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Oligomers of the Cytoplasmic Domain of the p62/E2 Membrane Protein of Semliki Forest Virus Bind to the Nucleocapsid In Vitro

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We analyzed the interaction between the nucleocapsid and synthetic peptides corresponding to the complete or truncated cytoplasmic protein domain of the Semliki Forest virus p62/E2 glycoprotein. We found that the peptide corresponding to the full-length domain efficiently bound nucleocapsids when coupled to a solid matrix via specific antibodies, whereas the shorter one did not. In solution, a substantial fraction of the full-length peptide associated into oligomers. Binding studies showed that it was mostly these oligomers, rather than the monomeric form of the peptide, which were able to interact with the nucleocapsid. Thus, our findings demonstrate a direct interaction between the spike proteins and the viral nucleocapsid. Furthermore, they suggest that this interaction is directed through formation of complexes containing several p62 or E2 subunits.

Semliki Forest virus (SFV) particles mature through budding at the cell surface (20). During this process, the nucleocapsid (NC) interacts with regions of the plasma membrane which contain viral membrane proteins. Several kinds of experimental data suggest that this interaction occurs through the cytoplasmic domains or tails of transmembrane protein p62 or its mature form, E2 (19). This domain contains 31 amino acid residues (7) and has been shown to contain structural complementarity to the NC surface by means of anti-idiotypic and antianti-idiotypic antibodies (22). Furthermore, the E2 protein has been cross-linked via this domain to the NC in the virus particle (8). The other membrane protein of SFV, E1, also spans the membrane but has only two arginine residues on the internal side (7). Thus, this protein is less likely to interact directly with the NC. Its incorporation into the viral envelope during budding is evidently dependent on the fact that it forms a heterodimeric complex with the p62/E2 protein (24).

To find direct biochemical proof for the p62/E2-NC interaction, we analyzed the binding of synthetic peptides corresponding to the whole of the cytoplasmic tail of p62 (31 amino acid residues) or its N-terminal part (13 amino acid residues) to viral and cell-associated NCs. Our results show that the cytoplasmic domain interacts with the NC and furthermore suggest that effective binding requires oligomerization of the peptide.

MATERIALS AND METHODS

Synthetic peptides. The synthetic peptides used were HN_2-RSKCLTPYALTPGAAVPWTLGILCCAPRAHA-COOH (referred to as the long peptide) and NH_2-RSKCLTPYAL TPG-COOH (short peptide). They were purified by high-pressure liquid chromatography and analyzed for amino acid composition to verify their identities. The long peptide was labeled with ^{125}I by the Bolton-Hunter method (1). When the long peptide was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in the absence of a reducing agent, an extensive ladder of bands was observed. After the peptide was boiled (1 min) in 100 mM dithiothreitol in sample buffer for PAGE containing 2% SDS (13), most of the higher oligomers dissociated, suggesting oligomerization through intermolecular disulfide bridges. However, a substantial fraction of oligomeric structures, especially trimers, remained (see Fig. 5A). The short peptide behaved as a monomer, even in the absence of a reducing agent. We used 10^{-5} M dithiothreitol in all incubations with the long peptide to avoid intermolecular disuldide bridge formation, unless otherwise stated.

Production of peptide-specific antibodies. Antibodies to the long and short peptides were raised. Before immunization of rabbits, the peptides were coupled with benzoquinone to keyhole limpet hemocyanin (9). Immunization was done intradermally three times at 2-week intervals with 50 μg in Freund adjuvant. The antiserum against the long peptide recognized the peptide monomer, as well as oligomers, when analyzed by immunoblotting (17) (serum dilution, 1:100). It recognized the SFV E2 membrane protein as well. However, it did not recognize the short peptide. One possible interpretation of the latter result is that the antigenic determinants of the long peptide are localized within its 18 carboxy-terminal amino acids. The antiserum to the short peptide recognized the long peptide, as well as the short one, by immunoblotting (serum dilution, 1:50), but it did not recognize the SFV E2 protein. It also did not immunoprecipitate the E2 protein, as the antiserum to the long peptide did.

