

High-Affinity Laminin Receptor Is a Receptor for Sindbis Virus in Mammalian Cells

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Sindbis virus is an alphavirus with a very wide host range, being able to infect many birds and mammals as well as mosquitoes. We have isolated a monoclonal antibody that largely blocks virus binding to mammalian cells. This antibody was found to be directed against the C-terminal domain of the high-affinity laminin receptor, a 67-kDa protein present on the cell surface that binds with high affinity to basement membrane laminin and that is known to be important in development and in tumor invasion. This receptor is believed to be formed from a 295-amino-acid polypeptide that is modified in some unknown way after translation. The primary sequence of this 295-amino-acid protein is highly conserved among mammals. We found the hamster amino acid sequence to be identical to a mouse sequence and to differ at only two amino acids from a human sequence and at two amino acids from a bovine sequence. To verify the importance of the laminin receptor for infection by Sindbis virus, hamster cells were stably transfected with the gene encoding the 295-amino-acid protein under the control of a high-efficiency promoter. Such transfected hamster cells overexpressed the laminin receptor at the cell surface, bound severalfold more Sindbis virions than did the parental cells, and became infected by Sindbis virus with a higher efficiency. In contrast, cells transfected with the antisense gene expressed less laminin receptor on the surface and were less susceptible to the virus. Binding of the virus varied linearly with the amount of laminin receptor on the cell surface, whereas infectivity measured with a plaque assay varied with the 1.4 power of the receptor concentration, suggesting that interaction with more than one receptor aids virus penetration. By these criteria, the laminin receptor functions as the major receptor for Sindbis virus entry into mammalian cells. We also found that the anti-laminin receptor antibody partially blocked Sindbis virus binding to mosquito cells, suggesting that the laminin receptor is conserved in mosquitoes and functions as a Sindbis virus receptor in this host. The wide distribution of this highly conserved receptor may be in part responsible for the broad host range exhibited by the virus, which infects a wide range of mammals and birds as well as its mosquito vector and can infect many different tissues within these hosts. Part of the broad host range of Sindbis virus also appears to result from an ability of the virus to utilize more than one different protein receptor, however, because the major receptor used by the virus to enter chicken cells appears to be a 63-kDa protein that is not the laminin receptor (K.-S. Wang, A. L. Schmaljohn, R. J. Kuhn, and J. H. Strauss, *Virology* 181:694-702, 1991).

The initial event in the interaction of a virus with the host cell is the attachment of the virus to receptors in the plasma membrane, and the presence of specific cellular receptors is a determinant of the species and tissue tropism of a virus and of its pathogenesis. The 26 registered alphaviruses (family *Togaviridae*) infect a wide variety of vertebrate hosts, including many species of birds and mammals, and are transmitted by mosquitoes, in which they also replicate (6). Many are pathogenic for humans and domestic animals, causing fever, arthralgia, or encephalitis (39). The viruses not only can infect very divergent organisms but can infect a wide variety of cells within the organism, including neurons and glial cells, striate and smooth muscle cells, lymph cells, synovial cells, brown fat cells, hepatocytes, and others (19, 39). The mechanisms by which the viruses can infect such a spectrum of cells and organisms is of considerable interest. Possible mechanisms include use of a receptor that is highly conserved across phylogenetic boundaries, the ability to use multiple receptors, or both. Influenza virus attaches to the widely distributed sialic acid and is capable of infecting a

wide variety of birds and mammals (52). Alphaviruses, however, have been found to use protein receptors (24, 31, 43). Most other viruses for which protein receptors have been characterized exhibit a narrow host range with respect both to the animal host and to the tissues within the host that are infected. Viruses whose protein receptors have been characterized include human immunodeficiency virus, which infects lymphocytes and brain cells that express its receptor, CD4 (9, 25, 29); human rhinoviruses, restricted in replication to the upper respiratory tract of humans, which use the cellular adhesion molecule ICAM-I (18, 44); the human herpesvirus Epstein-Barr virus, which uses the C3d complement receptor CR2 that is expressed on human B lymphocytes (13, 14); and the primate-specific poliovirus, whose receptor is a member of the immunoglobulin superfamily of unknown function (36).

Sindbis virus, the prototypic alphavirus, has a plus-strand RNA genome of 11,703 nucleotides complexed with a capsid protein to form an icosahedral nucleocapsid. This structure is surrounded by a lipid bilayer of host cell origin containing two virus-specific transmembrane glycoproteins (E1 and E2) organized in spikelike projections on the external surface of the virus particle (15, 46). The spikes form the organs of attachment to cell surface receptors, and the virus enters the cell by receptor-mediated endocytosis. Following endocyto-

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sis, fusion of the viral membrane with the endosomal membrane releases the viral nucleocapsid into the cytoplasm (24).

We have previously reported the use of anti-idiotypic antibodies to study Sindbis virus receptors (49). We found that an anti-idiotypic antibody made to a neutralizing antibody reactive with glycoprotein E2 functions as an antireceptor antibody in chicken cells but not in hamster cells. Here we report the isolation of a monoclonal antibody (MAb) that functions as an antireceptor antibody in mammalian cells. We have used this antibody to establish that the high-affinity laminin receptor serves as a major mammalian receptor for Sindbis virus. We show in addition that the laminin receptor is different from the protein that we previously identified as being a major receptor in chicken cells (49).

MATERIALS AND METHODS

Isolation of MAb 1C3. Two 6-week-old BALB/c female mice were injected intraperitoneally eight times at 3-week intervals with 10^7 whole BHK cells harvested from monolayer culture with 4 mM EDTA, and one mouse was injected on the same schedule with 200 μ g of sucrose gradient-purified BHK membranes, with adjuvant. Spleens were then removed, and lymphocytes were fused with the myeloma cell line NS-1, using standard methods (22). Hybridoma supernatant fluids were assayed for the ability to block infection of BHK cells by Sindbis virus (strain AR339) in a plaque reduction assay (49). To prepare purified antibodies, the hybridoma-producing MAb 1C3 and several control hybridomas were injected intraperitoneally into athymic NCR 6-week old-female nude mice (Simonsen Laboratory) that had been primed for 1 week by injection with pristane (2,6,10,14-tetramethyldecanoic acid). Ascites fluids were collected after 7 to 10 days, and the MAbs were purified by affinity chromatography. Immunoglobulin Ms (IgMs) (determined by Ouchterlony immunodiffusion assays) were purified by affinity chromatography on rat anti-mouse IgM coupled to Sepharose 4B (Zymed), and IgGs were purified on protein G-Sepharose columns (Pharmacia). The concentrations of MAbs were determined by a modified Bradford assay (Bio-Rad) or by optical density at 280 nm. The yield of MAb 1C3 was very low, even in nude mice, possibly because of the very close relationship between the mouse laminin receptor and the hamster laminin receptor.

