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A Putative Receptor for Venezuelan Equine Encephalitis Virus from Mosquito Cells

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We have identified a cellular protein from a continuous mosquito cell line (C6/36) that appears to play a significant role in the attachment of Venezuelan equine encephalitis (VEE) virus to these cells. VEE virus bound to a 32-kDa polypeptide present in the C6/36 plasma membrane fraction, and binding to this polypeptide was dose dependent and saturable and competed with homologous and heterologous alphaviruses. These observations suggest that this polypeptide binds virus via a receptor-ligand interaction. The 32-kDa polypeptide was expressed on the surfaces of C6/36 cells, and monoclonal antibodies directed against either this cell polypeptide or the VEE virus E2 glycoprotein, which is thought to be the viral attachment protein, interfered with virus attachment. Collectively, these data provide evidence suggesting that the 32-kDa polypeptide serves as a receptor for VEE virus infection of cells. We have characterized this cell polypeptide as a laminin-binding protein on the basis of its ability to interact directly with laminin as well as its immunologic cross-reactivity with the high-affinity human laminin receptor.

The ability of viruses to bind to host cells is controlled by the interaction of viral attachment proteins with cellular receptor proteins (CRP). The interaction between viral attachment proteins and CRP is thought to influence tissue tropism and/or host range for many viruses (12, 14, 20, 22, 36, 39, 40). This phenomenon is particularly evident for some mammalian viruses, for which the ability of the virus to bind to distinct host cells (1, 4, 9, 30) plays an important role in viral pathogenesis (5).

The Venezuelan equine encephalitis (VEE) virus complex is a group of serologically related alphaviruses in the family *Togaviridae*. In nature, these viruses are transmitted between susceptible vertebrate hosts by a variety of mosquito species (40). Virions are spherical (60 to 65 nm in diameter) and are composed of an icosahedral nucleocapsid containing a positive-stranded RNA genome. The nucleocapsid is surrounded by a lipid envelope containing two structural glycoproteins, E1 and E2. Certain domains of these two glycoproteins are exposed on the surface of virions and are responsible for the interaction of virus with neutralizing antibody. It has been reported previously that at least one of the E2 domains modulates the attachment of virus to cells (35).

VEE virus is typical of arboviruses in that transmission, dissemination, and amplification require cyclic passage through vertebrate and invertebrate hosts. The fact that infections of unrelated organ systems occur in such dissimilar hosts suggests either that these viruses utilize different strategies for attachment to different host cells or that they attach to cellular components which are highly conserved among phylogenetically distinct species. An understanding of the molecular basis of host specificity, vector competence, and tissue tropisms of VEE virus would be greatly facilitated by a better understanding of virus-receptor interactions.

Studies have shown that alphavirus attachment to cells is strongly dependent on the ionic strength. Pierce et al. (32) studied the effects of altering the ionic concentration on Sindbis (SIN) virus attachment to chick cells. They found that loosely bound virus could be washed off cells with buffers with an ionic strength of 0.2 or greater, whereas tightly bound virus remained attached under such conditions. They hypothesized that the loose binding of virus might be nonspecific adsorption of the virus (unrelated to virus infection) or that attachment might involve both loose and tight binding, perhaps sequentially.

Several investigators have identified potential alphavirus receptor proteins. Helenius et al. (18) suggested that murine and human histocompatability antigens serve as alphavirus receptors. Ubol and Griffin (42) identified two proteins that may serve the same function on mouse neuronal cells. Other investigators have studied the characteristics of virus attachment to cells without attempting to identify the receptor molecule (27, 38). In the most recent research on alphavirus receptors, Wang et al. (43) suggested that the high-affinity laminin receptor functions as a CRP for SIN virus infection of BHK cells. Here we describe the identification of a polypeptide from mosquito cells that appears to serve as an important mediator of VEE virus attachment and discuss the implications of these findings for the biology and ecology of VEE virus.

MATERIALS AND METHODS

Virus. A second BHK cell culture passage of the epizootic VEE virus subtype 1A Trinidad donkey strain was used. This strain, V3000, was derived from a full-length molecular clone (8) and was indistinguishable from the parent Trinidad donkey strain in terms of its pathogenicity in mice, hamsters (7), and horses (unpublished data). Some experiments used a second BHK cell culture passage of SIN virus that was similarly obtained from a full-length molecular clone (TR2000) (33). The type 1 poliovirus Maloney strain was used as a control in competition studies.

VEE and SIN viruses were grown in 95% confluent monolayers of BHK or Vero cells. The medium was removed from cell culture flasks or roller bottles. VEE virus was diluted in minimum essential medium with Earl's salts (EMEM) with nonessential amino acids (GIBCO/BRL, Gaithersburg, Md.) containing 5% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (50 μg/ml), and amphotericin B (0.25 μg/ml) and then adsorbed to cell monolayers at a multiplicity of infection of 10. Cells were incubated at 37°C, and supernatant fluids were harvested at 24 to 28 h postinfection for virus stocks or purification.

Poliovirus was grown in 95% confluent monolayers of HeLa cells in roller

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bottles essentially as described above for VEE and SIN viruses, except that (i) the incubation period after infection was reduced from 24 to 28 h to 12 to 15 h and (ii) virus was released from cells in a freeze-thaw cycle prior to purification.

For some experiments, VEE virus was intrinsically labeled with either [^{35}S]cysteine or $^{32}P_i$. Cell infection proceeded as described above, except that cell monolayers were washed once in cysteine-free EMEM (^{35}S label) or phosphate-free EMEM (^{32}P label) prior to infection. After infection, cysteine- or phosphate-deficient medium containing 25 μCi of [^{35}S]cysteine per ml and 1% fetal bovine serum or containing 36 μCi of $^{32}P_i$ per ml with 1% fetal bovine serum was added to flasks. Cells were then harvested as described above.