Coupling of peptides to solid supports. Peptides were immobilized on fixed *Staphylococcus aureus* cells (Pansorbin; Calbiochem-Behring, La Jolla, Calif.) via anti-peptide antibodies which bind to staphylococcal protein A. Pansorbin was first incubated with serum (2 ml/ml of packed cells) and then incubated with the peptide (50 mg/ml of packed cells). To evaluate the relative amount of bound peptide, the Pansorbin matrix was boiled in SDS-PAGE sample buffer and then subjected to electrophoresis on 15% polyacrylamide gels (13), followed by transfer to nitrocellulose. Ponceau S staining of the nitrocellulose sheet revealed that both the C-terminal and N-terminal antibodies bound comparable amounts of the long peptide. The N-terminal

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antibodies also efficiently coupled the short peptide to Pansorbin.

Isolation of viral NC. SFV was grown in BHK cells, and virions were purified by gradient centrifugation (10). Rubella virus was grown in Vero cells (14) and purified as was SFV. Viral NCs were isolated by solubilizing virions (100 μg/ml, with a [^{35}S]methionine-labeled virus marker) in TN buffer (0.5% Triton X-100 in 50 mM Tris buffer [pH 7.4] containing 100 mM NaCl), followed by centrifugation in a 5 to 20% sucrose gradient in TN buffer (Beckman SW40 rotor; 24 × 10^3 rpm, 90 min, 4°C). The fractions containing NC particles were used for assays with the peptides.

Binding assays. For binding of free peptide to NCs from viral particles, we used a 1:3 dilution of the NC preparation described above. Free peptide was added to the diluted NCs in 500 μl and incubated at 37°C for 15 min. Peptide-NC complexes were analyzed by first separating NCs from unbound peptide through centrifugation in a 5 to 20% sucrose gradient. Fractions containing NCs were then further analyzed for bound peptide by Western blotting (immunoblotting) using anti-peptide antibodies. A mixture of control peptides was made from [^{35}S]methionine-labeled vesicular stomatitis virus (VSV) particles. The VSV particles were trypsinized (1 mg/ml) for 30 min at 37°C. The reaction was stopped with soy bean trypsin inhibitor, and virions were pelleted. SDS-PAGE, followed by autoradiography of the supernatant, showed a broad band in the 2,000- to 3,000-dalton range and some higher-molecular-mass components too. The supernatant was used for binding assays with SFV NC.

For binding of NCs to Pansorbin-immobilized peptides, NCs were isolated from purified [^{35}S]methionine-labeled SFV. Binding to the solid support was quantified by scintillation counting after first pelleting the Pansorbin and then washing it twice with 1 ml of the incubation buffer.

To study the effect of peptide oligomerization on NC binding, we first separated the peptide forms by SDS-PAGE (15% acrylamide). These were then transferred onto nitrocellulose and assayed for NC binding by blotting. This was done with [^{35}S]methionine-labeled NC in phosphate-buffered saline containing 0.1% Triton X-100 and 1% fetal bovine serum (30 min, 37°C). The nitrocellulose was then washed twice with the same buffer and dried, followed by exposure to Fuji RX film for 3 days.

For assays with the cellular NC, confluent BHK cells (5-cm-diameter dish) were infected with SFV at a multiplicity of 5. The cells were then pulsed for 20 min with [^{35}S]methionine (50 μCi/ml) at 2 h postinfection. The early time point was chosen to label both cellular and viral proteins. Cells were chased for 20 min, washed, and lysed in 2 ml of 1% Triton X-100 in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) pH 7.0, containing 100 mM KCl, 40 mM NaCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Nuclei were spun down, and the postnuclear supernatant was used for assays with immobilized peptides. Further purification of the NCs from the lysate was considered unnecessary, as it has been shown that pulse-labeled capsid protein is chased into NC structures very efficiently (21). Incubation with immobilized peptides (5 μl of Pansorbin per assay) was for 60 min at 10°C in 100 μl of cell lysate. As a control, we used a VSV-infected cell lysate. This was prepared by using a VSV stock at a multiplicity of infection of 5 in the same way as SFV-infected cells.

