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The cytoplasmic domain of alphavirus E2 glycoprotein contains a short linear recognition signal required for viral budding

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Intracellular alphavirus nucleocapsids express a binding site for the cytoplasmic domain of the viral E2 spike glycoprotein. This binding site is recognized by the anti-idiotype monoclonal antibody, F13. The monoclonal anti-anti-idiotype antibody, raised against F13 and designated 3G10, recognizes the carboxy-terminal eight residues of the E2 cytoplasmic domain in Semliki Forest virus (SFV), identifying this as the signal for nucleocapsid interaction. F13 binding to cells infected with SFV or a second alphavirus, Sindbis virus, is inhibited by a synthetic peptide corresponding to the entire 31 residue cytoplasmic domain (E2c), and also by a synthetic peptide corresponding to the eight residue epitope recognized by 3G10. Both E2c and the eight residue peptide inhibited viral budding in microinjection experiments and when conjugated to colloidal gold are bound specifically to nucleocapsids in infected cells. These results identify a short linear signal in the E2 cytoplasmic domain required for the interaction with nucleocapsids which leads to budding of at least two alphaviruses from infected cells.
Key words: anti-idiotypes/epitope mapping/synthetic peptides/viral phenotypic mixing

Introduction

The alphaviruses are a well studied family of enveloped viruses with a single positive strand RNA genome (Schlesinger and Schlesinger, 1986). The cell biology of alphavirus infection has been widely studied, especially for two members of the family, Semliki Forest virus (SFV) and Sindbis virus (SN). The translation of the viral structural polyprotein together with its subsequent processing and the delivery of the spike glycoproteins to the plasma membrane of the infected cell have been described in detail (Simons and Garoff, 1980). Relatively less is kown about the assembly of the nucleocapsid.

Virus budding occurs at the plasma membrane of the infected cell and results in enclosure of the nucleocapsid within a layer of viral spike glycoproteins embedded in a host cell derived lipid bilayer. Host cell membrane proteins are almost completely excluded from this forming envelope, and considerable interest has focussed on the sorting mechanism responsible for this selectivity (Simons and Warren, 1984). A specific interaction between the cytoplasmic domains of the SFV spike glycoprotein complex and the surface of the nucleocapsid was proposed by Garoff and Simons, initially on the basis of crosslinking experiments (Garoff and Simons, 1974; Ziemiecki and Garoff, 1978). Similar results were also obtained for the closely related Sindbis virus (Brown *et al.*, 1974). Further experimental support came from the observation that when all of the phospholipid was removed from the SF virus particle with octyl β-D-glucopyranoside at low ionic strength, more than half of the spike glycoprotein complexes remained attached to the nucleocapsid (Helenius and Kartenbeck, 1980). Morphological observations on the effect of antisera on SN spike glycoproteins suggest that it is possible to aggregate the viral membrane proteins into a cap, and that when this happens the intracellular nucleocapsids are also found in the cap (Smith and Brown, 1977).

More recently, a specific interaction between the nucleocapsid and the cytoplasmic domain of the E2 spike glycoprotein was demonstrated using an anti-idiotype approach (Vaux *et al.*, 1988). A synthetic peptide corresponding to the 31 residue cytoplasmic domain of the E32 glycoprotein of SFV (E2c) was used to immunize Balb/c mice. The resulting antiserum recognized both the immunizing peptide and the E2 glycoprotein from virus or virally infected cells. A monoclonal internal image anti-idiotype antibody, designated F13, was produced by two consecutive rounds of *in vitro* immunization, beginning with the same E2c synthetic peptide. F13 immunoprecipitates SFV nucleocapsids and gives a punctate staining pattern in permeabilized cells infected with any one of a large number of alpha- or flaviviruses. A monoclonal internal image anti-idiotype antibody designated 3G10 (a 'third round' response) was raised against F13. This antibody recognizes the starting E2c peptide and the F13 IgM and is able to inhibit the binding of F13 to infected cells (Vaux *et al.*, 1988).

The paired *in vitro* immunization technique makes use of a polyclonal response against the initial antigen as the immunogen in a second round of immunization (Vaux, 1990). Anti-idiotype reagents are produced directly, without requiring the prior isolation of a relevant idiotype. Since the relevant idiotype recognizes the 'ligand' of the interaction under reconstruction it is useful to have access to this reagent. This may be obtained by production of monoclonal third round antibodies. For example, 3G10 is equivalent to the initial idiotype and therefore should recognize the exact structural motif in the E2c peptide which interacts with the nucleocapsid. Here, we show that the third round antibody 3G10 can be used to map the signal in the cytoplasmic domain of E2, and that the eight residue region identified in this way has all the properties expected of such a signal.

Results

Conservation of features in the E2c domain among alphaviruses

Figure 1 shows an alignment of the available sequences of the cytoplasmic domains of E2 spike glycoproteins from different alphaviruses. The level of conservation is high throughout this domain but two regions are particularly conserved. The first is the region around the CLTPY motif and the second is close to the carboxy terminus. Based simply on this comparison, these two regions would both seem good candidates for a linear–nucleocapsid interaction signal.

Neither sequence comparison nor structural prediction methods enable us to narrow down the region within E2c responsible for interaction with the nucleocapsid. Methods which would enable a more exact study include mutagenesis of the E2c region and reintroduction of these mutants into an infectious clone of SFV, or a synthetic peptide based inhibition experiment. We have employed an alternative approach based on the use of the anti-idiotype antibody F13 and the anti-anti-idiotype antibody 3G10 to analyse the E2c domain in more detail.

Epitope mapping within the E2c peptide

In order to analyse the 31 residue E2c peptide in detail, a series of 24 overlapping eight residue peptides with an offset of one residue per step were synthesized using a solid phase method which results in covalent linkage to polypropylene pins (the first pin carries the sequence RSKCLTPY, the second SKCLTPYA and so on; the one letter code is used). This array would be expected to reconstitute simple linear epitopes up to eight residues in length, but must obviously exclude longer simple linear epitopes and all discontinuous epitopes, as well as short epitopes with a dependence upon a remote region for their conformation. Despite these limitations, this approach revealed a considerable immunological complexity in E2c (Figure 2). A polyclonal rabbit antiserum raised against the unconjugated E2c peptide recognized a single strong linear epitope with the required core sequence PYALT (Figure 2A), while a Balb/c mouse polyclonal antiserum also generated by immunization with unconjugated peptide recognized three strong distinct epitopes (Figure 2B), overlapping only partially with the rabbit epitope. Strikingly, the monoclonal anti-anti-idiotype antibody 3G10 recognized a region only weakly recognized by the polyclonal Balb/c antiserum, consisting of the extreme carboxy terminus of the E2c peptide, with the sequence CCAPRAHA (Figure 2C).

