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Alphavirus Assembly and Entry: Role of the Cytoplasmic Tail of the E1 Spike Subunit

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The alphavirus Semliki Forest virus (SFV) matures by budding at the cell surface. This process is driven by interactions of its membrane protein heterodimer E2-E1 and the nucleocapsid. The virus penetrates into new cells by an E1-mediated membrane fusion event. The E1 subunit has a short, strongly positively charged cytoplasmic tail peptide (Arg-Arg) which is very conserved among different alphavirus E1 proteins. In this work, we have used in vitro mutagenesis of a full-size cDNA clone of SFV to study the role of the tail peptide of the E1 subunit in virus budding and fusion processes in baby hamster kidney cell culture. Our results suggest that the E1 tail plays no major role in SFV multiplication in animal cell culture.

Semliki Forest virus (SFV) is an alphavirus which has proven to be a very useful model system with which to study the budding and fusion processes of animal membrane viruses. The SFV membrane contains 240 spike proteins in a T=4 icosahedral arrangement (5, 23). Each spike contains three copies of a heterodimeric complex, which is made in the infected cell as a p62-E1 precursor complex (24, 28). Both p62 and E1 are transmembrane glycoproteins with C-terminal cytoplasmic tails (7). The p62 tail is 31 amino acid residues long, whereas that of E1 is composed of only two arginine residues. After synthesis in the endoplasmic reticulum, the p62-E1 protein complexes are routed to the cell surface, where they are used in the formation of an envelope around the viral nucleocapsid (NC) (1, 10). This so-called budding process is driven by the interaction of the spike proteins with the NC surface (21). Before budding, the spike heterodimer is proteolytically processed by cleavage of the p62 subunit into the mature E2 form (4). SFV enters new cells via the receptor-mediated pathway for endocytosis (12). During uptake, the virus is exposed to a mildly acidic condition which induces a reorganization of the tertiary and quaternary structure of the spike proteins. In this process, the E1 subunit dissociates from the E2 subunit to form a homotrimeric complex, which probably represents the active SFV fusion protein (13, 16, 25, 26).

An important unresolved question in our understanding of the SFV budding and fusion processes is the nature and regulation of the spike-NC interaction. A completely new approach to the study of these processes involves the use of infectious RNA that has been transcribed from full-size cDNA copies of the viral genome (15, 18). Using this technique, Gaedigk-Nitschko and Schlesinger showed that Sindbis virus with p62 (E2) tail mutations displayed a decreased efficiency of virus budding and also led to the production of multicore particles (6). The result suggests that the structure of the E2 tail is important for virus budding. In this work, we have used the SFV cDNA expression system to examine the role of the short E1 tail in alphavirus replication. The two arginine residues of the E1 tail represent a very conserved feature of alphaviruses (Table 1). One possibility is that they provide a good anchoring of the E1 subunit in the lipid membrane. This anchoring might be required when the newly formed E1 trimer performs the membrane fusion reaction in the endosomes. Another possibility is that the positively charged tail of E1 is required to interact with the viral NC during budding, possibly in cooperation with the p62 tail. An interesting hypothesis is that the arginine residues of the E1 subunit could make contacts with negatively charged phosphate groups of the viral RNA in the NC. It is known that the RNA is accessible to RNase in isolated NCs (19). Furthermore, image processing of electron microscopic pictures of Sindbis virus show that the spike proteins penetrate through the viral membrane into depressions or holes on the NC surface (5). To test these possible functions of the E1 tail, we determined the phenotypes of SFV mutants in which the consensus features of the E1 tail have been altered.

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Design of E1 cytoplasmic tail mutants. Figure 1 shows the SFV E1 tail variants generated in this study. The strategy was either to decrease the number of arginine residues or to substitute the arginines with one or two lysine residues or with neutral serine residues. For mutagenesis, the entire 26S coding region of the SFV genome was subcloned as a 3,800-bp *Eco*RI-*Hin*dIII fragment into M13mp18, and this plasmid was used for production of single-stranded templates (8, 27). Mutagenesis was done as described previously (14). The *Asu*II-*Spe*I fragment containing the E1 target region for mutagenesis was exchanged with the corresponding part in plasmid pSP6-SFV4 (15). This plasmid harbors the complete SFV genome as cDNA and can be transcribed with SP6 polymerase into full-length infectious RNA.

