Spike–nucleocapsid interaction in Semliki Forest virus reconstructed using network antibodies

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Using in vitro *immunization, we have reconstructed three consecutive steps of an idiotype network to show that the nucleocapsid of Semliki Forest virus contains a specific 'receptor' for the cytoplasmic tail of the E2 spike glycoprotein. This interaction could be the basis for the highly selective inclusion of viral glycoproteins—and exclusion of host cell surface proteins—during virus budding.*

THE budding of enveloped animal viruses from infected cells is characterized by the selective inclusion of the viral genome and its accessory proteins[1-3]. The budding mechanism is presently best understood for Semliki Forest virus (SFV), an alphavirus[4-6]. The first step of SFV assembly is the packaging of a 42S single-stranded genomic RNA into an icosahedral nucleocapsid containing 180 copies of a single capsid protein. The capsids attach to the plasma membrane of the infected cell and progressively associate with 240 copies of the heterotrimetric (E1, E2, E3) spike glycoprotein complex during formation of the nascent virion. Host cell proteins are almost completely excluded during this process. Finally, the virus is released by pinching off from the plasma membrane.

Although the mechanism for the selective inclusion of viral protein into the budding membrane remains uncertain, a specific interaction between the assembled nucleocapsid and the cytoplasmic domain of the spike glycoprotein complex seems likely[7]. Chemical cross-linking[8] and detergent extraction of intact virus particles[9] have provided evidence for a physical association between the capsid and spike proteins. Electron microscopy has revealed 80 regularly spaced depressions in the surface of the nucleocapsids, possibly corresponding to the putative binding sites for spike glycoprotein trimers[10].

To characterize the spike–capsid interaction further, we have used the structural mimicry of internal image anti-idiotype antibodies (see. ref. 11 for review). If the E2 cytoplasmic tail contains a determinant for binding to a specific site on the nucleocapsid, it should be possible to generate antibodies which would recognize that determinant. Anti-idiotypes to the anti-E2 tail antibodies would be expected to recognize the E2 binding site on the capsid. Finally, if anti-anti-idiotypes were made, some of these should bind to the original antigen—the E2 cytoplasmic domain. Starting with a synthetic peptide corresponding to the E2 cytoplasmic tail, we found that all three steps of this antibody network—the idiotype, the anti-idiotype and the anti-anti-idiotype—could be produced as monoclonal antibodies by immunization *in vitro*. This showed that there is a specific interaction between the cytoplasmic domain of the E2 glycoprotein and the cytoplasmic nucleocapsid.

Polyclonal idiotype response production

As the SFV E2 glycoprotein is the only spike protein component with a significant cytoplasmic domain, we synthesized a peptide corresponding to its 31-residue cytoplasmic tail (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). To demonstrate its immunogenicity in the BALB/c mouse, conventional polyclonal antibodies were prepared first by repeatedly immunizing BALB/c mice with 25 μg of the unconjugated peptide. The resulting antisera reacted specifically with the peptide as well as the intact E2 spike glycoprotein and its uncleaved precursor (p62) by immunoprecipitation (Fig. 1). The antisera were also weakly positive by indirect immunofluorescence (not shown).

To generate idiotype network antibodies, we used paired immunizations performed *in vitro*. Although anti-idiotype antibodies have been produced by conventional immunization procedures *in vivo*[11], *in vitro* immunization offers several advantages. The antigen is delivered directly to the immune cells, reducing the risk of antigen sequestration or proteolysis. The response to antigen is a primary one, resulting in immunoglobulin (Ig) M production, and occurs before antigen-specific T suppression[12,13]. The antigen should therefore elicit responses from every relevant member of the B-cell repertoire, unaffected by the T-cell related tolerance effects often seen *in vivo*. Thus, an *in vitro* polyclonal response to an antigen should be an optimal source of the most complete 'library' of idiotype antibodies against *all* of the immunogenic epitopes of a given antigen. A further advantage is that syngeneic, second-round immunizations to generate anti-idiotype antibodies can be performed using the crude supernatant from the first *in vitro* immunization as antigen. This eliminates the need for identification and purification of a relevant monospecific idiotype before anti-idiotype production.

Unconjugated E2 peptide was used for immunization *in vitro* and, at the end of the five-day immunization period, immunofluorescence on SFV-injected cells was used to show that the culture supernatant was weakly positive. To confirm that anti-peptide antibodies were indeed produced, non-adherent cells were then used to generate hybridomas that were screened by immunofluorescence and an enzyme-linked immunosorbent assay (ELISA) against the starting peptide. Several positive clones were identified and a stable cell line secreting an anti-E2 IgM was isolated.

Monoclonal anti-idiotype preparation

To produce anti-E2 peptide anti-idiotypes, the complete culture supernatant from the first round immunization was concentrated, dialysed to remove any remaining peptide (determined using ^{125}I-labelled peptide) and added directly to a second culture of naive, syngeneic spleen cells. After five days, non-adherent cells were collected and fused with the SP-2 myeloma cell line. Wells containing hybridomas were first screened for reactivity by indirect immunofluorescence on uninfected and SFV-infected baby hamster kidney (BHK) cells (see below). Of the 120 wells initially plated after the fusion, 110 contained viable hybridomas and five exhibited virus-specific reactivity; each of these yielded stable, IgM-secreting hybridoma cell lines after repeated cloning in agarose. The results described below were obtained with the antibody secreted by a hybridoma line designated F13.