Immunofluorescence of infected cells was done as described previously (24), except that ice-cold methaonol was used for fixation instead of paraformaldehyde (22). F13 antibody was a generous gift from David Vaux (European Molecular Biology Laboratory).

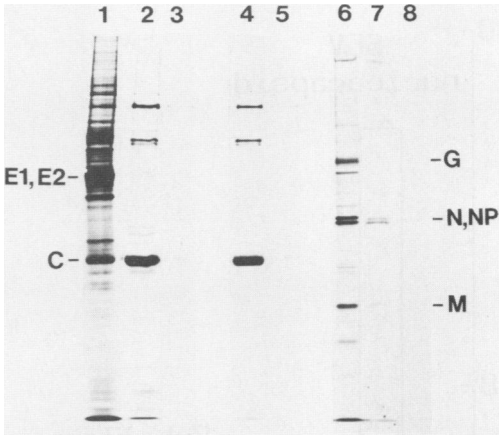

FIG. 1. Binding of NC particles of SFV-infected cell lysate to synthetic p62/E2 tail peptide coupled to a protein A matrix via specific antibodies. C- or N-terminal antibodies were used to couple the long-tail peptide to Pansorbin. The immobilized peptides were then incubated with a [^{35}S]methionine-labeled, postnuclear SFV-infected cell lysate (60 min at 10°C in a 100-μl volume; 5 μl of Pansorbin per assay) (lanes 2 to 5). After incubation, the Pansorbins were washed twice and subjected to SDS-PAGE, followed by fluorography. Lanes: 2, peptide coupled via C-terminal antibodies; 3, C-terminal antibody in Pansorbin without peptide; 4, peptide coupled via N-terminal antibodies; 5, N-terminal antibody without peptide. Lane 1 contained the infected postnuclear cell lysate (20 μl). A postnuclear VSV-infected cell lysate (lanes 6 to 8) was used as a control. Lane 7 contained the material bound to the C-terminal antibody-coupled peptide, while lane 8 contained material bound to a matrix with no peptide. Lane 6 contained the VSV-infected cell lysate (20 μl).

RESULTS

Immobilized long peptide binds NC. In our first set of experiments, we used antipeptide antibodies to fix the p62/E2 tail peptides to Pansorbin before testing for NC binding. In this way, the peptides were presented to the NCs as a layer with multiple interacting sites. This might mimic the condition during natural budding at the plasma membrane of infected cells. NC particles from infected cell lysate bound very efficiently to the long peptide when this had been bridged to the matrix via antibodies directed to its C- and N-terminal portions (Fig. 1, lanes 2 and 4). About 30% of the NCs in the lysate were bound under these conditions (100 μl of lysate and 5 μl of Pansorbin-bound peptide), and almost all could be bound by using more matrix-bound peptide. Several host proteins were still clearly visible in the lysate of the cells, which were pulse-labeled at a very early time point (2 h) postinfection (lane 1). These were not bound to the long peptide matrix (lanes 2 and 4). This was also true for host proteins pulse-labeled in mock-infected cells (data not shown). The material in the two bands of slower mobility in lanes 2 and 4 was not identified, but we know that it is specific for SFV-infected cells. The apparent molecular weight of the material corresponds well to those of the C protein dimers and trimers sometimes seen when analyzing SFV NCs by SDS-PAGE (H. Garoff, unpublished data). Thus, we conclude that the long peptide matrix specifically bound NC particles from an SFV-infected cell lysate. In

