Association of Sindbis Virus Capsid Protein with Phospholipid Membranes and the E2 Glycoprotein: Implications for Alphavirus Assembly†

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ABSTRACT: A late stage in assembly of alphaviruses within infected cells is thought to be directed by interactions between the nucleocapsid and the cytoplasmic domain of the E2 protein, a component of the viral E1/E2 glycoprotein complex that is embedded in the plasma membrane. Recognition between the nucleocapsid protein and the E2 protein was explored in solution using NMR spectroscopy, as well as in binding assays using a model phospholipid membrane system that incorporated a variety of Sindbis virus E2 cytoplasmic domain (cdE2) and capsid protein constructs. In these binding assays, synthetic cdE2 peptides were reconstituted into phospholipid vesicles to simulate the presentation of cdE2 on the inner leaflet of the plasma membrane. Results from these binding assays showed a direct interaction between a peptide containing the C-terminal 16 amino acids of the cdE2 sequence and a Sindbis virus capsid protein construct containing amino acids 19−264. Additional experiments that probed the sequence specificity of this cdE2−capsid interaction are also described. Further binding assays demonstrated an interaction between the 19−264 capsid protein and artificial vesicles containing neutral or negatively charged phospholipids, while capsid protein constructs with N-terminal truncations displayed either little or no affinity for such vesicles. The membrane-binding property of the capsid protein suggests that the membrane may play an active role in alphavirus assembly. The results are consistent with an assembly process involving an initial membrane association, whereby an association with E2 glycoprotein further enhances capsid binding to facilitate membrane envelopment of the nucleocapsid for budding. Collectively, these experiments elucidate certain requirements for the binding of Sindbis virus capsid protein to the cytoplasmic domain of the E2 glycoprotein, a critical event in the alphavirus maturation pathway.

Viral budding from the plasma membrane is a key maturation step for many enveloped viruses, including rhabdoviruses, bunyaviruses, paramyxoviruses, retroviruses, and togaviruses (*1*, *2*). The alphaviruses, a genus within the Togavirdae family that includes Sindbis virus (SINV),[1] Semliki Forest virus (SFV), and Ross River virus (RRV), are plus-stranded RNA viruses. Alphaviruses are spread by mosquitoes and have a variety of mammalian hosts, including man (*3*, *4*). Factors known to influence alphavirus budding efficiency include ionic strength conditions (*5*−*7*), plasma membrane cholesterol content (*8*−*10*), glycoprotein palmitoylation (*11*−*15*), lateral protein−protein interactions between the glycoproteins E2 and E1 (*16*−*19*), and protein−protein interactions between an assembled intracellular nucleocapsid and the cytoplasmic domain of E2 (cdE2) (*20*−*24*).

Both envelope glycoproteins and capsid protein are required for alphavirus budding from the plasma membrane (*25*). The capsid protein (∼30 kDa) binds viral RNA and assembles in the cytoplasm into nucleocapsids having $T = 4$ icosahedral symmetry (*26*). The envelope transmembrane glycoproteins E2 and E1 (∼50 kDa each) constitute the outer protein shell with spikes formed from a trimer of E2-E1 heterodimers; 80 such spikes are arranged upon a $T = 4$ icosahedral lattice that coincides with the nucleocapsid subunit arrangement (*26*). Both E2 and E1 are transported to the plasma membrane and have a single membrane-spanning helix followed by a cytoplasmic stretch of amino acids. The cytoplasmic stretch within E1 is predicted to be 2−4 amino acids in length (*27*, *28*), while a longer sequence of 31−33 amino acids composes cdE2 that interacts with capsid protein in a 1:1 stoichiometry in the mature virion (*26*, *28*). cdE2 is palmitoylated at three conserved cysteine residues (Figure 1) (*12*, *13*, *15*, *29*), and the lipophilic palmitoyl groups are hypothesized to anchor this domain to the inner leaflet of the plasma membrane, with the resulting

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[1] Abbreviations: SINV, Sindbis virus; RRV, Ross River virus; cdE2, cytoplasmic domain of E2; SCP, Sindbis virus capsid protein; RRCP, Ross River virus capsid protein; TNBS, 2,4,6-trinitrobenzenesulfonic acid; BSA, bovine serum albumin; TFE, trifluoroethanolamine; PC, L-α-phosphatidylcholine; PE, L-α-phosphatidylethanolamine; SM, sphingomyelin; PI, L-α-phosphatidylinositol; PS, L-α-phosphatidyl-L-serine; FABS, fast atom bombardment mass spectrometry; PDMS, plasma-desorption mass spectrometry; MLV, multilamellar vesicles.

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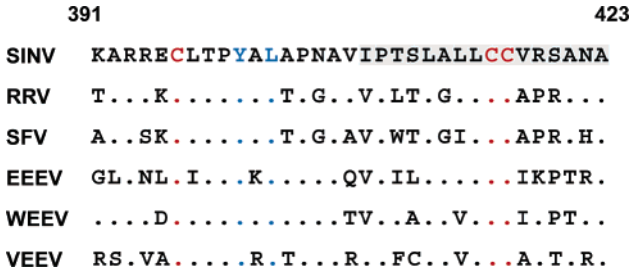

FIGURE 1: cdE2 sequences from different alphaviruses. The amino acid numbering scheme for Sindbis virus is used. A "." indicates a conserved residue at that position. Conserved cysteines are shown in red, and the conserved Y400 and L402 within the cdE2 tyrosine motif are shown in blue. Abbreviations: SINV, Sindbis virus; RRV, Ross River virus; SFV, Semliki Forest virus; EEEV, eastern equine encephalitis virus; WEEV, western equine encephalitis virus; VEEV, Venezuelan equine encephalitis virus. The region within the SINV E2 sequence found to recognize the SINV capsid protein in this study is shaded.

conformational restriction favoring subsequent cdE2−capsid protein interactions.

Studies to date have not provided a complete picture of the capsid protein−cdE2 interaction. Various reports have identified either the N-terminal or the C-terminal region of cdE2 as the critical determinant for binding nucleocapsids. In vitro binding assays using structural components from SFV indicate that the C-terminal amino acids of cdE2 are involved in capsid protein binding, since the full-length cdE2 domain but not the N-terminal region of cdE2 can support nucleocapsid binding (*21*).

Conversely, synthetic peptides corresponding to the N-terminal region of SINV cdE2 [e.g., E2 397−402 (LTPYAL)] have been shown to inhibit viral release when added to infected cells (*30*). While the mechanism by which these peptides effect inhibition of viral replication was never precisely determined, the peptides were hypothesized to traverse the plasma membrane and subsequently block viral release through direct competition with cdE2 for capsid protein binding within the cell cytoplasm. In addition, mutagenesis studies showed that a "tyrosine motif" including Y400 and L402 in the N-terminal region of cdE2 is important for the recruitment of nucleocapsids to the inner leaflet of an infected cell's plasma membrane, as well as for the efficient budding of infectious virus particles from the cell (*15*, *22*). The findings from these mutagenesis studies were interpreted as evidence that this tyrosine motif participates directly in capsid protein binding. The crystal structure of the C-terminal domain (residues 114−264) of the Sindbis virus capsid protein (SCP) reveals the existence of a hydrophobic pocket on the surface of the protein, and this pocket has been implicated as the binding site for the cdE2 tyrosine motif (*22*, *31*, *32*). The mutagenesis and crystallographic data prompted a structural model for the cdE2 tyrosine motif docked within this hydrophobic pocket (*22*, *31*−*33*). In particular, these modeling studies describe in detail the disposition of conserved cdE2 residues Y400 and L402 within the SCP pocket. More recently, a fit of the crystallographic structure of SCP into the 11 Å resolution cryoEM map of Sindbis virus was reported (*28*) and finds SCP oriented with the hydrophobic pocket near the transmembrane region of density assigned to E2, consistent with the above model. The structure of SCP residues 19−113 is unknown and thus cannot be fitted into the cryoEM density.

Although the budding of alphaviruses has long been considered to be directed by sequence-specific interactions between cdE2 and capsid protein (*23*, *25*), a direct demonstration of a sequence-specific interaction between cdE2 and capsid protein remains elusive. In vivo studies were performed with chimeric alphaviruses with either capsid protein or cdE2 from one alphavirus swapped with its counterpart from another alphavirus (*34*−*36*). Some chimeras displayed attenuated virus production, and their revertants showed adaptive amino acid changes in both cdE2 and capsid protein that restored budding efficiency to nearly wild-type levels. Such observations suggest specific cdE2−capsid contacts. On the other hand, other chimeras were generated from these experiments that replicated normally in the absence of amino acid changes. For instance, a RRV chimera where the cdE2 sequence was replaced with the corresponding sequence from SINV was observed to bud with wild-type efficiency (*35*). This result demonstrates that cross-reactivity between different alphavirus cdE2 and capsid proteins can occur in vivo, with some sequence variance in cdE2 or in the capsid protein being tolerated by the replicating virus. Moreover, in vitro cross-reactivity of cdE2 with nucleocapsids from rubella virus and with vesicular stomatitis virus (VSV) has also been reported (*21*), indicating that cdE2 can support interactions with capsid proteins from other virus families. The collective evidence for interactions among structural proteins from different viruses, observed both among different alphavirus structural proteins and between structural proteins of alphaviruses and other virus families, raises the possibility that the determinants for capsid−glycoprotein recognition in alphaviruses are not strongly sequence-based.