Protein synthesis by SFV mutants. Mutant and wild-type (wt) SFV RNAs were transcribed in vitro and electroporated into BHK-21 cells for phenotype analyses (15). This direct expression analysis we considered important, since multiple passaging of mutant virus, e.g., for the purpose of virus stock generation, can easily result in the selection of virus revertants or second-site mutations. After plating of electroporated BHK-21 cells into several parallel 35-mm-diameter dishes and incubation for 8 h, viral proteins were labeled with [^{35}S]methionine (50 μCi) for 30 min and chased in 10 times the normal concentration of methionine for a short (15-min) and a long (5-h) time period for each mutant (24). Cells were lysed in Nonidet P-40 buffer and analyzed for viral spike protein heterodimers by immunoprecipitation with an anti-E1 monoclonal antibody (UM 8.139) (2, 24). Media were centrifugated at 3,000 × g at 4°C for 5 min to

TABLE 1. Amino acid sequences of the C-terminal region of SFV and other alphavirus E1 spike subunits

Virus	Sequence[a]
SFV	--IGL|RR
Sindbis virus	--TST|RR
Ross River virus	--ITM|RR
Western equine encephalitis virus	--INT|RR
Ockelbo virus	--TST|RR
O'nyong-nyong virus	--SFS|RH
Eastern equine encephalitis virus	--FFH|RH
Venezuelan equine encephalitis virus	--TNQ|KHN|

[a] In single-letter code. The vertical line indicates separation of internal C-terminal tail regions from the membrane-spanning peptides. Note the conserved Arg-Arg sequence of the tail region (in boldface type).

pellet cell debris and then used for analysis of virus particles, using a monoclonal antibody (UM 5.1) against the E2 subunit of the virus spike. Figure 2 shows virus protein band patterns after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide gel) in a Hoefer Mighty Small II minigel system and autoradiography (3, 21). It can be seen that all virus variants produce p62 and E1 spike subunits, that p62 is efficiently cleaved to E2, and that after the 5-h chase, E2, E1, and capsid (C) protein-containing particles are released into the medium.

Spike protein subunits of viral mutants assemble into heterodimers. Association of p62 or E2 subunits with E1 subunits was monitored by measuring the p62 (E2) coimmunoprecipitation with E1, using the anti-E1 monoclonal antibody. Quantitation was done after SDS-PAGE by counting radioactivity in protein bands (24). Table 2 shows the amount of coimmunoprecipitated p62 and E2 radioactivity (normalized to the methionine content of the respective polypeptide chain) with that of E1 from [^{35}S]methionine-labeled cells which had been chased for 15 min. Approximately equal amounts (E1/p62 + E2 = 1.1 to 1.4) are precipitated in the cases of all SFV mutants tested. This result suggests that the Arg-Arg sequence in the E1 tail is not involved in heterodimerization of spike subunits.

Spike protein subunits of viral mutants are transported to the cell surface. The cell surface transport of p62-E1 heterodimers was monitored by measuring cleavage of the p62 subunit into the E2 form. This cleavage occurs at a very late stage of the transport process, that is, after the complex has left the *trans* Golgi network (4). Table 3 shows the quantitation of all p62 and E2 material in 5-h-chase cell and medium samples. The amount of E2 as a percentage of the sum of p62 plus E2 is about 70 to 90% for all of the mutants,

```
SFV [wt] Leu Arg Arg stop
         ctc cgc aga taa
SFV [R]  Leu Arg stop
         ctc cgc TAa taa
SFV [-]  Leu stop
         ctc TAG aga taa
SFV [K]  Leu Lys stop
         ctc AAG TAa taa
SFV [KS] Leu Lys Ser stop
         ctc AAG TCT taa
SFV [KK] Leu Lys Lys stop
         ctc AAG aAG taa
SFV [SS] Leu Ser Ser stop
         ctc TCT TCT taa
```

FIG. 1. Mutants of the E1 C-terminal region. Amino acid sequences are shown in three-letter code. Below are shown the corresponding gene sequences as they occur in cDNA. Mutated nucleotides are shown in uppercase. Note that all amino acid changes involve two or more changes in the corresponding codon. "stop" indicates translation stop in the corresponding RNA.