Since 3G10 is a third round response it should recognize

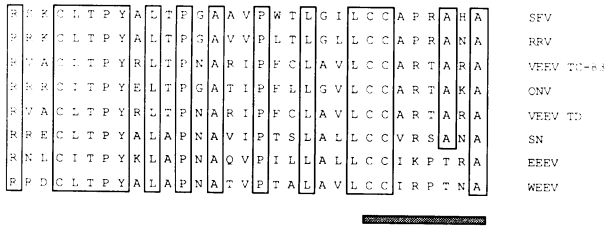

Fig. 1. Alignment of the E2 cytoplasmic domain amino acid residues for eight alphaviruses. The cytoplasmic domain sequence of the E2 glycoprotein from SFV (Garoff *et al.*, 1980) is shown aligned with the E2 cytoplasmic domains from Ross River virus (Dalgarno *et al.*, 1983), Eastern equine encephalitis virus (Chang and Trent, 1987), Western equine encephalitis virus (Hahn *et al.*, 1988), Venezuelan equine encephalitis virus reference strain (Johnson *et al.*, 1986), Venezuelan equine encephalitis virus Trinidad donkey strain (Kinney *et al.*, 1986), O'Nyong Nyong virus (Levinson *et al.*, 1990) and Sindbis virus (Russell *et al.*, 1989; Strauss *et al.*, 1984). Residues perfectly conserved in all eight sequences are boxed. The 3G10 epitope is marked with an underscore beneath the alignment.

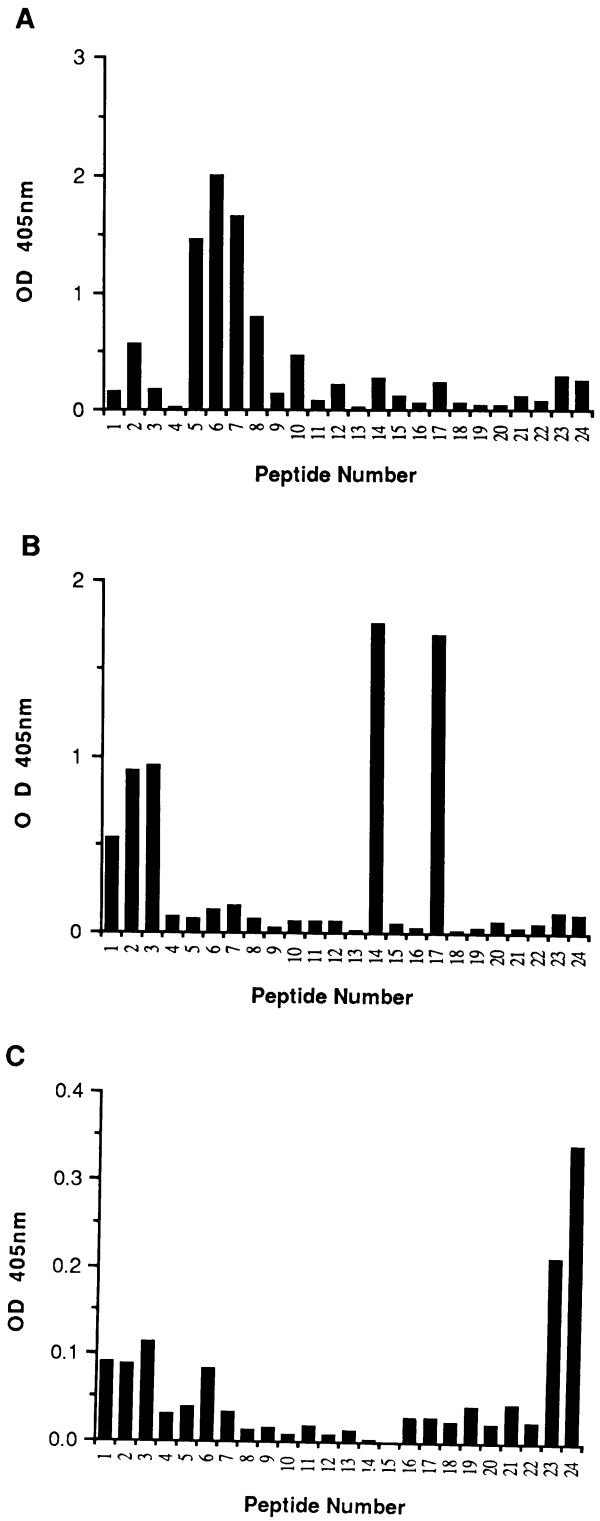

Fig. 2. Identification of epitopes within the SFV E2 cytoplasmic domain. An overlapping series of eight residue peptides (RSKCLTPY, SKCLTPYA, etc.) was used to map the responses of rabbit (**A**) and Balb/c mouse (**B**) to immunization with unconjugated E2c peptide. The monoclonal anti-anti-idiotype antibody 3G10 was also mapped (**C**), revealing a single discrete linear epitope at the extreme carboxy terminus.

the region of the initial antigen responsible for the protein–protein interaction being reconstructed. This suggests that the 3G10 epitope is the region of the tail of the E2 spike glycoprotein which interacts with the viral nucleocapsid to ensure specificity in the budding process. Thus, predicting that the terminal CCAPRAHA sequence of the SFV E2 spike glycoprotein interacts with the viral nucleocapsid and is responsible for the specificity of SFV budding.