Plaque reduction assay. Buffer (180 μ l) containing purified MAb at the concentrations indicated in the figures was added to cell monolayers in 12-well plates for 1 h at 22°C, followed by about 100 PFU of Sindbis virus AR339 purified by sedimentation through sucrose gradients. After a further 1 h of incubation at 22°C, the incubation buffer was removed and replaced with Eagle medium containing 1% agarose. Plaques were visualized by staining with 1% neutral red after 18 h in BHK cells, 24 h in Vero cells, 32 h in SW13 cells, or 13 h in secondary chicken cells.

Binding of 35 S-labeled Sindbis virus. Confluent monolayers of BHK cells in 96-well plates were treated with purified MAb in a volume of 25 μ l of phosphate-buffered saline (PBS) (10) containing 1% fetal calf serum, 1 mM CaCl_2 , and 1 mM MgCl_2 for 1 h at 10°C; 25 μ l of 35 S-labeled Sindbis virus (5,650 cpm/ μ l; 4×10^8 PFU/ μ l) purified by sedimentation in a sucrose gradient was then added to each well, and incubation continued for 1.5 h. The monolayers were washed and dissolved in 0.5% sodium dodecyl sulfate, and bound radioactivity was determined by liquid scintillation counting. The amount of labeled virus applied was shown in preliminary

saturation experiments to be sufficient to saturate the cell monolayers in the absence of inhibitory antibodies, and the amount of virus bound in the absence of MAb was taken as 100%.

Binding experiments with CHO cells were similar in design, but binding was performed at 8°C. Details are given in the legend to Fig. 8. For binding studies with mosquito C6/36 cells, 35 S-labeled Sindbis virus was added to 10^5 cells suspended in 300 μ l of binding buffer, and the virus was allowed to attach for 1.5 h at 4°C with gentle shaking. Cells were collected by centrifugation and washed three times, and the cell-bound radioactivity was assayed.

Immunoprecipitation. Cells were labeled with [35 S]methionine, and a crude membrane fraction was prepared as described previously (49). In some cases (Fig. 3B and C), the crude membrane pellet was resuspended at 4°C in 50 mM Tris-Cl (pH 7.5)–0.3 M NaCl–200 μ g of bovine serum albumin per ml–4 mM EDTA containing 3% Triton X-100 and protease inhibitors and used without further purification. In other cases (Fig. 3A), the pellet was resuspended by Dounce homogenization in 1 ml of 53% (wt/wt) sucrose in 10 mM NaPO_4 (pH 7.0), and membranes were further purified by sedimentation through a step sucrose gradient (2). Immunoprecipitation was done as described previously (21, 49, 57).

Isolation of phage lambda clones immunoreactive with 1C3. A λ gt11 cDNA expression library was constructed from oligo(dT)-primed cDNA made to poly(A)-selected RNA from BHK cells. Lambda plaques were screened with MAb 1C3 as the primary antibody and 125 I-conjugated goat anti-mouse IgM (Dupont) as the secondary antibody. Fifteen reactive clones were isolated from 10^6 λ gt11 plaques. Six of the fifteen clones were found to cross-hybridize, and the sequences of five of these clones were determined; they were found to start at various points in the laminin receptor sequence and terminate with the 3' poly(A). To isolate clones containing the 5' end of the sequence, the insert from clone 26 was excised with *EcoRI*, labeled by nick translation with [32 P]dCTP, and used to screen the λ gt11 library for clones which overlapped the clone 26 sequence. From 5×10^4 λ gt11 plaques, 124 reactive clones were found. Of these, 52 were analyzed by the polymerase chain reaction for the 5' end of the laminin receptor coding region, using the 17-mer oligonucleotide primer 5'-GCAAACCTCAGCACAGC-3' complementary to the 5' end of clone 7 and either the 24-mer λ gt11 forward primer or the 24-mer λ gt11 reverse primer. Amplified inserts were characterized by electrophoresis and DNA sequencing, and three inserts that contained the 5' end of the gene were chosen for detailed sequencing to obtain the complete sequence of the hamster laminin receptor.

Expression of the laminin receptor sense or antisense RNA. Inserts from clones 3 and 26 were excised from the λ gt11 phages with *EcoRI*, purified, and digested with *SacI*. After purification by agarose gel electrophoresis, the 5' end of the laminin receptor from clone 3 and the 3' end from clone 26 were joined in a three-piece ligation with pcDNA-1/neo vector DNA (Invitrogen) which had been partially digested with *EcoRI*. Constructs containing the full-length insert in both orientations were obtained, and plasmids pcDNA/neo-LR1 (sense) and pcDNA/neo-LR7 (antisense) were selected for transfection. BHK cells were transfected with 5 μ g of either the plus-sense or antisense construct or with the vector alone, using the Lipofectin (Life Technologies Inc.) transfection method (11). After 24 h, the cells were placed in medium containing 400 μ g of Geneticin (Life Technologies Inc.) per ml, and G418-resistant colonies were selected 2 weeks after transfection. Cells were prepared from multiple

independent colonies and screened for plaquing efficiency of Sindbis virus AR339. Most cell lines transfected with the sense gene overexpressed the laminin receptor and were more susceptible to infection by Sindbis virus by severalfold (see below). With continued passage, these cell lines became less susceptible to the virus (although remaining more susceptible than the parental cells), presumably because cells overproducing the laminin receptor are unstable and variants expressing less receptor are selected.

In a second experiment, the laminin receptor insert was excised from pcDNA/neo-LR1 and inserted into the *EcoRI* site of the vector pEE14 (a gift from Celltech). CHO-K1 cells (American Type Culture Collection) were transfected with 10 μ g of pEE14 or with pEE14 containing the laminin receptor insert, using Lipofectin. After 24 h, the cells were placed in medium containing methionine sulfoximine, and resistant colonies were selected.

RESULTS

Isolation of an antireceptor antibody (1C3) for mammals.

To isolate a MAb which recognized the Sindbis virus receptor on hamster cells, hybridomas were prepared from mice immunized with whole BHK cells or sucrose gradient-purified BHK membranes (2). This approach was used previously to isolate a MAb reactive with the HeLa cell receptor for poliovirus (38) or for rhinoviruses (8). Approximately 3,600 hybridoma culture supernatants were screened by plaque assay for the ability to protect BHK cells against infection by Sindbis virus. The conditions for the plaque assay are suboptimal, and the number of plaques obtained has been shown previously to be limited by the number of receptors available on the surface of the cells, as determined by direct virus binding (49). One hybridoma (from a mouse immunized with whole cells) was obtained that secreted an IgM (MAb 1C3) that partially blocked virus binding to BHK cells. An experiment illustrating the inhibition of plaque formation in BHK monolayers by 1C3 as a function of the concentration of purified MAb is shown in Fig. 1A. Following treatment of the cells with 20 μ g of 1C3 per ml, plaque formation was inhibited by 80%. Control MAbs did not reduce the plaque titer.

