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Sindbis Virus Attachment: Isolation and Characterization of Mutants with Impaired Binding to Vertebrate Cells

JEAN DUBUISSON AND CHARLES M. RICE*

Department of Molecular Microbiology, Washington University School of Medicine, 660 South Euclid Avenue, Box 8230, St. Louis, Missouri 63110-1093

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Sindbis virus can infect a broad range of insect and vertebrate cell types. The ability to restrict tissue tropism and target virus infection to specific cell types would expand the usefulness of engineered alphaviruses as gene expression vectors. In this study, virus pools derived from libraries of full-length Sindbis virus cDNA clones containing random insertion mutations in the PE2 or E1 virion glycoprotein gene were screened for mutants defective for binding to vertebrate cells. Binding-competent mutants were depleted by serial adsorption to chicken embryo fibroblast (CEF) monolayers at 4°C, and the remaining population was amplified by immune-enhanced infection of P388D1 cells. From the PE2 libraries, 12 candidate mutants showing reduced cytopathic effects on CEF monolayers were isolated and three representative mutants, NB1, NB2, and NB12, were characterized in detail. Insertion mutations for NB1 and NB12 were found near the PE2 cleavage site, whereas the insertion in NB2 occurred between residues 69 and 74 of E2. Although virion assembly and release occurred normally for all three mutants, PE2 cleavage was completely (NB1) or partially (NB12) blocked for the mutants with insertions near the PE2 cleavage site. Both NB1 and NB2 were defective for binding to CEF and BHK-21 cells. Mild trypsin digestion of isolated NB1 virions resulted in PE2 cleavage and partially restored binding to CEF. Besides defective binding, NB1 also exhibited slower CEF penetration kinetics. Consistent with previous work, these results implicate PE2 cleavage and domains in the N-terminal portion of E2 as important determinants of alphavirus binding and penetration. Binding-defective mutants such as NB2, which exhibit normal particle assembly, release, and penetration, may be useful for future efforts to target Sindbis virus infection.

The ability to target virus infection to specific cell types in vitro and in vivo should expand the uses for viral vectors in gene expression studies and therapy. A number of previous studies have explored the use of engineered derivatives of alphaviruses Sindbis virus and Semliki Forest virus (SFV) for transient expression of heterologous gene products in animal cells (reviewed in reference 2). These enveloped animal RNA viruses (46) contain a single positive-strand genomic RNA and are propagated in nature via a mosquito-vertebrate transmission cycle (reviewed in reference 34). Advantages of alphavirus-based gene expression vectors include rapid engineering of expression constructs, production of high-titered stocks of infectious particles, and high levels of expression (2). However, given the broad range of vertebrate and invertebrate tissues infected by these viruses, targeting of infection to specific cell types is not yet possible. One potential approach is to disrupt the receptor-binding domain(s) on the virion spikes and substitute heterologous peptide ligands capable of directing virus attachment to alternative receptors. Ideally, these manipulations should not compromise the efficiency of later stages in the entry process or the assembly and release of infectious particles. In this study, we have begun to explore this approach by isolating and characterizing Sindbis virus mutants with defects in binding to vertebrate cells.

The mature Sindbis virion is an icosahedron (6, 12, 16, 56) composed of 240 copies each (6, 12, 32) of three proteins: the capsid protein, C, and two transmembrane spike glycoproteins, E2 and E1. Virus particles are assembled in a series of steps beginning with translation of a subgenomic mRNA which encodes the structural protein precursors as part of a polyprotein (47, 52). This polyprotein is cleaved co- and posttranslationally to produce the precursors and final products (13, 53). The N-terminal protein, C, acts as an autoprotease to cleave itself from the nascent translation product, allowing the N terminus of the following protein, PE2, to function as a signal sequence for cotranslational translocation into the lumen of the endoplasmic reticulum. Tandem stop transfer and signal sequences at the C terminus of PE2 result in a type I membrane protein topology for PE2, as well as for the following protein, E1. Shortly after synthesis, PE2 and E1 form a heterodimeric complex which is transported to the plasma membrane (40, 64, 65). The C protein selectively encapsidates the genomic RNA (62) to form an iscosahedral nucleocapsid (6) which matures by budding from the plasma membrane via interactions with the cytoplasmic tail of PE2 (27). Shortly before or concomitant with virion release, PE2 is cleaved to produce the E3 glycoprotein (the N-terminal domain of PE2) and E2. E3 can either be secreted into the culture fluid (Sindbis virus; 26) or remain virion associated (SFV; 14). The PE2 cleavage is not required for assembly or release of alphavirus particles (37, 42, 44) but seems to play an important role in regulating virus entry by altering the stability of the E1-E2 interaction (44).

The initial step in the entry process is attachment or binding of the virion to receptors on susceptible host cells. For mammalian cells, at least one Sindbis virus receptor is a protein previously identified as the high-affinity laminin receptor, whose wide distribution and highly conserved nature may be in part responsible for the broad host range of the virus (60). However, different receptors have been identified on chicken embryo fibroblasts (CEF) (61) and

* Corresponding author.

mouse neuronal cells (55), suggesting that the virus can utilize more than one receptor. The domain(s) of the virion spike important for receptor binding has not been defined, and it is unknown whether single or multiple receptor-binding sites are present. Monoclonal antibodies (MAbs) (5, 30, 41, 49, 50) or polyclonal antibodies (7) capable of neutralizing virus infectivity are usually E2 specific, and mutations in E2, rather than E1, are more often associated with altered host range and virulence (24, 36, 50, 57). These findings indicate that E2 may be directly involved in receptor binding but do not exclude a role for E1.