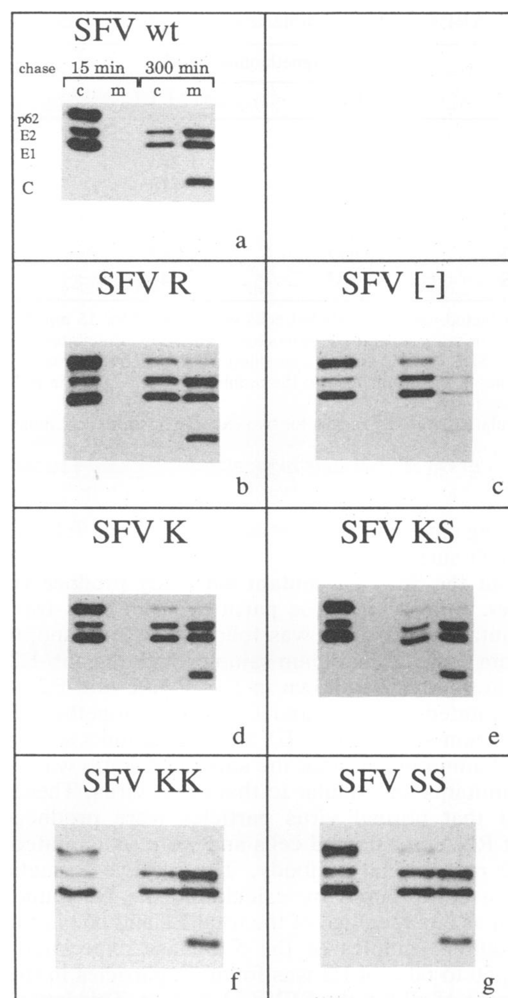

FIG. 2. Protein synthesis and particle formation in SFV mutant-infected cells. For each phenotype analysis, transfected cells were incubated for 8 h before being labeled with [^{35}S]methionine for 30 min and chased for 15 min or 5 h. The cells were lysed with Nonidet P-40 lysis buffer, and spike protein heterodimers (p62-E1) were precipitated with a monoclonal antibody against E1. Media were treated with a monoclonal antibody against E2 to precipitate virus particles in the absence of solubilizing agents. Precipitates were analyzed by SDS-PAGE; gels were processed for autoradiography. Exposure time was 24 h. Lanes (same order in all panels): c, cell sample; m, medium sample.

TABLE 2. Heterodimerization of p62 (E2) and E1

Virus	[35S]methionine cpm[a]		E1/(p62 + E2)[b]
	E1	p62 + E2	
SFV wt	1,073	932	1.15
SFV R	1,405	1,233	1.14
SFV (−)	777	718	1.08
SFV K	1,139	1,054	1.08
SFV KS	1,061	724	1.46
SFV KK	412	287	1.43
SFV SS	787	641	1.23

[a] Transfected and pulse-labeled cells were chased for 15 min before lysis and reaction with an anti-E1 monoclonal antibody. Precipitates were subjected to SDS-PAGE, and [35S]methionine radioactivity was quantitated. Values shown are normalized to the number of methionines in p62, E2, and E1, respectively (11).

[b] Calculated ratios are means for two experiments for each mutant and the wt.

suggesting efficient transport of all variant p62-E1 complexes to the cell surface.

All but the SFV (−) mutant efficiently produce virus-like particles. Release of virus particles from cells transfected with mutant virus RNA was followed by immunoprecipitation from 5-h-chase medium samples with the anti-E2 monoclonal antibody. As shown in Fig. 2, the anti-E2 antibody coprecipitated both E1 and C subunits together with E2. Quantitation showed that E1 and E2 subunits were present in equal amounts and that the spike-to-C ratio was constant in the mutants and similar to that of wt virus. These results suggest that normal virus particles were produced from mutant RNA-transfected cells and were precipitated by the anti-E2 monoclonal antibody. The particle formation efficiency was measured by calculating the E2 found in the medium as a percentage of the total E2 and p62 in the lysate and medium samples of the 5-h-chase experiment. Altogether, 50 to 60% of E2 was found in particles in the media in the case of all but the SFV (−) mutant. This latter variant released only about 12% of E2 into the medium after 5 h. Thus, we conclude that all but the SFV (−) mutant support efficient particle formation.

Particles produced by the mutant RNA-transfected cells are infectious. To measure the infectivity of the particles produced by the RNA-transfected cells, a plaque assay was used (21). All but the SFV (−)-transfected cell media contained 1×10^9 to 2×10^9 PFU/ml of medium after a 5-h chase time. The SFV (−) sample contained about 2×10^8 PFU/ml. This value corresponds to the lower yield of released virus protein described above. If the number of PFU is expressed in relation to the amount of released virus protein, all virus variants are observed to produce 2×10^4 to 5×10^4 PFU/cpm of released E2. This finding suggests that all released virus particles are approximately equally infectious, including the SFV (−) particles, which are more slowly released from infected cells.