The ability of 1C3 to block the binding of saturating amounts of 35 S-labeled Sindbis virus to BHK cells was also determined. Following treatment of the cells with 20 μ g of MAb 1C3 per ml, only 28% as much Sindbis virus bound to BHK cells as was bound in the absence of competing MAb or in the presence of control MAbs (Fig. 1B). Thus, MAb 1C3 blocked Sindbis virus binding to BHK cells as well as virus infection of these cells, and the virus blocking assay and plaque assay gave comparable results.

To determine whether the receptor identified by 1C3 also serves as a Sindbis virus receptor in other cells, we compared the ability of 1C3 to inhibit plaque formation in hamster cells with its effect in monkey (Vero), human (SW13), and chicken embryo cells (Fig. 1C). Plaque formation was inhibited by this antibody in all three mammalian cell lines, indicating that the epitope recognized by 1C3 is a component of a major Sindbis virus receptor in all three lines and, presumably, in all mammalian cells. The antibody had only a slight effect in chicken cells, however, suggesting that the major receptor used by the virus to enter chicken cells is different (see also below).

We also assayed the ability of 1C3 to block the binding of 35 S-labeled virus to mosquito cells, which serve as another host for Sindbis virus in nature. As shown in Table 1, 1C3

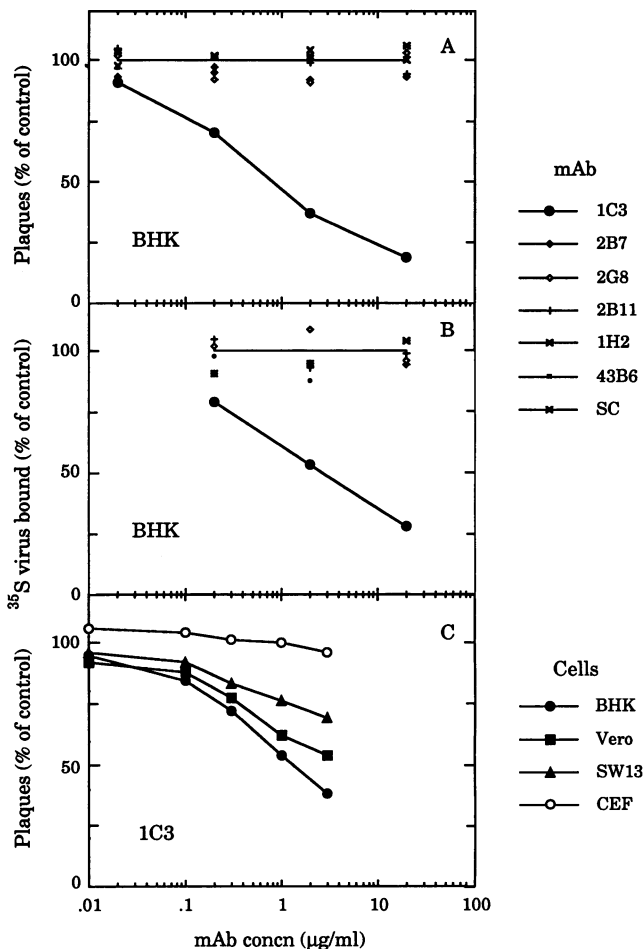


FIG. 1. Inhibition of Sindbis virus binding to cells by anti-BHK MAbs. MAbs 1C3 (IgM), 2B7 (IgM), 2G8 (IgM), 2B11 (IgG1), and 1H2 (IgG1) are various MAbs derived from mice immunized with whole BHK cells. 43B6 (IgM) is an irrelevant MAb derived from a different screening experiment used here as a control, and SC (IgG1) is an anti-rat membrane protein MAb also used as a control. These MAbs were affinity purified, and their abilities to block Sindbis virus binding were determined at different antibody concentrations. Assays were performed as described by Wang et al. (49). (A) Effects of the MAbs on virus binding to hamster BHK cells as determined by plaque reduction; (B) effects of the MAbs on binding to BHK cells as assayed by reduction in the amount of 35 S-labeled virus bound; (C) effects of MAb 1C3 on virus binding to different types of cells as assayed by plaque reduction.

does block in part the binding of Sindbis to mosquito cells, but not as effectively as it blocks binding to BHK cells (compare Table 1 with Fig. 1B). This result suggests that the receptor recognized by 1C3 is conserved in mosquito cells and that this same receptor functions as a Sindbis virus receptor in mosquito cells.

A control experiment is shown in Fig. 2. Using flow cytometric analysis, we found that 1C3 bound to the surface of BHK cells (Fig. 2A), as predicted if it functions as an antireceptor antibody. Two other IgM MAbs isolated during the screening procedure, 2G8 (Fig. 2B) and 2B7 (Fig. 2C), were also found to bind to BHK cells as well as did 1C3. These two MAbs had no effect on the binding of virus to BHK cells, however (Fig. 1A and B). Thus, the ability of

TABLE 1. Binding of ^{35}S -labeled Sindbis virus to mosquito cells in the presence of competing antibodies

MAb	^{35}S bound (% of control) ^a at concn of:	
	3 $\mu\text{g}/\text{ml}$	20 $\mu\text{g}/\text{ml}$
1C3	78	59
43B6	104	101
2B7	112	94

^a Virus stock contained 7,854 cpm/ μl and 6×10^8 PFU/ μl ; 40 μl of this stock was used to measure binding to 10^5 cells in a total volume of 300 μl . This amount of virus was found to saturate the cells.

1C3 to block virus adsorption is specific and not just an indirect consequence of cell surface binding.

Immunoprecipitation of a membrane-associated protein by 1C3. We next examined whether 1C3 would immunoprecipitate a protein from hamster cell membranes. Purified ^{35}S -labeled BHK cell membranes were dissociated and immunoprecipitated with 1C3 or various control MAbs. A labeled polypeptide with an apparent molecular mass of 67 kDa was specifically precipitated by MAb 1C3, whereas only faint background bands were seen in the absence of MAbs or upon immunoprecipitation by two control MAbs (Fig. 3A). As shown in Fig. 3B and C, a 67-kDa protein was also precipitated by 1C3 from mouse neuroblastoma (N18) cells and monkey kidney (Vero) cells, and a 71-kDa protein was precipitated from human adenocarcinoma (SW13) cells. A 71-kDa protein was also immunoprecipitated by 1C3 from chicken cells, which is of interest since this MAb does not block virus binding to chicken cells. The 71-kDa chicken protein, which is presumably the chicken homolog of the hamster 67-kDa protein, is different from the 63-kDa chicken protein previously identified from its reactivity with rabbit polyclonal anti-idiotypic antibodies as being a probable Sindbis virus receptor in chicken cells (49). First, the 71- and 63-kDa chicken proteins differ in electrophoretic mobility, and second, the 71-kDa protein is precipitated by MAb 1C3 but not by the rabbit polyclonal anti-idiotypic antibody 49, whereas the 63-kDa protein is precipitated by anti-idiotypic antibody 49 but not by MAb 1C3 (Fig. 3B and C).

