E2 transport to the cell surface in all of the mutants were similar to the wild type. Note that E1 could not be efficiently captured in this assay. This is due to inefficiency of biotinylation of E1 and subsequent loss of E1 from its heterodimeric association during the streptavidin–agarose precipitation (Zhao and Garoff, 1992). We conclude that the heterodimeric spike complexes in all p62 tail mutants were transported to the cell surface with normal kinetics.

Virus particle formation

Since the biosynthesis of the mutant proteins appeared unaffected, we examined whether the mutations had any effect on virus budding. Infected cells were pulse-labelled for 30 min and chased for 3 h after which the growth medium was analyzed by immunoprecipitation using an E2 monoclonal antibody. Since the immunoprecipitation was done in the absence of detergent, complete virus particles (consisting of the E1, E2 and capsid proteins) were recovered (Figure 3, lane M). In this experiment all mutants appeared to be budding deficient with the exception of the W variant, although the level of virus production for this mutant was significantly lower than for the wild type. TCA precipitation of total protein from the post-immunoprecipitation supernatant showed that the recovery of virions had been quantitative (data not shown).

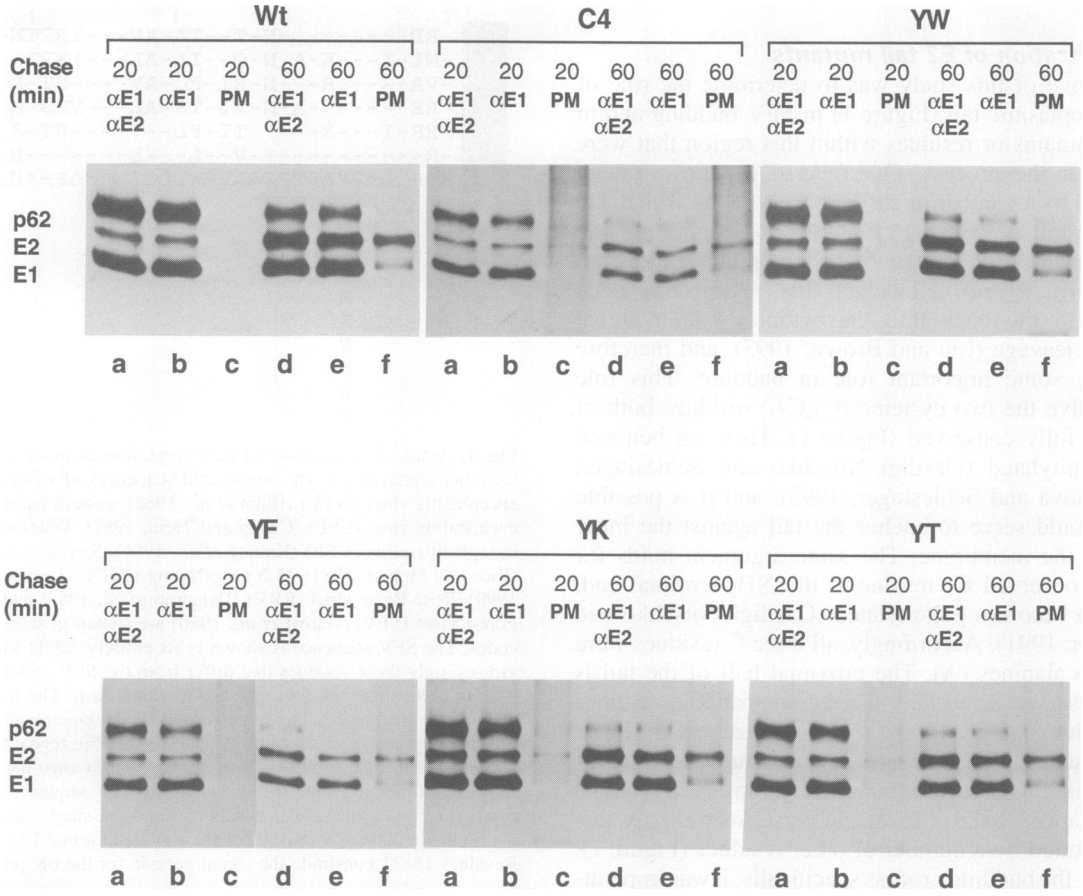

Fig. 2. Pulse–chase analysis of SFV-infected cells. BHK-infected cells were pulse-labelled for 10 min and chased for 20 or 60 min. Total spike proteins were immunoprecipitated from the lysates using a mixture of E1- and E2-specific monoclonal antibodies (lanes a and d), or spike protein coprecipitation analyzed by precipitation with an E1-specific monoclonal antibody alone (lanes b and e). Cell surface proteins were labelled with biotin on ice. After solubilization with detergent the biotin–protein complexes were captured with streptavidin–agarose (lanes c and f). Since all mutants displayed identical phenotypes, only selected ones are shown.