Binding of F13 to infected cells is inhibited by E2c and CCAP peptides

Since F13 is an internal image anti-idiotype antibody arising from E2c, the structure it recognizes on the surface of the intracellular nucleocapsid is the E2c binding site. Consequently, if the E2c peptide exhibits a similar solution structure to the authentic E2 cytoplasmic domain it should compete with F13 for binding to nucleocapsids. Figure 3 shows the result of quantitative immunofluorescence experiments which demonstrate that this is the case (for details of the peptides used see Table I). Compared with two separate control peptides, the E2c peptide exhibits a specific dose dependent inhibition of the binding of F13 to permeabilized SFV infected cells (Figure 3A).

If the epitope mapping has correctly identified the relevant region within E2c, then the eight residue peptide CCAP should also show this inhibition. A similar dose dependent inhibition of F13 binding is indeed also observed with the eight residue peptide (Figure 3A). The final extent of inhibition observed is similar for the two peptides, making it unlikely that a second binding signal is embedded in E2c.

The presence of a second signal within the E2c domain was also excluded by testing a synthetic peptide corresponding to the amino-terminal half of the SFV E2c sequence (RSKC). This peptide includes the CLTPY sequence which is strikingly conserved among alphaviruses. No inhibition of F13 binding was observed with this peptide (Figure 3A).

Since the F13 binding site is conserved among alphaviruses (Vaux *et al.*, 1988) we would expect that these peptides should show the same results in cells infected with SN instead of SFV, and this is the case (not shown). Moreover, if the signal resides in the C-terminal eight residues then the corresponding sequence from the E2 tail of SN should also bind specifically to nucleocapsids and inhibit binding of F13 to cells infected with SFV. Figure 3B shows that a synthetic peptide corresponding to the carboxy-terminal 14 residues of the SN E2c sequence (KTSL) is able partially to inhibit the binding of F13 to SFV infected cells in a dose dependent manner.

These quantitative inhibition results confirm that alphavirus E2 cytoplasmic domains contain a single short linear sequence responsible for interaction with viral nucleocapsids. This signal is located at the extreme carboxy terminus of the cytoplasmic domain, as predicted from epitope mapping with the anti-anti-idiotype 3G10. The signal shows effective crosstalk between alphaviruses, with peptides corresponding to the signal from SFV or SN inhibiting F13 binding to cells infected with SN or SFV. This result is consistent with the conservation of the E2c binding site on nucleocapsids from many alphaviruses, and previous observations of efficient phenotypic mixing between different alphaviruses. We next sought direct evidence for this E2c–nucleocapsid interaction by attempting to locate the sites to which the peptides bound in infected cells.

E2c and CCAP peptides bind to nucleocapsids in infected cells

In order to visualize the intracellular sites at which peptides corresponding to cytoplasmic domains bind in SFV infected cells we took advantage of the observation that it is possible to stabilize homogeneous colloidal gold particle suspensions with synthetic peptides. Several probes were prepared by stabilization of 7 nm gold particles with synthetic peptides including E2c and CCAP. Figure 4 shows the result of using these probes to decorate thawed frozen thin sections of BHK-21 cells infected for 6 h with SFV. Gold particles coated with the E2c peptide label intracellular nucleocapsids, as well as partially budded virus particles at the cell surface (Figure 4A). Not all morphologically distinct nucleocapsids

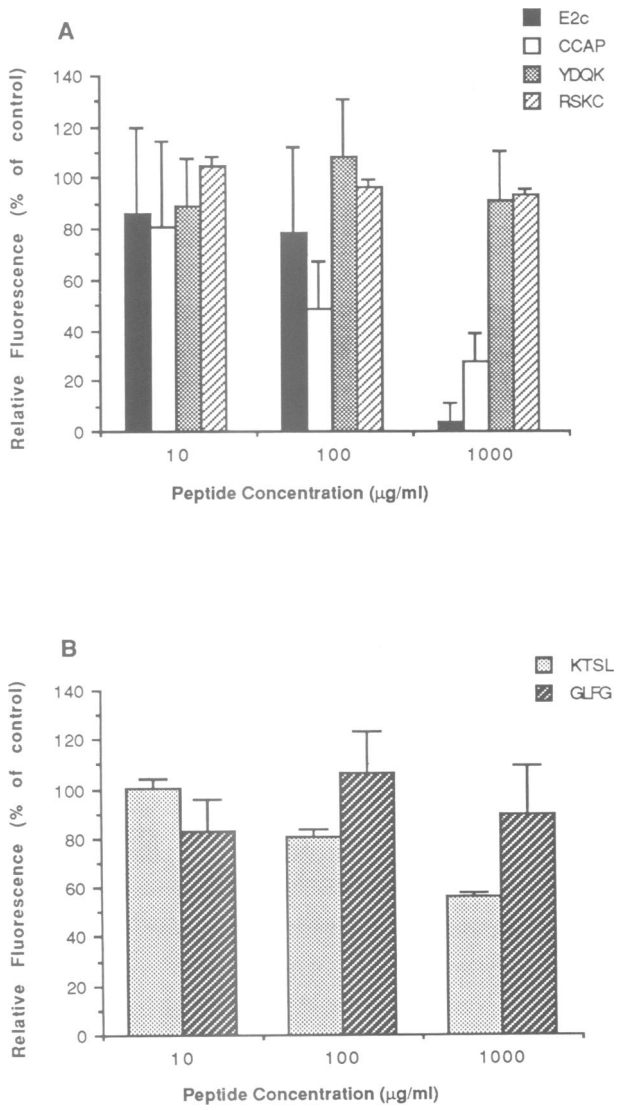

Fig. 3. Peptide inhibition of binding of the anti-idiotype antibody F13 to SFV infected cells. Fixed permeabilized SFV infected BHK-21 cells were stained with the anti-idiotype antibody F13 after preincubation with varying concentrations of synthetic peptides. Quantitation of the fluorescence signal (see Materials and methods) reveals that the binding of F13 shows a dose dependent inhibition by E2c and CCAP peptide, but is not inhibited by two control peptides (YDQK shown here) (**A**). A peptide corresponding to the N-terminal half of SFV E2c (RSKC) also has no effect (**A**). In contrast, a peptide corresponding to the C-terminal half of the SN E2c sequence (KTSL) does inhibit binding of F13 to SFV infected cells (**B**, which also shows the second control peptide, GLFG).