Successful interaction with the receptor(s) precedes penetration of the virion, uncoating of the nucleocapsid, genome translation, and initiation of RNA replication. A widely accepted model suggests that alphaviruses penetrate cells via receptor-mediated endocytosis, followed by fusion of the virion envelope with the endosomal membrane and subsequent release of the nucleocapsid into the cytoplasm (reviewed in reference 25). Fusion is preceded by a structural reorganization of the virion surface which exposes hydrophobic fusogenic domains at the virion surface. In the endocytosis model, this reorganization is triggered by the low pH of the endosome (63) and recent work suggests that the E1 hemagglutinin (7, 31) forms a homooligomer under these conditions (58, 59). Changes in alphavirus structure after exposure to low pH have been detected as alterations in the antigenic profile and physical properties of the virion and by differential protease sensitivity of the glycoproteins (9, 18, 49). However, reorganization of the virion surface can also be detected at the plasma membrane after receptor binding (3, 11, 28), leading to an alternative hypothesis in which fusion occurs at the plasma membrane rather than in an acidic endosomal compartment (3).

The aim of this work was to select and characterize Sindbis virus mutants which are defective in attachment to vertebrate cell surface receptors. Previous work demonstrated that viable mutants expressing heterologous epitopes on the virion surface could be recovered after random insertion mutagenesis of the PE2 gene in a full-length Sindbis virus cDNA clone (23). In this study, similar libraries (22, 23), containing random insertions in the PE2 or E1 glycoprotein gene, were used to select mutants with impaired binding to CEF monolayers. Several candidate mutants were identified, the sites of insertion were mapped, and the effects of the mutations on assembly, release, binding, and penetration were characterized. Two of the mutants exhibiting severe binding defects may prove useful in future efforts to target Sindbis virus infection.

MATERIALS AND METHODS

Cell culture and MAbs. Tissue culture cells, both BHK-21 and CEF, were maintained in Eagle's minimal essential medium (MEM) containing 10% (for BHK-21) or 4% (for CEF) fetal bovine serum (FBS). P388D1 cells were maintained in Dulbecco's modified essential medium containing 10% FBS. Anti-E2 (MAbs 49 and 50) and anti-E1 (MAb 33) MAbs were kindly provided by A. L. Schmaljohn and have been characterized previously (48, 49). Anti-E2 MAbs (R6 and R13) were a gift from R. E. Johnston and R. Schoepp and have been described previously (29, 30). MAbs that react with the 4D4 and G1 epitopes of Rift Valley fever virus (RVFV) were kindly provided by J. Smith (1).

Random insertion mutagenesis. Libraries of random insertions in the PE2 or E1 gene of a full-length Sindbis virus cDNA clone have been described previously (22, 23). Briefly, the insertional mutagen was a 45-bp oligonucleotide which encodes the RVFV 4D4 epitope in one of two possible open reading frames (23). Two different insertion libraries have been produced for PE2 which differ in the agent used to produce breaks in the target DNA, either DNase I in the presence of Mn^{2+} or methidiumpropyl-EDTA-Fe^{2+} (MPE) (22). MPE treatment was used for the E1 insertion library. The parental virus for these experiments, TR2001, was derived from transcripts of pTR2001. pTR2001 is a derivative of pTR2000 (36) in which the *Sst*I recognition site upstream from the SP6 promoter has been eliminated. The PE2 DNase I library represents more than 7×10^3 independent clones (23), and the PE2 and E1 MPE libraries represent 3×10^5 and 4×10^5, respectively (22).

Selection for mutants with impaired binding capacity. Plasmid DNAs from the final full-length insertion libraries were linearized by using *Xho*I and used as transcription templates for SP6 RNA polymerase (38). Populations of mutant viruses were generated by electroporation of 5×10^6 BHK-21 cells (20) with 2 μg of the resulting capped RNA transcripts. This method results in nearly 100% transfection efficiency of the surviving cells (20). Virus stocks were produced in MEM without $NaHCO_3$ and supplemented with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (final pH, 7.0). Culture supernatants, harvested 7 or 8 h after transfection, were adsorbed for 2 h at 4°C in six-well plates (600 μl per well) containing CEF. Five cycles of 2 h of adsorption plus overnight adsorption were done before amplification of the residual inoculum by using the immune enhancement method described by Chanas et al. (5). After adsorption, the residual inoculum was incubated with P388D1 cells after addition of a mixture of anti-E2 (MAb 49 ascitic fluid, 1/10,000 dilution) and anti-E1 (MAb 33, 1/10,000 dilution) MAbs or hyperimmune anti-E1+E2 ascitic fluid (1/5,000 dilution). Optimal conditions for immune enhancement using these antibodies were determined by using the parental virus. Where indicated, some mutants were selected by using an anti-4D4 MAb in the immune-enhanced amplification steps. Virus was allowed to amplify in P388D1 cells for 3 days.

After multiple cycles of CEF binding and P388D1 amplification, individual mutants were cloned by end-point dilution. Tenfold dilutions of the residual inoculum from the last CEF-binding depletion cycle were added to 96-well plates containing P388D1 cells and the appropriate dilution of enhancing antibody. After 2 or 3 days of incubation at 37°C in Dulbecco's modified essential medium containing 10% FBS, an aliquot from each well was transferred to CEF monolayers in 96-well plates and the rest of the supernatant was stored at −80°C. Dilution end points were determined by virus-specific immunofluorescence. P388D1 cells in 96-well plates were fixed with isopropanol for 10 min at 4°C and incubated successively with anti-E1+E2 rabbit serum (dilution, 1/1,000) and then rhodamine-conjugated goat anti-rabbit immunoglobulins (dilution, 1/100; Boehringer Mannheim), each for 30 min at 37°C. Infected CEF monolayers in 96-well plates were incubated for 2 days at 37°C, and the last dilution showing a cytopathic effect was determined. Dilutions which showed a positive signal by immunofluorescence and no cytopathic effect on CEF were amplified on P388D1 cells and checked a second time for absence of a cytopathic effect. Then, a second limiting dilution selection was performed. Some mutants showing a partial cytopathic effect on CEF after 2 days were also amplified and characterized.

Generation of mutant cDNA clones. Standard recombinant DNA techniques were used to generate cDNA clones (45).