Mutant viruses have a pH threshold for cell fusion similar to that of wt virus. The ability of E1 of the virus variants to mediate membrane fusion was studied indirectly by measuring the pH threshold for virus-induced cell-cell fusion. For this purpose, we infected cell cultures in six-well dishes with coverslips with wt or recombinant SFV at 0.1 PFU per cell. The virus stocks used represented medium samples of cells that had been electroporated with the corresponding RNA. Five hours postinfection, the medium was removed and cells were incubated with buffers varying in pH for 2 min at 37°C. We used minimum essential medium without bicarbonate, supplemented with either 20 mM MOPS (4-morpholinepropanesulfonic acid; pH 7.0), 20 mM MES (4-morpholineethanesulfonic acid; pH 6.5 and 6.0), or 20 mM sodium succinate (pH 5.5). The pH conditions were chosen such that one (6.5) was slightly above the pH threshold for inducing wt virus fusion (6.2) and another was slightly more acidic (6.0). After this 2-min incubation, buffers were replaced with complete BHK medium; cells were kept for another hour at 37°C, then fixed in methanol, and analyzed for polykaryon formation after staining for virus-infected cells, using indirect immunofluorescence with an anti-E2 polyclonal rabbit antiserum (24). As shown in Fig. 3, all variants as well as wt virus induced cell-cell fusion at pH 6.0 and 5.5 but not at pH 7.0 or 6.5. In these immunofluorescence analyses, nonfused infected cells were seen as separate E2-positive cells in the monolayer. In contrast, the fused cells were large, less intensely stained polykaryons with several (dark) nuclei. These results suggest that the two arginine residues at the C terminus of E1 have no important role in the virus fusion reaction.

Role of E1 tail. We have speculated that a role for the conserved Arg-Arg tail peptide of the alphavirus E1 protein might be to enhance the E1 membrane-anchoring effect of the adjacent transmembrane peptide region. This function could be important for biosynthesis of the SFV spike and for the membrane fusion activity of E1. Clearly, our experimental results do not support this hypothesis. E1 variants in which the Arg-Arg tail peptide was changed to one arginine residue, one or two lysine residues, or two serine residues or was completely deleted were all used for heterodimer formation with p62 in the infected cells and could be incorporated into virus particles. Further, all E1 variants supported low pH-induced cell-cell fusion in an in vitro assay with a pH threshold typical for the wt virus. The virus variants also appeared to penetrate normally into new host cells, as shown by the plaque assay.

As a third possible role for the Arg-Arg tail peptide of E1, we have speculated about its possible involvement in the virus budding process. However, this possibility seems also not to be the case. The SFV variants in which the Arg-Arg tail peptide was changed were, with one exception, as budding competent as wt SFV. The single exception was the variant with an E1 protein with the Arg-Arg tail peptide deletion, which budded with about one-fifth the efficiency of wt virus. Interestingly, this mutated E1 subunit was quite normal in its heterodimerization reaction with p62, p62E1

TABLE 3. Cleavage of the p62 subunit in the spike heterodimer

Virus	Radioactivity (cpm)[a]		Cleavage efficiency (%)[b]
	p62 + E2	E2	
SFV wt	528	456	86
SFV R	1,146	861	75
SFV (−)	604	433	72
SFV K	991	816	82
SFV KS	671	592	88
SFV KK	429	383	89
SFV SS	744	639	86

[a] Transfected and pulse-labeled cells were chased for 5 h before lysis and reaction with a monoclonal antibody against E1 to precipitate spike heterodimers. Corresponding media were reacted with a monoclonal antibody against E2 to precipitate virus. Immunoprecipitates were analyzed by SDS-PAGE, and radioactivity was quantitated.

[b] E2 radioactivity as a percentage of the sum of p62 and E2 radioactivity; mean of two experiments.