To see whether the defect in virus particle production was due to an inability of the heterodimer to interact with the NC or due to an inability to complete particle envelopment and release from the cell surface, infected cells were studied by electron microscopy (Figure 4). No budding intermediates or any attachment of NCs at the plasma membrane could be found with any budding negative mutant. During alphavirus infection three types of cytopathic structures can be seen [Griffiths *et al.* (1983) and references therein]. Cytopathic vacuoles of type I (CPV-I) form early in infection and represent sites of virus replication (Froshauer *et al.*, 1988). Such structures were readily detected in both wild type and mutant infected cells (Figure 4, A-D). Type II (CPV-II) vacuolar structures form late in infection and consist of numerous NCs attached to their cytoplasmic face. This is probably due to spike proteins which tend to accumulate in the Golgi complex late in infection and start to bind NCs. In the present study, CPV-II structures were seen only in cells infected with wild type virus (Figure 4A), but not in cells infected with mutant virus (Figure 4C and D). Structures of the third type are dense formations with NCs lined up against the periphery of internal membranes. The basis for these structures is presumably the same as for the CPV-II vacuoles. Again, these structures were only found in cells infected with wild type virus (Figure 4A). The

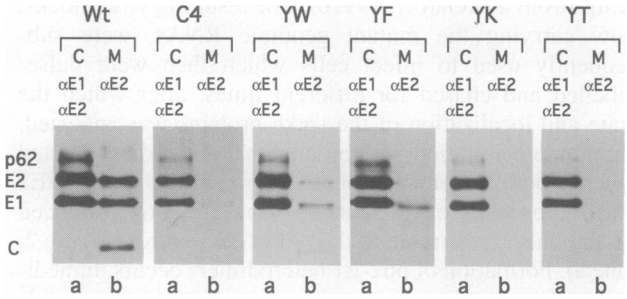

Fig. 3. Production of progeny virus from infected BHK cells. Infected BHK cells were pulse-labelled for 30 min and chased for 3 h. Total cell lysates immunoprecipitated with a mixture of E1- and E2-specific monoclonal antibodies (lane a). The clarified growth medium was used for immunoprecipitation with an E2 specific monoclonal antibody (lane b). The faint bands in the b lanes (and the slightly faster migrating band below the E1 protein in YW, lane b) represent soluble E1 and E2 fragments. We have previously shown that E2–E1 heterodimers do not form stable pools at the cell surface but that they are immediately incorporated into budding virions. Heterodimers that are not incorporated quickly become degraded or released as soluble (anchor-less) fragments into the growth medium (Zhao and Garoff, 1992).

abundance of the CPV-I structures was evidence for efficient virus replicated, and taken together with the biochemical data, these results suggest that the observed