Table I. Synthetic peptides used in this study

Name	Sequence
E2c	NH_2-R-S-K-C-L-T-P-Y-A-L-T-P-G-A-A-V-P-W-T-L-G-I-L-C-C-A-P-R-A-H-A-COOH
RSKC	NH_2-R-S-K-C-L-T-P-Y-A-L-T-P-G-A-COOH
KTSL	NH_2-K-T-S-L-A-L-L-C-C-V-R-S-A-N-A-COOH
MDDQ	NH_2-M-D-D-Q-R-D-L-I-S-D-D-E-Q-L-P-M-L-G-R-R-P-G-A-P-E-S-K-COOH
GLFG	NH_2-G-L-F-G-A-I-A-K-COOH
CCAP	NH_2-C-C-A-P-R-A-H-A-COOH
YDQK	NH_2-Y-D-Q-K-A-V-K-D-E-L-COOH

are labelled, consistent with the observation that late in an SFV infection there are many nucleocapsids which will never bud from the cell. After 5 h infection with SFV, BHK-21 cells contain a population of F13 positive structures which form a subset of the particles labelled using a monospecific polyclonal antiserum to the capsid protein (Vaux *et al.*, 1988). Colloidal gold particles coated with the CCAP peptide shown an identical staining pattern (Figure 4B). Colloidal gold particles coated with a control peptide (Figure 4C) or with BSA (not shown) did not label nucleocapsids.

A striking feature of alphavirus infection in tissue culture cells is the appearance of tubular membrane bound structures coated on the cytoplasmic face with viral nucleocapsids. These structures were originally described in early studies of the morphology of alphaviral infections, where they were called cytopathic vacuoles type II or CPV IIs. They were later shown to be SFV modified parts of the trans-Golgi network (TGN) where spike glycoproteins accumulate to a very high density (Griffiths *et al.* 1982, 1989). Since these structures arise by frustrated budding of nucleocapsids at membranes which carry high spike glycoprotein densities, all of the nucleocapsids associated with a CPV II should be budding competent. They should therefore all be capable of recognizing the E2c signal. Figure 5 shows that CPV IIs are intensely labelled by E2c coated gold particles (Figure 5A) and by CCAP coated cold particles (Figure 5B). The dense packing of viral spike glycoproteins in the membrane of this organelle may be clearly seen as regular transverse striations, especially in the upper part of Figure 5B.

Microinjection assay for inhibitors of viral budding

The naked 42S single positive strand genome RNA of SFV is infectious when introduced into the cytoplasm of susceptible cells (Rice *et al.*, 1987). When genomic RNA is microinjected into a very small percentage of cells on a coverslip a productive infection occurs, with the release of infectious virions from the injected cells. These virions spread the infection to non-injected cells on the coverslip. This spread of infection may be assessed by fixing coverslips at intervals and staining the cell monolayer for surface expression of viral spike glycoproteins. Spread of infection may be monitored by counting infected cells and also by counting infectious particles in the medium using a plaque titration assay. Co-microinjection of RNA together with peptides that might affect virus formation permits an estimate of inhibition of viral budding to be made.

Table II shows that the E2c peptide is an effective inhibitor of viral budding in this assay and that control peptides do not have the same effect. Thus, the interaction between the E2 cytoplasmic domain and the viral nucleocapsid is essential for viral budding. The eight residue peptide corresponding to the 3G10 epitope also inhibits the spread of SFV infection in this assay. This result confirms that the essential interaction between the SFV E2 cytoplasmic domain and the SFV nucleocapsid is mediated by the carboxy-terminal eight residues.

The 14 residue peptide including the carboxy-terminal residues of SN E2 (KTSL) was also tested in this assay and found to cause significant inhibition of viral budding when SFV genomic RNA was used to start the infection. Thus, the sequence from SN E2 is capable of competing for E2c binding sites on SFV nucleocapsids, and this competition leads to inhibition of viral budding. The SN and SFV carboxy-terminal signals are therefore functionally interchangeable.

Taken together, these experimental results clearly identify a short linear sequence at the extreme carboxy terminus of the cytoplasmic domain of the E2 spike glycoprotein as the signal required for viral budding. This signal is functionally interchangeable between at least two alphaviruses.

Discussion

The budding of an alphavirus at the plasma membrane of an infected cell is a good example of a highly efficient membrane sorting event. Since it is mediated by a simple set of viral structural proteins it has been recognized as an important model for understanding endogenous cellular sorting mechanisms (Simons and Warren, 1984). The efficiency of the sorting event, together with the stringent requirements for budding which prevent phenotypic mixing by incorporation of spike glycoproteins from other viral families (Burge and Pfefferkorn, 1966; Lagwinska *et al.*, 1975), prompted the idea of a specific interaction between the intracellular nucleocapsid and a signal in the cytoplasmic domain of the viral spike glycoprotein complex.

We began this search for a budding signal by noting that the structure recognized by F13 is strongly conserved among alphaviruses. This suggests that a conserved structural motif might serve as a signal. The network antibody study localized this signal to the cytoplasmic domain of the E2 spike glycoprotein (Vaux *et al.* 1988). A comparison of the deduced amino acid sequence of the E2 cytoplasmic domains for eight alphaviruses revealed a generally high level of conservation with two short perfectly conserved regions (Figure 1). The high level of conservation prevents the identification of a possible short linear signal by sequence comparison alone.