For virus purification, infected cell culture supernatants were pooled and clarified by centrifugation at 10,000 × *g* for 30 min. Polyethylene glycol (average molecular weight, 8,000) and NaCl were added to clarified supernatant to 7 and 2.3%, respectively. Virus was then precipitated with stirring overnight at 4°C. Precipitated virus was pelleted by centrifugation at 10,000 × *g* for 30 min and suspended in Dulbecco's phosphate-buffered saline (PBS). Virus was then purified by centrifugation through a 20 to 60% (wt/wt) sucrose density gradient for 3.5 h at 100,000 × *g*. Sucrose solutions were prepared in Hanks' balanced salt solution (HBSS) without $NaHCO_3$ containing 0.02 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.2). After centrifugation, the virus band was harvested and its purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (21).

Cell membrane protein preparation. Cells were scraped from cell culture flasks or roller bottles, pelleted at 300 × *g* for 5 min, and washed once in cell homogenization buffer (10 mM Tris-HCl, 2 mM EGTA, 250 mM sucrose, and protease inhibitor cocktail [0.5 μg of pepstatin per ml, 5 μg (each) of leupeptin, chymotrypsin, antipain, and aprotinin per ml, and 9 μg of phenylmethylsulfonyl fluoride per ml (15)] [pH 7.4]). Cells were suspended in 10 ml of cell homogenation buffer and disrupted with 100 strokes of a stainless steel Dounce homogenizer. The homogenate was then sequentially centrifuged at 800 × *g* for 10 min (nuclear pellet), at 10,000 × *g* for 15 min (mitochondrial pellet), at 30,000 × *g* for 30 min (microsomal pellet), and at 100,000 × *g* for 90 min (membrane pellet). Each pellet was suspended in 5 ml of deionized water containing protease inhibitors (as described above), divided into 0.5-ml aliquots, and frozen at −70°C until needed. For some experiments, proteins were solubilized directly from pellets in 0.1% trifluoroacetic acid.

Binding assays. To identify cell polypeptides capable of binding virus, a protein blot–virus-binding assay was developed. For this assay, cell membrane proteins from fractionated pellets were separated by SDS-PAGE. Polypeptides were then blotted onto nitrocellulose sheets by using a semidry blotting apparatus (Pharmacia/LKB, Piscataway, N.J.) in 48 mM Tris–39 mM glycine–10% (vol/vol) methanol. The nitrocellulose was then blocked overnight at 4°C in PBS containing 5% skim milk (Difco, Detroit, Mich.). After blocking, the nitrocellulose membrane was washed once for 5 min with wash solution (PBS–1% skim milk) and once in high-salt wash solution (PBS–1% skim milk–220 mM NaCl [final]). The membrane was incubated for 1 h at 37°C with high-salt wash buffer containing 25 μg of purified VEE virus per ml and then washed three times with high-salt wash buffer and once with wash buffer. To detect virus binding directly, radiolabeled virus was used and the nitrocellulose was dried and exposed to X-ray film. In other experiments, virus binding was detected indirectly by immersing nitrocellulose membranes in anti-VEE virus hyperimmune mouse ascitic fluid (ATCC VR-1249 AF) diluted 1:10,000 in wash buffer for 1 h at 37°C, followed by three 5-min washes in wash buffer. A secondary sheep anti-mouse immunoglobulin conjugated with horseradish peroxidase (Amersham, Arlington Heights, Ill.) was diluted 1:4,000 in wash buffer, incubated with nitrocellulose for 1 h at 37°C, and then washed one time in wash buffer and three times in PBS. Virus binding was visualized on high-speed X-ray film (OMC; Eastman Kodak, Rochester, N.Y.) by using an enhanced chemiluminescent substrate (Amersham) according to the manufacturer's recommendations. Immunobinding assays were performed as described above, except that wash solution was used in place of the high-salt wash solution in all steps and monoclonal antibody supernatants or polyclonal sera were used in place of virus.

The protein blot–laminin-binding assay was performed under the same conditions and protocol used for the protein blot–virus-binding assay. Laminin and the anti-laminin detector antibody were purchased from Sigma Chemical Co. (St. Louis, Mo.; catalog no. L-2020 and L-9393, respectively).

Direct binding of virus to cells. Direct binding studies were carried out to characterize the attachment of VEE virus to C6/36 and BHK cells. For these studies, confluent monolayers of C6/36 or BHK cells were prepared in 96-well plates. Before incubation with virus, the medium was removed from monolayers and 300 μl of blocking buffer (HBSS adjusted to 220 mM NaCl–20 mM HEPES–1% fish gelatin [Sigma] [pH 7.4]; for some experiments, the NaCl concentration of HBSS was not adjusted) was added to each well to prevent nonspecific binding of virus to plates. After 1 h at room temperature, the blocking buffer was removed, the plates were cooled to 4°C, and 60 μl of radiolabeled, gradient-purified virus suspended in ice-cold blocking buffer was added to appropriate wells. Virus was adsorbed to cells for 1 h at 4°C, and cells were then washed three times with ice-cold blocking buffer. After being washed, cells were solubilized in 100 μl of PBS containing 1% SDS, and bound radioactivity was counted. Assays were conducted in quadruplicate.

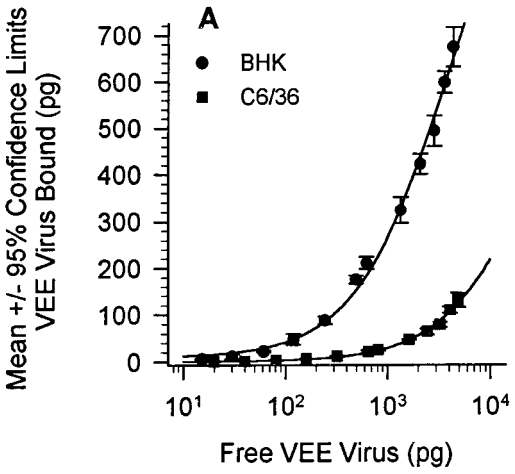
Monoclonal antibody production. BALB/c mice were twice immunized intraperitoneally with 100 μg of crude C6/36 membrane preparation emulsified in Freund's complete adjuvant for primary immunization and Freund's incomplete adjuvant for secondary immunization. Two weeks later, mice received an intravascular immunization of 100 μg of C6/36 membrane preparation. Three days later, mice were euthanized, and splenocytes were fused with Sp2/0-Ag14 myeloma cells as described previously (11). Hybridoma cultures were incubated at 37°C with several changes of hypoxanthine-aminopterin-thymidine medium, and the supernatant fluids were screened by enzyme-linked immunosorbent assay (ELISA), with live C6/36 cells as the antigen as described below. Selected hybridoma cultures were cloned by limiting dilution. All selected monoclonal antibodies were of the immunoglobulin M isotype.