In the current study, the capsid protein sequence requirements for direct binding of SINV cdE2 were explored in solution using NMR spectroscopy and more extensively using binding assays where a variety of E2 cytoplasmic domain constructs were reconstituted into phospholipid vesicles. Synthetic cdE2 peptides were modified with C_{16} alkyl groups at cysteine positions prior to reconstitution into liposomes to mimic the palmitoylation of cdE2 found in vivo. These studies probe the direct interaction between cdE2 and SCP at a membrane interface to investigate structural requirements for an initial event in assembly. This in vitro system does not incorporate higher order interactions and thus cannot capture features present in later events of assembly. SCP constructs containing residues 19−264 (SCP 19−264), 81−264 (SCP 81−264), and 114−264 (SCP 114−264) were tested for binding to the reconstituted cdE2 peptides to better define the regions in SCP and cdE2 that might participate in cdE2−capsid protein contacts. In addition, the experiments demonstrate that SCP can bind to neutral or negatively charged artificial vesicles in the absence of reconstituted cdE2. This SCP−membrane interaction appears analogous to the interaction of other viral matrix or capsid proteins with membranes in other enveloped RNA virus systems (*37*−*43*). On the basis of this observation, we introduce an alphavirus assembly model that features capsid protein−membrane interactions as well as capsid protein−cdE2 interactions in the viral assembly process.

MATERIALS AND METHODS

Materials. E2/6 (NH_3^+-PYALAP-NH_2), E2/12 (Ac-KARRECLTPYAL-NH_2), E2/33 (Ac-KARRECLTPYALAP-

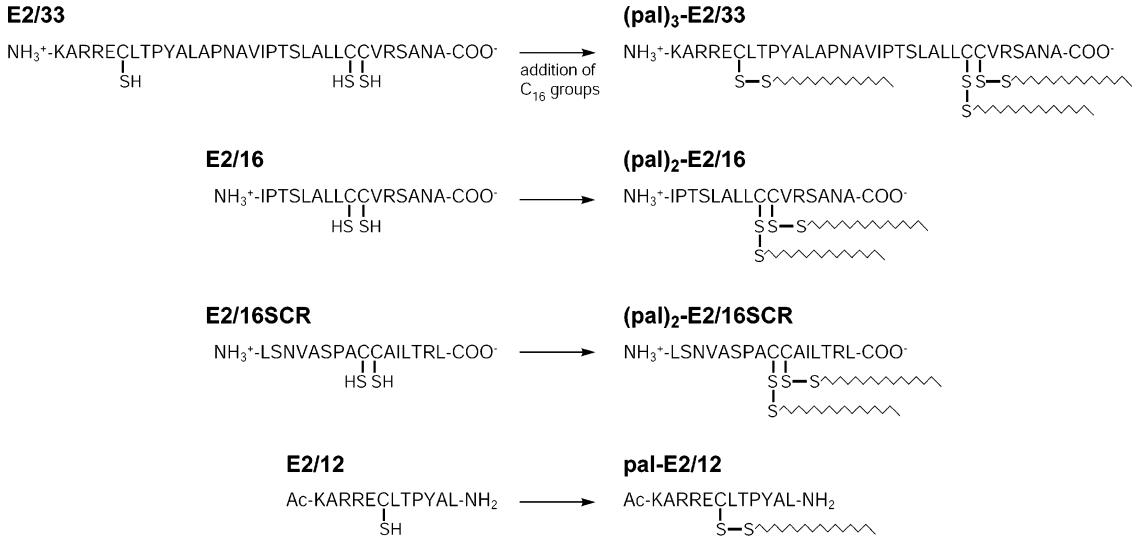

FIGURE 2: Alkylation of synthetic cdE2 peptides with C_{16} groups specifically at cysteine positions.

NAVIPTSLALLCCVRSANA-NH_2), and scrambled E2/16SCR (NH_3^+-LSNVASPACCAILTRL-COO^-) peptides were synthesized at the Purdue University Peptide Synthesis Facility, using either Fmoc (for E2/6, E2/12, and E2/16SCR) or tBoc (for E2/33) chemistry, and were further purified using reverse-phase chromatography on either a C_4 or a C_{18} column. E2/16 (NH_3^+-IPTSLALLCCVRSANA-COO^-) was a gift from Dr. James Strauss (California Institute of Technology). Hexyldecyldithiopyridine (DTP-C16) was prepared as described elsewhere (*44*). Trifluoroethanolamine (TFE), L-α-phosphatidylcholine (PC), L-α-phosphatidylethanolamine (PE), sphingomyelin (SM), L-α-phosphatidyl-L-serine (PS), L-α-phosphatidylinositol (PI), L-α-phosphatidic acid (PA), cholesterol, Triton X-100, and electrophoresis grade bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Formic acid (98−100%) and spectroscopic grade acetonitrile were obtained from VWR Scientific, and 2,4,6-trinitrobenzenesulfonic acid (TNBS) was purchased from Aldrich Chemical Co. The constructs SCP 19−264, 81−264, and 114−264 and Ross River virus capsid protein (RRCP) construct 1−270 were expressed in *Escherichia coli* and purified as described previously (*45*).

NMR Data Acquisition. One-dimensional ^{1}H titration spectra of SCP 114−264 with E2/6 were measured at 22 °C using a Varian VXR-500 spectrometer. The water signal was suppressed with presaturation using the decoupler channel. An initial reference ^{1}H spectrum of 0.40 mM SCP 114−264 in 50 mM sodium phosphate buffer (90% D_2O/10% H_2O, pH 7.5), 50 mM NaCl, and 1 mM NaN_3 was collected, and aliquots of E2/6 were added to the SCP sample to yield 0.5:1 and 1.6:1 molar ratios of E2/6:SCP 114−264. A ^{1}H spectrum of the E2/6:SCP 114−264 mixture was collected following each addition of E2/6. All spectra were obtained using 600 transients, a 60° excitation pulse, and a 3 s recycle delay. Data were Fourier transformed using an exponential line broadening parameter of 0.7 Hz with the Varian VNMR software package. Difference spectra were calculated using VNMR. E2/6 assignments were based upon random coil ^{1}H chemical shifts for amino acids (*46*) and inspection of peak splitting patterns.

Synthesis and Purification of Alkylated E2 Peptides. The cysteine-specific alkylation of synthetic SINV cdE2 peptides was accomplished using a thioalkylating reagent (DTP-C16) that was developed in this laboratory and described previously (*44*). In this procedure, C_{16} groups were covalently attached to cysteine residues within the cdE2 peptides via a disulfide linkage (Figure 2). The resulting alkylated E2/12 (pal-E2/12), E2/16 [$(pal)_2$-E2/16], and E2/16SCR [$(pal)_2$-E2/16SCR] peptides were purified using reverse-phase chromatography (C_{18} column) with a 5−95% water/acetonitrile gradient developed over 30 min. The triply alkylated E2/33 [$(pal)_3$-E2/33] peptide was similarly purified using reverse-phase chromatography with water/acetonitrile gradients containing 20% formic acid as outlined previously (*44*). Successful synthesis and purification of the alkylated peptides (Figure 2) were confirmed by fast atom bombardment mass spectrometry (FABS) and by plasma-desorption mass spectrometry (PDMS).

Preparation of Large Multilamellar Vesicles (MLV's) and Reconstitution of Alkylated E2 Peptides. Stocks of multilamellar PC liposomes were prepared by dissolving egg PC in chloroform and drying the lipid to a thin film in a round-bottomed flask under a stream of nitrogen gas. After the sample was placed under vacuum for at least 2 h, the lipid was resuspended to 20 mg/mL in 10 mM sodium phosphate (pH 7.2), 140 mM NaCl, and 0.02% NaN_3 (buffer A); several cycles of freeze−thawing and vortexing resuspended all dried lipid from the flask and yielded multilamellar vesicles. These liposomes were centrifuged at 4500*g* for 5 min at 18−20 °C, and the supernatant containing smaller vesicles was removed. The pellet was gently resuspended, and the sample was centrifuged and resuspended twice more to isolate the larger vesicles. The MLV's were resuspended in a final volume of buffer A equal to that for dissolution at the initial 20 mg/mL. Lipid loss from resuspension was judged to be minimal since there was no apparent change in the size of the lipid pellet over the course of the preparation. *Het*erogeneous lipid (Het) vesicles, containing a 0.9:1 molar ratio of cholesterol:phospholipid and molar ratios of PC:PE:SM:PS:PI:PA of 6:5:4:3:1:1, were prepared in the same fashion.