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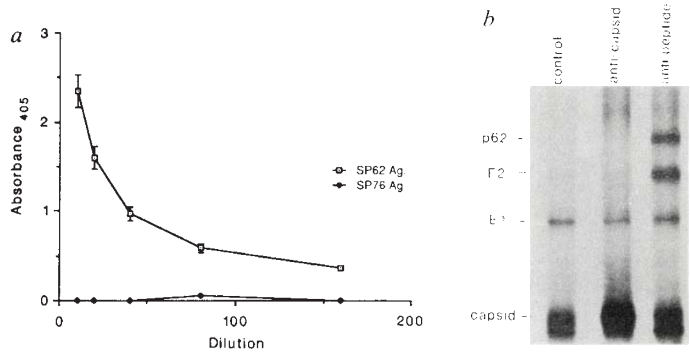


Fig. 1 Reactivity of anti-cytoplasmic domain antibodies. *a*, ELISA of mouse anti-E2 cytoplasmic domain antiserum on the synthetic E2 tail peptide (open squares) and on a control synthetic peptide (closed squares). *b*, Immunoprecipitation of capsid, E2 and p62 from metabolically [^{35}S]methionine-labelled lysates of SFV-infected BHK-21 cells.

Methods. Synthetic peptides were purified by preparative HPLC and their identity confirmed by amino-acid analysis. For ELISA, solid-phase substrates were prepared by drying 0.5 μg peptide in distilled water on 96-well plates (Nunc Immulon) overnight at 42 °C. Other substrates were plated at 50 μl per well of 10–100 μg ml^{-1} protein in a 0.1 M sodium carbonate buffer at *p*H 8.5 and incubated overnight at 4 °C. Nonspecific sites were blocked by pre-incubation with PBS (Ca^{2+}/Mg^{2+}-free) containing 10% normal goat serum for 2 h at room temperature. Wells were washed with PBS and incubated with the first antibody diluted in the blocking solution (50 μl per well) for 2 h at room temperature. The plates were washed in five changes of PBS over 30 min and then re-blocked for 30 min. Alkaline phosphatase-conjugated, affinity-purified goat second antibodies (Zymed) were then added at 50 μl per well at dilutions from 1:300 to 1:800 in PBS containing 10% goat serum. After 2 h, the plates were washed and then incubated with phosphatase substrate (disodium p-nitrophenyl phosphate; Sigma) in 50 mM sodium glycinate *p*H 8.7 containing 4 mM $MgCl_2$ at room temperature. Plates were read in an automatic plate scanner after 2 h. The result of a representative experiment is shown as means of triplicate determinations with error bars to plus and minus 1 standard deviation. Control peptides included a 22-residue synthetic peptide corresponding to part of the LDL receptor cytoplasmic domain (NH_2-K-N-W-R-L-K-N-I-N-S-I-N-F-D-N-P-V-Y-Q-K-T-T-COOH; closed squares) and two water-soluble synthetic peptides corresponding to parts of the ectodomain of the human Fc receptor (ref. 30; data not shown). Specific polyclonal antisera raised against the control peptides were used as positive controls in these control experiments to check adequate peptide retention by the ELISA plates. For the immunoprecipitation experiments, BHK-21 cells were plated to 70% confluence in 100-mm tissue culture dishes and infected the following day with SFV at a multiplicity of infection (MOI) of 500. After 2 h at 37 °C, the plates were washed twice in RPMI and incubated for 30 min at 37 °C in methionine-free medium containing 2 μg ml^{-1} actinomycin D. This medium was then removed and replaced with 5 ml of fresh medium containing 0.5 mCi [^{35}S]methionine. The plates were incubated at 37 °C for 150 min, washed in PBS, and collected by scraping in 4 ml per dish of cold homogenization buffer (PBS with 300 mM NaCl, 2 mM EDTA, 20 mM NaN_3, 1 mM phenylmethylsulphonylfluoride, 0.22 U ml^{-1} aprotinin and 200 μg ml^{-1} bovine serum albumin (BSA), *p*H 8.2). The cells were homogenized by eight strokes of a stainless steel Dounce homogenizer on ice and centrifuged in a Beckman microfuge at 4 °C for 5 min. The postnuclear homogenate was stored at −70 °C until used, then thawed and brought to 1% with NP-40. The lysate was cleared by a 2 h incubation with 5% (w/v) washed fixed *Staphylococcus aureus* on a microshaker at 4 °C and microfuged for 10 min. Aliquots (50 μl) were incubated with 3–10 μl of mouse or rabbit polyclonal antisera at 4 °C in an Eppendorf microshaker. After 2 h, 20 μg of a goat anti-mouse IgG was added to precipitations involving murine first antibodies. After another 2 h, 50 μl of a 10% (w/v) suspension of *Staph. aureus* was added to all samples. Incubation was continued for 2 h and the immunoadsorbant was washed three times in 0.6 M NaCl, 10 mM Tris-HCl *p*H 7.2 at 4 °C. Finally, the pellets were washed once in distilled water and resuspended in sample buffer containing 5 μg cold SFV per sample, heated to 95 °C for 5 min and run unreduced on 10% polyacrylamide gels. Gels were stained with Coomassie blue, neutralized in PBS, and incubated in salicylate (Chamberlain, 1979) before drying and autoradiography (from 12 h to 5 days). Under these conditions, minor nonspecific precipitations of E1 and capsid protein are seen in all lanes, whereas only the anti-cytoplasmic domain antibody precipitates any E2 or p62.

As shown in Fig. 2, F13 IgM exhibited a punctate immunofluorescence pattern throughout the cytoplasm of SFV-infected BHK-21 cells (Fig. 2*b*). The pattern was distinct from that obtained with a rabbit anti-SFV spike glycoprotein antiserum which had a characteristic staining pattern of the endoplasmic reticulum and Golgi region on methanol-fixed and permeabilized cells (Fig. 2*a*). It was, however, similar to the distribution of nucleocapsids visualized by a rabbit polyclonal anti-capsid antiserum (Fig. 2*c*). The F13 staining was known to be due to virus infection, as no staining was observed on mock-infected cells (Fig. 2*d*) or an SFV-infected cells not permeabilized before antibody addition, indicating that the antigen was intracellular.