ELISA. Hybridoma culture supernatants were screened for anti-C6/36 cell activity by an ELISA using live C6/36 cells. Cells were placed into wells of a 96-well plate (10^5 cells per well) and centrifuged at 1,000 × *g* for 2 min. The cell culture medium was removed, and plate blocking buffer (PBS–5% skim milk) was added to each well. Plates were blocked for 1 h at room temperature. After the blocking step, cells were again centrifuged to the bottom of the plate, blocking solution was removed, and 50 μl of hybridoma supernatant was pipetted into each well. Supernatants were incubated with cells for 1 h at 4°C. Then cells were washed twice in ice-cold wash buffer (PBS–1% skim milk) and were centrifuged between each wash. Fifty microliters of goat anti-mouse horseradish peroxidase conjugate (Kirkegaard and Perry, Gaithersburg, Md.) diluted 1:800 in wash buffer was added to each well. Plates were again incubated for 1 h at 4°C and then washed twice in ice-cold wash buffer and once in cold PBS, with centrifugation again between washes. Antibody binding was detected with 50 μl of ABTS substrate (Kirkegaard and Perry) per well.

RESULTS

Interactions of virus with cells. A series of virus-binding studies were performed to determine the importance of receptor-ligand interactions for the attachment of VEE virus to C6/36 cells, a continuous mosquito cell line derived from *Aedes albopictus* (Diptera: Culicidae) embryonic tissue (37). These cells are susceptible to VEE virus infection and produce high titers of virus when infected. In the first set of experiments (Fig. 1), a constant number of cells was incubated with various amounts of ^{35}S-labeled VEE virus. Bound virus was quantified in quadruplicate by scintillation counting. Counts were averaged, and the results were converted to mass bound and expressed in the form of a Klotz plot (13). The data show that under isotonic conditions, C6/36 cells bound between 2 and 5% of the total input virus. In comparison, the same number of BHK cells bound between 15 and 40% of the total input virus, showing that these cells bind virus much more efficiently than do C6/36 cells under the conditions used. The data also show that the binding of VEE virus to C6/36 and BHK cells was dose dependent but not saturable (Fig. 1A). When the conditions of the test were altered by increasing the ionic strength to 220 mM, the amount of virus bound to C6/36 cells was less (0.2 to 0.7% of total input virus). Under these more stringent conditions (equivalent to those of Pierce et al. [32]), virus bound to C6/36 cells in both a dose-dependent and saturable fashion (Fig. 1B). The proportion of virus bound to cells was less than that observed in other studies of alphavirus attachment (32). This discrepancy is most likely a function of differences in the techniques used, although the possibility remains that it is due to differences in the relative binding affinities of different alphaviruses for the cells used.

Identification of cell membrane proteins with VEE virus-binding activities. Proteins with VEE virus-binding activities were identified by a protein blot–virus-binding assay. Under isotonic conditions, virus appeared to bind to proteins in a nonspecific manner (data not shown). However, when the ionic strength of the wash buffer was increased to the more stringent 220 mM used in the cell interaction studies described above, binding studies with ^{32}P-labeled VEE virus revealed a 32-kDa polypeptide from C6/36 cells that bound virus with a high degree of specificity (Fig. 2). In addition, the relative intensities of the bands seen in Fig. 2 suggest that the extent of virus binding to this protein was dose dependent. In subsequent experiments, binding to the 32-kDa polypeptide was quanti-



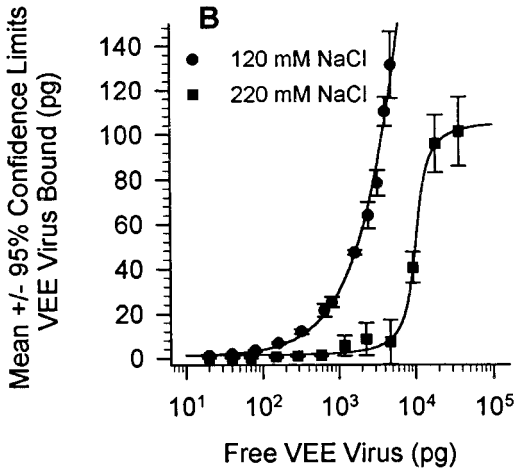


FIG. 1. Klotz plot of data from direct VEE virus-binding studies with BHK and C6/36 cells (A) and VEE virus-binding studies at two ionic concentrations in C6/36 cells (B). Twofold serial dilutions of ^{35}S-labeled VEE virus were incubated with confluent monolayers of C6/36 cells in 96-well plates. After incubation and washing, cells were lysed in 1% SDS and solubilized proteins were counted for radioactivity by scintillation counting. The counts from four replicates were averaged, and average counts were converted to picograms of virus bound on the basis of the specific activity of the intrinsically labeled virus.

tated by scintillation counting of radiolabeled bands excised from nitrocellulose membranes. The results confirmed that the binding of VEE virus to the 32-kDa polypeptide was both dose dependent and saturable (Fig. 3). In some experiments, less efficient binding to 12- to 14-, 40-, and 60-kDa polypeptides was also observed (Fig. 4 and 5). The relative amount of 32-kDa polypeptide observed and the relative proportion of 32-kDa polypeptide to other virus-binding proteins from a given membrane preparation were variable and appeared to differ with cell passage level and culture age. In all experiments, the 32-kDa polypeptide was the predominant band observed; as a result, we focused our attention on this protein. To avoid potential problems associated with these inconsistencies, all quantitative data were obtained with the same membrane preparation.