In order to locate the exact signal we used the monoclonal anti-anti-idiotype antibody 3G10 to probe the cytoplasmic

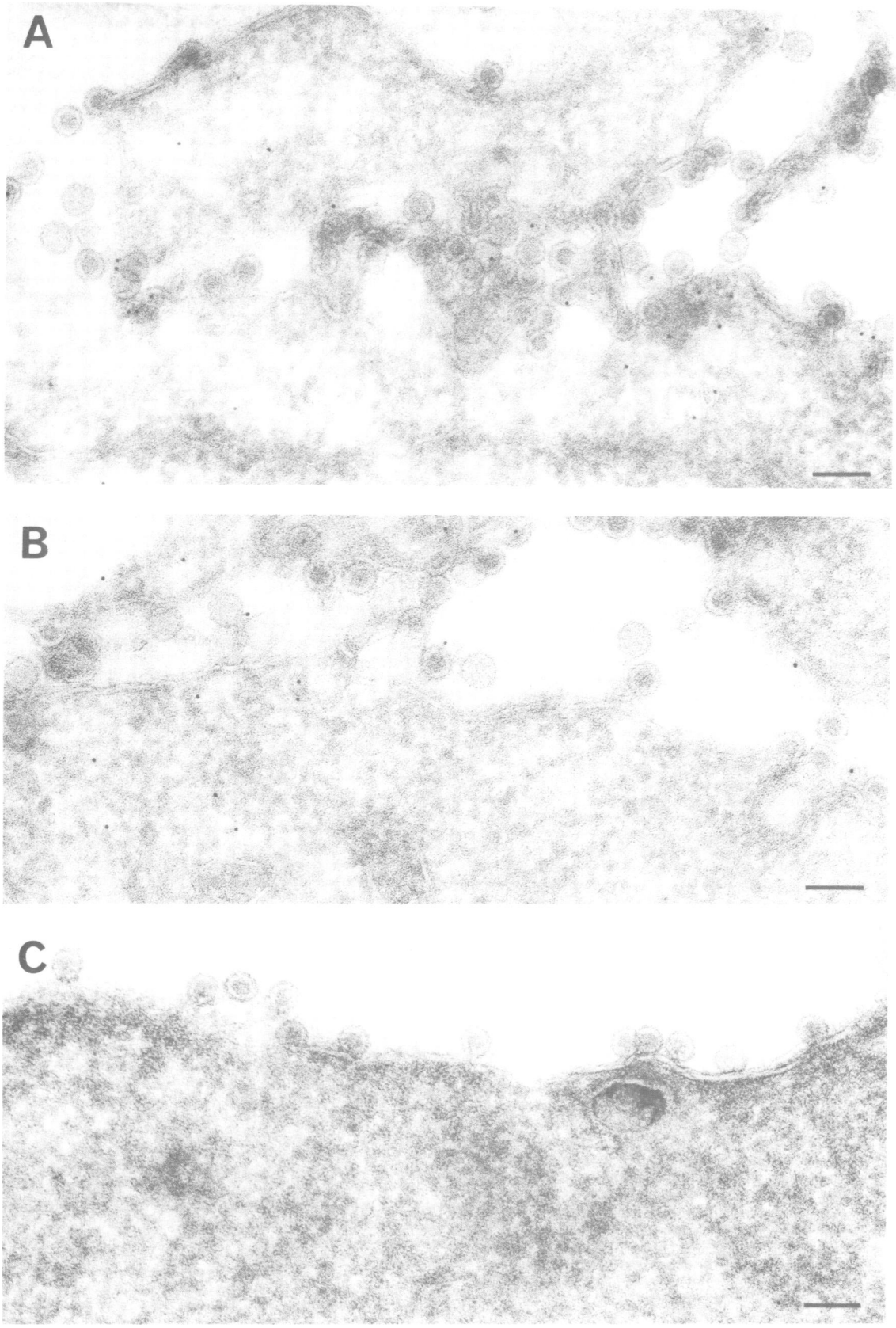

Fig. 4. Colloidal gold conjugated E2 peptides label nucleocapsids in SFV infected cells. Thawed frozen sections of SFV infected BHK-21 cells were labelled with colloidal gold conjugates of the E2c peptide (**A**), the eight residue CCAP peptide (**B**) and the control KET peptide (**C**). Both the E2 tail peptides labelled nucleocapsids in the cytoplasm and partially budded virions at the plasma membrane. No labelling was seen with the control peptide or with BSA–gold or protein A–gold. Bar = 0.1 μm.