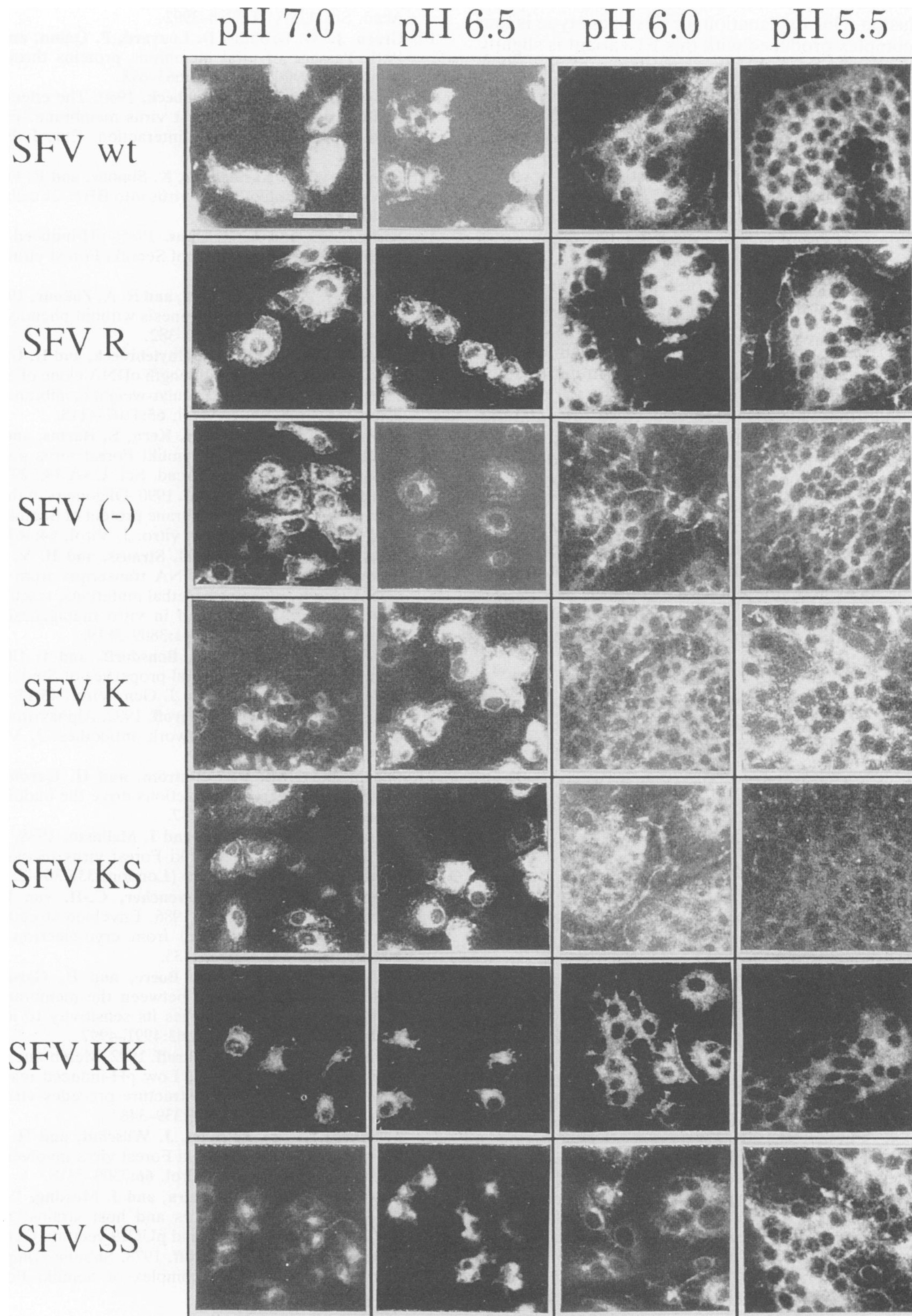

FIG. 3. pH threshold for induction of cell-cell fusion by SFV variants. Monolayer cultures of BHK-21 cells were infected with wt virus and SFV variants at 0.1 PFU per cell. After 5 h of incubation, cells were treated for 2 min with buffers of indicated pH. The buffer was then replaced with BHK medium, and cells were incubated further for 1 h at 37°C before being fixed and stained for E2. Note the formation of large E2-positive polykaryons after treatment with pH 6.0 and 5.5 buffers. The bar represents 50 μm.

transport, and cleavage as well as in its capacity to support membrane fusion. One explanation for this phenotype is that the p62-E1 complex produced with this E1 variant is slightly abnormal in comparison with the complexes of the other variants and that the alphavirus budding reaction is more sensitive to this aberrant structure than are spike subunit heterodimerization, cell surface transport, and virus entry functions.

In summary, we conclude that the E1 tail does not play any important role either in virus budding or in virus penetration when grown in in vitro cell cultures. However, it is still possible that the Arg-Arg tail of E1 has some more subtle role in virus multiplication which is not possible to hdetect in our assays. Such a role could possibly be reflected in detailed virus growth rate analyses or during multiplication in mosquito cells (the arthropod host of alphaviruses). Alternatively, the conserved features of the E1 tail might be important for the persistence of alphaviruses in nature. The major value of our results with the E1 tail virus variants lies in the fact that they have excluded several speculations about the role of the E1 subunit in alphavirus budding or entry processes. The results relating to virus budding also point to the crucial role played by the other subunit of the spike heterodimer, p62, in NC binding, a role which is clearly supported by p62 tail mutagenesis experiments (6). It will be important in future studies to define this interaction in great detail despite the difficulties encountered so far with use of biochemical and immunological approaches (9, 11, 20–22). This interaction is an important key to our understanding of both virus assembly during budding and virus disassembly during fusion.

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