To support the concept that the 32-kDa polypeptide may

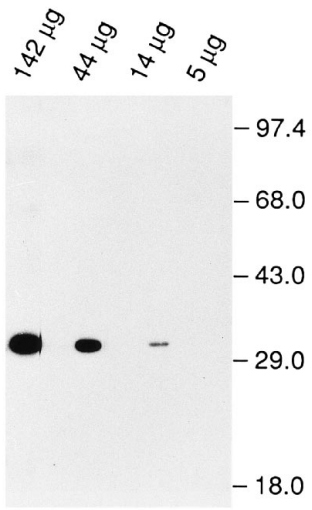


FIG. 2. Protein blot–virus-binding assay in which 100 μg of crude C6/36 membrane preparation per lane was separated by SDS-PAGE. Separated proteins were blotted onto nitrocellulose, and the nitrocellulose was probed with various amounts of ^{32}P-labeled VEE virus (shown above the lanes). Apparent molecular mass standards (in kilodaltons) are on the right.

function as a receptor for VEE virus, we performed competition experiments using homologous and heterologous alphaviruses and poliovirus (Fig. 6). In these studies, constant amounts of ^{35}S-labeled VEE virus and various amounts of

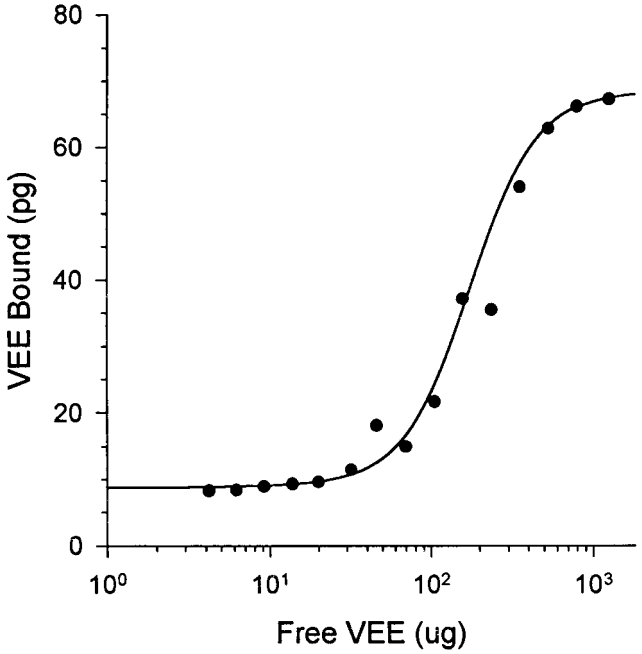


FIG. 3. Dose-response curve prepared from the results of a protein blot–virus-binding assay in which 100 μg of crude C6/36 membrane preparation per lane was separated by SDS-PAGE. Separated proteins were blotted onto nitrocellulose, and the nitrocellulose was probed with twofold serial dilutions of ^{32}P-labeled VEE virus. The 32-kDa polypeptides from all lanes, along with attached ^{32}P-labeled VEE virus, were excised and counted for radioactivity by scintillation counting. The counts per minute were converted to mass bound on the basis of ^{32}P-labeled VEE virus-specific activity. The results are presented in the form of a Klotz plot.

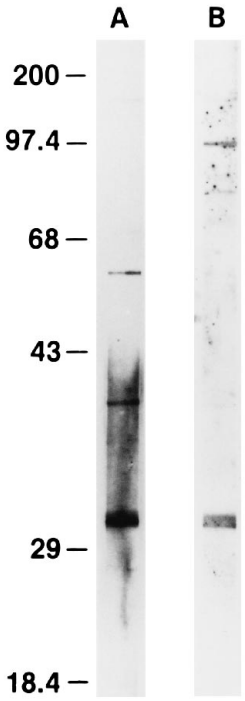


FIG. 4. Cross-reactivities of anti-human laminin receptor polyclonal antibodies with the 32-kDa polypeptide. Polyclonal rabbit antisera directed against the human high-affinity laminin receptor protein were used in an immunobinding assay. C6/36 membrane proteins were separated by SDS-PAGE and transferred to nitrocellulose. Then proteins were probed with antisera, and binding was detected indirectly (B). The results of a protein blot–VEE virus-binding assay (A) are included for comparison. Apparent molecular mass standards (in kilodaltons) are on the left.

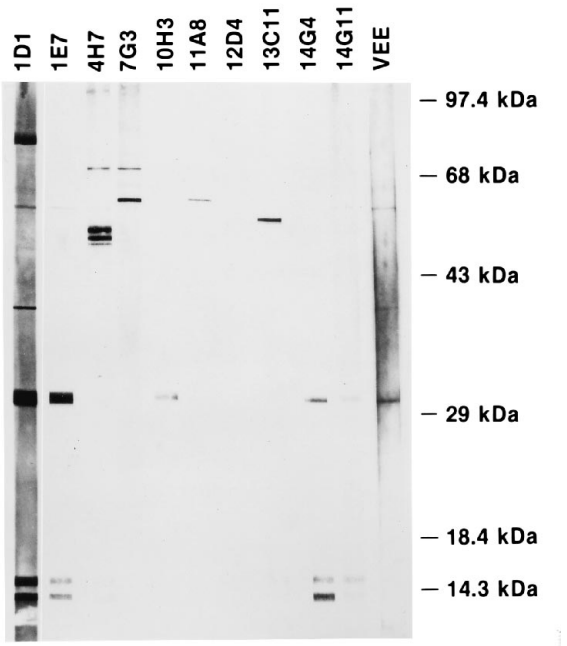


FIG. 5. Immunobinding assay with monoclonal antibodies directed against C6/36 membrane proteins. The transfer of C6/36 membrane proteins to nitrocellulose was completed as described above after separation by preparative SDS-PAGE. Undiluted hybridoma supernatants were incubated with strips of nitrocellulose, and binding was detected indirectly. Apparent molecular mass standards are on the right.

unlabeled VEE virus, SIN virus, or poliovirus were incubated with nitrocellulose strips containing electrophoretically separated C6/36 membrane proteins as described above. As the amount of unlabeled alphavirus increased, a corresponding decrease in the amount of labeled virus that bound to the 32-kDa polypeptide was observed. No such competitive relationship was observed with poliovirus. These studies, together with the observations described above, support the concept that the interaction of VEE virus with the 32-kDa polypeptide may be receptor mediated.