1C3 reacts with the laminin receptor. A cDNA library in $\lambda\text{gt}11$ was prepared from BHK mRNA and screened with MAb 1C3, using ^{125}I -labeled goat anti-mouse IgM as a secondary antibody (57, 58). Fifteen reactive phage clones were found upon screening of 10^6 plaques. Of these, six clones (numbers 5, 7, 11, 26, 30, and 51) were found by Southern analysis to contain inserts that cross-hybridized, and sequence analysis of five of these clones (Fig. 4) revealed that they all contained the C-terminal coding region of the hamster high-affinity laminin receptor, as described in more detail below. Four additional clones reactive with 1C3 were also sequenced and found to contain apparently irrelevant inserts; one insert encoded part of the ribosomal protein S26, one insert encoded a protein related to elongation factor EF2R, and two contained sequences not present in the protein data base.

The insert from clone 26 (Fig. 4) was then used to screen the $\lambda\text{gt}11$ library, and 52 reactive clones were further screened by a polymerase chain reaction technique for the presence of the 5' end of the gene as described in Materials and Methods. Several clones containing the 5' end of the gene were identified and sequenced. The clones sequenced, including five of the six clones immunoreactive with 1C3 and three clones selected to contain the 5' end of the gene, are

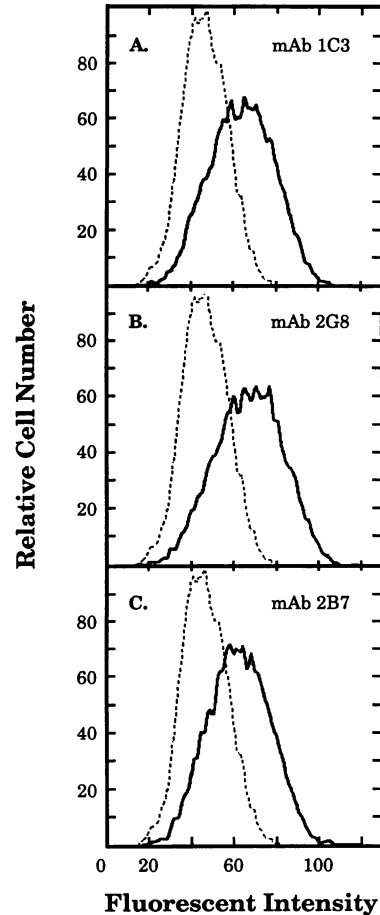


FIG. 2. Binding of MAbs to BHK cells as determined by flow cytometry. BHK cells were treated with different MAbs and then stained with fluorescein isothiocyanate-conjugated goat anti-mouse IgM (μ chain), and the amount of MAb bound was assayed by flow cytometry as described previously (49). Dashed lines, profiles of cells stained with the secondary antibody only; solid lines, cells first treated with the indicated MAb.

schematically illustrated in Fig. 4. The composite sequence obtained is shown in Fig. 5. This sequence includes 64 nucleotides of 5' nontranslated region, the entire 885-nucleotide sequence encoding the laminin receptor, and the 68-nucleotide 3' nontranslated region. At least two different copies of the gene were sequenced, and six silent transitions between the two copies are identified in Fig. 5. The deduced amino acid sequence of the hamster laminin receptor is 295 residues in length and is identical to that of the mouse protein as determined by Makrides et al. (30); a second mouse gene has been found to differ at two amino acids (40). The hamster protein differs at two amino acids from a human counterpart (53, 59) and at two amino acids from a bovine protein (20). The nucleotide sequence within the coding region is 92.5% identical between hamster and mouse genes and 89% identical between hamster and human genes. As stated, there are six nucleotide differences, all silent transitions, between the two copies of the hamster gene that we sequenced (0.6% divergence). In view of the identity in amino acid sequence of the hamster and mouse genes, it is of interest that we were able to obtain an antibody in mice directed against the hamster protein.

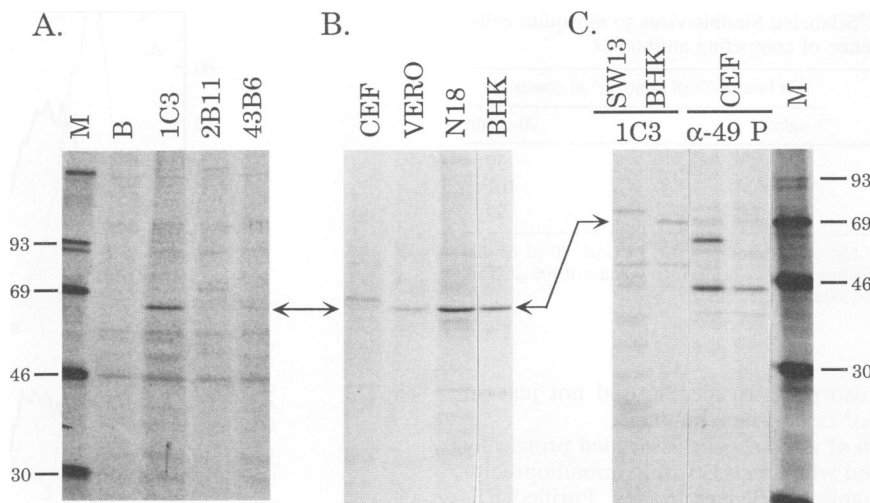


FIG. 3. Immunoprecipitation of a 67-kDa protein from cell membrane preparations by MAb 1C3. Membrane preparations from ³⁵S-labeled BHK cells, N18 mouse neuroblastoma cells, Vero cells, or chicken fibroblast cells were immunoprecipitated with 5 μg of purified MAb or with a rabbit polyclonal anti-idiotypic antiserum. (A) BHK membranes precipitated with various MABs (see Fig. 1). Immunoprecipitates in lanes B (control with no MAB added), 1C3, and 43B6 were collected by treatment with rat anti-mouse IgM coupled to Sepharose 4B (Zymed). The immunoprecipitate with 2B11 was collected by treatment with protein G-Sepharose (Pharmacia). Lane M, labeled molecular weight standards (indicated in kilodaltons). (B) Membrane preparations from chicken, Vero, N18, and BHK cells precipitated with MAb 1C3. (C) Membrane preparations from BHK and SW13 cells immunoprecipitated with 1C3. Membrane preparations from chicken cells were immunoprecipitated with rabbit polyclonal anti-idiotypic antibody 49 (α-49) or with preimmune serum (P) from the same rabbit (49). Lane M, molecular weight markers (indicated in kilodaltons).

The five sequenced laminin receptor clones that are immunoreactive with MAb 1C3 contain in common the nucleotide sequences encoding the 48 C-terminal amino acids of this protein (residues 248 to 295); it seems clear that 1C3 is directed against this C-terminal domain, and therefore this domain must be extracellular (see also references 5 and 54). The inhibition in the binding of Sindbis virus that results

from binding of 1C3 to mammalian cells suggests that the virus also binds to the C-terminal region. Laminin and the synthetic peptide YIGSR, both of which bind to the laminin receptor (7, 16, 17, 55), were examined for the ability to interfere with virus binding to cells. Neither of these polypeptides had any effect at 80 μg/ml on virus binding (data not shown), suggesting that the binding sites on the laminin receptor for laminin and for Sindbis virus are different. Castronovo et al. (5) reported that laminin bound to residues 161 to 180 of the laminin receptor.