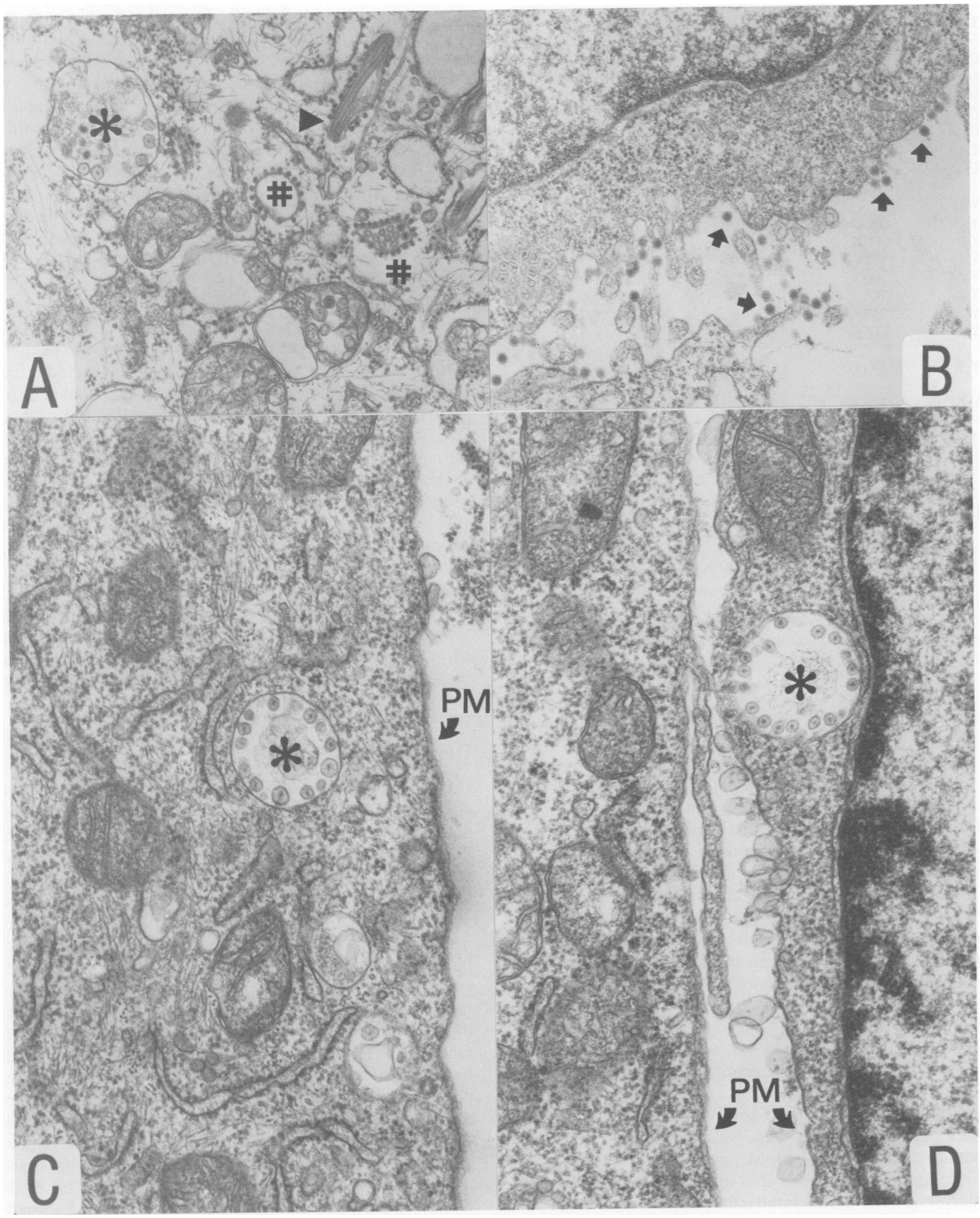

Fig. 4. Cell sections (5 h after infection) analyzed by electron microscopy. (**A**) Cell infected with wild type virus shows CPV-I (*), CPV-II (#) and type-III structures (arrowhead). (**B**) Cell infected with wild type virus showing budding virus at the plasma membrane (arrows). (**C**) Cell infected with Y_8K mutant virus, showing CPV-I (*) vacuole and plasma membrane (PM). (**D**) Cell infected with Y_8S mutant virus, showing CPV-I (*) vacuole and plasma membrane (PM). Magnification is ×40 000.

budding deficiencies of the mutants were due to an inability of spike proteins to react with the NC.