The F13 antigen

For biochemical identification of the antigen, the F13 antibody was used for immunoprecipitation from [^{35}S]methionine-labelled, SFV-infected cells. In preliminary experiments, the use of ELISA showed that the antigen was sensitive to low concentrations of non-ionic detergent (for example 0.1% Triton X-100, Triton X-114, β-D-octylglucoside, CHAPS, Tween 20). Therefore, immunoprecipitation was performed by first allowing the antibody to bind to the antigen in crude post-nuclear supernatants before detergent solubilization for the immunoadsorption and washing steps. As shown in Fig. 3, F13 IgM precipitated the capsid protein; no other viral or host cell proteins were immunoprecipitated. Although there was some nonspecific sticking, insignificant amounts of capsid were precipitated by control monoclonal IgMs. This background precipitation was not concentration-dependent and occurred if the control IgM was omitted entirely (Fig. 3). F13 was also found by ELISA not to react with the E2 cytoplasmic domain peptide (see below).

These results indicated that F13 recognized a detergent-sensitive epitope on the SFV nucleocapsid and did not recognize a host cell antigen or the starting synthetic peptide. Thus, it was likely that F13 arose as an internal image anti-idiotype against the combining site of an antibody against the cytoplasmic domain peptide.

Anti-anti-idiotype production

The formal possibility remained that the F13 hybridoma represented the immortalization of an irrelevant B-cell specificity from the naive BALB/c spleen which happened to react with an epitope on the SFV capsid. To exclude this, we used F13 cells as antigen for preparation of anti-anti-idiotype monoclonals. *In vivo* immunization was used to obtain IgG antibodies to screen for binding to F13 IgM using γ-chain-specific second reagents.

Syngeneic BALB/c mice were immunized and boosted intravenously once with washed, glutaraldehyde-fixed F13 cells. They received an intravenous boost of purified F13 IgM four days before fusion. The resulting hybridoma supernatants were tested for their ability to recognize the starting F13 IgM and the E2 cytoplasmic domain peptide. Results obtained for the 16 highest-binding supernatants are shown in Fig. 4*a*. There were two classes of positive reactivity. Well 14 is an example of the first class: strong binding to F13 IgM (Fig. 4*a*, top) but no reactivity to the starting cytoplasmic domain peptide (Fig. 4*a*, bottom). This pattern of reactivity was consistent with 'framework' anti-idiotypes[11]: antibodies which detect novel epitopes on the immunizing antibody outside the antigen combining site.

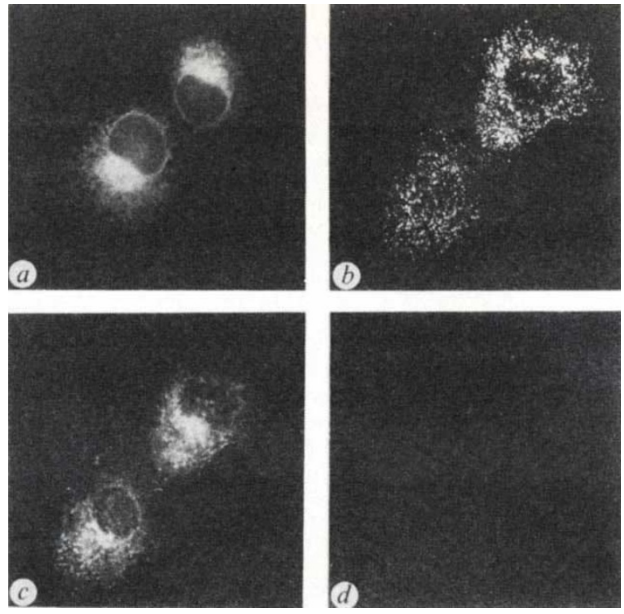


Fig. 2 Intracellular localization of viral proteins in infected cells. SFV-infected (*a*, *b* and *c*) or mock-infected (*d*) BHK-21 cells were fixed, permeabilized and labelled with a rabbit polyclonal antibody to SFV spike glycoproteins (*a*), the monocloncal anti-idiotype antibody F13 IgM (panels *b* and *d*), or a rabbit polyclonal antibody against isolated SFV nucleocapsids (panel *c*). No F13 reactivity was seen if infected cells were stained without permeabilization (unfixed cells at 4 °C or cells fixed with 3% (w/v) paraformaldehyde without subsequent methanol treatment). The apparent lack of cell surface staining in *a* reflects the fixation used, because methanol decreases the amount of spike protein surface fluorescence. *b* and *c* were obtained by double immunofluorescence and represent the same field exposed for fluorescein and rhodamine, respectively.