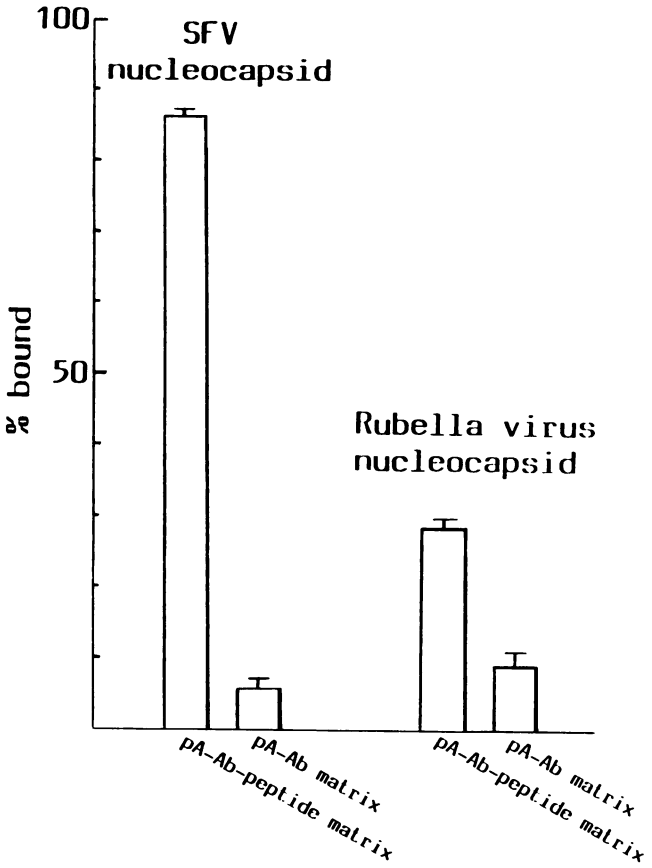

FIG. 2. Binding of NC particles of the virus particle to the synthetic p62/E2 tail peptide coupled to a protein A (pA) matrix via specific antibodies (Ab). [^{35}S]methionine-labeled NC (1.5 μg/ml) isolated from virions was incubated with the long-tail peptide coupled to Pansorbin via C-terminal antibodies. Incubation was in 500 μl with 1 μl of packed Pansorbin cells. Pansorbin with the coupled peptide (pA-Ab-peptide matrix) bound SFV NCs, while an identical matrix without the peptide (pA-Ab matrix) showed negligible binding. Rubella virus NCs (about 0.75 μg) tested similarly bound less efficiently to the peptide matrix than those of SFV.

preliminary tests aimed at characterization of this binding, we found that it was resistant to a 0.5 M NaCl wash but was abolished at higher salt concentrations.

The long peptide matrix also bound NC particles isolated from SFV virions. Figure 2 shows that almost all of the NC particles (0.75 μg) were bound to 1 μl of matrix-bound peptide, while almost no binding to a matrix without peptide was observed. Binding of viral NC to the Pansorbin-bound long peptide was lowered significantly by the presence of high concentrations of the soluble long peptide (Fig. 3). This suggests that specific peptide-binding sites exist on the NC surface. This is also supported by the fact that the long peptide was able to compete for NC binding with antiidiotypic tail peptide antibody F13 (22). This was shown by peptide concentration-dependent suppression of F13 antibody staining of infected cells. Suppression was obtained at 100 μg of the peptide per ml but not at 10 μg/ml (Fig. 4A and B). Control staining with anti-E2 monoclonal antibody 5.2 (24) was not inhibited by the long peptide (Fig. 4C and D). Staining with neither antibody was suppressed by the short peptide.

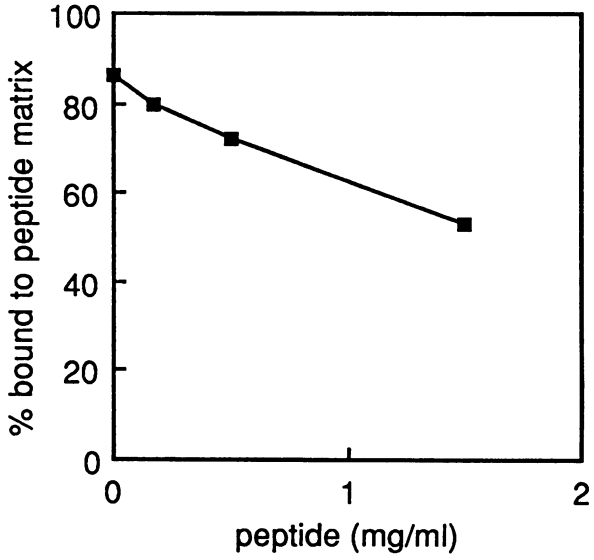

FIG. 3. Inhibition of NC binding to immobilized long-tail peptide with excess soluble peptide. The long-tail peptide was coupled to Pansorbin via C-terminal antibodies and incubated with [^{35}S]methionine-labeled viral NC particles in the presence of the indicated concentrations of the soluble peptide.