Total RNA from infected P388D1 cells was extracted at 24 h by the RNazol B method as recommended by the manufacturer (Tel-Test). cDNA was synthesized by using an oligo(dT) primer (39) and Superscript reverse transcriptase (GIBCO-BRL). This cDNA was used for polymerase chain reaction (PCR) amplification (43) of the gene encoding PE2 by using a plus-sense oligonucleotide which hybridized 5′ to the *Stu*I site in Sindbis virus cDNA (position 8569) and a negative-sense oligonucleotide hybridizing 3′ to the *Bss*HII site (position 9804). For each mutant, the resulting product was digested with *Stu*I and *Bss*HII, isolated, and used to replace the corresponding region in pTR2001. The position of each insertion mutation was localized by appropriate restriction enzyme analyses, and the region containing the insert was sequenced (45). For the three mutants selected for extensive characterization, the sequence of the entire *Stu*I-*Bss*HII region was determined to eliminate clones that had secondary mutations introduced during cDNA cloning or PCR amplification. The full-length cDNA clone for the double mutant NB1+2 was constructed by subcloning appropriate restriction fragments from the cDNA clones of NB1 and NB2.

Production of virus stocks and virus purification. As described above, mutant or parental virus stocks from cDNA clones were produced by electroporation of BHK-21 cells. At 12 h after transfection, culture supernatants were harvested and aliquots were stored at −80°C.

For virus purification, BHK-21 cells were infected with about 1 PFU per cell (PFU determined as described below in the binding assay section). The culture supernatant was harvested 15 h after infection and clarified by centrifugation for 30 min at 8,000 rpm in a Sorvall SS34 rotor, and virus was precipitated with polyethylene glycol as described previously (35). The virus was resuspended in TNE (0.2 M NaCl, 0.05 M Tris [pH 7.4], 1 mM EDTA) and centrifuged through a 15 to 45% (wt/vol) sucrose gradient in TNE for 90 min at 35,000 rpm in a Beckman SW50.1 rotor at 4°C. The virus band was collected, diluted in TNE, and concentrated by centrifugation through a cushion of 15% sucrose in TNE for 90 min at 35,000 rpm in an SW50.1 rotor. The pellet was resuspended in phosphate-buffered saline (PBS) and stored at −80°C. In some experiments, virus was labeled with ^{35}S-translabel (ICN). At 4 h postinoculation, the medium was replaced with MEM containing 1/40 of the normal concentration of methionine, 2% FBS, and 50 μCi of ^{35}S-translabel (ICN) per ml and incubation was continued for an additional 12 h at 37°C.

Analysis of virus maturation. To compare the release of progeny virions between the mutants and parental virus, RNAs transcribed in vitro were use to transfect BHK-21 cells by electroporation. Transfected cells were plated in 60-mm-diameter tissue culture plates and incubated at 37°C, and at 2-h intervals, a portion of each supernatant was harvested and stored at −80°C. Titers were determined in parallel by end-point dilution on P388D1 cells in the presence of MAbs 33 and 49 (1/10,000 dilution of each). After 48 h at 37°C, the presence of virus was revealed by immunofluorescence as described above. Titers are expressed in 50% tissue culture-infective doses ($TCID_{50}$) per milliliter.

Binding assays. For the nonradioactive binding-entry assay, 10-fold dilutions of virus in PBS containing 1% FBS were incubated with CEF in six-well plates (200 μl per well) for 1 h at 4°C. Monolayers were then rinsed three times with PBS to remove unbound particles and overlaid with 1% agarose in MEM containing 2% FBS. Plates were fixed after 2 days, and plaques were revealed by crystal violet staining (for the parental virus and mutants NB2 and NB12) or immunostaining (as described below). This binding assay was done in parallel for the parental virus and all of the mutants by using equivalent $TCID_{50}$ titers. A reduced number of PFU indicates that the mutant has an impaired ability to enter cells, if maturation is similar to that of the parental virus.

To differentiate between defects in binding versus penetration, radioactive binding assays were also performed. Two hundred microliters of labeled purified virus particles in PBS containing 1% FBS was adsorbed in triplicate to CEF or BHK-21 cells in six-well plates for 1 h at 4°C. Plates were rinsed three times with PBS to remove unbound particles, and the cells were lysed with 0.5% sodium dodecyl sulfate (SDS). The associated radioactivity was measured by scintillation counting.

Penetration assay. Virus penetration was measured by using the technique described by Flynn et al. (11), with minor modifications. Briefly, virus was allowed to attach to approximately 10^6 CEF in 60-mm tissue culture plates for 60 min at 4°C. Virus titers were adjusted to give approximately 100 to 200 PFU after removal of the inoculum and three washes with PBS. The virus-cell complexes, in cold MEM, were then shifted to 37°C. At intervals between 0 and 40 min, two plates were chilled by replacing the medium with ice-cold PBS and incubated for 45 min at 4°C in the presence of 2 ml of PBS containing 0.5 mg of proteinase K per ml. Protease digestion was terminated by addition of 10 ml of PBS containing 10% FBS and 1 mM phenylmethylsulfonyl fluoride. The cell suspensions were removed from the tissue culture plates, pelleted by centrifugation at 600 × *g*, washed once with PBS containing 10% FBS and 1 mM phenylmethylsulfonyl fluoride, and resuspended in 20 ml of MEM containing 4% FBS. A 200-μl sample of the cell suspension was plated in each well of a 96-well plate, and the wells containing infectious centers were revealed by immunostaining after 24 or 48 h. The number of infectious centers in the original suspension was calculated by using the Poisson distribution. In this procedure, only those PFU resistant to removal or inactivation by proteinase K are counted as infectious centers. The number of PFU that had attached were determined by plaque assay by using a parallel set of 60-mm tissue culture plates which had been overlaid with agarose. The proportion of PFU that penetrated cells (became resistant to proteinase K) was calculated by dividing the number of infectious centers by the number of PFU as measured by plaque assay.

Digestion of virus with exogenous trypsin. Trypsin treatment of virus was carried out in PBS, pH 7.4. Between 1×10^6 and 4×10^6 $TCID_{50}$ (10^5 to 2×10^5 cpm) of virus was incubated with 15 μg of trypsin (Boehringer Mannheim) per ml for 30 min on ice. After protease digestion, soybean trypsin inhibitor (Boehringer Mannheim) was added to 100 μg/ml and incubation was continued for 10 min on ice. This mixture was used directly for subsequent experiments.