Function of the 32-kDa polypeptide. Recent studies by Wang et al. (43) suggest that the 67-kDa high-affinity laminin receptor of BHK cells serves as a receptor for SIN virus. Our results, which show that SIN virus can competitively inhibit the binding of VEE virus to a 32-kDa polypeptide, suggest either that SIN virus can bind to an alternate receptor molecule in mosquito cells or that the 32-kDa polypeptide is related to the 67-kDa laminin-binding protein. Therefore, we carried out studies to determine if the 32-kDa polypeptide could function as a laminin-binding protein. Under the same conditions used for the VEE virus-binding studies, laminin appeared to bind to the same 32-kDa polypeptide as did VEE virus (Fig. 7). These data suggest that the 32-kDa polypeptide is a laminin-binding molecule. In competition studies, however, laminin did not prevent or reduce the attachment of VEE virus to the 32-kDa polypeptide (data not shown). Polyclonal antibody directed against the high-affinity laminin receptor from human cells, provided by Hynda Kleinman, Laboratory of Developmental Biology and Anomalies, National Institute of Dental Research, National Institutes of Health, Bethesda, Md. (3), also reacted with the 32-kDa polypeptide from C6/36 cells in immunobinding assays (Fig. 4). Collectively, these data provide evidence that the 32-kDa polypeptide is a laminin-binding protein, although VEE virus and laminin appear to attach to different domains on this polypeptide. These data also suggest that the 32-kDa C6/36 polypeptide shares immunological and functional domains with laminin receptors from other host species.

Effects of anti-VEE virus monoclones on virus attachment. Monoclonal antibodies directed against the VEE virus E2c epitope, developed by John Roehrig, Centers for Disease Control and Prevention, Fort Collins, Colo., reduce the ability of ^{35}S-labeled VEE virus to bind to Vero cells (35). Monoclonal antibodies directed against other E2 epitopes had little or no effect on VEE virus binding to these cells, which suggests that the E2c domain serves as the major viral attachment domain. We determined the effects of two of these anti-E2c monoclonal antibodies, 1A4A-1 and 3B4C-1, on VEE virus attachment to the electrophoretically separated 32-kDa polypeptide as described above. For these experiments, VEE virus was preincubated with various dilutions of anti-E2c monoclonal antibody. The effects of antibody binding on virus attachment were then analyzed in a protein blot–virus-binding assay. The results obtained with 1A4A-1 (Fig. 8) show that this monoclonal antibody completely inhibited the binding of virus to the 32-kDa polypeptide over a range of dilutions (1:4 to 1:256), while an isotype-matched anti-E1 monoclonal antibody had no effect on virus binding. Results essentially the same as those obtained with monoclonal antibody 1A4A-1 were observed when we tested another E2c monoclonal antibody, 3B4C-1 (data not shown). Unexpectedly, however, we also observed that virus binding to the 32-kDa polypeptide was enhanced at moderate antibody dilutions, an effect that was reduced as the antibody was diluted further. Enhanced binding may have been caused by the cross-linking of virus particles by antibody in slight

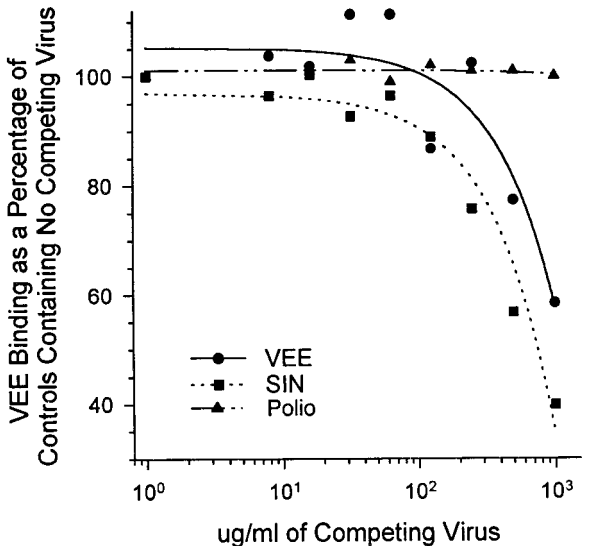


FIG. 6. Homologous and heterologous alphavirus competition with VEE virus binding to the 32-kDa polypeptide. For this experiment, 25 μg of ^{35}S-labeled VEE virus was mixed with twofold serial dilutions of unlabeled VEE or SIN virus and incubated with strips of nitrocellulose containing SDS-PAGE-separated C6/36 membrane proteins. After incubation and washing, radioactive protein bands corresponding to the 32-kDa polypeptide were excised and counted by scintillation counting. The results are expressed as percentages of controls in which no competing virus was used. Competition studies with poliovirus (Polio) utilized unlabeled VEE virus, whose binding was detected indirectly and quantified by lasar scanning densitometry. Both detection systems have been shown to return essentially identical results in multiple experiments. Data from both experiments were standardized and combined on one graph for the sake of brevity.