When the internal components of rubella virus (another togavirus) and VSV (a rhabdovirus) were tested for binding to the immobilized long peptide, some binding was observed. Rubella NCs bound one-third as efficiently as SFV NCs to the peptide matrix (Fig. 2). There was a low level of binding of the NP and M components of a VSV-infected cell lysate to the same matrix (Fig. 1, lanes 6 to 8). These results show that the NCs of other viruses, especially related ones, can bind to the tail of the SFV p62/E2 protein.

The long peptide oligomers bind NC particles. SDS-PAGE of the long peptide revealed oligomeric forms, even in the presence of a reducing agent (Fig. 5A). To see whether the oligomeric state of the tail peptide affected NC particle binding, the peptide preparation was fractionated by SDS-PAGE under reducing conditions, followed by transfer to nitrocellulose and subsequent blotting with [^{35}S]methionine-labeled NC particles. The different forms of the long peptide showed weak binding (Fig. 5B). Interestingly, the trimeric and higher oligomeric peptide forms showed more binding than the monomer, relative to the amount of each form on the nitrocellulose. Alkylation of the peptide after reduction abolished binding to the monomeric form, while that of the oligomers remained. These results suggest that the NCs bind preferentially to the peptide in an oligomerized form. That there was some binding to the monomeric form could possibly be explained by some disulfide bridge-mediated oligomerization of these molecules (that could be blocked by alkylation) on the nitrocellulose during immunoblotting. When the short peptide (which moved as a single band on SDS-PAGE) was tested in similar NC blotting experiments, no binding was observed. In a control experiment, the signal peptide of chicken oviduct lysozyme did not bind SFV NCs, suggesting that binding by the long peptide was specific.

Oligomers of the long peptide in solution bind NCs. To analyze whether the free soluble long peptide could bind to viral NC particles, we incubated isolated [^{35}S]methionine-labeled NC particles with the peptide. The incubation mixture was then subjected to centrifugation in a 5 to 20%