Alkylated E2 peptides were reconstituted into PC MLV's by adding 1 μg/μL pal-E2/12 dissolved in TFE or 1 μg/μL

$(pal)_2$-E2/16, $(pal)_2$-E2/16SCR, or $(pal)_3$-E2/33 dissolved in 95% TFE:5% 1:2:1 acetic acid:chloroform:ethanol ("ACE") to the PC/chloroform solution prior to drying the lipid with nitrogen gas. Identical amounts of TFE or TFE:ACE solution were added to PC/chloroform mixtures that were used to make PC vesicles that lacked alkylated E2 peptide. The molar ratios of PC:E2 peptide that were used in binding assays were 160−190:1, and the total amount of E2 peptide that was added to the lipid mixture was typically 150−200 μg. After addition of the alkylated peptide to the PC/chloroform solution, PC MLV's were prepared as described above.

Binding Assays in a Phospholipid Membrane Environment. PC MLV preparations (4 mg/mL PC, 0.4% BSA) with or without reconstituted E2 peptides were incubated with a particular SCP construct (SCP 19−264, 81−264, or 114−264) for 1 h at room temperature in buffer A. Binding experiments were performed using 100−400 ng of protein in reaction volumes of 100 μL. Control reactions containing only SCP (no MLV's or E2 peptide), along with negative control reactions containing PC vesicles with and without reconstituted E2 peptides (but no SCP), were also performed. Following centrifugation at 4500*g* for 5 min at 18−20 °C, the resulting pellet and supernatant fractions were separated and brought up to equal volumes. BSA was added to the pellet fractions to match the BSA concentration in the supernatant fractions. Equal volume aliquots of each fraction were then blotted to nitrocellulose using a dot blot manifold (Bio-Rad). To assay for SCP content in the pellet and supernatant fractions, the nitrocellulose blot was subsequently probed with a polyclonal anti-SCP antibody and then developed using a chemiluminescent system (Amersham). Binding assays using Het vesicles were performed in a similar fashion. Dot blots were quantitated using a Bio-Rad GS-710 densitometer.

Detection of pal-E2/12 in PC Liposomes. A colorimetric assay that uses TNBS to detect protein amino groups was employed to verify that pal-E2/12 partitions to PC membranes when reconstituted into vesicles, as well as to estimate the fraction of pal-E2/12 that partitions to the outer leaflet of the PC MLV's (*47*, *48*). The amount of pal-E2/12 on the outer leaflet of the PC MLV's was assessed by diluting aliquots of PC MLV's containing pal-E2/12 to 0.6 mL with a solution of 150 mM NaCl, followed by addition of 0.2 mL of 0.8 M Na_2HPO_4 (pH 8.5). Twenty microliters of 1.5% TNBS was then added to the samples (termed "surface-accessible pal-E2/12" samples), which were incubated in the dark for 30 min. After this incubation period, 0.4 mL of 1.2% Triton X-100 in 1.5 M HCl was added to quench the reactions, and absorbances at 410 nm were measured within 1 h.

The total amount of pal-E2/12 present within the inner lamellae of the PC vesicles, as well as on the outer leaflet, was assayed by diluting aliquots of PC MLV's containing pal-E2/12 to 0.6 mL with 150 mM NaCl, followed by addition of 0.2 mL of (thoroughly mixed) 1.6% Triton X-100 in 0.8 M Na_2HPO_4 (pH 8.5). Twenty microliters of 1.5% TNBS was added to these samples (termed "total pal-E2/12" samples), which were then incubated in the dark for 30 min. After this incubation period, 0.4 mL of 0.4% Triton X-100 in 1.5 M HCl was added to quench the reactions, and absorbances at 410 nm were measured within 1 h. Sample blanks consisted of identical preparations of aliquots of PC liposomes lacking pal-E2/12. The ratio of the optical densities of the surface-accessible pal-E2/12 and total pal-E2/12 samples ($OD_{surface}/OD_{total}$) showed that approximately 39% of pal-E2/12 is surface-accessible when reconstituted into PC membranes as described above.

RESULTS

NMR-Detected Binding Using E2/6 and SCP 114−264. Binding of cdE2 to SCP was monitored by NMR spectroscopy using E2/6 (Figure 3a), a peptide containing Y400 and L402 within the conserved cdE2 tyrosine motif (Figure 1), and SCP 114−264, a protein fragment containing the hydrophobic pocket previously hypothesized to be the cdE2 binding site. Regions of a 1D ^1H spectrum of E2/6 containing the δ- and ϵ-proton signals from Y400 (7.15 and 6.85 ppm, respectively), the methyl H^{β} signals from A401 and A403 (1.3−1.4 ppm), and the methyl H^{δ} signals from L402 (0.9−1.0 ppm) are shown in Figure 3e. Any association of E2/6 with SCP 114−264 would lead to line broadening and/or chemical shift changes of the E2/6 peptide signals. In particular, such spectral changes are expected to occur for peaks from the Y400 and L402 side chains upon binding to a hydrophobic pocket on SCP 114−264.

Successive aliquots of E2/6 were added to 0.4 mM SCP 114−264 as described in Materials and Methods. The difference spectrum corresponding to subtraction of the free SCP 114−264 spectrum (Figure 3b) from the spectrum measured from the E2/6 peptide in the presence of SCP 114−264 (Figure 3c) is shown in Figure 3d. NMR data for two titration points (0.5:1 and 1.6:1 molar ratios of E2/6:SCP 114−264) were collected, and the results from the "1.6:1 titration point" are shown. Neither chemical shift changes nor line broadening of the peptide signals is observed in the presence of SCP 114−264 (Figure 3d vs Figure 3e). The lack of such spectral changes shows that E2/6 does not bind to SCP 114−264 under the conditions tested (0.4 mM SCP 114−264, 0.6 mM E2/6, 50 mM potassium phosphate, 50 mM NaCl, 1 mM NaN_3, pH 7.5).

Detection of SCP−cdE2 and SCP−Membrane Interactions in a Phospholipid Membrane Environment. The lack of binding of soluble E2/6 to SCP 114−264 motivated an expanded search for other cdE2 and SCP sequences that can mediate capsid protein−glycoprotein interactions. In particular, amino acid sequences corresponding to the entire cdE2 sequence (E2/33), as well as the C-terminal 16 amino acids (E2/16) and the N-terminal 12 amino acids (E2/12) of cdE2, were examined for binding to SCP 114−264. Furthermore, larger capsid protein constructs containing residues 19−264 and 81−264 were also investigated for binding to cdE2 peptides. The poor solubility of E2/16 and E2/33 precluded an NMR analysis as was performed with E2/6 and SCP 114−264. As such, the various capsid protein constructs were tested for cdE2 binding in a lipid membrane system containing cdE2 peptides that were reconstituted into phospholipid vesicles, as described below.

Binding assays with phospholipid vesicles were performed using synthetic cdE2 peptides that were alkylated at cysteine positions with C_{16} groups to mimic the palmitoylation modification found at these sites in vivo (see Materials and Methods and Figure 2). Following reconstitution of these cdE2 peptides into MLV's, SCP was incubated with the