Plaque immunostaining. One hour after virus inoculation, CEF monolayers in six-well plates were overlaid with 1% agarose in MEM containing 2% FBS. After 2 days at 37°C, the agarose plugs were carefully removed and the monolayers were fixed with isopropanol for 10 min at 4°C. Monolayers were incubated successively with rabbit anti-E1+E2 serum (diluted 1/10,000 in PBS) followed by biotinylated goat anti-rabbit immunoglobulins (1/200 dilution; Sigma), each for 30 min at 37°C. Plates were then incubated with the Vectastain ABC alkaline phosphatase kit (Vector Laboratories) for 30 min at room temperature and revealed by using

Alkaline Phosphatase Substrate kit II (Vector Laboratories). Between steps, plates were rinsed three times with PBS and the final color reaction was stopped by rinsing with distilled H_2O.

ELISA. Anti-E2 MAbs were tested by enzyme-linked immunosorbent assay (ELISA) (15) for the ability to bind to mutant and parental virions. Briefly, wells of 96-well plates (high binding, type II; Costar) were incubated overnight at 4°C with 100 μl of purified virus in PBS (0.5 μg/ml). Following adsorption of virus, plates were blocked with 5% (wt/vol) skim milk for 2 h at room temperature. A 1-h incubation of serial 10-fold dilutions of the MAbs (in PBS–0.5% skim milk) was followed by incubation (1 h) with goat anti-mouse peroxidase-conjugated immunoglobulins (Boehringer Mannheim). Plates were developed by using the ABTS substrate (Boehringer Mannheim) as described by the manufacturer. Between steps, plates were rinsed three times with PBS. A MAb-binding index was calculated for each mutant by normalizing the binding of a given MAb to the level of binding of anti-E1 MAb 33. This value was normalized to that obtained for the parental virus. The MAb X binding index was calculated with the following formula: (OD_{405} of MAb X on mutant Y/OD_{405} of MAb 33 on mutant Y)/(OD_{405} of MAb X on parental virus/OD_{405} of MAb 33 on parental virus), where OD_{405} is optical density at 405 nm.

An ELISA was also performed to compare the level of expression of the viral glycoproteins of the double mutant with that of the parental virus. CEF monolayers in 96-well plates were washed with PBS and transfected with RNA transcripts (100 ng of RNA per well) by using 50 μl of RNase-free PBS containing 2.5 μl of lipofectin (GIBCO-BRL) per well (10). After 10 min at room temperature, the transfection mixture was removed and replaced with MEM containing 2% FBS. Four or 5 h after transfection, cells were fixed with isopropanol for 10 min at 4°C and relative expression of the viral glycoproteins was determined by ELISA using anti-E1+E2 hyperimmune rabbit serum as described above. Nontransfected wells were used as negative controls.

Immunoprecipitation. The antigen used for immunoprecipitation analyses was radiolabeled purified virus diluted with 5 volumes of immunoprecipitation buffer (0.15 M NaCl, 0.05 M Tris-Cl [pH 7.2], 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 0.1% NaN_3). The immunoprecipitation procedure, using fixed *Staphylococcus aureus* Cowan I (Calbiochem), was done as described by Harlow and Lane (15). Labeled proteins were separated by SDS–8% polyacrylamide gel electrophoresis (PAGE) (19). After electrophoresis, gels were treated with sodium salicylate (4), dried, and exposed to Kodak XAR-5 film.

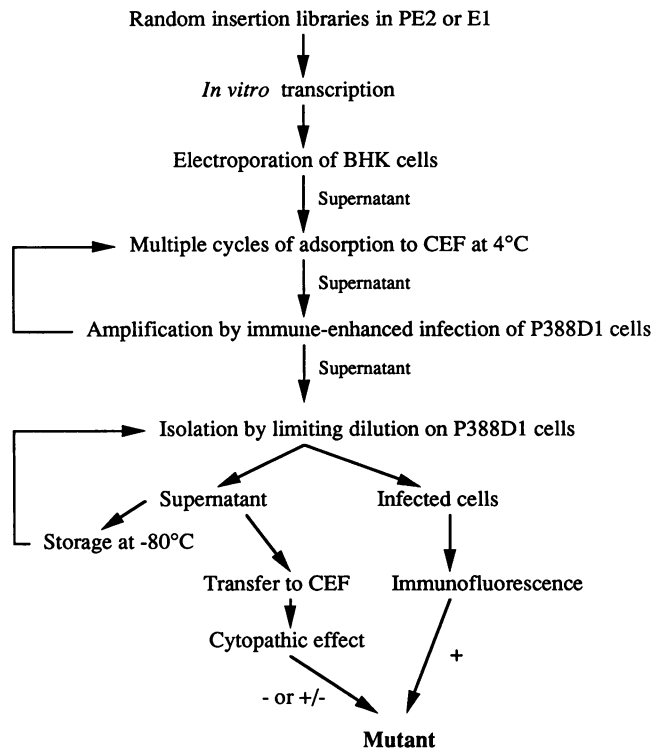

FIG. 1. Scheme for isolation of mutants with impaired binding to CEF. See Materials and Methods and Results for further details.