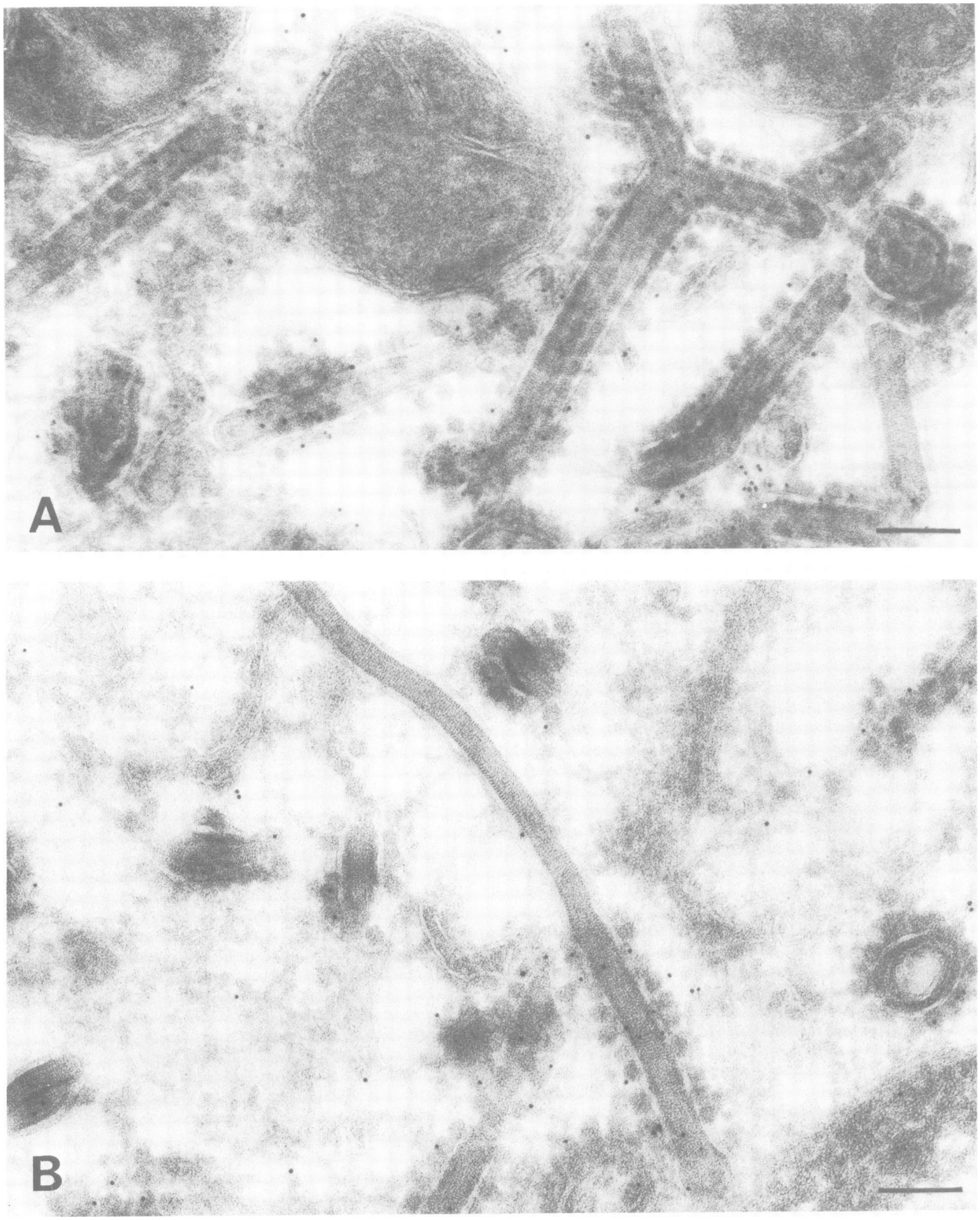

Fig. 5. Cytopathic vacuole labelling by E2 peptide–gold conjugates. Both E2c–gold (**A**) and CCAP–gold (**B**) strongly labelled nucleocapsids aligned along the cytoplasmic face of virally modified elements of the TGN, the type II cytopathic vacuoles. Note that in areas of the CPV II which are not decorated with nucleocapsids no peptide gold labelling is seen. The high density packing of viral spike glycoprotein in the membrane of the CPV II is seen particularly well in the upper part of (**B**). Bar = 0.1 μm.

domain of the E2 spike glycoprotein. This reagent recognized a single linear epitope at the extreme carboxy terminus of a peptide corresponding to the entire cytoplasmic domain of the E2 spike glycoprotein of SFV. The epitope recognized by the anti-anti-idiotype antibody was present in a monomeric eight residue synthetic peptide. The epitope mapping experi-

Table II. Peptide inhibition of viral budding

Experiment	Peptide	Titre[a] (range)	Percentage
1	MDDQ (control)	12500	100
	E2c	1250	10
2	MDDQ (control)	6250 (6250)	100
	CCAP	50 (40–50)	1
3	GLFG (control)	500 (250–1000)	100
	E2c	0 (0)	0
	KTSL	0 (0)	0
	CCAP	20 (10–20)	4
4	GLFG (control)	9300 (6250–12500)	100
	CCAP	460 (10–1250)	5
	E2c	0 (0)	0
	KTSL	0 (0)	0

[a]Plaque forming units per ml of culture supernatant obtained 8 h post-injection, see Materials and methods for complete description of micro-injection protocol.

ment also revealed the 31 residue E2c to be immunologically complex, with major differences between the responses of rabbits and mice. The observation that polyclonal antisera raised against E2c in mice or rabbits recognize strong epitopes remote from the 3G10 epitope suggests that it would have been difficult if not impossible to carry out the original network antibody study using polyclonal reagents.

The identification of the C terminal eight amino acids of SFV E2c as the signal for nucleocapsid interaction by epitope mapping was confirmed by three additional experiments. Firstly, the binding of F13 to nucelocapsids in infected cells is specifically inhibited by the CCAP peptide, which corresponds to this epitope. Secondly, CCAP peptide conjugated to colloidal gold labels SFV nucleocapsids in thawed frozen thin sections of SFV infected BHK-21 cells. Finally, the CCAP peptide shares with the E2c and KTSL peptides the ability to inhibit viral budding when co-microinjected into cells along with infectious SFV genomic RNA. These results unequivocally identified the site of a signal which interacts with the nucleocapsid and confirmed that this signal does not have to be present on a trimeric structure to be recognized.

Several studies have addressed the importance of the E2 cytoplasmic domain for virion formation. Recently, it has been reported that a full length E2 cytoplasmic domain peptide can bind to isolated SFV nucleocapsids *in vitro* (Metsikko and Garoff, 1990). In this *in vitro* binding study, a full length E2c peptide was compared with a peptide corresponding to the membrane-proximal amino-terminal half of the domain. No binding was seen with the amino-terminal peptide, consistent with a simple model in which the site for nucleocapsid interaction is found in the C-terminal membrane-distal half of E2c. In a preliminary communication describing inhibition of virus formation by synthetic peptides added to the medium bathing infected cells (Schlesinger, 1990), Schlesinger has implicated the membrane proximal conserved PYALT region in viral budding, but in this study peptides corresponding to the carboxy-terminal region were not tested. The most detailed information on the role of the E2 domain comes from recent work involving mutations in this region introduced into an infectious cDNA clone of Sindbis virus (Gaedigk-Nitschko and Schlesinger, 1991.) In this study, three point mutations and one double mutation were made in the E2 tail. Mutation of cysteine 395 (residue 4 in sequence of Figure 1) to serine did not reduce virus production in chick embryo fibroblasts compared with wild type. Similarly, mutation of tyrosine 399 (residue 8 of sequence in Figure 1) to phenylalanine resulted in wild type levels of virus production in these cells. In marked contrast, a double mutant which converted the pair of cysteines at 415 and 416 (residues 24 and 25 of sequence in Figure 1) to serine and alanine resulted in the total loss of virus production. A single mutant of serine 419 (residue 28 of sequence in Figure 1) to cysteine also caused a profound reduction in virus production to $<1\%$ of wild type in chick embryo fibroblasts at up to 6 h of infection. Thus, each of the mutations which has a strong effect on virus production maps into the eight residue region identified by the anti-anti-idiotype antibody as the signal for nucleocapsid interaction.

Functional organization of the E2 cytoplasmic domain

Several functional features of the E2 cytoplasmic tail are now recognizable. The membrane-proximal three residues form a charged region characteristic of a membrane anchor, and the data presented here show that the carboxy-terminal eight residues form a budding recognition signal.