Methods. To prepare thymus-conditioned medium (TCM), single cell suspensions from the thymuses of three-week-old BALB/c and C57/B1 mice were co-cultured at a density of 2×10^6 thymocytes of each strain per ml in a medium consisting of alpha-modified Eagle's medium (MEM) with 50 μM 2-mercaptoethanol and 2% type-100 rabbit serum (Diagnostic Biochemistry. After 48 h, the supernatant was clarified by centrifugation and 0.22 μm filtered before storage at −20 °C. The TCM retained the ability to support *in vitro* immunization for at least six months when prepared in this way. *In vitro* immunizations were performed by a modification[30] of the method of Borrebaeck and Moller[31]. Briefly, the spleens were dissected from two 12-week-old BALB/c mice and a single-cell suspension was prepared by teasing the organs apart with two 18-gauge needles. The cells were washed twice in alpha-MEM containing 50 μM 2-mercaptoethanol and resuspended to a final volume of 30 ml in 50% TCM and 50% normal lymphocyte medium (NLM) (alpha-MEM, 2 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 1× Gibco non-essential amino acids, 10 mM HEPES *p*H 7.2 and 20% heat inactivated fetal calf serum (JR Scientific). The cell density was approximately 1×10^7 nucleated cells per ml. 10 μg of sterile-filtered E2 cytoplasmic domain peptide in 0.1 ml was then added and the mixture cultured in a T-75 flask at 37 °C in 5% CO_2 without disturbance for five days. Non-adherent cells were then released by brick shaking and collected by centrifugation at 400*g* for 5 min. The cells were used for hybridoma production by fusion with SP2/0-Ag14 myeloma cells (refs 32, 33 and Fig. 4 legend). The supernatant from this *in vitro* immunization was concentrated 15-fold by dialysis against solid polyethylene glycol (M_r, 20,000), dialysed extensively against PBS and used as the antigen for an identical repeat *in vitro* immunization. The non-adherent cells from this second immunization were fused with SP2 myeloma cells and the resulting hybridomas screened by indirect immunofluorescence. BHK-21 cells were plated at 80% confluence on multi-well slides and infected at an MOI of 10–100 with SFV in RPMI supplemented with 1 mg ml^{-1} BSA and 10 mM HEPES (*p*H 6.8) for 2 h, washed and incubated in growth medium for 3 h. Mock-infected cells were incubated in the same buffer without virus. The cells were washed with PBS and fixed with methanol for 6 min at −20 °C. Nonspecific sites were blocked with PBS containing 10% goat serum for 30 min and the wells were incubated with first antibody for 1 h. For double-label immunofluorescence, both first antibodies were applied together (20 μg ml^{-1} for purified F13 IgM). The slides were washed and reblocked for 30 min. Fluorochrome-conjugated second antibodies (Zymed) diluted 1:40 in PBS with 10% goat serum were then added for 1 h. After washing in PBS and water, the slides were mounted in Moviol and photographed with Tri-X Pan on a Zeiss Photomicroscope III equipped with epifluorescence illumination.

In contrast, wells 3, 6 and 9 contained antibodies with strong reactivity to both purified F13 IgM (Fig. 4*a*, top) and cytoplasmic domain peptide (Fig. 4*a*, bottom). None of these antibodies gave signals above background when assayed by ELISA on bovine servum albumin (BSA) or a range of other water-soluble peptides of approximately similar size and composition, including a peptide corresponding to the cytoplasmic domain of the low-density lipoprotein receptor (data not shown). This pattern of reactivity was consistent with 'internal image' anti-idiotypes[11]. A representative IgG-secreting hybridoma was cloned (well 9) and designated 3G10. Purified 3G10 IgG bound to the cytoplasmic domain peptide (Fig. 4*b*, bottom). As expected, the anti-idiotype F13 IgM with anti-capsid reactivity did not bind to the E2 tail peptide (Fig. 4*b*, middle).

The anti-anti-idiotype 3G10 IgG not only recognized the synthetic peptide by ELISA, but also labelled permeabilized SFV-infected BHK-21 cells. Although its distribution was less extensive than that of the polyclonal antisera to the spike glycoprotein complex (Fig. 2*c*), it was similar to the weaker pattern observed using the murine anti-peptide antisera (not shown) or a rabbit polyclonal antiserum against the same synthetic peptide[14].

An immunofluorescence assay has been used to confirm that, as an internal image anti-idiotype to F13 IgM, 3G10 IgG also recognizes the antigen combining site on F13 IgM and competes for binding to the SFV nucleocapsid. When F13 IgM was preincubated with excess goat serum (Fig. 5*a*) or monoclonal mouse IgG against an irrelevant epitope (Fig. 5*b*), the normal F13 pattern and intensity was observed. However, if F13 was preincubated with purified 3G10 IgG, there was a marked loss of the usual F13 staining pattern when this mixture was used to stain SFV-infected cells (Fig. 5*c*).

Structures bearing the F13 epitope

The F13 determinant reflects a functional domain on the nucleocapsid involved in its interaction with viral spike glycoproteins and thus the intracellular distribution of capsids being the F13 determinant may be related to the mechanism of virus assembly. Nucleocapsids are overproduced during alphavirus infections with only a fraction of the most recently synthesized capsids being incorporated into new virions[15]; thus, it seems likely that the F13-positive nucleocapsids include the functionally active subset. The relationship between the F13-positive capsids and the total capsid population was examined by double-label immunofluorescence. Five hours after infection, BHK-21 cells were stained with F13 IgM (detected using fluorescein isothiocyanate-(FITC)-conjugated second antibody) and a rabbit anti-capsid antiserum (visualized using tetramethylrhodamine isothiocyanate (TRITC)-conjugated second antibody). Superimposition of the two images clearly showed that almost all of the F13-positive structures (green) were also stained by the anti-capsid antibodies (red) and thus appeared as yellow spots (Fig. 6). As expected, a majority of the structures were reactive only with the anti-capsid antibody, reflecting the accumulating pool of presumably aberrant nucleocapsids which do not form virions.