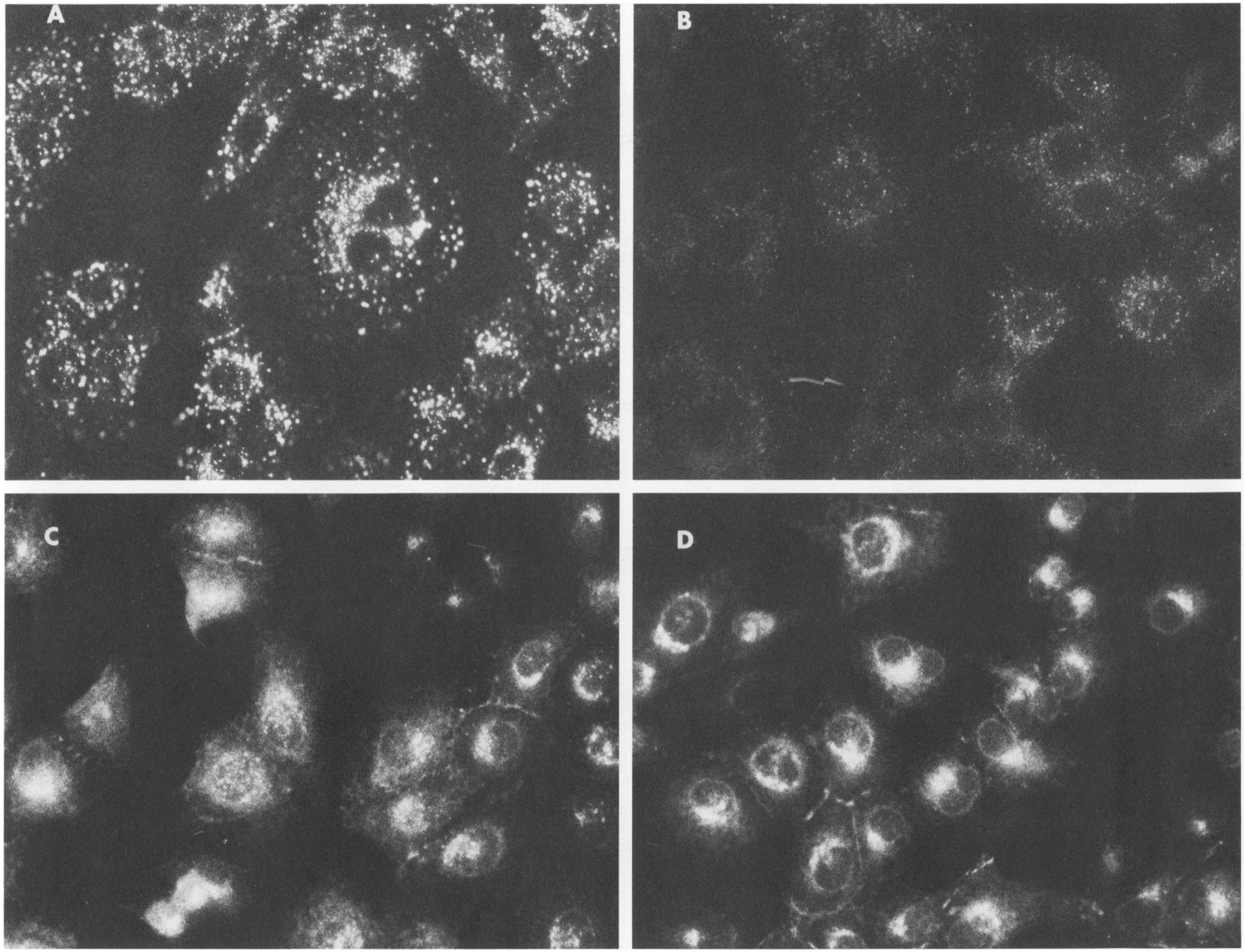

FIG. 4. Competition of NC binding between the p62 long-tail peptide and F13 antibody. After fixation in ice-cold methanol, infected cells were treated first with phosphate-buffered saline–gelatin (24) (30 min at room temperature), then with either capsid-reactive anti-idiotypic antibody F13 (22) (450 μg/ml in phosphate-buffered saline–gelatin for 1 h at room temperature) or E2-reactive monoclonal antibody 5.2 (24) (ascites fluid diluted 1:50 in phosphate-buffered saline–gelatin), and finally with the secondary antibody. For competition analysis, the long peptide was added into the preincubation and primary antibody reaction mixture at concentrations of 10, 100, and 1,000 μg/ml. Panels: A, staining with F13 antibody with no competing peptide; B, suppression of F13 reaction in the presence of 100 μg of the peptide; C, staining of E2 protein with the 5.2 antibody; D, lack of suppression of 5.2 antibody staining by the long peptide (100 μg/ml).