What other functions might be mediated by the E2 cytoplasmic domain? Romand and Garoff (1986) analysed a series of carboxy-terminal truncation mutations and showed that most of the E2 cytoplasmic domain is not required for transport competence. The p62 mutants with shortened cytoplasmic domains also showed wild type polarized expression in the MDCK epithelial cell line. These results suggest that the E2 cytoplasmic domain is not required for transport through the exocytic pathway, nor for delivery to the appropriate domain of a polarized epithelium. The p62 truncations all become palmitoylated, suggesting that the whole E2 cytoplasmic domain is not required for this post-translational modification.

An important requirement for viral infection is the uncoating of the incoming virion to release RNA from the nucleocapsid and begin the process of RNA replication in the cytoplasm of the infected cell. There is good evidence that alphavirus normally enter cells via the endocytic pathway and that a low pH mediated fusion event is an important part of this process (reviewed in Kielian and Helenius, 1986 and Marsh and Helenius, 1989). Comparatively little is known about disassembly during viral entry, and it is possible that disruption of the E2c–nucleocapsid interaction is required for the establishment of infection by an incoming virus particle. It is probable that viral entry is associated with the transient re-exposure of the F13 epitope, implying that the E2c signal may become dissociated some time prior to the loosening or disassembly of the capsid shell (D.J.T.Vaux, I.Mellman and A.Helenius, manuscript in preparation).

Implications for viral budding

The envelopment of the nucleocapsid by the lipid bilayer is thought to be driven by multiple interactions between the cytoplasmic domains of the membrane-embedded glycoproteins and the surface of the nucleocapsid itself (Fuller, 1987; Metsikko and Garoff, 1990). Thus, disruption of this interaction should effectively prevent virion formation. The data presented here show that a short synthetic peptide containing the signal for this interaction competes with E2 glycopro-

tein cytoplasmic domains for nucleocapsid sites and inhibits budding of the virus. This budding mechanism involving a specific conserved signal also offers an explanation for the selectivity of viral budding, with the inclusion of an exact icosahedral array of spike glycoproteins into the viral envelope and the near complete exclusion of host cell proteins.

Moreover, the signals present in E2c from SFV and SN both block F13 binding to cells infected with SFV or SN. This interchangeability also extends to the function of the signal during viral budding. Experiments are in progress to map the extent of this functional interchangeability.

With the identification of a signal, the exclusion of host proteins from the forming virion may be studied in greater detail. Cellular proteins may be excluded because they lack the cytoplasmic signal which enables them to interact with the nucleocapsid, or because they are unable to enter patches of viral spike proteins at the plasma membrane. If the interaction signal is necessary and sufficient for inclusion then it should be possible to prepare mixed phenotype viral particles by infecting cells engineered to express a reporter protein which carries this signal. If lateral interactions at the plasma membrane are also strong determinants of inclusion then the signal may be necessary but not by itself sufficient.

Finally, this budding signal offers a sensitive point in the viral life cycle which may be amenable to therapeutic attack. In principle, the introduction of the eight residue peptide into cells is sufficient to inhibit viral budding. This is unlikely to be practical as a therapeutic method because of problems of peptide antigenicity and the difficulty of delivering the peptide across the plasma membrane and maintaining adequate intracellular levels. Nevertheless, the successful use of the network antibody approach in this study implies that the signal has a characteristic three dimensional structure. Our long term goal is to identify this structure at high resolution and use this information for the rational design of membrane permeable non-peptide, non-immunogenic mimetic substances with testable therapeutic potential.

Materials and methods

Reagents

Affinity-purified goat antibodies to mouse immunoglobulin heavy chains were obtained from Zymed Inc (San Francisco, USA) as fluorescein or alkaline phosphatase conjugates and used at the manufacturer's recommended dilutions. Fetal calf serum was obtained from Gibco–BRL (7514 Eggenstein, FRG). All other reagents were obtained from Sigma unless otherwise stated and were of analytical grade.

Peptides

Peptides were synthesized in an automated solid phase synthesizer using continuous flow Fmoc chemistry, cleaved and purified to homogeneity by preparative reverse phase HPLC (Frank and Gausepohl, 1987). Product identity was confirmed by sequence determination using automated Edman degradation. All peptides used were freely water soluble and were prepared as 5 mg/ml aqueous solutions. The sequences of the synthetic peptides used in this study are given in Table I. Epitope mapping peptides covalently attached to polyethylene rods were prepared using the Pepscan method (Geysen *et al.*, 1987), modified for automation with *in situ* activation of Fmoc amino acid derivatives (Gausepohl *et al.*, 1990).

Cells

BHK-21 cells were propagated in DMEM basal medium supplemented with 10% (v/v) fetal calf serum, subcultured twice a week. Hybridoma cell lines were propagated in alpha MEM supplemented with 20% fetal calf serum 2 mM glutamine, 2 mM sodium pyruvate, 10 mM HEPES pH 7.2, 1× Gibco non-essential amino acids and 50 μM 2-mercaptoethanol. Antibodies were concentrated from culture supernatants by ammonium sulphate precipitation and used without further purification. The F13 hybridoma line was also propagated as an ascitic tumour in Balb/c mice primed with 2,6,10,14 tetra-methyl-pentadecane. Clarified ascites was then treated by ammonium sulphate precipitation.

Viruses

Semliki Forest virus (SFV) and Sindbis virus (SN) were standard strains obtained from the laboratory of Professor Ari Helenius, Yale University School of Medicine, New Haven, CT, USA. Titred viral stocks were maintained in single-use aliquots at −80°C. Plaque titrations were carried out in triplicate using a modified microtitre plate method with carboxymethyl cellulose and BHK-21 cells (Precious and Russell, 1985).

Epitope mapping

The polyethylene rods carrying the peptides were mounted on a plastic holder with 96 well plate format so that incubations could be carried out in a 96 well plate. The pins were first incubated in Blocking solution [BS; 10% (v/v) goat serum in phosphate buffered saline without divalent cations (PBS.CMF) containing 10 mM sodium azide) for 30 min at room temperature. The pin array was then dipped into first antibodies diluted in BS for 60 min at room temperature. The pins were washed in five changes of 200 ml PBS.CMF over 20 min on an orbital shaker, reblocked for 30 min and dipped into appropriate alkaline phosphatase conjugated second antibodies for 60 min. After washing as before, the pins were dipped into a 96 well plate containing substrate (Sigma 110-104) in 50 mM sodium glycinate pH 9.8 for 60 min. Finally, the optical density at 405 nm was read in a Cambridge Technologies densitometer. Background binding was measured on three irrelevant peptide pins and subtracted from the values shown (in no case was this background more than 0.2 OD).