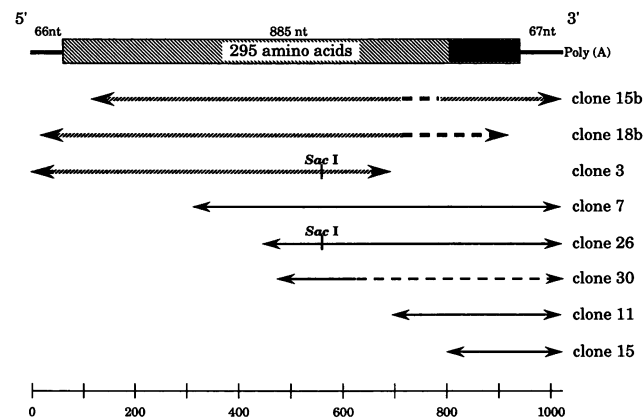


FIG. 4. Evidence that the Sindbis receptor identified by 1C3 is the high-affinity laminin receptor. The open box depicts the coding region of the BHK laminin receptor; the dark box represents the C-terminal 48 residues common to clones reactive with 1C3. Five clones (7, 26, 30, 11, and 15) selected by their immunoreactivity with MAb 1C3 are illustrated to scale (solid lines). Agt11 clones containing the 5' end of the gene (clones 3, 18b, and 15b; patterned lines) were selected by screening with a probe from clone 26 followed by polymerase chain reaction analysis. Clones were sequenced except in those regions shown as dashed. The *Sac*I restriction site used to construct a full-length laminin receptor clone is indicated on clones 3 and 26. nt, nucleotides.

BHK cells overexpressing the laminin receptor are more susceptible to Sindbis virus. Sindbis virus has a very broad host range, and attempts to identify cell lines completely lacking in receptors for the virus have not been successful. Thus, it is not possible to transform a receptor-negative cell to one that is susceptible to the virus, as has been done in other virus systems (18, 29, 35, 44). However, we reasoned that if the laminin receptor functions as a receptor for Sindbis virus, cells that expressed increased amounts of this receptor might become more susceptible to virus infection. To test this possibility, we constructed a full-length cDNA clone of the BHK laminin receptor and inserted it in both the sense and antisense orientations into an expression vector (pcDNA-1/neo) containing a neomycin resistance gene for selection of stable transfectants in mammalian cells, in which the inserted gene is expressed under the control of a high-efficiency promoter.

BHK cells were transfected with both the sense and antisense constructs, as well as with the vector only as a control, and neomycin-resistant transformants were isolated. The efficiency of plaque formation was determined on multiple independent clones. Of 73 clones transfected with the plus-sense gene, more than 90% demonstrated a greater than twofold increase in efficiency of plaquing of Sindbis virus, and 19% demonstrated a greater than fourfold increase in efficiency, compared with vector-only transformants (Fig.

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1      CTGGGACGGGTCATACCGCGTGTCTTCTGATTCCCGTCGTAACCTAAAAGGGAA 55
56    ACTTACACAATGTCGGAGCCCTTAGCTCCCTGCAGATGAAGGAGGAAGATGCTCTCAAG 115
1      M S G A L D V L Q M K E E D V L K 17
116   TTCCTTGTCTCAGGAACCCACTTAGTGGCCACCACTTACTTTCAGATGGAGCGATAC 175
18    F L A A G T H L G G T N L D F Q M E Q Y 37
176   ATCTACAAAAGAAAAGTGAAGGATATCATACATAAATCTAAGAGGACATGGGAGAAG 235
38    I Y K R K R K S D G I Y I I N L K R T W E K 57
236   CTGTTGTGGCAGCCCGGCCCATCGTGTCTATTGAGAACCCGGCTGATGTTAGCGTCATC 295
58    L L L A A R A I V A I E N P A D V S V I 77
296   TCCTCAGGAACACTGGGCGAGCTGTGTGAAGTTTCCCGCTGCCACCGGAGCCACT 355
78    S S R N T G Q R A V L K F A A A T G A T 97
356   CCAATTGCTGGCGTTTCACTCTCGCCACCTTCAACAACAGATCCAAGCAGCGTCCGG 415
98    P I A G R F T P G T F T N Q I Q A A F R 117
416   GAGCCTCGGCTTCTGGTGTCTCACTGACCCAGGGCTGACCACCAGCCCTCACAGAGGGC 475
118   E P R L L V V T D P R A D H Q P L T E A 137
476   TCTTACGTCACCTGCCACCACTGCTGTGTAAACACAGACTCTCTCTGGCTACGCTG 535
138   S Y V N L P T I A L C N T D S F L R Y V 157
536   GACATTGCCATCCCGTCAACAACAGGAGCTCACTCAGTGGGTCTGATGTGGTGGATG 595
158   D I A I P C N N K G A H S V G L M W W M 177
596   CTGCCCGGGAAGTACTCCGATGCGTGGCCTATCTCCCGAGAGCACTCCATGGGAGGTC 655
178   L A R E V L R M R G T I S R E H P W E V 197
656   ATGCTGACCTTACTTCTCAGAGCCAGAGGAGATTGAGAAGGAAGAGCAGCGCTGCC 715
198   M P D L Y F R E E I E K E E Q A A 217
716   GCCGAGAAGCTGTGACCAAGGAGAAATTCAGGCGAATGGAGCGCCAGCCTCTGAG 775
218   A E K A V T K E E F Q G E W T A P A P E 237
776   TTCAGTGTCTCAGCCAGAGGTGGCCGACTGGTCTGAAGGTGTGACAGGTGCCCTCTGTA 835
238   F T A Q P E V A D W S E G V Q V P S V 257
836   CCCATCCAGCAGTTTCTACTGAAGACTGGAGTCCAGCCAGCCACTGAGGAGGTGCA 895
258   P I Q Q P F T E D W S A Q P T E D W S 277
896   GCAGCTCCACGGCGAGCCCACTGAGTGGGTTGGACCACCAGTGGTCTGCTGAGCT 955
278   A A P T A Q A T E W V G T T W S 295
956   GCTTGGGACACCCGAGCAAGGAAAAGGTGGAAGGAAAATAAGTTTCTACAGCTG 1017
    
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FIG. 5. Translated nucleotide sequence of the hamster laminin receptor. Sequences of two hamster genes were determined, and six nucleotide differences, all silent transitions, were observed (boxes). The amino acid sequence for the mouse high-affinity laminin receptor as determined by Makrides et al. (30) is identical to that shown here for BHK. There are two amino acid differences (shaded squares) between the BHK sequence and the sequence of the human laminin receptor (53, 59), Ala-241_{BHK} → Thr-241_{human} and Glu-293_{BHK} → Asp-293_{human}, and two differences (shaded circles) from the bovine sequence (20), Ala-272_{BHK} → Ser-272_{cow} and Ala-290_{BHK} → Thr-290_{cow}. A second published sequence for the mouse laminin receptor (40) differs in two amino acids (Phe-18 and Arg-155) from the sequence of Makrides et al. (30).