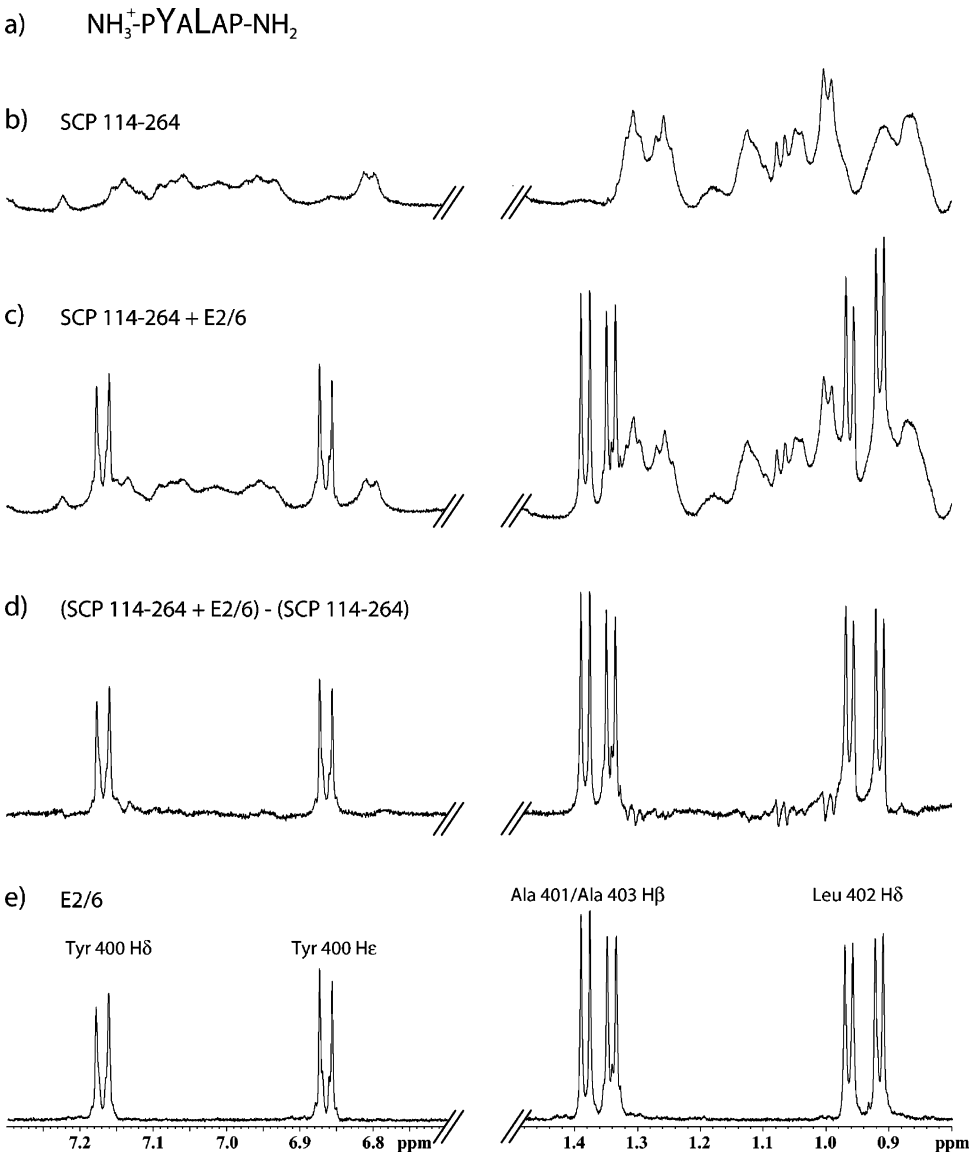

FIGURE 3: E2/6 titration with SCP 114−264. (a) The synthetic E2/6 peptide, with residues hypothesized to interact with SCP 114−264 (Y400 and L402) highlighted; (b) 1D ^{1}H spectra of 0.4 mM SCP 114−264 and (c) SCP 114−264 + E2/6 (1.6:1 molar ratio of E2/6:SCP); (d) a difference ^{1}H spectrum of (SCP 114−264 + E2/6) − (SCP 114−264) to reveal only the E2/6 signals; (e) free E2/6 peptide. Assignments of the tyrosine, alanine, and leucine signals are based upon random coil ^{1}H chemical shift values (*46*) and inspection of peak splitting patterns.

cdE2-containing vesicles to test for binding. In parallel, incubation of SCP with PC liposomes lacking cdE2 peptides was performed to assess the extent of background binding of SCP to the phospholipid membranes. Following centrifugation, pellet and supernatant fractions of a reaction mixture were analyzed for capsid protein content using a dot blot assay as described in Materials and Methods. The presence of increased amounts SCP in pellet fractions of reactions containing cdE2 peptides in vesicles, relative to that found in pellet fractions of reactions containing vesicles lacking cdE2, indicates binding of SCP to reconstituted cdE2. Both cdE2−SCP interactions and SCP−membrane interactions were characterized.

SCP−Membrane Interactions. SCP constructs 19−264, 81−264, and 114−264 were tested for binding to PC vesicles and to Het vesicles having a 0.9:1 molar ratio of cholesterol: phospholipid with a phospholipid molar ratio of PC:PE:SM: PS:PI:PA of 6:5:4:3:1:1 (prepared as described in Materials and Methods). The composition of these Het vesicles mimics the heterogeneous lipid composition of the Sindbis virus envelope (*49*); about 18% of the total lipid in these artificial vesicles is negatively charged. After incubation of SCP 19−264 with Het vesicles and subsequent centrifugation, SCP 19−264 is found mostly in the pellet fraction (∼95% bound), while incubation of SCP 19−264 with PC vesicles results in roughly 60% of SCP 19−264 partitioning to the pellet fraction (Figures 4a,b and 5a). A control experiment containing SCP 19−264 but no phospholipid membranes shows that SCP remains in the supernatant fraction when membranes are absent from the solution (Figure 4g). Additional experiments using PC or Het membranes without capsid protein rule out the possibility of cross-reactivity of the anti-SCP antibody with phospholipid membranes (Figure 4h). In contrast, the shorter fragment SCP 114−264 incubated with either Het or PC vesicles is found only in supernatant fractions. These observations indicate that SCP 19−264 has stronger affinity for Het vesicles than for PC vesicles and that SCP 114−264 does not display detectable affinity for

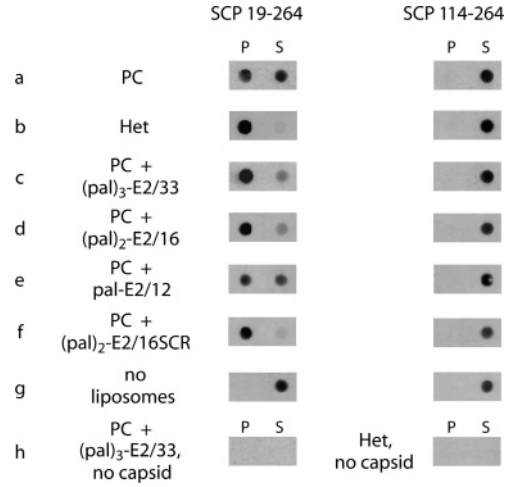

FIGURE 4: Dot blot results from binding assays in a phospholipid membrane environment. P = pellet fraction; S = supernatant fraction. (a−f) Binding of SCP 19−264 and SCP 114−264 to (a) PC vesicles, (b) Het vesicles, (c) PC vesicles with reconstituted $(pal)_3$-E2/33, (d) PC vesicles with reconstituted $(pal)_2$-E2/16, (e) PC vesicles with reconstituted pal-E2/12, and (f) PC vesicles with reconstituted $(pal)_2$-E2/16SCR. (g) Control reactions containing SCP 19−264 or SCP 114−264 but no liposomes or reconstituted peptides. (h) Control reactions containing either PC vesicles with reconstituted $(pal)_3$-E2/33 or Het vesicles but lacking capsid protein. Results shown are representative of at least three independent binding reactions. All reactions were performed in sodium phosphate buffer (10 mM sodium phosphate, 140 mM NaCl, 1 mM NaN_3, pH 7.2) using 1, 2, or 4 ng/μL capsid protein in a 100 μL reaction volume. The molar ratio of PC:reconstituted cdE2 peptide is 160−190:1 in binding assays where alkylated cdE2 peptides were used; assuming that approximately 40% of the total reconstituted cdE2 peptide is surface-accessible (as for pal-E2/12), the estimated cdE2:SCP molar ratio in binding reactions is 2000−8000:1.

phospholipid (0% bound; Figures 4a,b and 5a). Similar experiments with SCP 81−264 demonstrate that SCP 81−264 has significantly less affinity to either Het or PC membranes than does SCP 19−264 (only 5−10% bound; Figure 5a), suggesting that the "membrane-association" sequence lies primarily within the first 20−80 amino acids of SCP.

SCP−cdE2 Interactions. The alkylated cdE2 peptides pal-E2/12, $(pal)_2$-E2/16, and $(pal)_3$-E2/33 were each reconstituted into PC vesicles. The sequence of the triply alkylated peptide $(pal)_3$-E2/33 corresponds to the entire amino acid sequence of the SINV cdE2 (Figures 1 and 2). The pal-E2/12 peptide corresponds to the N-terminal 12 residues of cdE2 and contains Y400 and L402, and the $(pal)_2$-E2/16 peptide corresponds to the C-terminal 16 residues of cdE2 (Figure 2). Phospholipid vesicles containing reconstituted alkylated peptides were incubated with either SCP 19−264, 81−264, or 114−264. Typical results from such experiments are shown in Figure 4c−e, and all results are summarized in Figure 5a.

Binding assays using PC vesicles with reconstituted $(pal)_3$-E2/33 show a marked increase of SCP 19−264 in the pellet fraction (~90% bound) compared to corresponding assays with PC vesicles lacking $(pal)_3$-E2/33 (~60% bound; Figures 4a,c and 5a). Control experiments show that the increase in signal from the pellet fraction does not arise from cross-reactivity of the anti-SCP antibody with reconstituted $(pal)_3$-E2/33 (Figure 4h). Therefore, the observed increase in SCP in the pellet fraction indicates a direct interaction between