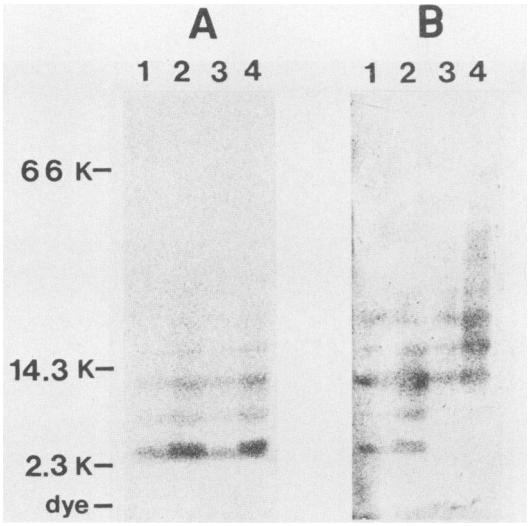

FIG. 5. Binding of [^{35}S]NC to different oligomeric forms of the long peptide. Four peptide samples were subjected to SDS-PAGE on a 15% gel and transferred onto nitrocellulose. This was first stained with Ponceau S to show the different oligomeric forms found in SDS buffer under reducing conditions (A) and subsequently blocked with fetal bovine serum (5%) and blotted with [^{35}S]methionine-labeled NCs (5,000 dpm/μg of protein) isolated from SFV virions. The corresponding autoradiogram is shown in panel B. Lanes 1 and 3 contained 20 μg of the peptide, while lanes 2 and 4 contained 40 μg. All samples were reduced with dithiothreitol, and the samples in lanes 3 and 4 were alkylated with iodoacetamide. The size standards used were the lysozyme signal peptide (2.3 kilodaltons), lysozyme (14.3 kilodaltons), and bovine serum albumin (66 kilodaltons).

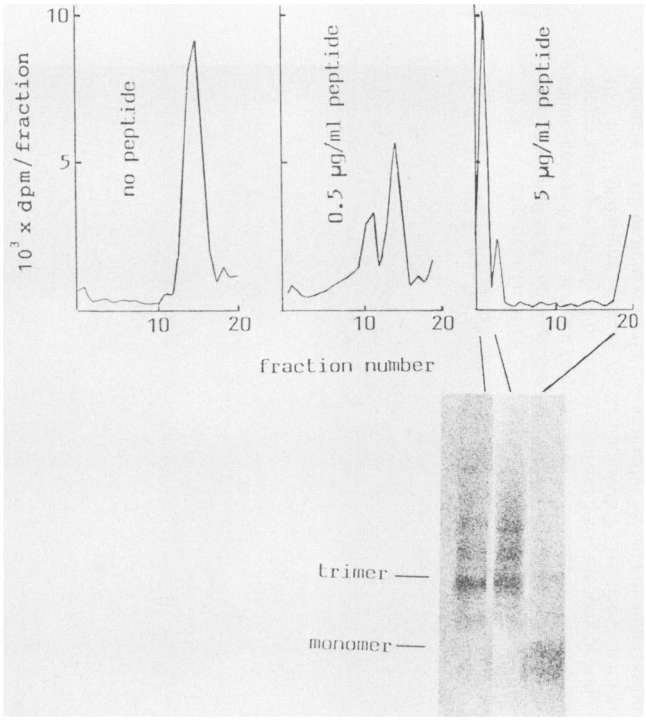

FIG. 6. NC particles bind oligomeric forms of the E2 tail peptide. Isolated viral [^{35}S]methionine-labeled NC particles (2 μg of protein per ml) were incubated with the indicated concentrations of the long peptide corresponding to the whole p62/E2 cytoplasmic tail. The incubation mixture was then applied to the top of a 5 to 20% sucrose gradient in TN buffer. After centrifugation for 90 min at 25,000 rpm (Beckman SW40 rotor), fractions (500 μl) were collected and analyzed for ^{35}S radioactivity. In the experiment using the peptide at a concentration of 5 μg/ml for incubation with NC, fractions containing radioactivity were recentrifuged for 60 min at 17,000 rpm (Beckman JA18.1 rotor) to pellet NC particles with the bound peptide. The pellets and part (1/10) of the gradient top fraction were subjected to SDS-PAGE on a 15% gel, followed by transfer to nitrocellulose and immunoblotting with specific antipeptide antibodies (lower panel).

sucrose density gradient. At a low peptide concentration (0.5 μg/ml), we found, in addition to NC monomers, apparent dimers of the NC. Higher peptide concentrations caused aggregation of the NC particles (Fig. 6). SDS-PAGE of [^{35}S]methionine-labeled NC-containing fractions was then performed under reducing conditions, followed by immunoblotting with antibodies against the long peptide. This analysis showed that the aggregated NCs did indeed contain the bound peptide. SDS-resistant trimeric and higher oligomeric forms of the peptide preparation (see above) were especially prevalent (Fig. 6). Similar analysis with the short peptide showed no interaction with the nucleocapsid, even at a high (0.5 mg/ml) peptide concentration.