Due to the relative insensitivity of the biochemical assays, budding was also followed using a more sensitive plaque assay. Since RNAs of alphaviruses are expected to mutate at high frequency, this assay could not employ

Table I. SFV E2 tail mutant characteristics

Construct[a]		Titer[b]	Reversion[c]	
W.t.	TGC	1.8×10^7	NA	
C_4A	GCC	3.0×10^1	4	(C/TGC)
$C_{24}A$	GCC	4.4×10^4	3	(C/TGC)
$C_{25}A$	GCC	1.1×10^5	3	(C/TGC)
$C_{24,25}A$	GCC	2.2×10^2	3	(C,C/TGC)
W.t.	TAT	1.8×10^7	NA	
Y_8W	TGG	3.0×10^6	3	(W/TGG)
Y_8F	TTT	1.3×10^5	3	(Y/TAT)
Y_8L	CTT	3.6×10^4	3	(Y/TAT)
Y_8M	ATG	2.0×10^4	4	(Y/TAT)
Y_8K	AAA	3.6×10^2	3	(Y/TAT)
Y_8S	TCT	3.3×10^2	3	(Y/TAT)
Y_8N	AAC	9.5×10^1	2	(Y/TAT)
			2	(W/TGG)
Y_8R	CGT	5.5×10^1	2	(Y/TAT)
			2	(L/CTT)
Y_8T	TCC	2.2×10^1	8	(Y/TAC)

[a]Mutant residues are given as, for example, C_4A, where C indicates the original, wild type (w.t.) residue at position 4 of the E2 tail (see Figure 1), and A is the corresponding mutant residue in that position. Codons for the individual wild type or mutant constructs are given. The wild type C codon was in all cases TGC.
[b]Titer indicates amount of virus (p.f.u.) per ml produced from 10^6 cells between 6 and 8 h after transfection. It was determined by plating supernatant on monolayers of BHK cells and staining with neutral red for detection. Plaque assays were done four times and the titers represent the mean value.
[c]Number indicates number of revertant plaque isolates sequenced. The brackets indicate revertant residue found and corresponding codon. NA, not applicable.

infection by mutant virus stocks. Instead, *in vitro*-made genomic RNAs were directly transfected into cells with >95% efficiency (Liljeström *et al.*, 1991) and production of virions monitored between 6 and 8 h after transfection at a time when the kinetics of virion production is linear. The results indicated that all mutations hampered particle formation (Table I). The C_4 residue appeared to be essential, whereas C_{24} or C_{25} were at least partly dispensable. However, the $C_{24}C_{25}$ double mutant was lethal. The Y substitutions fell roughly into two categories. Mutant residues with aromatic or bulky hydrophobic side chains were somewhat proficient in budding, whereas all other were severely defective, if not lethal (see below).

Reversion analysis

Since all mutants produced at least a few plaques, it was of interest to analyze their genotypes with respect to the E2 tail region. The plaque sizes of the mutants correlated well with observed titers, where variants with the lowest titers gave plaques that were hardly visible to the extent that in some experiments (depending on the density of the cell monolayer) they could only be detected under the microscope. More importantly, the plaques for each mutant were more heterogenous in size than those found for the wild type virus. Accordingly, several individual plaques of at least two different sizes were isolated and passaged twice to amplify the amount of virus. The virus stock was concentrated, the genomic RNA isolated, PCR amplified and sequenced (E2 tail region only). The result showed that all cysteine mutants had reverted to the wild type genotype, although all substitutions were based on two nucleotide changes (Table I). Even the double mutant, with a total of four base changes in two codons, had fully reverted. This indicated a very high reversion frequency combined with a strong selective pressure. For the Y substitutions, revertants were found in all cases except for the W variant. Apparently the negative effect of this mutation was not severe enough to generate a strong enough disadvantage for it to be detected after only a few passages. This was further underlined in the case of the two N for W suppressor mutants, which were found to retain their W genotypes even after two additional passages. In contrast, the two R for L suppressors, when passaged for two more cycles, further evolved to a Y genotype.