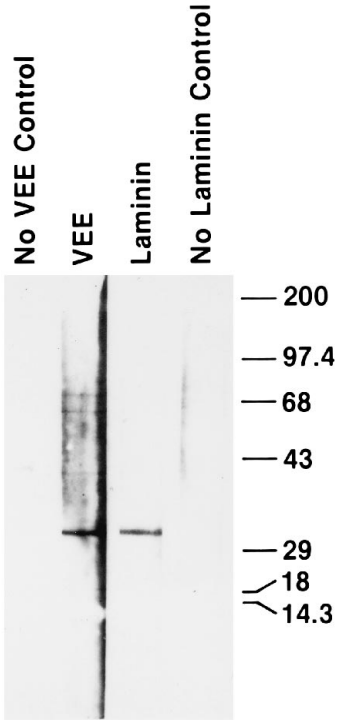


FIG. 7. Results of protein blot–virus-binding and protein blot–laminin-binding assays. This experiment demonstrates the ability of both 25 μg of gradiant-purified VEE virus and 350 ng of laminin to bind to the 32-kDa C6/36 polypeptide; these amounts represent approximately equal molar concentrations (3.85×10^{-10} M). These assays were completed as described above and detected indirectly. Control lanes included all detection reagents in the absence of VEE virus or laminin. Apparent molecular mass standards (in kilodaltons) are on the right.

antigen excess, resulting in the binding of aggregates to the membrane. Alternatively, binding enhancement may have resulted from antibody-induced modification of viral spikes (16), yielding an increased affinity for the 32-kDa polypeptide. In any event, these data demonstrate clearly that monoclonal antibodies known to neutralize virus in vitro by inhibiting attachment to vertebrate cells can also prevent virus binding to the 32-kDa polypeptide from invertebrate cells.

Characterization of the 32-kDa polypeptide with monoclonal antibodies. To analyze the 32-kDa polypeptide antigenically, monoclonal antibodies against the cell membrane proteins of C6/36 cells were prepared. Hybridoma supernatants were screened initially by ELISA against live cells to identify surface-reactive antibodies. Positive monoclonal antibodies were used in immunoblotting assays against C6/36 membrane proteins resolved by SDS-PAGE and blotted to nitrocellulose membranes (Fig. 5). Three classes of 32-kDa polypeptide-reactive monoclonal antibody were identified. The first class (e.g., 10H3) reacted specifically with the 32-kDa polypeptide. The second class (e.g., 1D1) reacted with 12- to 14-, 40-, 60-, and 80-kDa polypeptides. Monoclonal antibodies of the third class (e.g., 14G4) cross-reacted only with the 12- to 14-kDa polypeptides. Additional antibodies that reacted specifically with the 60-kDa polypeptide (e.g., 13C11) were identified. Monoclonal antibodies 1D1, 10H3, and 14G4 reacted strongly with C6/36 cell surface proteins in immunofluorescence assays of live cells and with paraffin-embedded vector mosquito midgut cross sections (data not shown). The results of binding studies with the 32-kDa polypeptide-specific antibody 10H3 demonstrate that the 32-kDa polypeptide was present on the plasma membrane of both continuous cell lines and vector midgut epithelial cells, where it would be available to bind virus during the initial stages of viral infection. Additionally, the results of these binding studies suggest that other proteins found in the membrane fraction of C6/36 cells possess epitopes in common with the 32-kDa polypeptide, which may explain the binding of VEE virus to these same bands as described above.

Infection inhibition assays. While our data demonstrate that the 32-kDa polypeptide could bind VEE virus in a solid-phase binding assay, data at the whole-cell level were necessary to confirm the biological significance of this finding. Monoclonal antibodies reactive with the 32-kDa polypeptide were used in a plaque reduction assay in which the antibodies were incubated with monolayers of C6/36 cells before infection with a known concentration (100 PFU) of virus. These experiments demonstrated that some monoclonal antibodies could reduce dramatically the ability of virus to infect cells (Fig. 9). Monoclonal antibody 1D1, which cross-reacted with multiple membrane proteins, including the 32-kDa polypeptide, had the greatest inhibitory effect on VEE virus infection of C6/36 cells, reducing viral plaques by 73% in comparison to an isotype-matched negative control antibody. Antibody 14G4, directed against the 12- to 14- and 32-kDa polypeptides, reduced plaque formation less efficiently (60% inhibition) than did 1D1. One monoclonal antibody, 10H3, which reacted with an epitope specific to the 32-kDa polypeptide, had no effect on the plaquing efficiency of VEE virus on C6/36 cells. These results suggest that the domain responsible for virus attachment is common to multiple polypeptides. Similar results were observed in plaque reduction assays of Vero cells (data not shown), suggesting that

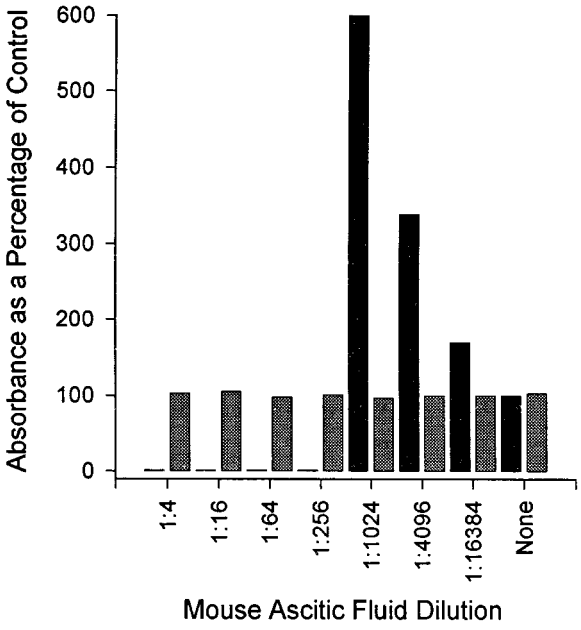


FIG. 8. Ability of anti-VEE monoclonal antibody to prevent VEE virus binding to the 32-kDa polypeptide. Various dilutions of anti-VEE virus monoclonal antibody 1A4A-1 (solid), directed against the E2c domain, or an isotype-controlled anti-E1 monoclonal antibody (hatched) were incubated with virus and used in a protein blot–virus-binding assay as described above. The results were measured by scanning densitometry and expressed in terms of absorbance as a percentage of the control.