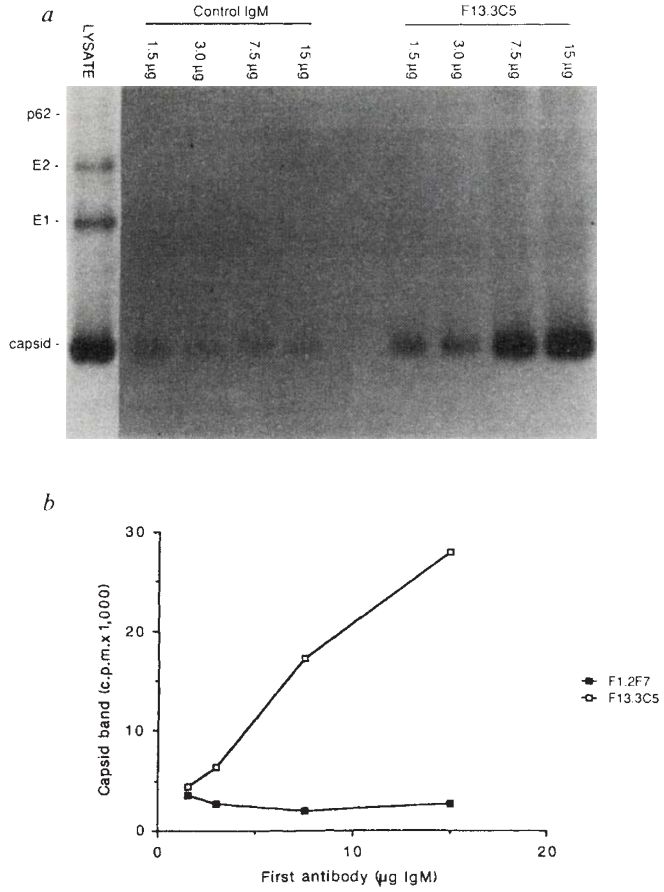


Fig. 3 F13 IgM specifically immunoprecipitates the SFV capsid protein. *a*, Autoradiograph of immunoprecipitation from metabolically ^{35}S-labelled, SFV-infected, BHK-21 cell homogenates using increasing amounts of first antibody. *b*, Quantitation of F13 capsid immunoprecipitation.

Methods. Radiolabelled homogenates (prepared as in Fig. 1 legend) were incubated in aliquots of 50 µl. With a range of identical doses of an irrelevant mouse IgM monoclonal antibody (*a*, left) or F13 IgM (*a*, right; clone F13.3C5). All incubations were at 4 °C on an Eppendorf microshaker. After 2 h to allow antibody–antigen interaction to occur, NP-40 was added to a final concentration of 1% and the lysates were cleared with fixed *Staph. aureus*. The cleared lysates containing pre-bound first antibody were incubated for 2 h with 20 µg per sample of goat anti-mouse IgG or IgM as appropriate and then treated exactly the same as immunoprecipitations from lysates (see Fig. 1 legend). F13 recognizes the capsid protein in a dose-dependent way and does not recognize any other cellular or viral protein (labelled SFV structural proteins are shown in the lane at left). Labelled capsid bands from the gel autoradiographed in *a* were excised and counted by liquid scintillation spectroscopy. Background radioactivity, determined using gel strips of the same size from empty lanes, contained less than 100 c.p.m.

Discussion

We have used internal image anti-idiotype antibodies to show that there is a specific receptor–ligand-like interaction between the nucleocapsid and the cytoplasmic domain of the E2 spike glycoprotein of Semliki Forest virus. This interaction, originally predicted by Simons and Garoff[7,8], is likely to be critical in the organization of the budding of SFV and related viruses from infected cells. Our results support a mechanism whereby newly formed nucleocapsids attach to the cytoplasmic face of the plasma membrane by recognizing the E2 endodomain and recruit additional spike glycoprotein complexes into the nascent bud. Host cell membrane proteins, whose cytoplasmic domains lack the determinant involved in nucleocapsid binding, would not be recruited; in fact, they might be sterically *excluded* as a result of the close association of the spike glycoprotein trimers[2,10]. The spike–nucleocapsid interaction is likely to reflect the mechanism of virus budding, but its possible role in other functions—such as the stability or infectivity of the virus particle—cannot be excluded.

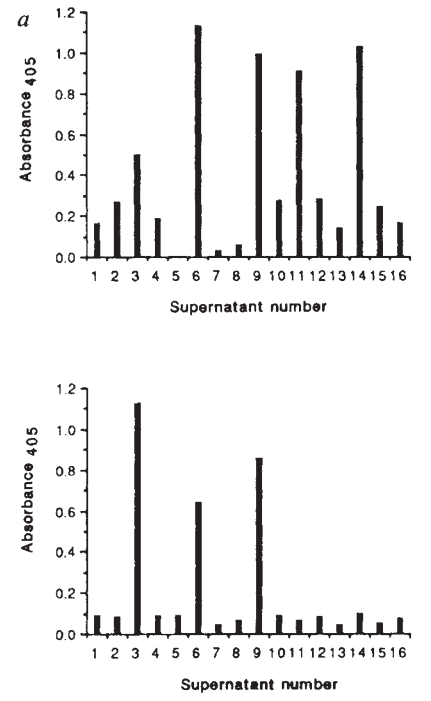
These results also demonstrate that it is possible to use internal image anti-idiotypic antibodies to reconstruct interactions between membrane protein 'ligands' and potential cytoplasmic 'receptors' which may specify membrane traffic events. Moreover, the development of a method to produce monoclonal anti-idiotypes by *in vitro* immunization provides a rapid and practical new approach to a wide variety of problems related to protein interactions and intracellular recognition.

Production of monoclonal anti-idiotypes *in vitro*. Although the SFV E2 cytoplasmic domain only contains 31 amino acids, it is long enough to have a number of distinct antigenic epitopes, only one of which can be the site for capsid binding. No assay exists for the identification of the relevant epitope. The generation of an idiotype library of anti-peptide antibodies which was assumed to contain antibodies against the relevant epitope overcame this problem. The library was then used as the antigen for a second *in vitro* immunization, resulting in the production of monoclonal anti-idiotype antibodies (for example F13 IgM), the functionally relevant determinant. Critical to the process, however, was the confirmation that F13 IgM represented an authentic internal image anti-idiotype by isolating monoclonal anti-F13 antibodies and showing that a subset of these were specifically reactive with both F13 IgM and the starting cytoplasmic domain peptide. These monoclonal antibodies were thus 'anti-anti-idiotype' with respect to the starting peptide and could only have arisen if F13 was a true internal image anti-idiotype. Additionally, these internal image anti-anti-idiotypes (for example 3G10 IgG) must recognize the specific epitope within the peptide that interacts with the nucleocapsid. The final result is monoclonal antibodies against the active sites of both the nucleocapsid 'receptor' (F13 IgM) and the E2 tail 'ligand' (3G10 IgG).