Although the sequence analysis of the mutants involved PCR, we do not believe that the results are artefactual. Extreme care was taken in all steps to exclude the possibility of contamination. In addition, the mutants were analyzed in 10 separate experiments involving groups of five isolates. In no case was the wild type construct present during the assays. The facts that the W mutants never reverted, and that we were able to isolate suppressor mutations also support our results. The many (eight) T for Y revertants are particularly illustrative. Although phenotypically revertants, they were not genetically so, but rather had taken a short cut by changing only one base to produce the other possible codon for Y, i.e. TAC (Table I). As an additional control, revertant and wild type virus stocks were used to infect cells and virus production assayed between 6 and 8 h after infection. All revertants now produced wild type levels of progeny (data not shown).

Discussion

Our biochemical, genetic and electron microscopy data strongly suggest that the cytoplasmic domain of the alphavirus E2 spike glycoprotein directly interacts with the NC and that this interaction is required for virus budding. The results further suggest that the spike–NC interaction is dependent on two different determinants within the E2 tail. The first determinant involves the single Y residue which appears to be absolutely required for budding. Although residues with hydrophobic bulky side chains may partially act as substitutes for Y, they are so impaired in budding that reversions rapidly establish themselves in the population. Given the high rate of mutation, even the most proficient W substitute is expected to revert to the more efficient Y. The budding negative phenotypes of the different Y substitutions could be due to a number of reasons. The most straightforward explanation is that they define the residue and/or region which is directly interacting with the NC. Another possibility is that they affect the overall conformation of the E2 tail and thereby interfere with the presentation of a second site binding domain. Although this model cannot be totally ruled out, we find it improbable since all Y substitutions would not be expected to give such clear negative effects. The possibility that the substitutions would interfere through some sort of steric hindrance also appears unlikely for the same reasons. Finally, one could speculate that the mutations might affect lateral protein–protein interactions and thereby somehow interfere with the budding process.

We have recently shown that trimeric complexes $(E2–E1)_3$ indeed form before the NC is encountered, a direct proof for the existence of such lateral interactions (Ekström *et al.*, 1994). In experiments where wild type and mutant spikes (many of which were those described in this study) were coexpressed in the cell, mutant spikes interfered with budding (probably by complexing with wild type counterparts) and to some extent were incorporated into virions, suggesting that their budding negative phenotype did not depend on impaired lateral interactions. Altogether, our results support a picture where the tyrosine motif is directly involved in NC binding. The fact that the W, F, L and M mutants did produce virus at low levels suggests that residues with bulky hydrophobic side chains at the Y position can to some extent be tolerated.

In another study using Sindbis virus (SIN), Schlesinger and colleagues (Gaedigk-Nitschko and Schlesinger, 1991; Ivanova and Schlesinger, 1993) analyzed the effects of various *in vitro*-made E2 tail substitutions. They found that P_7G, Y_8F, A_9I, A_9K and $P_{12}G$ substitutions were viable (see Figure 1 for SIN sequence), but produced virus at levels below that of wild type. The problem with their study was that the initial transfection levels only reached 1% as (compared with >95% in our study) and thus required plaque purification and passaging of the virus to amplify a stock for phenotype analysis. From what we now have learned, mutations of essential residues change extremely rapidly and therefore it is unclear to what extent revertants or suppressor mutations blur the interpretation of their results. Nevertheless, since all of their mutants were at least somewhat impaired in replication, possible revertants or suppressors might not have been fully established in the population at the time of assay, and thus the residues defined in their study probably define a domain important for efficient budding. In another approach, the same group used synthetic peptides corresponding to the E2 tail region to inhibit production of virus from infected cells. Interestingly, the most effective inhibitor was a peptide spanning residues 4–10, exactly overlapping with the first part of the repeat motif with the unique tyrosine residue (Collier *et al.*, 1992).