6). Conversely, BHK cells transfected with the antisense gene demonstrated a decreased efficiency of plating, with almost half of the clones yielding only 35 to 50% as many plaques as did parental BHK cells (Fig. 6). Thus, overexpression of the laminin receptor leads to increased susceptibility of BHK cells to infection by Sindbis virus, whereas expression of antisense RNA from the gene decreases the susceptibility of the cells, presumably by leading to decreased expression of the laminin receptor.

To confirm that increases or decreases in laminin receptor concentration had occurred on the surface of cells transfected with the plus-sense or antisense laminin receptor gene, we used flow cytometry to assay the ability of various cells to bind MAb 1C3 (Fig. 7). Two cell lines, 41 and 52, that exhibited a fourfold increase in sensitivity to Sindbis virus after transfection with the plus-sense gene bound more of the MAb (Fig. 7E and F, respectively) than did the parental BHK cells (Fig. 7D) or BHK cells transfected by the vector only (Fig. 7E and F). The increase in binding was estimated to be about threefold by comparison with fluorescent standards. Conversely, two cell lines that were only 40% as susceptible to the virus after transfection with the gene in the antisense orientation, clones 4 and 38, bound less MAb 1C3 (Fig. 7B and C, respectively), on the order of 60% as much as cells transfected with the vector only (Fig. 7B and C).

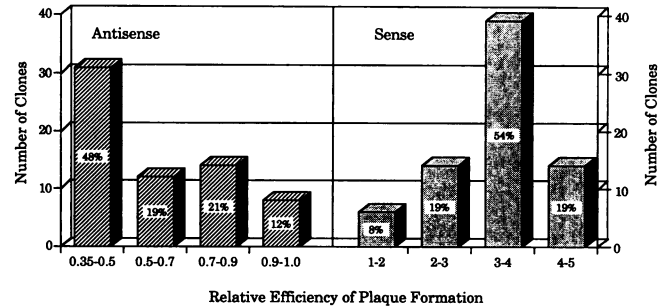


FIG. 6. Correlation of expression of the laminin receptor with susceptibility to Sindbis infection. BHK cells were transfected with an expression vector in which a full-length BHK laminin receptor cDNA had been inserted in either the sense or antisense orientation. Independent G418-resistant colonies were selected 2 weeks after transfection and screened for plating efficiency of Sindbis virus AR339. The efficiency of plaque formation by Sindbis virus on monolayers of cells derived from independent colonies is shown relative to the efficiency on cells transformed with the vector only.

Thus, the concentration of laminin receptor expressed on the cell surface as measured by the binding of 1C3 is correlated with the efficiency with which the cells can be infected by Sindbis virus, and we conclude that this protein functions as a major receptor for Sindbis virus in mammalian cells.

As a control, several transfected cell lines were examined by Southern blot analysis and found to contain a new 1.4-kB *EcoRI* fragment from the transfected gene when compared with nontransfected cells (41).

Transfection of CHO cells with the laminin receptor. To further illustrate the increased binding of Sindbis virus to cells overexpressing the laminin receptor, we transfected CHO cells with the laminin receptor by using the expression vector pEE14, which is reported to lead to very high expression of the inserted gene (45). Cell lines transfected with the laminin receptor, CHO-LR, and with the vector alone, CHO-pEE, were selected and compared with the parental CHO cells. A fluorescence-activated cell sorting (FACS) assay demonstrated that the transfected cell line overexpressed the laminin receptor, as shown by increased binding of 1C3 (data not shown). Saturation binding curves for the binding of ³⁵S-labeled Sindbis virus to CHO and to CHO-LR cells are shown in Fig. 8. Binding was saturable, and the transfected cells bound much more Sindbis virus; at saturation, the increase in binding was 4.6-fold.

The relationship between increased binding of radioactive virus and increased sensitivity to plaque formation for CHO, CHO-LR, and CHO-pEE cells is shown in Table 2. CHO and CHO-pEE cells were equivalent in the virus binding and plaque assay experiments. CHO-LR cells bound 4.6-fold more radiolabeled virus at saturation and produced about 7.7-fold more plaques in the standard plaque assay.

Our results make clear that overexpression of the laminin receptor on the surface of hamster cells leads to increased binding of Sindbis virus and to more efficient infection of these cells by the virus. The increase in laminin receptor concentration at the cell surface measured by binding of 1C3, the increase in binding of radiolabeled Sindbis virus, and the increase in sensitivity to infection measured in a plaque assay are all correlated with one another. In Fig. 9 is presented a log-log plot of the relative plaque titer obtained on the independently transfected BHK and CHO cells versus the relative amount of laminin receptor present on the surface of each cell line. The analysis is described in detail in

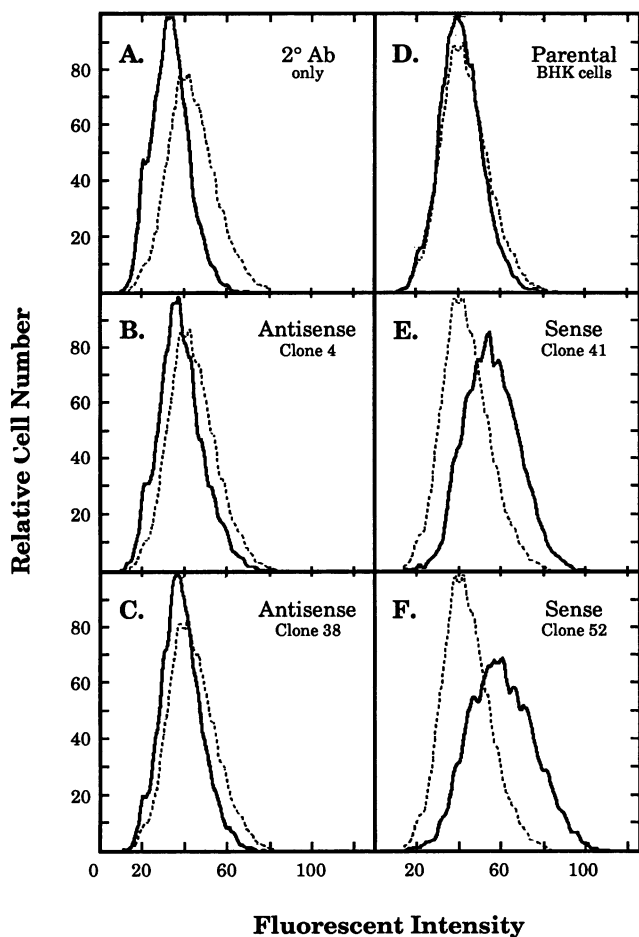


FIG. 7. Increased or decreased expression of the laminin receptor on the surface of BHK cells after transfection with a plus-sense or antisense laminin receptor gene. BHK cells transfected with the plus-sense laminin receptor gene (clones 41 and 52), the minus-sense gene (clones 4 and 38), or with vector containing no insert, or the nontransformed cells, were examined for the ability to bind MAb 1C3 by flow cytometry. The dashed line in each panel is the reference profile of BHK cells transfected with the pcDNA/neo vector containing no insert and stained with MAb 1C3. In panel A, the solid line represents nontransfected cells stained with the secondary antibody only. In panels B to F, the solid line is the fluorescence profile of the cells identified in the panel stained with MAb 1C3. The increase or decrease in the amount of antibody bound was estimated by using fluorescent standards.