Indirect immunofluorescence

BHK-21 cells were grown overnight on 20 mm square coverslips, washed twice with PBS.CMF and infected with SFV at an approximate m.o.i. of 20 p.f.u./cell for 60 min in MEM containing BSA but no serum. The cells were then washed once in PBS.CMF and incubated for a further 4 h in normal growth medium. The coverslips were then washed twice in PBS.CMF and fixed in methanol at −20°C for 6 min. Duplicate coverslips were preblocked in BS alone or BS containing peptide at the appropriate concentration for 30 min and incubated for 60 min in F13 diluted in BS and containing various concentrations of peptides. The F13 was used at a concentration shown to be saturating in this assay. Coverslips were washed five times in PBS.CMF, reblocked and incubated for 60 min in fluorescein conjugated goat anti-mouse IgM (mu chain specific) second antibody. After washing five times in PBS.CMF the coverslips were mounted in Moviol and one of each pair examined in a Leitz Axiophot with 100× objective. Photographs were taken using Kodak TMY 400 rated at 1600 ASA with exposures ranging from 10 to 30 s. The second coverslip of each pair was examined with a Zeiss inverted fluorescence microscope equipped with a Hamamatsu SIT camera. Digitized images were quantitated using an IBMS image processing system (Zeiss) to give values for mean fluorescence intensity per unit area within the manually defined cell periphery. All values shown are arithmetic means, plus and minus one standard deviation based upon measurements of at least 120 cells (range 125–210) per point. A total machine background (maximally 0.2) has been subtracted from each value.

Microinjection budding assay

Genomic 42S RNA was isolated from purified virus by the technique of Davis *et al.* (1986). Preliminary experiments confirmed the infectivity of microinjected SFV RNA, and the absence of infection if the RNA was only released into the medium. 1 mm diameter circles were marked at the centre of 10 mm round glass coverslips with a diamond pencil. BHK-21 cells were plated in individual 35 mm dishes to give ~50% confluent monolayers on the coverslip after overnight incubation. A small number of cells within the marked circle (5–20, constant within an experiment) were injected with ~0.1 pl of an aqueous solution of 1 μg/ml genomic RNA, either alone or containing 5 mg/ml of the synthetic peptide under test (Ansorge and Pepperkok, 1988; Pepperkok *et al.*, 1988). Peptide and RNA solutions were mixed at the microscope and loaded and injected within 3 min to minimize RNA degradation, using a Zeiss AIS computer controlled microinjection system. The injection introduces ~15 copies of the viral genome (assuming all of the RNA in the preparation to be full length genomic RNA) and between 1 and 3 × 10^8 peptide molecules per cell. Coverslips were then incubated in normal growth medium at 37°C for various time intervals (3, 6, 9 and 18 h) and then processed. The medium in the 35 mm dishes was collected and retained for plaque assay of viral titre and the cells were washed twice in PBS.CMF and prepared for indirect immunofluorescence as described above. A rabbit polyclonal antiserum raised against SFV 29S

complexes was used to detect cells expressing viral glycoproteins. All plaque titrations were carried out in triplicate, and the data shown in Table II include the range of titres obtained.

Preliminary experiments showed that microinjection of genomic RNA into only 10 cells at the centre of a 10 mm coverslip containing ~10 000 cells resulted in 100% infection of 18 h post-injection. The appearance of remote foci of infected cells surrounded by uninfected cells at intermediate time points confirmed that the spread of infection was not due to cell fusion or migration. Measurable titres of infectious virus in the medium surrounding the cells confirmed that infectious virions were being released. In every case, reduction in plaque titre was associated with reduced numbers of infected cells by the immunofluorescence assay.

As an internal control to ensure that an apparent inhibition, manifest as the absence of progressive infection, was not simply a toxic effect on the small number of cells actually injected, every experiment included at 3 h time point. At this time only injected cells should express viral spike glycoprotein on their cell surface. Data were only collected from experiments in which the microinjected cells themselves survived to express surface viral glycoproteins at 3 h. This control also ensured that a peptide preparation which caused degradation of the viral RNA was not misinterpreted as causing inhibition of viral budding. In addition, at late time points (18 h post-injection or longer) all of the cells become infected, because the first budded virion to escape the inhibition will produce a wild type infection in a non-injected cell which does not contain the inhibitor. This confirms that inhibitory peptides are indeed inhibiting budding and virion release and not altering the virions produced so as to prevent re-infection of non-injected cells. Only experiments in which >500 infected cells were counted at 18 h post-injection were included. The variation in control titre between experiments is a consequence of the number of cells injected (although this was constant within an experiment), and the use of different batches of isolated viral genomic RNA.

In most experiments a time point of 6 or 8 h post-injection clearly showed the effects of inhibitors. At this time in the absence of inhibitors about 100–200 cells strongly express the viral spike glycoprotein and a reproducible plaque titration value may be obtained. Table II summarizes the results for this time point from a number of experiments which met the control criteria outlined above.

Electron microscopy

7 nm nominal diameter colloidal gold suspensions were prepared by the tannic acid method (Slot and Geuze, 1985), buffered to the pI of the peptides using 0.1 M sodium hydroxide (Horrisberger and Clerc, 1985) and stabilized with synthetic peptides in aqueous solution, at the lowest concentration sufficient to prevent aggregation to a 0.2 M salt challenge. The suspensions were concentrated by centrifugation in a Beckman airfuge and maintained as stocks at OD_{520} 3.5–4.0. These probes were diluted in 0.2% (w/v) bovine serum albumin (BSA) and applied to thawed cryo-sections of SFV infected BHK cells for 90 min on 10 μl drops at room temperature (Griffiths *et al.*, 1984). Grids were washed in 0.2% BSA and distilled water and embedded in uranyl acetate/methocellulose mixture (Griffiths *et al.*, 1984) prior to examination and photography.

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