The second tail determinant involves the three C residues which also appear to have an important function, perhaps related to the presentation of the tyrosine motif to the NC. The Schlesinger group has shown that these residues become palmitylated, and that all three cysteines are important for budding (Gaedigk-Nitschko and Schlesinger, 1991; Ivanova and Schlesinger, 1993). Due to the palmitylation, the cysteines probably do not interact with the NC directly, but instead insert the bound fatty acids into the inner leaflet of the lipid bilayer thus anchoring the tail from both ends. In our study mutation of the most NH_2-terminal C_4 residue was lethal, suggesting that anchoring at this end is critical for function. The same seems to be the case at the distal end containing two adjacent C residues, although one fatty acyl group is sufficient to at least partially restore function. The fact that the mutation of the more proximal C_{24} residue of the two gave significantly less progeny suggests that a tight anchoring of the COOH-terminal end to the membrane at the correct distance from the Y motif is critical for proper function.

In a different approach, the spike–NC interactions were studied using SIN and Ross River Virus (RRV) chimeric viruses, where the capsid protein-encoding region was derived from SIN and the rest of the genome from RRV (Lopez *et al.*, 1994). Since the basic construction was virtually non-productive, it allowed for the analysis of *in vitro*-made SIN E2 substitutions towards the RRV genotype. Their results indicated that residues corresponding to the V_{16}, $W_{18}T_{19}$, G_{21} and $A_{26}P_{27}R_{28}$ of SFV were not important for budding. This suggests that the exact sequence of the 6K signal peptide region (residues 15–31) is not crucial for budding. In contrast, they showed that a change of residues RRV T_{11}/G_{13} for the SIN A_{11}/N_{13} partly restored budding proficiency, suggesting a central role of the repeat motif structure for budding.

An earlier study used internal-image anti-idiotype network antibodies in an attempt to show and define the interaction between the NC and the spike complex of SFV (Vaux *et al.*, 1988). The same antibodies were later used to map the recognition site in more detail, with the conclusion that it would be the COOH-terminal eight amino acid residues of the E2 tail (CCAPRAHA) that constitutes the recognition domain for the NC (Kail *et al.*, 1991). However, later it turned out that the anti-idiotype antibodies used did not have the assumed specificity, but instead recognized the RNA replication sites in the host cell (Suomalainen and Garoff, 1992). Thus, these antibodies cannot be used for mapping of the NC-interacting domain of E2 and, in fact, several of the results discussed above speak against their mapping conclusions.

The alphavirus particle consists of 240 copies of the capsid protein which form the NC ($T = 4$). This is surrounded by an envelope with 240 copies of the heterodimeric (E2 + E1) transmembrane proteins arranged into 80 trimeric $(E2–E1)_3$ spike structures ($T = 4$) (Fuller, 1987; Choi *et al.*, 1991; Tong *et al.*, 1993). The envelopment of these particles can be divided into several steps. The E2–E1 heterodimers first trimerize to $(E2–E1)_3$ spikes before being incorporated into the viral bud (Ekström *et al.*, 1994). The trimerization could be important to express a strong multivalent NC binding domain which could catalyze the envelopment process. The tyrosine motif in the cytoplasmic domain of the spikes then binds to the NC and this probably constitutes the main driving force of the budding process. Finally, it is possible that the envelopment of the NC might also involve additional lateral interactions between spikes in the plane of the membrane, since the spikes of isolated alphavirus membranes have been found to maintain their regular hexagonal assays (von Bonsdorff and Harrison, 1978).

The mechanism guiding the incorporation of spike proteins into budding virus would appear to be fundamentally similar to that involved in the selective retrieval of transmembrane proteins into carrier vesicles for signal-mediated intracellular transport. In both cases interactions between cytoplasmic domains of transmembrane proteins (viral spike proteins versus cellular receptor proteins) and signal recognition elements of peripheral membrane components (NC versus adaptin/clathrin) are involved. The structural feature guiding the incorporation of cellular proteins appears in many cases to be a critical tyrosine residue in a context of what is thought to be a reverse-turn motif. Whether this structure is present in the native protein remains to be determined, but it is clear that residues flanking the tyrosine are important in determining