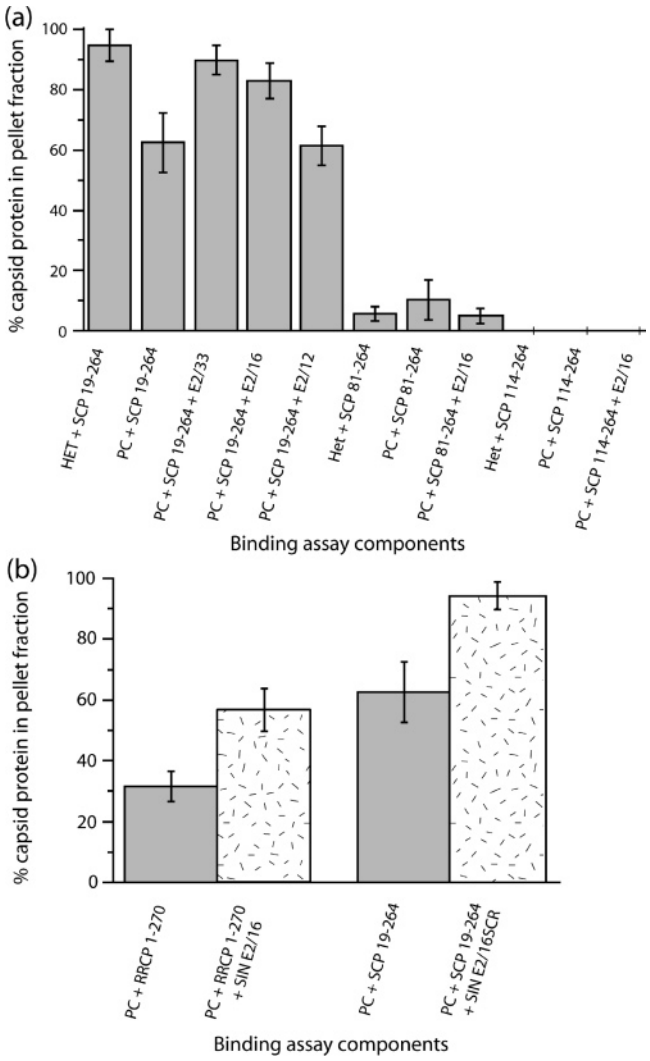

FIGURE 5: (a) Results from binding experiments with various Sindbis virus cdE2 and SCP constructs. (b) Results from experiments that probe the specificity of the SIN cdE2−SCP interaction.

SCP 19−264 and reconstituted $(pal)_3$-E2/33. A similar increase of SCP 19−264 in the pellet fraction is observed in experiments using PC liposomes with reconstituted $(pal)_2$-E2/16 (~83% bound; Figures 4d and 5a). As with $(pal)_3$-E2/33, the increase in signal in the pellet fraction does not arise from cross-reactivity of the anti-SCP antibody with reconstituted $(pal)_2$-E2/16 (data not shown). The data thus indicate that the C-terminal 16 amino acids of cdE2 are sufficient for binding SCP 19−264. SCP 114−264 is not recovered in the pellet fraction from experiments using PC vesicles containing either $(pal)_2$-E2/16 or $(pal)_3$-E2/33 (Figures 4c,d and 5a), suggesting that the component that promotes cdE2 binding must lie in the N-terminal region of SCP. Finally, incorporation of E2/16 into PC membranes does not enhance SCP 81−264 affinity for these vesicles (Figure 5a), further delineating the cdE2 recognition element to lie within the first 20−80 amino acids of SCP.

Binding assays to examine SCP interactions with the N-terminal region of cdE2 used pal-E2/12 mixed with PC liposomes. These assays show no increase in the levels of either SCP 19−264 or 114−264 in the pellet fraction (Figures 4e and 5a). The failure to detect increased amounts of capsid protein in the pellet fraction does not stem from an inability of pal-E2/12 to partition to phospholipid

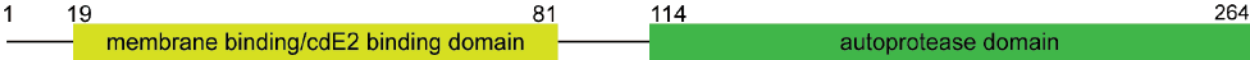

FIGURE 6: SCP sequence with the proposed membrane binding/cdE2 binding region shown relative to the SCP protease domain.

membranes, as the capacity of pal-E2/12 to bind to phospholipid vesicles has been previously demonstrated by HPLC analysis (*44*). Moreover, the presence of reconstituted pal-E2/12 in PC liposomes was also determined via absorbance measurements from a colorimetric assay that uses TNBS (*2*, *14*) to detect the ϵ-amino group of the lysine residue in pal-E2/12 (see Materials and Methods); the absorbance measurements from this assay further indicate that 39% of the total reconstituted pal-E2/12 partitions to the outer leaflet of the PC vesicles (data not shown). This assessment of surface-accessible pal-E2/12 leads to an estimation of a molar ratio for pal-E2/12:SCP in binding reactions of approximately 4000:1. Collectively, these results suggest that pal-E2/12 partitions to PC vesicles and is present on the membrane surface in great excess of SCP, yet is insufficient to bind SCP 19−264.

Specificity of the cdE2−Capsid Interaction. The specificity of the SINV cdE2−capsid protein interaction was examined by assessing the binding of full-length RRCP 1−270 to $(pal)_2$-E2/16 reconstituted in PC membranes. Under the assay conditions employed, RRCP 1−270 exhibits somewhat less affinity for PC membranes than does SCP 19−264 (Figure 5b). A significant increase in RRCP content in the pellet fraction was observed in the presence of $(pal)_2$-E2/16 (57% RRCP bound), relative to parallel experiments where $(pal)_2$-E2/16 is not included in PC MLV's (32% RRCP bound; Figure 5b). This observed enhancement in binding is analogous to that observed in experiments with SCP 19−264 (Figure 5a). Such data indicate that RRCP (having 56% sequence identity with SCP) can interact with E2/16 from SINV, demonstrating cross-reactivity between the structural components of RRV and SINV in vitro.

To further probe the sequence requirements of the cdE2−capsid protein interaction, binding assays were performed using $(pal)_2$-E2/16SCR reconstituted in PC membranes. While this peptide has the same amino acid composition as $(pal)_2$-E2/16, the amino acids are located at different positions in the sequence except for the two cysteines, whose positions were conserved in $(pal)_2$-E2/16SCR (Figures 1 and 2). The cysteine positions were retained to allow comparable anchoring of the alkylated peptides $(pal)_2$-E2/16 and $(pal)_2$-E2/16SCR to the PC membrane and similar presentation of these two peptides to the target capsid protein.

Binding assays using PC vesicles with reconstituted $(pal)_2$-E2/16SCR result in a significant increase of SCP 19−264 in the pellet fraction (~94% bound; Figures 4f and 5b). This result indicates that $(pal)_2$-E2/16SCR recognizes SCP 19−264 as readily as $(pal)_2$-E2/16 (Figure 5a), an unexpected finding given the prevailing hypothesis that the cdE2−capsid protein interaction is sequence-specific (*23*, *25*). Additional binding assays using SCP 114−264 and $(pal)_2$-E2/16SCR show that this SCP construct is only detected in supernatant fractions, indicating that SCP 114−264 has no affinity for $(pal)_2$-E2/16SCR (Figure 4f).

DISCUSSION

The present study provides biochemical evidence that pinpoints the regions within cdE2 and SCP that participate in the alphavirus E2−capsid protein interaction and may be important in an early event of assembly. The direct binding measurements on purified fragments of SCP and cdE2 reported here elucidate certain recognition elements between SCP and cdE2. The NMR spectroscopy and liposome binding assays expand upon previous mutagenesis, structural, and modeling studies that examine capsid protein−cdE2 association (*15*, *22*, *31*−*33*).

Capsid−cdE2 Association. Overall, the results support an alphavirus assembly model in which the C-terminal region of cdE2 (E2 residues 408−423) is important for recruiting SCP through association with the N-terminal region of SCP (residues 19−80; Figure 5a). Binding experiments show that both $(pal)_3$-E2/33 and $(pal)_2$-E2/16 that are reconstituted into liposomes can enhance the binding of SCP 19−264 to phospholipid membranes, indicating that an SCP recognition element lies within the last 16 residues of cdE2. This observation is in accord with earlier findings (*21*). The association of SCP 114−264 to membranes was not enhanced by any of the alkylated cdE2 peptides (Figures 4 and 5a), and membrane association of SCP 81−264 was not enhanced by $(pal)_2$-E2/16 (Figure 5a). These results suggest that a cdE2 recognition element lies within the N-terminal domain (residues 19−80) of SCP (Figure 6).

In addition, the observation that cdE2 peptides can enhance the binding of SCP 19−264 to liposomes demonstrates that SCP can interact with membranes prior to formation of nucleocapsid core particles. This result is consistent with previous studies that find that SFV capsid protein mutants are defective in forming cytosolic nucleocapsids but, nonetheless, support SFV budding from infected cells (*50*, *51*). Taken together, these results and those reported here suggest that while assembled nucleocapsids are observed in the cytoplasm of infected cells, the assembly of SCP into nucleocapsid core particles might not be a strict requirement for SCP−cdE2 association. Furthermore, as nucleocapsid attachment to internal cellular membranes within infected cells has been previously observed (*22*), the site of cdE2−SCP complex formation may not be simply restricted to the plasma membrane.

Mutagenesis studies have demonstrated that Y400 and L402 are key cdE2 residues involved in the recruitment of nucleocapsids to the plasma membrane of infected cells, presumably through direct protein contacts with nucleocapsids (*15*, *22*). On the basis of these mutagenesis data and SCP−SCP interactions observed in the SCP crystal structure, the conserved cdE2 residues Y400 and L402 have been hypothesized to bind within a hydrophobic pocket on the surface of the SCP 114−264 proteolytic domain (*22*, *31*−*33*). To examine this aspect of the alphavirus assembly model, cdE2 peptides containing the tyrosine motif were tested for binding to SCP constructs containing the hydrophobic pocket.