the figure legend. From this analysis, we conclude that (i) the binding of radiolabeled virus is linearly related to the number of laminin receptors on the cell surface and (ii) the number of plaques formed varies as the 1.4 power of the number of laminin receptors, suggesting that there is some cooperativity in virus entry.

DISCUSSION

The high-affinity laminin receptor. We have demonstrated that a cell adhesion molecule, the 67-kDa high-affinity laminin receptor, serves as a major receptor for Sindbis virus in several mammalian cell lines. Laminin is a major component of basement membranes from almost all tissues and plays an important role in cellular adhesion, morphology, spreading,

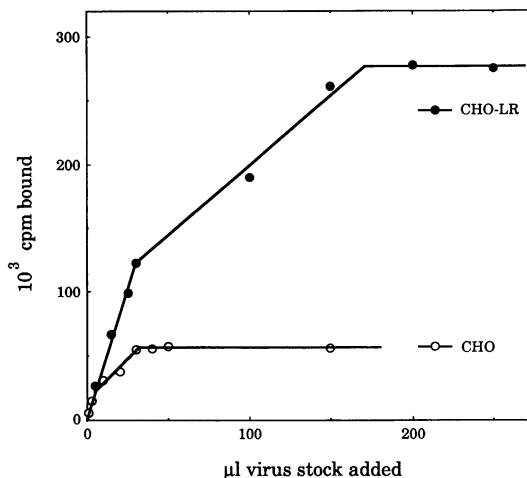


FIG. 8. Saturation binding of ^{35}S -labeled Sindbis virus to CHO cells or to CHO cells overexpressing the laminin receptor. CHO-K1 cells (CHO) or CHO-K1 cells transfected with pEE14 containing the laminin receptor gene (CHO-LR) were examined for the ability to bind ^{35}S virus. Sindbis virus labeled with [^{35}S]methionine was purified in sucrose gradients and dialyzed against PBS. Cells in 96-well plates were covered with the indicated amounts of this purified virus preparation in a total volume of 200 μl (except that the 250- μl sample was in a total volume of 250 μl), and the virus was allowed to bind for 1.5 h at 8°C. After washing, the amount of radioactivity bound to the cell monolayers was determined. One microliter of the virus preparation contained 7,140 cpm and 4×10^8 PFU. At the lowest concentrations of virus tested, 75% of the radioactivity added was bound by the cells. Note that much higher concentrations of virus were required to saturate the cells overexpressing the laminin receptor than to saturate the parental cells.

migration, differentiation, and metastasis (26). A number of different laminin receptors have been described, some of which belong to the integrin family (reviewed in reference 33). The 67-kDa high-affinity receptor, which has been proposed to be identical to a 67-kDa receptor for elastin (34), was first identified because it binds with very high affinity to immobilized laminin during affinity chromatography (reviewed in reference 32), and it is thought to be responsible for mediating many of the interactions of cells with laminin. Interest in this receptor is heightened by the fact that it is overexpressed in tumor cells, especially tumor cells that are poorly differentiated and invasive (40, 53, 54, 59). The gene has been found to be single copy in birds (1) but multicopy in mammals, being present in about 18 copies in humans (1) and either 6 (12) or 21 (1) copies in mice. It is highly conserved, at least among mammals; we found the hamster gene to be identical in amino acid sequence to a mouse sequence (30) and to differ at only two amino acids from a human (59) or a bovine (20) sequence.

TABLE 2. Relative efficiency of virus binding and plaque formation by CHO cells transformed with the laminin receptor

Cells	Plaques		cpm bound ^a	
	No.	Ratio ^b	No.	Ratio ^b
CHO-LR	411	7.7	260,600	4.6
CHO-pEE	58	1.1	55,800	1.0
CHO	53	1.0	56,300	1.0

^a Virus bound with use of 150 μl of virus stock (Fig. 8).

^b Of the value for CHO-LR or CHO-pEE to the value for CHO.

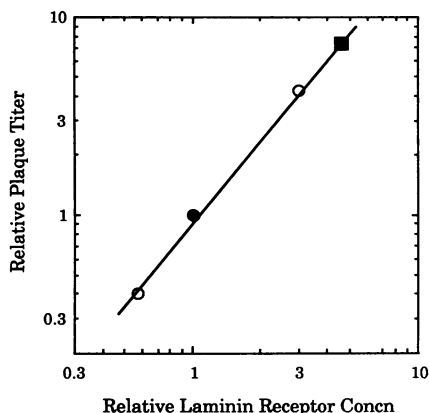


FIG. 9. Relative plaque titer on different hamster cell lines as a function of the relative amounts of laminin receptor on the cell surface. The relative plaque titer on the three transformed BHK cell lines is plotted with circles as a function of the amount of laminin receptor on the cell surface as determined by quantitation of the FACS analysis shown in Fig. 7 (the open circle at the lower left is for cells transformed with the antisense gene, the solid circle is for cells transformed with vector alone, and the open circle at the upper right is for cells transformed with the plus-sense vector). On this log-log plot, these three points determine a straight line with a slope of 1.38. When the point (solid square) for the relative plaque titer found on CHO-LR cells (7.7) is plotted on this line, a relative laminin receptor concentration of 4.4 is determined for these cells, in good agreement with the relative amount of ^{35}S -labeled virus bound at saturation (Fig. 8 and Table 2).