Although the SFV spike is a heterotrimer containing three copies of the E2 glycoprotein, F13 IgM was produced starting with a synthetic E2 tail peptide which is not likely to have any oligomeric organization. Thus, the relevant signal specifying E2 tail–capsid interaction must be present on the synthetic peptide in aqueous solution. This implies that the trimeric state of the E2 tail in the spike glycoprotein complex cannot be essential for expression of this signal, although it is possible that positive cooperativity between three sites enhances its effectiveness *in vivo*.

Conservation of the spike–nucleocapsid signal. As the E2–nucleocapsid interaction probably reflects a functionally important recognition step in virus budding, we expected that the F13 determinant might be conserved on the capsids of alphaviruses other than SFV. Indeed, strong labelling was seen in BHK-21 cells infected with each of 14 different alphaviruses tested (including Sindbis, Ross river, Eastern equine encephalitis, Whataroa and O'Nyong Nyong viruses; D.J.T.V. *et al.*, manuscript in preparation). This reactivity was also observed in cells infected with viruses from the related flavivirus group (Japanese encephalitis, West Nile, Tembusu and Banzi viruses). Thus, the nucleocapsid determinant recognized by F13 IgM seems to be conserved through virus evolution. However, the structural organization and budding of flaviviruses is more complex (and less well understood) than alphaviruses. At least some flaviviruses have a matrix protein intercalated between the nucleocapsid and spike-containing lipid envelope[16]. It is not known whether the site recognized by F13 IgM in flaviviruses interacts with the spike glycoprotein itself or with an intervening matrix protein.

Although the extent of the conservation of the F13 epitope is not yet known, it is not a pan-viral structure because it is not expressed in cells infected with influenza virus (an



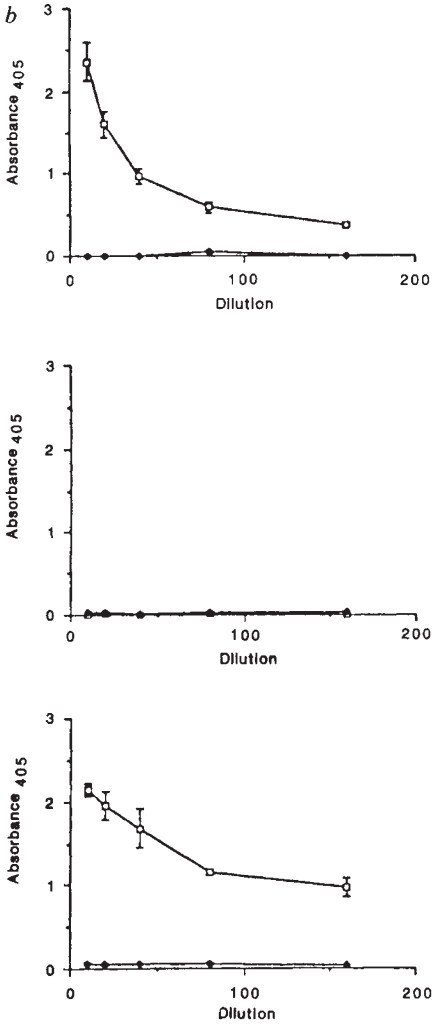


Fig. 4 Identification of anti-F13 anti-idiotype monoclonal antibodies. *a*, Screening for anti-F13 anti-idiotype reactivity. Supernatants from hybridoma-containing wells were assayed by ELISA using purified F13 IgM (top) and synthetic E2 cytoplasmic domain peptide (bottom) as solid-phase antigen. Wells 3, 6 and 9 contained antibodies which reacted with both the antigen (F13 IgM) and the starting peptide whereas others (wells 11 and 14) contained antibodies that recognized only F13 IgM. The positive control (mouse anti-peptide antiserum at 1:200 dilution) had a mean absorbance of 2.5 and the blank (medium only) had a mean absorbance of 0.07. *b*, Reactivity of the anti-peptide idiotype, anti-idiotype and anti-anti-idiotype monoclonal antibodies. The reactivity of the anti-E2 cytoplasmic domain peptide mouse polyclonal antiserum (top), the monoclonal anti-peptide anti-idiotype F13 IgM (middle) and the monoclonal anti-anti-idiotype 3G10 IgG (bottom) against the starting E2 peptide was determined by solid-phase ELISA. Open squares indicate reactivity to the synthetic E2 cytoplasmic domain peptide and closed squares indicate reactivity to an irrelevant peptide (see Fig. 1 legend).
Methods. Hybridomas were produced from the spleens of mice hyper-immunized with purified F13 IgM and fixed F13 cells as described[33]. Feeder layers consisting of syngeneic mouse total peritoneal exudate cells at 1×10^5 per well in 0.3 ml NLM were established in 24-well plates. The fusion mixture was plated at 1×10^5 spleen input cell per well in 0.3 ml NLM containing 100 μM (1×) hypoxanthine and 2 μg ml^{-1} (1×) azaserine. After 24 h, the wells were fed with 0.3 ml × NLM containing 2× hypoxanthine and 2× azaserine and on subsequent days with NLM containing 1× hypoxanthine.

orthomyxovirus) or vesicular stomatitis virus (a rhabdovirus). These results are consistent with previous observations that pseudotypes may form between two alphaviruses[17] or between an alphavirus and a flavivirus[18] but not between an alphavirus nucleocapsid and vesicular stomatitis virus glycoprotein G[19]. The mechanism of alphavirus budding, however, is not directly applicable to all enveloped viruses. Retroviruses such as Rous sarcoma virus, whose budding mechanism is poorly understood and probably very different from the alphaviruses, are able to produce virions using mutant forms of the *env* spike glycoprotein that lack a cytoplasmic tail[20]. Presumably other proteins (such as *gag*) participate in interactions which organize newly formed viral components during budding.