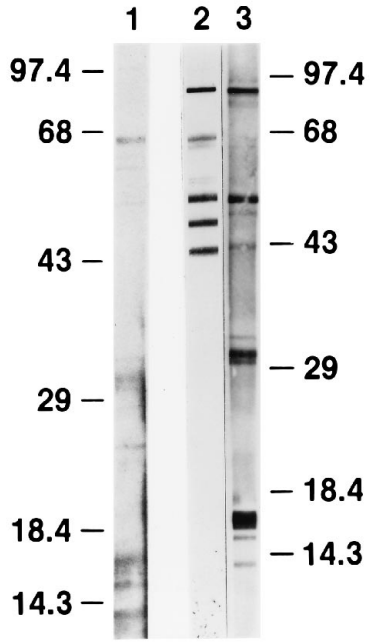


FIG. 10. Binding of VEE virus, anti-C6/36 monoclonal antibody, and anti-human laminin receptor polyclonal antibodies to BHK membrane proteins. BHK membrane proteins were separated and blotted to nitrocellulose as described above. Nitrocellulose strips were incubated with VEE virus (lane 1), rabbit anti-human high-affinity laminin receptor (lane 2), and monoclonal antibody 1D1 (lane 3). Binding was detected and visualized indirectly. Apparent molecular mass standards (in kilodaltons) are on both sides.

homologous VEE virus-binding domains exist on cells derived from phylogenetically distinct hosts.

Binding studies with BHK cell membrane proteins. To analyze the relationships among alphavirus-binding proteins from different cells, BHK membranes were isolated as described above for C6/36 cells and their component polypeptides were resolved by SDS-PAGE and then transferred to nitrocellulose membranes. Membranes were then probed with anti-C6/36 monoclonal antibody 1D1, virus, and anti-human high-affinity laminin receptor peptide antibodies (Fig. 10). Many cross-reactive polypeptides were identified in these studies. The protein blot–virus-binding assay showed that the 32-kDa BHK polypeptide and the 12- to 18- and 67-kDa species possessed VEE virus-binding activity (Fig. 10, lane 1). Polyclonal antibody directed against the human high-affinity laminin receptor peptide reacted with the 67-kDa VEE virus-binding BHK polypeptide, but not the 32-kDa VEE virus-binding BHK polypeptide (Fig. 10, lane 2). Anti-C6/36 monoclonal antibody 1D1 reacted with the 32-kDa BHK polypeptide, several additional polypeptides also identified by anti-human high-affinity laminin receptor peptide antisera, and the 12- to 18-kDa species. Antibody 1D1, however, failed to react significantly with the 67-kDa BHK polypeptide identified in the virus-binding assay (Fig. 10, lane 3).

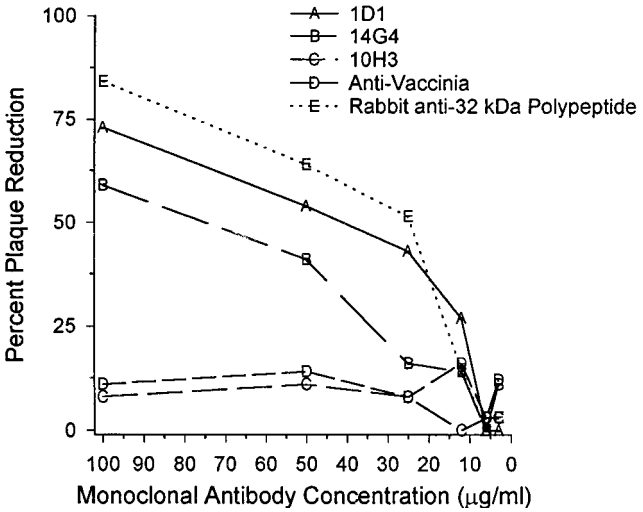


FIG. 9. Abilities of monoclonal antibodies directed against the 32-kDa polypeptide to inhibit VEE virus plaque formation. Antibodies were incubated in twofold serial dilutions with monolayers of C6/36 cells in six-well plates at 4°C. Cells were washed three times with ice-cold media and incubated for 1 h with 100 PFU of VEE virus at 4°C. After virus adsorption, cells were overlaid with 0.6% agarose in medium and incubated at 35°C. After 2 days, cells were fixed to plates in 10% buffered formalin and the agarose was removed. Plaques were observed indirectly by immunoperoxidase staining. The results are expressed as percent plaque reduction at a given antibody concentration.

DISCUSSION

This paper describes the identification of a protein from C6/36 cells that binds VEE virus in vitro and appears to modulate both virus attachment and infection of cells. VEE virus binds predominantly to a 32-kDa laminin-binding polypeptide from the plasma membrane fraction of C6/36 cells. Virus binding to this protein is dose dependent and saturable and can be competed with by homologous and heterologous alphaviruses. This polypeptide is found on the cell surface, and monoclonal antibodies directed against this polypeptide inhibit the infection of cells. Furthermore, monoclonal antibodies directed

against the attachment domain on the virus particle prevent attachment to this polypeptide. These later observations suggest that this laminin-binding polypeptide plays an important role in the attachment of VEE virus to live cells and functions as a CRP.

The attachment of VEE virus to cells appears to be a complex phenomenon. While the 32-kDa polypeptide was the predominant VEE virus-binding C6/36 cell membrane polypeptide, other polypeptides from these cells also bound VEE virus. Immunobinding and infection inhibition studies using anti-C6/36 monoclonal antibodies showed that these polypeptides are antigenically related and may be functionally related. Monoclonal antibody 1D1 blocked viral infectivity in C6/36 cells with greater efficiency than did 14G4. The former antibody cross-reacted with multiple VEE virus-binding polypeptides, while the latter monoclonal antibody reacted only with the 32-kDa polypeptide and proteins of approximately 12 to 14 kDa. These data suggest that the efficiency with which 1D1 blocks infection is a function of its ability to bind to multiple virus attachment proteins. Treating cells with antibody 10H3, which is specific for the 32-kDa polypeptide alone, did not result in a decrease in plaquing efficiency. This observation demonstrates the specificity of the blocking assays and shows that the unique epitope identified by this monoclonal antibody does not modulate viral attachment. Finally, these data suggest that the domain which appears to be responsible for virus attachment is not unique to the 32-kDa polypeptide but is found on multiple, possibly related polypeptides contained within the membrane fraction.