RESULTS

Selection and isolation of mutants with reduced binding to CEF. Libraries of random insertion mutations in the Sindbis virus PE2 or E1 glycoprotein gene (22, 23) were used to screen for viral mutants with impaired binding to CEF (Fig. 1). The insertional mutagen used to construct these libraries consisted of a 45-bp synthetic oligonucleotide which could encode an 11-amino-acid epitope of RVFV (reacting with MAb 4D4) in one of two possible open reading frames (23). Given the complexity of these libraries (which ranged from 7×10^3 to 4×10^5 independent insertion mutations) and the resulting virus populations produced by electroporation, we expected that some mutants might have totally or partially lost the ability to bind to vertebrate host cell receptors while retaining the ability to penetrate cells, initiate replication, and assemble progeny virions. To select for binding-defective mutants, virus populations were repeatedly adsorbed to CEF at 4°C. This procedure was first optimized by using the parental virus, TR2001. After 2 h at 4°C, infectivity was reduced by about 50%, and after several cycles of adsorption, the residual inoculum showed an about 2-order-of-magnitude reduction in infectivity compared with a control experiment conducted in the absence of CEF (data not shown). After these adsorption steps, the virus remaining in the supernatant was amplified in P388D1 cells by immune enhancement (Fig. 1) (5). In P388D1 cells, Sindbis virus infection is enhanced in the presence of low concentrations of glycoprotein-specific antibodies, presumably via interaction of opsonized virus particles with Fc receptors on the surface of this macrophage-like cell line. Hence, mutants blocked for binding to the normal virus receptor(s) can be propagated by this method if they retain both the ability to bind the enhancing antibodies and perform later steps in the entry process. Since some insertion mutations might be expected to disrupt binding of particular antibodies, we used several different antisera for P388D1 amplification, including a mixture of E1- and E2-specific MAbs, polyclonal serum to E1+E2, or MAb 4D4, which reacts with the RVFV epitope encoded by one reading frame of the insertional mutagen (Table 1).

Up to three cycles of CEF adsorption and P388D1 amplification were necessary to isolate mutants impaired in the ability to infect CEF. Mutants with possible defects in CEF binding were initially identified by screening for a severe reduction in cytopathic effect. Twelve mutants, all derived from the PE2 insertion libraries, were selected for further characterization and cloned by limiting dilution. These mutants (NB1 to NB12), the random insertion libraries from

TABLE 1. Derivation of CEF binding-defective Sindbis virus mutants[a]

Mutant	PE2 library[b]	No. of selection cycles[c]	Enhancing antibody[d]	Group[e]
NB1	MPE	3	49+33	1A
NB2	DNase I	3	49+33	2
NB3	DNase I	3	49+33	2
NB4	MPE	3	49+33	1B
NB5	DNase I	3	HI	2
NB6	DNase I	3	HI	2
NB7	DNase I	1	4D4	3
NB8	DNase I	1	4D4	3
NB9	DNase I	1	4D4	3
NB10	MPE	1	HI	1B
NB11	DNase I	1	4D4	3
NB12	MPE	1	4D4	3

[a] Selected CEF-nonbinding (NB) mutants were produced as described in Materials and Methods.
[b] PE2 random insertion library constructed in pTR2001 by using DNase I or MPE (22).
[c] Number of cycles of CEF adsorption and immune-enhanced amplification in P388D1 cells.
[d] Antisera used for selection during immune-enhanced amplification in P388D1 cells. 49+33, Sindbis virus E2- and E1-specific MAbs 49 and 33; HI, Sindbis virus-specific hyperimmune serum to E1+E2; 4D4, anti-RVFV MAb 4D4.
[e] Grouping is by plaque morphology and insertion site (see also Fig. 2 and 3).

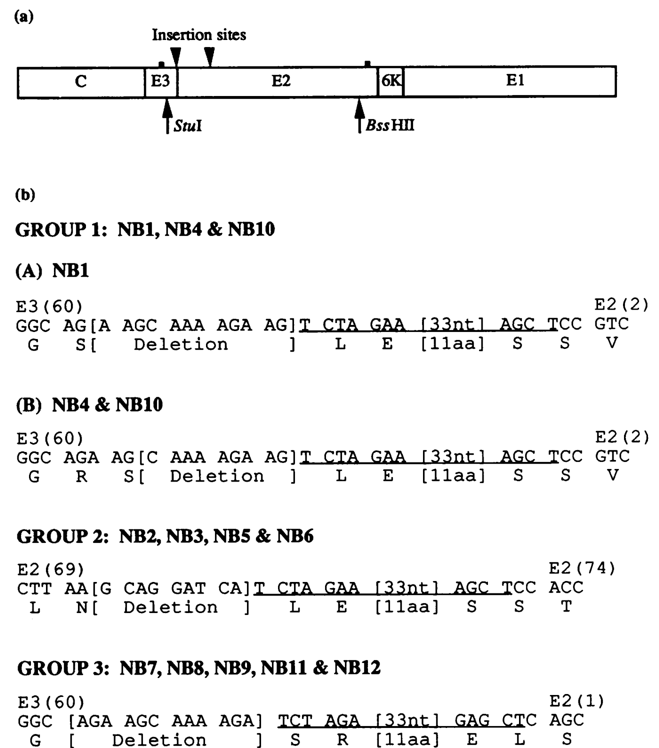

FIG. 2. Location and sequence of selected insertion mutations. (a) Diagram of Sindbis virus structural polyprotein organization (39). Insertion sites are indicated by arrowheads, and the *Stu*I and *Bss*HII restriction sites used to clone the mutagenized PE2 region into the full-length parental cDNA clone (TR2001) are indicated. The approximate locations of the two oligonucleotides used for PCR amplification of the PE2 gene are indicated by small squares. (b) Nucleotide (nt) and translated sequences of the insertion mutations and flanking regions. The PE2 sequences flanking each insertion site are shown. A partial sequence of the inserted oligonucleotide (23) is underlined. Nucleotides deleted from the PE2 sequence, produced as a result of the methods used to linearize and repair the target DNA (8, 17, 22, 23), are bracketed. Numbers refer to the positions of the indicated amino acid (aa) residues from the N termini of the respective proteins (39). The following single-letter code for amino acids is used: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. The 11-amino-acid sequences not shown are REPWTQVRLSA, for mutants in groups 1 and 2, and KGTMDSGQTKR, for mutants in group 3.

which they originated, the numbers of cycles of CEF adsorption and P388D1 amplification, and the antibodies used for immune enhancement are summarized in Table 1. A stock of each mutant was used for preliminary characterization and to infect P388D1 monolayers for production of total infected-cell RNA. The region of PE2 containing the insertion mutations was amplified by reverse transcription-PCR and cloned into the original parental plasmid to construct full-length cDNA clones for each mutant. Clones yielding infectious RNA transcripts were used to generate virus stocks for further characterization and to localize the insertion mutations.