Conventional antibodies to nucleocapsids of alphaviruses frequently show little, if any, cross-reactivity. For example, the rabbit antiserum against SFV capsid protein did not recognize the capsids of any of the other alphaviruses tested. In contrast, the broad cross-reactivity of F13 IgM probably results from its specificity for an epitope in the spike binding site. Functionally important sites on proteins are often highly conserved and are therefore good targets for cross-reactive antibodies. They are consequently good candidates as vaccine or diagnostic antigens. The anti-idiotypic route allows selection of antibodies to functional sites on proteins against which it may be difficult to obtain a strong immune response by conventional immunization. The ability of internal image anti-idiotype antibodies to identify such sites within viruses makes them potentially powerful tools in vaccine development.

Use of internal image anti-idiotype antibodies. Mimicry of receptor structure by anti-ligand anti-idiotypes was first demonstrated by Sege and Peterson using retinol binding protein as the ligand and human pre-albumin as the receptor[21]. Other groups have used anti-ligand anti-idiotype antibodies to probe receptor–ligand interactions at the cell surface. These include identification of the mammalian reovirus type 3 receptor[22,23], generation of antibodies to the acetylcholine receptor[24], insulin receptor[25], thyroid stimulating hormone (TSH) receptor[26], lymphocyte retroviral binding site[27] and the vasopressin receptor in rat brain[28]. An anti-idiotype approach using polyclonal rabbit antisera has also been used successfully to identify a receptor apparently involved in protein import into chloroplasts[29].

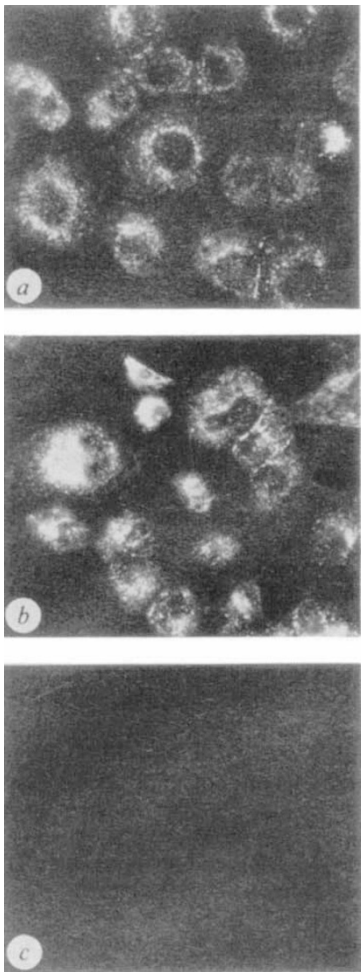


Fig. 5 Anti-anti-idiotype 3G10 competes with antigen for F13 binding. F13 IgM at 1 μg ml^{-1} was pre-incubated with 100 μg ml^{-1} of an irrelevant monoclonal mouse IgG (*b*) or 100 μg ml^{-1} 3G10 IgG (*c*) for 2 h and then used to stain methanol-fixed and permeabilized SFV infected BHK-21 cells. The pattern and intensity of labelling in *b* are indistinguishable from that in control wells stained with 1 μg ml^{-1} F13 without pre-incubation (*a*). Pre-incubation of F13 with 3G10 results in a marked loss of labelling (*c*). All three panels received identical exposures both during initial photography and subsequent printing.

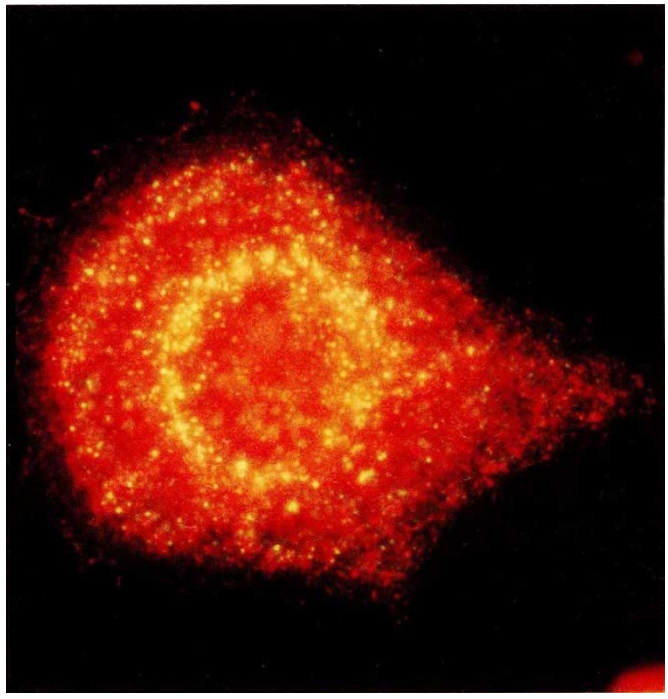


Fig. 6 Anti-idiotype F13 detects a subset of nucleocapsids in SFV-infected BHK-21 cells. BHK-21 cells were infected for 5 h with SFV and then fixed and permeabilized with methanol. Double-label indirect immunofluorescence was then carried out using a polyclonal rabbit anti-nucleocapsid antiserum (rhodamine label) and the anti-idiotype F13 IgM (fluorescein label). Cells were photographed by double exposure of single frames of colour slide film to assess the degree of co-localization of rhodamine- and fluorescein-positive structures. Structures staining yellow represent co-localization of both fluorochromes; some images were photographed as double exposures fractionally our of register to confirm the presence of both labels (not shown). Background levels for all antibody combinations were as shown for mock-infected cells (Fig. 2*d*), and there was no detectable cross-reactivity between second antibodies. Almost all fluorescein-staining F13 IgM-positive structures were contained within the more extensive population of structures labelling with the anti-capsid antibody. The few green fluorescein-staining structures probably resulted from out-of-register localization due to differences in optical properties associated with detecting fluorescein and rhodamine fluorescence.