The initial interaction between VEE virus and cells is further complicated by the observation that at least two forms of binding can occur, depending on the ionic strength of the buffer used in the assay. Our observations closely parallel those described by Pierce et al. (32) with SIN virus. We found that nonspecific VEE virus binding (loosely bound virus) could be prevented by using buffers with an ionic strength of 0.2 or greater, whereas binding to the 32-kDa polypeptide (tightly bound virus) was unaffected under such conditions. The biological data presented here, showing that monoclonal antibodies directed against the 32-kDa polypeptide can inhibit virus attachment and prevent the infection of cells, suggest that it is tight binding which plays the major role in virus attachment. Additional evidence for the importance of tight binding is the observation that the neutralizing anti-VEE virus monoclonal antibody 1A4A-1 prevents virus attachment to live cells (35) and to the 32-kDa polypeptide (Fig. 8) but has no effect on the nonspecific binding observed under isotonic conditions (data not shown).

From these observations, it is clear that multiple mechanisms for virus attachment may exist. Alternatively, a cascade of events, one of which is the interaction of virus with the 32-kDa polypeptide, may be required for the attachment of virus to cells. Further research is required to determine the relative importance of each of these factors in the attachment of VEE virus to cells.

This is the second report to suggest that laminin-binding proteins mediate the attachment of alphaviruses to susceptible cells. Wang et al. (43) identified a 67-kDa laminin-binding polypeptide that may serve as a SIN virus receptor for BHK cells. The 67-kDa SIN virus receptor from BHK cells and the 32-kDa VEE virus receptor from C6/36 cells may be structurally related. In our hands, VEE virus bound to both 32- and 67-kDa species in BHK cells. Monoclonal antibody 1D1, which reacted with the 32-kDa polypeptide from C6/36 cells, also reacted with nitrocellulose-bound membrane proteins from BHK cells, including a 32-kDa polypeptide (Fig. 10). However, unlike virus binding, monoclonal antibody 1D1 did not cross-react with the 67-kDa VEE virus-binding protein from BHK cells. The virus-binding data suggest a structural relationship between the 32-kDa C6/36 and 67-kDa BHK polypeptides. The immunological tools currently available, however, do not confirm this possibility. It is also interesting that the full-length gene identified in the studies of Wang et al. (43) is predicted to encode a polypeptide of 32.7 kDa, not 67 kDa. In those studies, the predicted polypeptide is hypothesized to be a precursor of the 67-kDa polypeptide containing the virus-binding domain. It is possible that the 32-kDa C6/36 polypeptide and the predicted 32.7-kDa BHK polypeptide are analogous in that they are posttranslationally modified or associate with other proteins to form the larger polypeptides identified in our studies (40 and 60 kDa) and those of Wang et al. (67 kDa) (43).

Laminin is an extracellular glycoprotein known to mediate cell attachment, morphology, differentiation, movement, and growth; it is also implicated as a factor in tumor metastasis (24). The biological function of laminin is mediated through its attachment to cells by means of a receptor-ligand interaction. Laminin-binding proteins are ubiquitous, being found in cells from a variety of organs and tissues. Many forms of laminin-binding proteins with various biochemical and biophysical characteristics have been identified (reviewed in reference 28). The laminin-binding proteins known as galactoside-binding lectins are of particular interest. This family includes the 67-kDa high-affinity laminin receptor (26, 34), which is presumably the protein identified by Wang et al. (43) as a receptor of SIN virus in mammalian cells. Other members of this family include 12- to 14- and 31- to 35-kDa proteins (2, 17, 19, 29). These proteins are known to share certain antigenic and functional characteristics on the basis of the observation that antibodies to one cross-react with the others (10).

On the basis of studies that show that the 32-kDa polypeptide from C6/36 shares epitopes with the human high-affinity laminin receptor (Fig. 4), it is possible that the 32-kDa C6/36 polypeptide is a member of the galactoside-binding lectin family of laminin-binding molecules. From the information described here and by Wang et al. (43), it is possible that this family of laminin-binding molecules plays an important role in the attachment of alphaviruses to both vertebrate and invertebrate cells.

Unlike many other arboviruses, VEE virus has an extremely broad host range. This virus infects cells from both higher and lower vertebrates (2, 31). More importantly, VEE virus infects many hematophagous insects, including at least five genera of mosquitoes and one genus of ticks (6, 23, 41). The use of laminin-binding proteins as receptors for virus attachment by VEE virus appears to provide a plausible explanation for the observed broad host range of this virus. Laminin-binding proteins isolated from different hosts are highly conserved. The deduced amino acid sequences of hamster and mouse laminin receptor proteins are identical and vary from that of a human laminin receptor by only two amino acids (43). On the basis of the cross-reactivity of monoclonal antibodies prepared against the C6/36 cell laminin-binding protein with similar proteins derived from vertebrate cells and the observation that monoclonal antibodies directed against the 32-kDa polypeptide from C6/36 cells inhibit virus infection of Vero and C6/36 cells with equal levels of efficiency, there may be a high degree of homology with mosquito analogs to vertebrate laminin receptor proteins as well. Such homology between laminin receptor proteins may contribute to the ability of VEE virus to infect a broad range of hosts.

From the data presented here, it seems likely that laminin-binding proteins play a role in the initial events of alphavirus

infection of many cells. From previous studies, it seems possible that this family of proteins may not be the only molecules which can modulate infection (25, 38, 42, 44). Further research on alphavirus receptors will help to determine how cell susceptibility to infection is controlled and the importance of receptor-mediated events in the regulation of virus tropisms and pathogenesis.

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