Mapping of insertion sites and preliminary characterization of the mutants. Digestion with appropriate restriction enzymes, followed by limited nucleotide sequence analysis, was used to determine the position and sequence of each insertion mutation. Along with short deletions, three insertion sites in the N-terminal portion of PE2 were identified (Fig. 2). Two groups of mutants contained insertions at or near the E3-E2 cleavage site. For mutants NB1, NB4, and NB10 (group 1), the inserted sequence was located between residue 60 (NB1) or 61 (NB4 and NB10) of E3 (E3 is 64 amino acids) and the second residue of E2. The sequences of mutants NB7, NB8, NB9, NB11, and NB12 (group 3) were found to be identical and the insert was located between residue 60 of E3 and the first residue of E2. The latter group was amplified by using the RVFV 4D4 MAb for immune enhancement, and as expected, these mutants encoded the 4D4 epitope in frame with the structural protein open reading frame. The sequences of mutants NB2, NB3, NB5, and NB6 (group 2) were also identical and contained the insert between residues 69 and 74 of E2. For mutants in both groups 1 and 2, the alternative open reading frame encoded by the inserted oligonucleotide was in frame with the structural-protein-coding sequences.

The plaque phenotypes of the mutants on CEF monolayers were also characterized. One representative from each group is shown in Fig. 3. CEF infected by NB1, NB4, and NB10 did not show any plaque formation as revealed by crystal violet staining (data not shown). However, by immunostaining, tiny plaques could be detected 2 days after inoculation (Fig. 3). Sometimes medium plaques were observed, suggesting that revertants had arisen; nevertheless, they appeared to represent only a minor population in these virus stocks. The relative sizes of the plaques produced by mutants NB2, NB3, NB5, and NB6 or NB7, NB8, NB9, NB11, and NB12 were medium or small, respectively, compared with the size of the plaques produced by the parental virus (Fig. 3). Given the results of the limited sequence analyses and the plaque assays (Fig. 2 and 3), only one representative from each group was selected for further characterization (NB1 [group 1], NB2 [group 2], and NB12 [group 3]). The PCR-amplified region used to construct the corresponding full-length clones for these mutants (the *Stu*I-

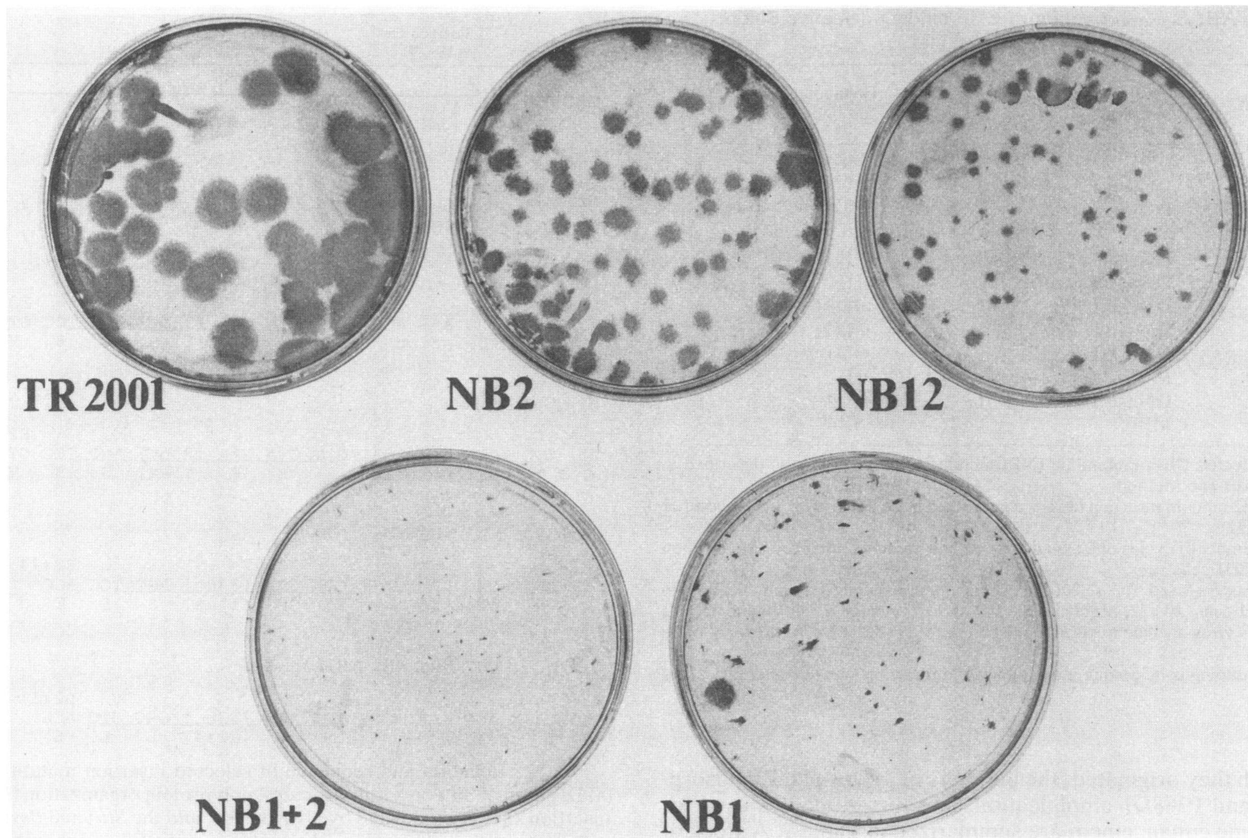

FIG. 3. Plaque phenotypes of parental and mutant viruses on CEF. Shown are plaques produced on CEF by the parental virus (TR2001); insertion mutants in groups 1 (NB1), 2 (NB2), and 3 (NB12); and the double mutant (NB1+2). Plaques were revealed by immunostaining (see Materials and Methods) after 2 days of incubation at 37°C.