NMR chemical shift is exquisitely sensitive to environment, and so NMR spectroscopy was used to investigate the association of SCP 114−264, which contains the hydrophobic pocket, with E2/6, which contains Y400 and L402 (Figure 3a). The lack of spectral changes for E2/6 resonances in the

presence of SCP 114−264 (Figure 3) establishes that E2/6 does not bind SCP 114−264. Furthermore, neither SCP 114−264 nor SCP 19−264 shows higher affinity for phospholipid membranes in the presence of pal-E2/12, a peptide that also contains Y400 and L402 (Figure 4e). Thus, the N-terminal residues of cdE2 do not appear to be sufficient for binding SCP. These findings are consistent with previously reported biochemical data that show that the first 13 amino acids of cdE2 are not sufficient for binding SFV nucleocapsids in vitro (*21*). The data from the present binding studies find no evidence that Y400 and L402 function in viral assembly through an initial protein−protein interaction with the SCP hydrophobic pocket, suggesting that these two critical residues must mediate viral assembly through some other mechanism. In addition, the binding data do not support the previously reported hypothesis that peptides corresponding to the N-terminal region of cdE2 block viral release in infected cells via competitive inhibition of SCP−cdE2 interactions (*30*). The binding data also fail to show that the SCP hydrophobic pocket is sufficient for establishing protein−protein contacts with other regions of cdE2, as neither $(pal)_3$-E2/33 nor $(pal)_2$-E2/16 enhances the binding of SCP 114−264 to phospholipid membranes in vitro.

Y400 and L402 are required for proper association of nucleocapsids with the plasma membrane of infected cells, but the binding studies described here suggest that association of this cdE2 tyrosine motif with the hydrophobic pocket of isolated SCP cannot be an initial step for such nucleocapsid association. One possible explanation for the role of Y400 and L402 in nucleocapsid recruitment may be that the cdE2 tyrosine motif functions by making critical lateral interactions with other transmembrane components within the trimeric spike complex (e.g., E1 or 6K) which would not be detected by the experiments reported here. Lateral interactions between spike protein components have been previously shown to control the recruitment of nucleocapsids to the inner leaflet of the plasma membrane (*16*, *18*, *19*) as well as play an important role in efficient viral budding (*17*, *19*, *52*). In this vein, lateral interactions involving the tyrosine motif may possibly influence nucleocapsid recruitment to the plasma membrane by modulating the presentation of the C-terminal region of cdE2 to the capsid protein, thus providing indirect control of cdE2−capsid protein binding. In support of such a hypothesis, construction of chimeric alphaviruses (*18*, *19*) or of an E1 deletion mutant (*16*) where lateral interactions between spike proteins are disrupted shows a phenotype identical to that produced by tyrosine motif mutations (*15*, *22*), i.e., assembled nucleocapsids dissociated from internal or plasma membranes of infected cells. Indeed, recent cryoelectron microscopy studies that show the close association of the E1 and E2 transmembrane domains, as well as contact between the E2 ectodomain and the E1 fusion domain, have brought a new appreciation for the role of lateral E1−E2 interactions in alphavirus structure (*28*, *53*).

Despite the lack of binding data to support the hypothesized interaction between the cdE2 tyrosine motif and the SCP hydrophobic pocket, the occurrence of this interaction is not completely ruled out: the SCP hydrophobic pocket could be the final lodging point of the cdE2 tyrosine motif in the mature virus as described in previous docking studies (*28*), but in such a case other factors must be required prior to such an interaction. Association of the tyrosine motif in the SCP pocket may involve the stabilization of collective interactions of glycoproteins and SCP in the assembled virion. An initial SCP contact with the C-terminal region of cdE2 could trigger further rearrangement events that ultimately dock the cdE2 tyrosine motif within the SCP hydrophobic pocket at a later assembly step. Conceivably, protein−protein interactions between cdE2 and SCP residues 114−264 may occur only in fully mature virions, not in intermediate stages of alphavirus budding, and hence would not be detectable by the binding assays employed in this study.

Specificity of the cdE2−Capsid Interaction. The capsid proteins of RRV and SINV show 56% sequence identity, and cross-reactivity between alphavirus structural components (*35*) as well as between SFV cdE2 and nucleocapsids from other enveloped viruses (*21*) has been previously demonstrated. We examined the ability of $(pal)_2$-E2/16 to enhance membrane association of RRCP 1−270 and found that $(pal)_2$-E2/16 increased affinity by amounts similar to that observed with SCP 19−264 (Figure 5b). The results from the present study suggest that both RRCP and SCP can associate with the C-terminal region of SINV cdE2, consistent with the previously reported cross-reactivity (*21*, *35*). Specificity of the cdE2−capsid interaction was further examined by using $(pal)_2$-E2/16SCR, a peptide construct containing a "scrambled" E2/16 sequence that preserved the position of $C_{416}C_{417}$ within the sequence. Results from these assays showed that $(pal)_2$-E2/16SCR could recognize SCP 19−264 (Figure 4f), a finding that runs counter to the prevailing hypothesis that the cdE2−capsid protein interaction is sequence-specific. However, the SCP−cdE2 interaction does not appear to be completely nonspecific, as SCP 19−264 fails to recognize the amino acid sequence contained in pal-E2/12 (Figures 4e and 5a). Furthermore, results from these assays show that the $(pal)_2$-E2/16SCR construct cannot recognize SCP 114−264 (Figure 4f), indicating that some particular recognition element within SCP 19−264 is required for complex formation.

Taken together, the results suggest that the alphavirus capsid−cdE2 interaction is not strictly sequence-specific. The absence of a clear, sequence-specific capsid−cdE2 recognition event in these experiments is consistent with previously reported in vitro and in vivo data that demonstrate cross-reactivity of alphavirus structural components (*21*, *35*). A possible explanation for these observations is that a stable capsid−cdE2 interaction may simply require a stretch of hydrophobic amino acids in cdE2, a modest criterion that can be satisfied by many different amino acid sequences. The notion of a cdE2−capsid interaction without strict sequence specificity directing alphavirus budding is not without precedence, as a similar mechanism has been previously proposed to drive VSV budding (*54*). Finally, another possible explanation for the above observations is that SCP somehow recognizes the tandem palmitoyl moieties present within both E2/16 and E2/16SCR, rather than a particular amino acid sequence within cdE2.

Protein−Membrane Interactions. Both RRCP and SCP are reported here to bind to PC liposomes and thus display an inherent affinity for membranes. The liposome binding assays show that SCP 19−264 has even higher affinity for Het membranes, which have a lipid composition similar to that found in the plasma membrane of eukaryotic cells. Con-

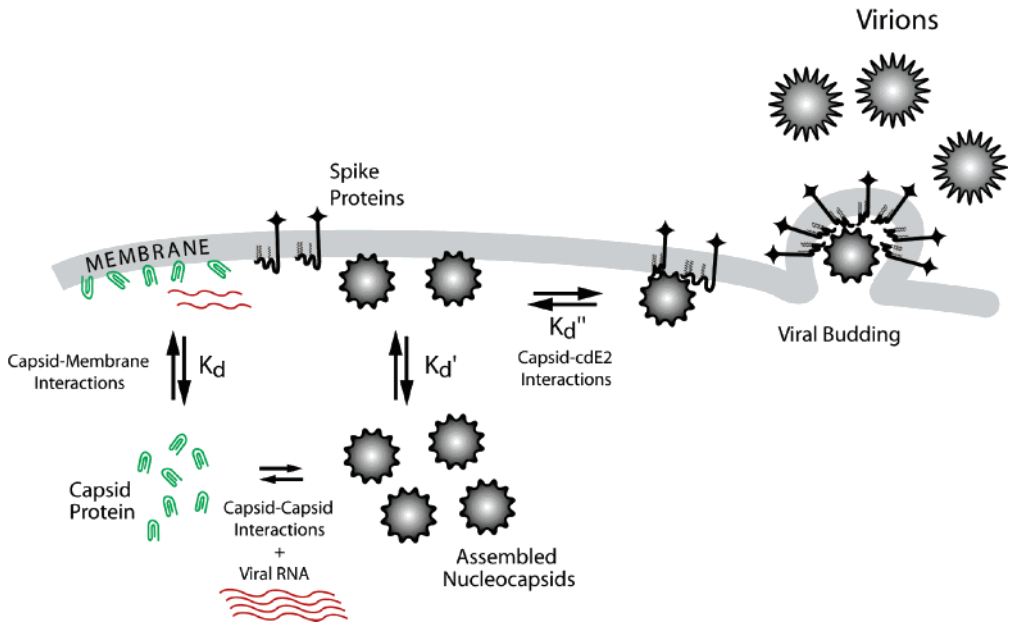

FIGURE 7: Possible model for membrane-facilitated alphavirus assembly and budding. The capsid protein is first recruited to the membrane, either as an isolated protein subunit (with a dissociation constant K_d) or in the context of a nucleocapsid (with a dissociation constant K_d'). After membrane attachment, capsid protein interactions with cdE2 take place with a dissociation constant K_d''.

versely, the constructs SCP 81−264 and SCP 114−264 display either slight or no affinity for membranes (Figures 4a,b and 5a), indicating that the membrane-targeting sequence lies within SCP 19−80 (Figure 6). The mechanism for the increased affinity of SCP 19−264 for Het membranes likely has a significant electrostatic component, as the N-terminal domain of the 19−264 construct contains numerous positively charged amino acids available to interact with the negatively charged surface of the Het vesicles. The SCP 81−264 and 114−264 constructs have fewer basic residues (Figure 6) and correspondingly have either little or no affinity for the negatively charged vesicles. These observations are consistent with expected results for an electrostatic mechanism of binding. In addition, hydrophobic interactions between SCP and phospholipid membranes also must contribute to binding since SCP 19−264 shows intermediate affinity for the electrically neutral surface of the PC vesicles (Figures 4a and 5a). Indeed, membrane association by mechanisms other than electrostatic attraction has been demonstrated for capsid or matrix proteins in other viral systems (e.g., the capsid protein of dengue virus) (*39*, *40*, *55*).