the correct conformation of the targeting motif [see Trowbridge *et al.* (1993) for a recent review]. Such motifs seem to be important in many sorting pathways. Some trigger the clustering of plasma membrane proteins into clathrin-coated pits for endocytosis as shown for numerous proteins including low density lipoprotein receptor (LDLR), lysosomal acid phosphatase (LAP), transferrin receptor (TR), asialoglycoprotein receptor (ASGP), polymeric immunoglobulin receptor (pIgR), and also for a mutant form of influenza virus hemagglutinin (HA) (Trowbridge *et al.*, 1993). In other cases, the internalization signal directs the retrieval of proteins into compartments such as the *trans*-Golgi network (TGN) or the lysosome, as shown for the TGN38/41 proteins (Ladinsky and Howell, 1992; Bos *et al.*, 1993; Humphrey *et al.*, 1993; Reaves *et al.*, 1993) and the lysosomal integral membrane protein (LAMP-1) (Guarnieri *et al.*, 1993), respectively. Other, equally distinct signals seem to be required for basolateral sorting in polarized cells as shown for LDLR (Hunziker *et al.*, 1991; Matter *et al.*, 1992; Yokode *et al.*, 1992), pIgR (Casanova *et al.*, 1991), LAP (Prill *et al.*, 1993), TR (Dargemont *et al.*, 1993) and HA (Brewer and Roth, 1991). The use of tyrosine sorting signals can now be extended to include viruses, and we have here described the first example of membrane virus employing this kind of a strategy to incorporate selectively its spike proteins into budding structures during assembly.

Materials and methods

Materials

Media and reagents for cell culture were purchased from Gibco Laboratories Life Technologies Ltd., Paisley, Scotland. The monoclonal antibodies against p62/E2 (UM 5.1) and E1 (UM 8.139) were used as ascites preparations and have been described earlier (Boere *et al.*, 1984; Wahlberg *et al.*, 1989). [^{35}S]methionine was from Amersham. Sulfosuccinimidyl-6-(biotin-amido)hexanoate (NHS-LC-biotin) was purchased from Pearse Chemicals. Streptavidin–agarose and phenylmethylsulfonylfluoride (PMSF) were from Sigma. Proteinase K was from Bethesda Research Laboratories, and protein A–Sepharose from Pharmacia.

Cells and viruses

BHK-21 cells were grown in BHK medium supplemented with 5% fetal calf serum, 10% tryptose phosphate broth, 2 mM glutamine and 20 mM HEPES. Stocks of SFV were propagated in baby hamster kidney (BHK-21) cells as described before (Wahlberg *et al.*, 1989). Packaging of *in vitro*-produced genomic RNA into infectious SFV particles has been described earlier (Liljeström and Garoff, 1991b; Zhao and Garoff, 1992). Berglund *et al.*, 1993).

Construction of mutants

Site-directed oligonucleotide mutagenesis was done according to Kunkel *et al.* (1987) as described previously (Liljeström *et al.*, 1991).

Metabolic labelling, biotinylation and immunoprecipitation

BHK cell monolayers in 35 mm dishes were infected with 10 p.f.u. per cell wild type SFV or 10 infectious units/cell of *in vivo* packaged SFV E2 tail mutant virus. After a 60 min adsorption at 37°C, the medium was replaced with fresh minimum essential medium (EMEM). Five hours after infection the cells were starved for 30 min, pulse-labelled for 10 min with [^{35}S]methionine and chased for 20 or 60 min. The viral membrane proteins on the cell surface were then labelled with 1 mg/ml NHS-LC-biotin in phosphate-buffered saline containing Ca^{2+} and Mg^{2+} (PBS^+) for 30 min at 4°C. After washing twice with cold 50 mM NH_4Cl in PBS^+ and once with PBS^+, the cells were lysed with Nonidet P-40 (NP-40) lysis buffer [1% NP-40, 50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1 μg of PMSF per ml]. Each lysate was divided into three parts. One part was immunoprecipitated with monoclonal antibodies against the E1 and E2 subunits. The second aliquot was immunoprecipitated with monoclonal antibody against the E1 subunit only. The third one was incubated with streptavidin–agarose to precipitate biotinylated surface proteins as previously described (Zhao and Garoff, 1992). Finally the samples were processed for SDS–PAGE.