*Bss*HII region, Fig. 2a) was checked to verify that only the sequence changes shown in Fig. 2b were present.

Kinetics of virus maturation and release. After CEF adsorption and P388D1 amplification, potential binding-defective mutants had been chosen on the basis of reduced cytopathic effects on CEF. This phenotype could also result from defects in other steps in the replication cycle besides binding to CEF receptors. To compare maturation and release of the parental virus with those of mutants NB1, NB2, and NB12, electroporation of BHK-21 cells with RNA transcripts was used to bypass the entry step. With this method, virus yield depends only on RNA amplification, particle assembly, and virus egress from transfected cells. A defect in one or more of these steps should reduce virus accumulation in the culture medium. Given that some mutants might be impaired for binding to CEF cells, virus yields were determined by end-point dilution assays using P388D1 cells in the presence of enhancing antibody (Fig. 4). The time course and yield of viral progeny were similar for NB1 (Fig. 4a), NB2 (Fig. 4b), NB12 (Fig. 4c), and parental strain TR2001, indicating that the insertion mutations did not adversely affect viral particle assembly and release.

Protein components of purified virions. To determine possible effects of the insertions on the maturation of E2, the structural proteins present in purified virions were analyzed by SDS–8% PAGE (Fig. 5a). Mutant NB2 showed the same protein profile as the parental virus. In mutants NB1 and NB12, a protein with a higher molecular weight, corresponding to uncleaved PE2, was detected. Partial PE2 cleavage was observed for mutant NB12 but not for NB1, as confirmed by immunoprecipitation using an E2-specific MAb (data not shown). Together with the data from the assembly-and-release assay (Fig. 4), these data indicate that PE2 cleavage is not necessary for efficient utilization of the NB1 PE2-E1 heterodimer complex for particle formation. Similar results had been obtained for the S.A.AR86 strain of Sindbis virus (42) and an SFV mutant with a mutation blocking cleavage at the E3-E2 site (44).

Binding of the mutants to CEF and BHK-21 cells. To determine whether the mutants were impaired for binding or at other steps in the entry process, several assays were performed. In the first experiment, nonradioactive virus was adsorbed to CEF for 1 h at 4°C and, after removal of the inoculum, the bound virus was measured in a plaque assay. Since the maturation of mutants NB1, NB2, and NB12 was similar to that of the parental virus, a reduction of infectivity by this assay indicates entry defects at the level of attachment or penetration or at both levels. Mutants NB1 and NB2 showed a large reduction in the number of PFU produced in this entry assay (Fig. 6), which was repeated by using independent virus stocks with similar results (data not shown). Their infectivities, compared with that of the parental virus, were reduced to 1.8% for NB1 and 7% for NB2. In contrast, NB12 showed only a modest defect in entry compared with the parent.

To determine whether the impaired entry of mutants NB1

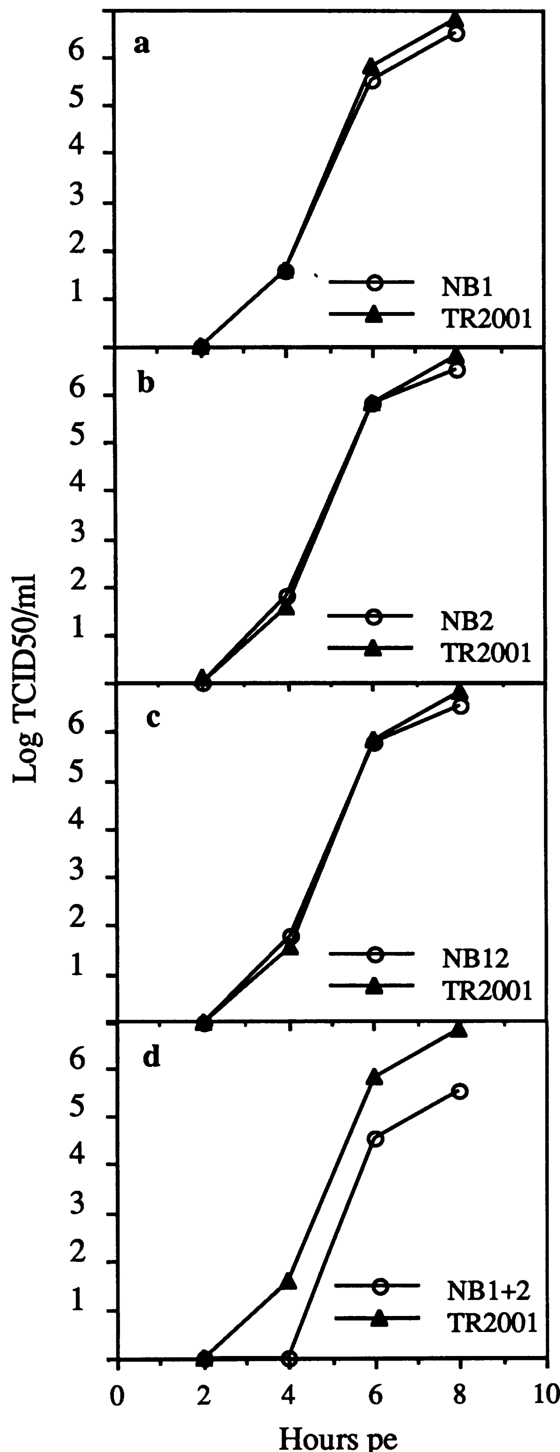

FIG. 4. Kinetics of virus release. Release of progeny virus was measured for the parental virus (TR2001); mutants in groups 1 (NB1), 2 (NB2), and 3 (NB12); and the double mutant (NB1+2). At the indicated times after electroporation (pe) of BHK-21 cells, released virus was measured by immune-enhanced titration on P388D1 cells as described in Materials and Methods.