The membrane-binding property of SCP is strongly reminiscent of the interaction of capsid or matrix proteins with membranes (observed both in vitro and in vivo in other enveloped RNA viruses, such as influenza virus (*38*, *39*, *41*), VSV (*37*, *56*, *57*), and human immunodeficiency virus (HIV) (*42*, *43*, *58*). The association of matrix protein with the plasma membrane has been hypothesized to be a step in the viral assembly pathway in these systems.

Assembly Model. A long-held view of alphavirus assembly, first proposed by Garoff and Simons approximately 30 years ago, describes viral budding as being triggered by nucleocapsid interactions with exposed cytoplasmic regions of spike glycoproteins located at the plasma membrane (*23*). In this model, the nucleocapsid serves as a nucleation site for additional viral glycoprotein interactions, ultimately leading to the release of a mature virion from the cell surface. Active participation of the membrane bilayer in functional macromolecular interactions is not a featured component of this particular model for alphavirus budding.

In light of the protein−membrane interactions demonstrated in the present study, we propose an extended model for alphavirus assembly that incorporates SCP−membrane interactions as well as SCP−cdE2 interactions (Figure 7). This model features initial SCP association with the membrane, either as an isolated protein subunit (with a dissociation constant K_d) or in the context of a fully assembled nucleocapsid (with a dissociation constant K_d'). We hypothesize that isolated SCP subunits can potentially participate in functionally important protein−membrane interactions, with formation of additional nucleocapsids from individual SCP subunits and viral RNA occurring in a subsequent assembly step at the membrane surface. In addition, given previous observations of nucleocapsids aligned along internal membranes within infected cells (*22*), we hypothesize that the initial SCP−membrane interaction can take place at internal cellular membranes (e.g., Golgi membranes) as well as at the plasma membrane.

Regardless of the precise intracellular site and biochemical pathway for nucleocapsid formation, we envision membrane recruitment mediated through cdE2 interaction with SCP to promote subsequent formation of nucleocapsids and eventual budding (with a dissociation constant K_d''; Figure 7). As we find no evidence that the tyrosine region of cdE2 has an affinity high enough to bind the hydrophobic pocket of SCP in the case of isolated molecules, we propose that the initial cdE2−SCP interaction is mediated through the C-terminal residues of cdE2 and residues 19−80 of SCP. A final rearrangement of trimeric E1/E2 spike complexes and nucleocapsid could orient SCP subunits so that the hydrophobic pocket is near the glycoprotein transmembrane helices, as indicated in the cryoEM fitted structure (*28*).

Our proposed alphavirus assembly model features virion maturation and budding as being driven by the combined contribution of low-affinity SCP−membrane and SCP−cdE2 binding events. A similar assembly pathway may be utilized by influenza virus: in vivo studies show that matrix protein association with the plasma membrane is measurably enhanced by the presence of the envelope glycoproteins hemagglutinin and neuraminidase (*38*). The role of the membrane in viral assembly has only recently been explored in alphavirus (*59*, *60*), and further studies are needed to fully understand capsid−membrane interactions during assembly and in the virus particle.

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REFERENCES

1. Garoff, H., Hewson, R., and Opstelten, D.-J. E. (1998) Virus Maturation by Budding, *Microbiol. Mol. Biol. Rev. 62*, 1171−1190.
2. Strauss, J. H., Strauss, E. G., and Kuhn, R. J. (1995) Budding of Alphaviruses, *Trends Microbiol. 3*, 346−350.
3. Schlesinger, S., and Schlesinger, M. J. (1986) *The Togaviridae and Flaviviridae*, Plenum Press, New York.
4. Strauss, J. S., and Strauss, E. G. (1994) The Alphaviruses: Gene Expression, Replication, and Evolution, *Microbiol. Rev. 58*, 491−562.
5. Waite, M. R. F., and Pfefferkorn, E. R. (1970) Inhibition of Sindbis Virus Production by Media of Low Ionic Strength: Intracellular Events and Requirements for Reversal, *J. Virol. 5*, 60−71.
6. Waite, M. R. F., Brown, D. T., and Pfefferkorn, E. R. (1972) Inhibition of Sindbis Virus Release by Media of Low Ionic Strength: an Electron Microscope Study, *J. Virol. 10*, 537−544.
7. Li, M.-l., and Stollar, V. (1995) A Mutant of Sindbis Virus Which Is Released Efficiently from Cells Maintained in Low Ionic Strength Medium, *Virology 210*, 237−243.
8. Vashishtha, M., Phalen, T., Marquardt, M. T., Ryu, J. S., Ng, A. C., and Kielian, M. (1998) A Single Point Mutation Controls the Cholesterol Dependence of Semliki Forest Virus Entry and Exit, *J. Cell Biol. 140*, 91−99.
9. Garry, R. F., Bostick, D. A., Schram, R., and Waite, M. R. F. (1985) The Ratio of Plasma Membrane Cholesterol to Phospholipid and the Inhibition of Sindbis Virus Maturation by Low NaCl Medium, *J. Gen. Virol. 66*, 1171−1177.
10. Marquardt, M. T., Phalen, T., and Kielian, M. (1993) Cholesterol Is Required in the Exit Pathway of Semliki Forest Virus, *J. Cell Biol. 123*, 57−65.
11. Gaedigk-Nitschko, K., Ding, M., Levy, M. A., and Schlesinger, M. J. (1990) Site-Directed Mutations in the Sindbis Virus 6K Protein Reveal Sites for Fatty Acylation and the Underacylated Protein Affects Virus Release and Virion Structure, *Virology 175*, 282−291.
12. Gaedigk-Nitschko, K., and Schlesinger, M. J. (1991) Site-Directed Mutations in Sindbis Virus E2 Glycoprotein's Cytoplasmic Domain and the 6K Protein Lead to Similar Defects in Virus Assembly and Budding, *Virology 183*, 206−214.
13. Ivanova, L., and Schlesinger, M. J. (1993) Site-Directed Mutations in the Sindbis Virus E2 Glycoprotein Identify Palmitoylation Sites and Affect Virus Budding, *J. Virol. 67*, 2546−2551.
14. Ryan, C., Ivanova, L., and Schlesinger, M. J. (1998) Effects of the site-directed mutations of transmembrane cysteines in Sindbis virus E1 and E2 glycoproteins on palmitoylation and virus replication, *Virology 249*, 62−67.
15. Zhao, H., Lindqvist, B., Garoff, H., von Bonsdorff, C.-H., and Liljeström, P. (1994) A tyrosine-based motif in the cytoplasmic domain of the alphavirus envelope protein is essential for budding, *EMBO J. 13*, 4204−4211.
16. Barth, B. U., and Garoff, H. (1997) The Nucleocapsid-Binding Spike Subunit E2 of Semliki Forest Virus Requires Complex Formation with the E1 Subunit for Activity, *J. Virol. 71*, 7857−7865.
17. Kim, K. H., Strauss, E. G., and Strauss, J. H. (2000) Adaptive Mutations in Sindbis Virus E2 and Ross River Virus E1 That Allow Efficient Budding of Chimeric Viruses, *J. Virol. 74*, 2663−2670.
18. Yao, J., Strauss, E. G., and Strauss, J. H. (1998) Molecular Genetic Study of the Interaction of Sindbis Virus E2 with Ross River E1 for Virus Budding, *J. Virol. 72*, 1418−1423.
19. Yao, J. S., Strauss, E. G., and Strauss, J. H. (1996) Interactions between PE2, E1, and 6K Required for Assembly of Alphaviruses Studied with Chimeric Viruses, *J. Virol. 70*, 7910−7920.
20. Suomalainen, M., and Garoff, H. (1992) Alphavirus Spike-Nucleocapsid Interaction and Network Antibodies, *J. Virol. 66*, 5106−5109.
21. Metsikkö, K., and Garoff, H. (1990) Oligomers of the Cytoplasmic Domain of the p62/E2 Membrane Protein of Semliki Forest Virus Bind to the Nucleocapsid In Vitro, *J. Virol. 64*, 4678−4683.
22. Owen, K. E., and Kuhn, R. J. (1997) Alphavirus Budding is Dependent on the Interaction between the Nucleocapsid and Hydrophobic Amino Acids on the Cytoplasmic Domain of the E2 Envelope Glycoprotein, *Virology 230*, 187−196.
23. Garoff, H., and Simons, K. (1974) Location of the Spike Glycoproteins in the Semliki Forest Virus Membrane, *Proc. Natl. Acad. Sci. U.S.A. 71*, 3988−3992.
24. Kail, M., Hollinshead, M., Ansorge, W., Pepperkok, R., Frank, R., Griffiths, G., and Vaux, D. (1991) The cytoplasmic domain of alphavirus E2 glycoprotein contains a short linear recognition signal required for viral budding, *EMBO J. 10*, 2343−2351.
25. Suomalainen, M., Liljeström, P., and Garoff, H. (1992) Spike Protein-Nucleocapsid Interactions Drive the Budding of Alphaviruses, *J. Virol. 66*, 4737−4747.
26. Cheng, R. H., Kuhn, R. J., Olson, N. H., Rossmann, M. G., Choi, H.-K., Smith, T. J., and Baker, T. S. (1995) Nucleocapsid and Glycoprotein Organization in an Enveloped Virus, *Cell 80*, 621−630.
27. Rice, C. M., Bell, J. R., Hunkapiller, M. W., Strauss, E. G., and Strauss, J. H. (1982) Isolation and Characterization of the Hydrophobic COOH-terminal Domains of the Sindbis Virion Glycoproteins, *J. Mol. Biol. 154*, 355−378.
28. Zhang, W., Mukhopadhyay, S., Pletnev, S. V., Baker, T. S., Kuhn, R. J., and Rossmann, M. G. (2002) Placement of the Structural Proteins in Sindbis Virus, *J. Virol. 76*, 11645−11658.
29. Liu, L. N., Lee, H., Hernandez, R., and Brown, D. T. (1996) Mutations in the Endo Domain of Sindbis Virus Glycoprotein E2 Block Phosphorylation, Reorientation of the Endo Domain, and Nucleocapsid Binding, *Virology 222*, 236−246.
30. Collier, N. C., Adams, S. P., Weingarten, H., and Schlesinger, M. J. (1992) Inhibition of enveloped RNA virus formation by peptides corresponding to glycoprotein sequences, *Antiviral Chem. Chemother. 3*, 31−36.
31. Lee, S., Owen, K. E., Choi, H.-K., Lee, H., Lu, G., Wengler, G., Brown, D. T., Rossmann, M. G., and Kuhn, R. J. (1996) Identification of a protein binding site on the surface of the alphavirus nucleocapsid and its implication in virus assembly, *Structure 4*, 531−541.
32. Lee, S., Kuhn, R. J., and Rossmann, M. G. (1998) Probing the potential glycoprotein binding site of sindbis virus capsid protein with dioxane and model building, *Proteins 33*, 311−137.
33. Skoging, U., Vihinen, M., Nilsson, L., and Liljeström, P. (1996) Aromatic interactions define the binding of the alphavirus spike to its nucleocapsid, *Structure 4*, 519−529.
34. Frolov, I., Frolova, E., and Schlesinger, S. (1997) Sindbis Virus Replicons and Sindbis Virus: Assembly of Chimeras and of Particles Deficient in Virus RNA, *J. Virol. 71*, 2819−2829.
35. Lopez, S., Yao, J.-S., Kuhn, R. J., Strauss, E. G., and Strauss, J. H. (1994) Nucleocapsid-Glycoprotein Interactions Required for Assembly of Alphaviruses, *J. Virol. 68*, 1316−1323.
36. Smyth, J., Suomalainen, M., and Garoff, H. (1997) Efficient Multiplication of a Semliki Forest Virus Chimera Containing Sindbis Virus Spikes, *J. Virol. 71*, 818−823.
37. Chong, L. D., and Rose, J. K. (1993) Membrane Association of Functional Vesicular Stomatitis Virus Matrix Protein In Vivo, *J. Virol. 67*, 407−414.
38. Enami, M., and Enami, K. (1996) Influenza Virus Hemagglutinin and Neuraminidase Glycoproteins Stimulate the Membrane Association of the Matrix Protein, *J. Virol. 70*, 6653−6657.