Immuno-isolation of virus particles or viral membrane proteins fragments from the infected cell medium

Media from infected and pulse-labelled (30 min) cell cultures were clarified after a 3 h chase and immunoprecipitated, in the absence of detergent, with a monoclonal antibody directed against the E2 subunit. Remaining proteins were recovered by precipitation with 10% TCA as previously described (Zhao and Garoff, 1992). The corresponding cell samples were lysed and treated with anti-E1 and anti-E2 monoclonal antibody to compare cell-associated spike protein with those incorporated into virions.

Plaque assay

To determine the exact amounts of virus particles produced from infections by SFV, *in vitro*-made RNA was electroporated into BHK-21 cells. Six hours after transfection the cells were washed three times with 37°C PBS and overlaid with fresh BHK medium, and the incubation was continued for 2 h. The medium was then collected, the cells washed once with PBS and the combined medium plus wash cleared for cell debris and cells by low speed centrifugation (2000 *g*). The number of p.f.u. present in the supernatant was determined by conventional plaque assay on BHK-21 cell monolayers.

Determination of genomic RNA sequences

Individual plaques from 2 h plaque assays were picked into EMEM + 0.2% BSA and used to infect a single 35 mm dish of BHK cells. After 24 h the growth medium was cleared of cells and then used to infect a T-160 flask of BHK cells. After another 24 h the medium was collected and cleared and the virus sedimented by centrifugation through a 20% glycerol in TNE (Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA) cushion using an SW28 rotor at 25 000 r.p.m. for 2 h at 4°C. The pellet was resuspended in 400 μl of TNE by incubation on ice overnight. Twenty μl of 10% SDS and 40 μl of NaAc (pH 4.9) were added and the genomic RNA extracted using 500 μl of phenol/chloroform/isoamylalcohol (Sigma). The RNA was ethanol precipitated from the water phase and resuspended in 8 μl H_2O, heated to 65°C and put on ice. Five μl of Bulk First Strand Reaction Mix containing RNAguard and murine reverse transcriptase (Pharmacia) was added and the mixture incubated for 60 min at 37°C. The cDNA was then heated to 90°C for 5 min and chilled on ice. Thirty pmol of downstream and upstream primers (flanking the E2 tail region) were added together with 2.5 U of *Taq* DNA polymerase to a total volume of 50 μl and the cDNA was PCR amplified in 30 cycles: (each cycle consisted of denaturation at 94°C for 1 min, annealling at 58°C for 1 min, and elongation at 72°C for 1.5 min. After a final extended elongation at 72°C for 15 min the sample was cooled and the cDNA run on a preparative 1% low melting point agarose gel. The amplified fragment was excised and the DNA isolated using Gelase technology (Invitrogen). The DNA was purified by phenol extraction and precipitated by adding 1 vol of 5 M NH_4Ac and 2 vols of ethanol. The pellet was resuspended in 10 μl H_2O and subjected to PCR sequencing by the DyeDeoxy-Terminator Cycle Sequencing protocol (Applied Biosystems), using AmpliTaq DNA polymerase for 25 cycles, each consisting of 30 s at 96°C, 15 s at 58°C and 4 min at 60°C. After the sequencing reactions H_2O was added to 100 μl and the material was phenol extracted twice and then ethanol precipitated. Sequence analysis was done using an automated DNA sequencer (Applied Biosystems 373A).

Electron microscopy

BHK cell monolayers were infected with SFV at an m.o.i. of 2 as described above. Five hours after infection the medium was replaced with 1 ml of 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2. The cells were then immediately scraped off and the suspension collected by pelleting in an Eppendorf centrifuge at 14 000 r.p.m. for 2 min. The tubes were left for 2 h at ambient temperature after which the supernatant was changed to 0.1 M sodium cacodylate containing 0.2 M sucrose. Post-fixation was with 1% OsO_4 in cacodylate buffer for 1 h at room temperature and then *en bloc* staining overnight in 1% uranyl acetate. After dehydration through an ethanol series the pellets were embedded in Epon 812 using propylene oxide as an intermediate. Thin sections were cut with a Sorvall MT-6000 ultramicrotome, the sections post-stained with lead hydroxide and/or uranyl acetate and examined in a Jeol 100CX II electron microscope.

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