We found that MAb 1C3 precipitated the 67-kDa laminin receptor from plasma membrane preparations but recognized clones in a λ gt11 library that encoded a protein of only 295 amino acids. Rabbit polyclonal antibodies made against fusion proteins containing part or all of this sequence precipitated the 67-kDa protein from cell membrane preparations, but these antisera also precipitated a protein with an apparent molecular mass of 37 kDa, as estimated from its electrophoretic mobility, that was produced upon translation of the 295-amino-acid open reading frame in reticulocyte lysates (51). Hybridization probes from the full-length laminin receptor cDNA clone reacted with a single mRNA of 1,400 nucleotides in Northern (RNA) blots (42). Similar results have been previously reported by others, and there is now a considerable body of evidence (summarized below) strongly suggesting that the 37-kDa protein is a precursor to the 67-kDa protein present on the cell surface (reviewed in reference 5) and that the apparent change in mass does not involve glycosylation (3, 20). (i) Many immune reagents made to the 295-amino-acid sequence, such as antipeptide antibodies made against various domains of the 295-amino-acid protein or antibodies made against fusion proteins produced from the 295-residue protein, are reactive with the 67-kDa protein (3, 5, 51, 54). Further, the sequence of a cyanogen bromide fragment obtained from the 67-kDa protein is present in the 295-amino-acid sequence (53). It is thus clear that the 67- and 37-kDa proteins contain extensive amino acid sequences in common. (ii) Hybridization probes react only with an mRNA of about 1,400 nucleotides on Northern blots, and there is no evidence for a larger mRNA, produced for example by differential splicing, that reacts with these probes (40, 42, 53). (iii) In pulse-chase experiments in human melanoma A2058 cells, the 37-kDa form was first labeled, followed by the 67-kDa form, consistent with a

precursor-product relationship (3). (iv) Overexpression of the 295-amino-acid sequence in BHK cells leads to increased expression of a cell surface protein reactive with MAb 1C3, as measured by FACS analysis (this report). Our expression results thus differ from those of Castronovo et al. (3), who found that overexpression of the 295-amino-acid laminin receptor gene in COS cells led to increased production of the 37-kDa precursor in the cytoplasm of transfected cells but did not lead to an increase in 67-kDa laminin receptor at the cell surface. The nature of the modification that could convert the 37-kDa precursor to the 67-kDa form of the laminin receptor is obscure. Castronovo et al. (3, 5) have proposed that the modification involves covalent linkage of the 37-kDa precursor to a second protein.

Although the data indicating that the 37-kDa polypeptide is a precursor to the 67-kDa laminin receptor are substantial, biochemical characterization of the 67-kDa protein is not complete. Furthermore, the mRNA for the 295-residue polypeptide is abundant, and it is conceivable that a larger mRNA could have been missed even though we (42) and others (40, 53) have searched overexposed Northern blots for such an mRNA. Grosso et al. (20) have proposed, in fact, that the 37-kDa protein is a cytoplasmic protein and that the gene or mRNA encoding the 67-kDa laminin receptor remains to be identified. Rigorous characterization of the 67-kDa laminin receptor is necessary to conclusively prove that the 37-kDa protein is a precursor to it and to determine the nature of the modification.

Castronovo et al. (5) found that a polypeptide comprising amino acids 161 to 180 of the 295-amino-acid open reading frame bound with high affinity to laminin, indicating that this domain of the laminin receptor is responsible for binding to laminin. Furthermore, we (MAb 1C3 binding; Fig. 4) and others (5, 54) found that antibodies that bound to the C-terminal half of the laminin receptor reacted with intact cells. Thus, this domain of the laminin receptor must be extracellular. From analysis of the amino acid sequence, the protein has been postulated to contain a short N-terminal cytoplasmic domain and a transmembrane segment comprising residues 86 to 101 (40). This orientation of the protein is supported by results with antipeptide antibodies, in which it was found that antibodies made to domains C terminal to residue 107 would bind to intact cells, whereas antibodies reactive with regions of the protein between residues 1 to 103 reacted only with permeabilized cells (5).

The laminin receptor is a receptor for Sindbis virus. The results with MAb 1C3 and with hamster cells that express various levels of the laminin receptor at their surface show that this protein is a major receptor for Sindbis virus in the mammalian cell lines tested and, by extension, in all mammalian cells. 1C3 is reactive with an epitope within the C-terminal 48 amino acids (residues 248 to 295) of the laminin receptor, as determined from its reactivity with the λ gt11 library. Because binding of this antibody blocks virus attachment, we propose that the virus also binds somewhere in this C-terminal domain. This hypothesis is consistent with the observation that laminin does not interfere with virus binding, since laminin binds to the high-affinity laminin receptor between residues 161 and 180 (5).

Expression of the 67-kDa laminin receptor has been found to be developmentally regulated, and less differentiated cells produce more of the receptor (4, 54, 56). It is intriguing that the severity of Sindbis virus infection of mice is age dependent, with more extensive virus replication in newborn mice than in mature mice (23). It remains to be seen whether this difference reflects altered expression of the laminin receptor

or whether it results from developmentally related expression of proteins of 110 and 74 kDa that appear to function as receptors for Sindbis virus in mouse neurons (48).

Multiple receptors for Sindbis virus. Sindbis virus has an extremely broad host range. It infects many mammals and birds and within these higher vertebrates is capable of infecting cells of many different tissues (6, 37). We have also tested a number of cell lines from lower vertebrates, including bluegill fish trunk (BF-2) cells, brown bullhead fish trunk (BB) cells, viper spleen (VSW) cells, and *Xenopus* kidney (A6) cells, and found that the virus would infect all of these cells (50). In addition, the virus can replicate in several tissues within its mosquito vector. Our findings indicate that Sindbis virus achieves this enormous host range in two ways: first, by using a receptor that is highly conserved, and second, by using more than one receptor. The high-affinity laminin receptor is highly conserved in mammals and is expressed in many tissues. Our results with 1C3 suggest that it is also conserved in mosquito cells. This conservation seems to be responsible for at least part of the broad host range of the virus. However, it seems clear that the major receptor used by the virus to enter chicken cells is not the laminin receptor. First, 1C3 does not block virus infection of chicken cells to a significant extent even though it does precipitate a 71-kDa protein presumed to be the chicken laminin receptor. Second, we have previously identified a 63-kDa chicken protein as a major Sindbis virus receptor on chicken cells. This 63-kDa protein is immunoprecipitated from chicken cells by a rabbit polyclonal anti-idiotypic antiserum that acts as an antireceptor antiserum in chicken cells but not in hamster cells (49). This 63-kDa protein has not been further characterized, but it is known to be immunologically and electrophoretically distinct from the 71-kDa chicken laminin receptor (Fig. 2C). Thus, although Sindbis virus may use the chicken laminin receptor to a limited extent to enter cells (Fig. 1C), it seems clear that the major protein receptors used by the virus in mammalian and avian cells are different. It is unclear whether a single binding site on the Sindbis virus glycoprotein spike recognizes a common structural feature on these different receptors or whether the glycoprotein spike contains multiple potential binding sites. The hypothesis that the virus can use multiple receptors is supported by previous results that suggest that more than one receptor for the virus is present on chicken cells (49) and on mammalian cells (43) and with results that suggest that the virus can use receptors other than the laminin receptor to enter mammalian cells (28, 48).

We have shown that hamster cells that express different numbers of laminin receptors at their surface differ in the efficiency with which they are infected by Sindbis virus. It is known, however, that even single amino acid changes in the envelope glycoproteins of Sindbis virus can alter the affinity with which it binds to neurons and thus the neurotropism of the virus (27, 47). The adaptation of the virus to different receptors could be responsible for such changes in tropism.

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