39. Faaberg, K. S., and Peeples, M. E. (1988) Association of Soluble Matrix Protein of Newcastle Disease Virus with Liposomes Is Independent of Ionic Conditions, *Virology 166*, 123−132.
40. Markoff, L., Falgout, B., and Chang, A. (1997) A Conserved Internal Hydrophobic Domain Mediates the Stable Membrane Integration of the Dengue Virus Capsid Protein, *Virology 233*, 105−117.
41. Sha, B., and Luo, M. (1997) Structure of a bifunctional membrane-RNA binding protein, influenza virus matrix protein M1, *Nat. Struct. Biol. 4*, 239−244.
42. Zhou, W., Parent, L. J., Wills, J. W., and Resh, M. D. (1994) Identification of a Membrane-binding Domain within the Amino-Terminal Region of Human Immunodeficiency Virus Type 1 Gag Protein Which Interacts with Acidic Phospholipids, *J. Virol. 68*, 2556−2569.
43. Zhou, W., and Resh, M. D. (1996) Differential Membrane Binding of the Human Immunodeficiency Virus Type 1 Matrix Protein, *J. Virol. 70*, 8540−8548.
44. Wilkinson, T. A., Yin, J., Pidgeon, C., and Post, C. B. (2000) Alkylation of Cysteine-Containing Peptides to Mimic Palmitoylation, *J. Pept. Res. 55*, 140−147.
45. Tellinghuisen, T. L., Hamburger, A. E., Fisher, B. R., Ostendorp, R., and Kuhn, R. J. (1999) In vitro assembly of alphavirus cores by using nucleocapsid protein expressed in *Escherichia coli*, *J. Virol. 73*, 5309−5319.
46. Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, John Wiley & Sons, New York.
47. Fields, R. (1971) The Rapid Determination of Amino Groups with TNBS, *Methods Enzymol. 25*, 464−468.
48. Barenholz, Y., Gibbes, D., Litman, B. J., Goll, J., Thompson, T. E., and Carlson, F. D. (1977) A Simple Method for the Preparation of Homogeneous Phospholipid Vesicles, *Biochemistry 16*, 2806−2810.
49. David, A. E. (1971) Lipid Composition of Sindbis Virus, *Virology 46*, 711−720.
50. Forsell, K., Griffiths, G., and Garoff, H. (1996) Preformed cytoplasmic nucleocapsids are not necessary for alphavirus budding, *EMBO J. 15*, 6495−6505.
51. Skoging-Nyberg, U., and Liljeström, P. (2001) M-X-I Motif of Semliki Forest Virus Capsid Protein Affects Nucleocapsid Assembly, *J. Virol. 75*, 4625−4632.
52. Ekström, M., Liljeström, P., and Garoff, H. (1994) Membrane Protein Lateral Interactions Control Semliki Forest Virus Budding, *EMBO J. 13*, 1058−1064.
53. Mancini, E. J., Clarke, M., Gowen, B. E., Rutten, T., and Fuller, S. D. (2000) Cryo-electron Microscopy Reveals the Functional Organization of an Enveloped Virus, Semliki Forest Virus, *Mol. Cell 5*, 255−266.
54. Schnell, M. J., Buonocore, L., Boritz, E., Ghosh, H. P., Chernish, R., and Rose, J. K. (1998) Requirement for a Non-specific Glycoprotein Cytoplasmic Domain Sequence To Drive Efficient Budding of Vesicular Stomatitis Virus, *EMBO J. 17*, 1289−1296.
55. Gregoriades, A. (1980) Interaction of Influenza M Protein with Viral Lipid and Phosphatidylcholine Vesicles, *J. Virol. 36*, 470−479.
56. Chong, L. D., and Rose, J. K. (1994) Interactions of Normal and Mutant Vesicular Stomatitis Matrix Proteins with the Plasma Membrane and Nucleocapsids, *J. Virol. 68*, 441−447.
57. Justice, P. A., Sun, W., Li, Y., Ye, Z., Grigera, P. R., and Wagner, R. R. (1995) Membrane Vesiculation Function and Exocytosis of Wild-Type and Mutant Matrix Proteins of Vesicular Stomatitis Virus, *J. Virol. 69*, 3156−3160.
58. Scarlata, S., Ehrlich, L. S., and Carter, C. A. (1998) Membrane-Induced Alterations in HIV-1 Gag and Matrix Protein−Protein Interactions, *J. Mol. Biol. 277*, 161−169.
59. Lu, Y. E., and Kielian, M. (2000) Semliki Forest Virus Budding: Assay, Mechanisms, and Cholesterol Requirement, *J. Virol. 74*, 7708−7719.
60. Kielian, M., Chatterjee, P. K., Gibbons, D. L., and Lu, Y. E. (2000) Specific Roles for Lipids in Virus Fusion and Exit. Examples from the Alphaviruses, *Subcell. Biochem. 34*, 409−455.

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