We used the ^{125}I-labeled long peptide to analyze whether the long peptide bound to the NC at low peptide concentrations at which NC dimerization of aggregation did not occur. Under nonreducing conditions, part (30%) of the label sedimented with the NCs. However, when the peptide preparation was reduced, only marginal binding was observed. The specificity of long peptide binding in these experiments was tested with a tryptic digest of [^{35}S]methionine-labeled VSV G protein. No significant fraction of the G peptides was found to bind to the SFV NC (data not shown). We concluded that oligomers of the long peptide bind to NC particles and that the aggregation of the NC particles probably occurs through interparticle cross-linking by the oligomeric peptides.

DISCUSSION

In this study, we showed that there is an interaction between the long peptide corresponding to the entire cytoplasmic domain of the p62/E2 protein and viral or cell-associated NCs. This interaction shares binding properties with the F13 anti-idiotypic antibody which was raised by using the p62 tail as the antigen and which has been shown to bind to NCs in infected cells (22). When NC binding to the matrix-bound long peptide was compared with the binding properties of a large number of cellular proteins, the NC-p62/E2 tail interaction appeared to be quite specific. However, significant binding was observed when the NC of the related rubella virus was tested. As there is no sequence homology between the cytoplasmic domain of p62/E2 of SFV and those of membrane proteins E1 and E2 of rubella virus (4, 5, 7, 23), this could mean that some features of the NC-spike interaction are based on rather degenerate amino acid sequences, such as those which prevail in signal sequence-signal recognition particle or polypeptide chain-chaperone interactions (15, 18). Such a degeneracy of binding is supported by the fact that SFV tail anti-idiotypic antibodies have been found to recognize NCs of many other alphaviruses, in particular, NCs of many flaviviruses (22).

The long peptide showed a tendency to form dithiothreitol-SDS-resistant oligomers, especially trimers, in solution. It was preferentially these oligomeric forms which bound the

SFV NCs. Thus, the binding apparently requires many contact sites. Efficient binding of NC particles to the matrix-bound long peptide can also be explained by presentation of multiple peptide contact sites to the NC. These results might reflect a general requirement for multimeric forms of the p62/E2-E1 heterodimers to drive SFV budding. The $(E2\text{-}E1)_3$ structure of the spike protein in the viral envelope, shown by cryoelectron microscopy (6), possibly represents such a form.

The short peptide corresponding to the amino-terminal part of the cytoplasmic domain of p62/E2 did not show efficient binding in our assays. A simple interpretation of our data would be that it is the C-terminal part of the cytoplasmic domain which mediates binding to the NC. However, a sequence comparison between the p62/E2 tails of different alphaviruses shows that it is the less hydrophobic N-terminal portion of the tail which is especially well conserved (2, 3, 7, 12, 16). Therefore, NC binding might still be mediated by this region. In view of this possibility, our results could be explained by assuming that the C-terminal region is required to maintain the binding-active form of the N-terminal part. For instance, the N-terminal portion of the tail might have to be oligomerized for efficient binding to occur, and this could be directed by the less conserved and hydrophobic C-terminal part of the tail.

A spike protein oligomerization-dependent interaction with the NC is interesting not only from the aspect of virus assembly but also from the disassembly point of view. Disassociation of the trimeric state of the spike during virus entry would be expected to weaken the membrane-NC interaction and thereby facilitate release of the NC after the virus-host membrane fusion in the acidic endosome compartment (11). Our recent finding that the E2-E1 heterodimeric association in the viral membrane is very sensitive to mildly acidic conditions in vitro could well reflect a spike oligomer dissociation process during virus entry (24). Membrane protein oligomerization might, therefore, be an important regulator of alphavirus assembly and entry events.

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