Previous studies involving anti-idiotype reagents to examine receptor–ligand-like interactions have required the identification and isolation of a single relevant idiotype before anti-idiotype production which is time consuming. Our approach does not require the identification or purification of ligand or receptor, nor does it require that a functionally relevant single idiotype be identified; it also can produce useful anti-idiotype reagents less than three weeks. In addition to the anti-viral antibodies described, we have used the approach to produce idiotype and potential anti-idiotype antibodies beginning with complex cellular organelles (for example lysosomes) as antigen, as well as with endogenous cellular proteins and derived synthetic peptides. By definition, internal image anti-idiotypes should recognize sites involved in intermolucular recognition events and so these antibodies should interfere with a functional interaction between the antigen ('receptor') and its presumptive 'ligand', be it another protein, organelle or viral component. Experiments are in progress to determine if the anti-idiotype F13 and the anti-anti-idiotype 3G10 interfere sufficiently with the SFV spike–capsid interaction to disrupt viral assembly and budding.

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A peptide model of a protein folding intermediate

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It is difficult to determine the structures of protein folding intermediates because folding is a highly cooperative process. A disulphide-bonded peptide pair, designed to mimic the first crucial intermediate in the folding of bovine pancreatic trypsin inhibitor, contains secondary and tertiary structure similar to that found in the native protein. Peptide models like this circumvent the problem of cooperativity and permit characterization of structures of folding intermediates.

ONE of the basic goals of protein folding studies is to determine the structures of folding intermediates, ultimately at atomic resolution, and to understand the thermodynamic basis of their formation. It is difficult to obtain folding intermediates of single-domain proteins at equilibrium because the cooperativity of folding is high. Folding intermediates can be populated kinetically but they are short-lived, so detailed structural analyses (using nuclear magnetic resonance spectroscopy or X-ray crystallography for example) are difficult. Amide proton labelling methods have provided insights into the structures of kinetic folding intermediates[1,2], but a high-resolution structure has not yet been determined for any folding intermediate of a single-domain protein.

The most well characterized protein folding pathway is described in terms of disulphide-bond formation in the oxidative refolding of bovine pancreatic trypsin inhibitor[3,4] (BPTI). Reduction of the three disulphide bonds in native BPTI unfolds the protein in the absence of denaturants. The pathway of disulphide-bond formation in the refolding of reduced BPTI has been determined by Creighton (for review, see ref. 5). The term 'pathway' has led to some confusion and we use it here in the same sense as Creighton: a pathway describes the kinetically most accessible routes for folding. Other routes for folding are possible but are not depicted because they are less probable.

A simplified version of the pathway for folding of BPTI (ref. 3) is shown in Fig. 1*a*. A crucial intermediate in this pathway contains a single disulphide bond between residues 30 and 51 (ref. 6) and is called [30–51]. Because disulphide-bond formation and protein folding are thermodynamically linked processes, the conformations that favour particular disulphide bonds in folding are stabilized by those disulphides[3]. Thus, structural characterization of intermediates trapped by blocking the remaining thiols may allow one to understand the pathway in structural terms, provided that the blocking groups do not alter the conformations of these intermediates. Unfortunately, solubility limitations and the presence of substantial unfolded regions have hindered detailed structural characterization of trapped BPTI folding intermediates. Nevertheless, several trapped intermediates have been surveyed using NMR[7]: spectra of the intermediates were found to resemble spectra of BPTI (ref. 8) more closely as the number of native disulphide bonds in the intermediates increased. A comparison of the resonance pattern in the NMR spectrum of [30–51] with that of native BPTI suggested[7] that at low temperatures [30–51] contains part of the central β-sheet found in BPTI.

We have designed and synthesized a small (30 residues) synthetic analogue of [30–51]. The analogue (called PαPβ) is a disulphide-bonded peptide pair in which two short peptides are connected by a disulphide bond corresponding to the 30–51 disulphide of BPTI (Fig. 1*b*). PαPβ is very soluble in aqueous solution and does not aggregate. As judged by circular dichroism (CD) and NMR, PαPβ is >90% folded in aqueous solution (pH 6) at 4 °C. The structure unfolds when the disulphide bond is reduced. Two-dimensional nuclear Overhauser effect spectroscopy (NOESY) indicates that PαPβ contains much of the secondary and tertiary structure present in the corresponding region of native BPTI. These results indicate that native-like structure can form early in protein folding, and that peptide models can be used to characterize the structures of transient folding intermediates. In future work, it may be possible to crystallize peptide models like PαPβ.

Design of a synthetic analogue of [30–51]

Creighton's pathway[3] for the folding of BPTI, also referred to as the rearrangement pathway, is shown in Fig. 1*a*. BPTI can refold into a native-like structure without the 30–51 disulphide[9], but the resultant species is a non-productive intermediate that must undergo unfavourable disulphide rearrangements to complete folding[4]. This non-productive intermediate [N(30SH, 51SH)] and minor species have been omitted from Fig. 1*a* (see Fig. 6 of ref. 4 for a more complete description). Although there are fifteen possible one-disulphide combinations, [30–51] accounts for 60% of the one-disulphide species observed. The importance of [30–51] is clear from the observation that all further intermediates in the pathway retain the 30–51 disulphide (Fig. 1*a*).

Of the four remaining cysteines in [30–51], residues 5, 14 and 38 participate in formation of the second disulphide bond with approximately equal probability. In contrast, Cys 55 does not participate to an appreciable extent[3,10]. None of the pre-