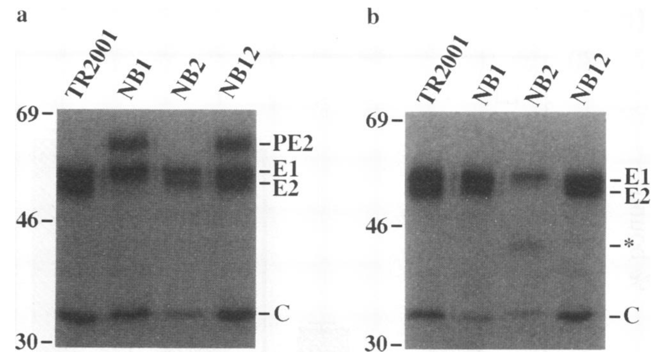

FIG. 5. Structural protein components of parental and mutant virions. Purified parental (TR2001) or mutant virions (NB1, NB2, and NB12 of groups 1, 2, and 3, respectively) were analyzed by SDS-PAGE. Purified virions were labeled with [^{35}S]Met and treated (b) or not treated (a) with trypsin prior to electrophoresis. The positions of the virion capsid protein (C), glycoproteins (E2 and E1), and the uncleaved form of E2 (PE2) are indicated. After trypsin treatment of NB2, a 41-kDa band (*) which correlated with the disappearance of E2 appeared. Molecular masses (in kilodaltons) are indicated at the left of each panel.

and NB2 was due to a reduction in the ability to attach to host cells, purified, radiolabeled virus preparations were used to assay binding to CEF or BHK-21 monolayers at 4°C. Mutants NB1 and NB2 showed similar reductions in the ability to bind to CEF cells (Fig. 7a) and, to a lesser extent, BHK-21 cells (Fig. 7b). Mutant NB12 was also examined in this assay and did not exhibit defective binding to either CEF or BHK-21 cells and hence was less interesting for the present study.

Since the nonradioactive entry assay (Fig. 6) had shown that the infectivity of mutant NB1 was significantly reduced

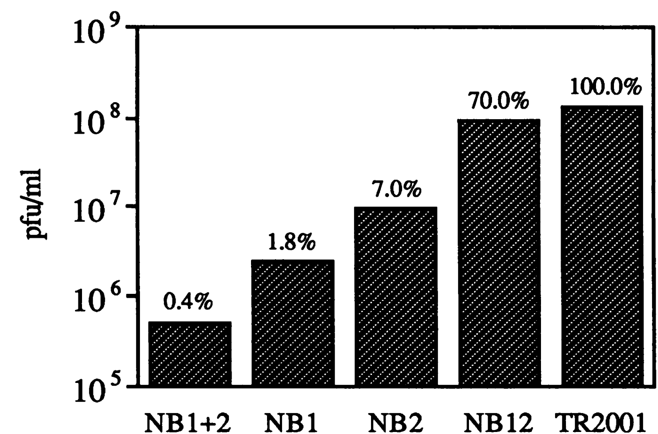

FIG. 6. CEF binding and entry by parental and mutant viruses. A nonradioactive assay was used to compare the entry process of the parental virus (TR2001) with those of mutants NB1, NB2, and NB12 and double mutant NB1+2. For each virus, 10-fold serial dilutions of an equivalent number of infectious particles (as determined by immune-enhanced titration on P388D1 cells; 3.5×10^6 $TCID_{50}$/ml) were allowed to adsorb to CEF at 4°C (see Materials and Methods). The number of PFU produced represents the number of infectious particles which were still able to enter (bind and penetrate) cells and initiate an infectious cycle. For NB1+2, the number of PFU estimated by this assay could be an underestimate given the tiny plaques produced by this mutant on CEF.

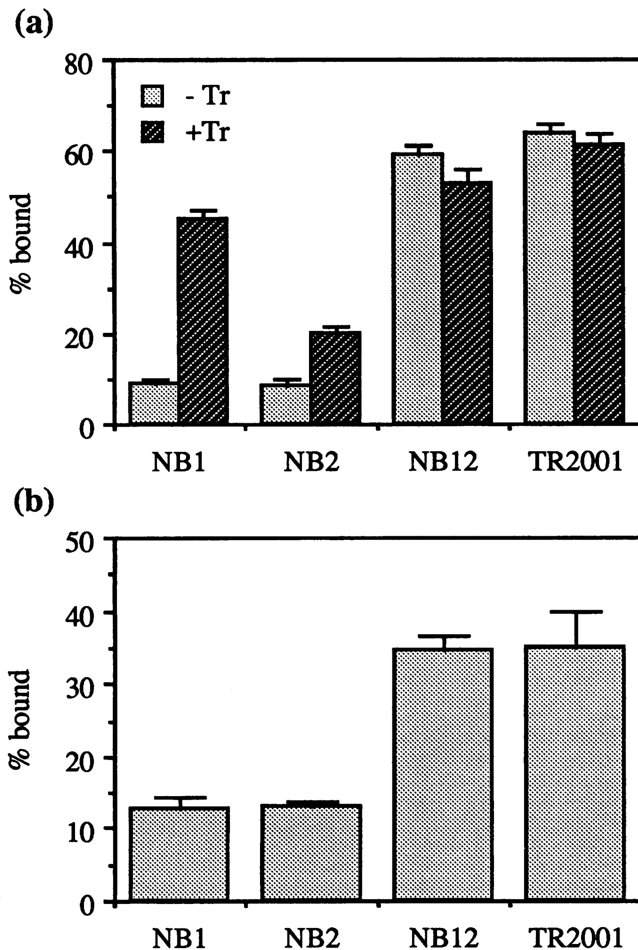

FIG. 7. Binding of purified virus to CEF and BHK cells. Purified preparations of radiolabeled parental virus (TR2001) and mutants NB1, NB2, and NB12 (10^5 to 2×10^5 cpm, corresponding to 1×10^6 to 4×10^6 $TCID_{50}$) were tested for binding to CEF (a) or BHK-21 (b). The results are expressed as percentages of the total input radioactivity that bound to cells after 1 h of incubation at 4°C, and standard deviations are shown above the bars. Virions were treated (+Tr) or not treated (−Tr) with trypsin.

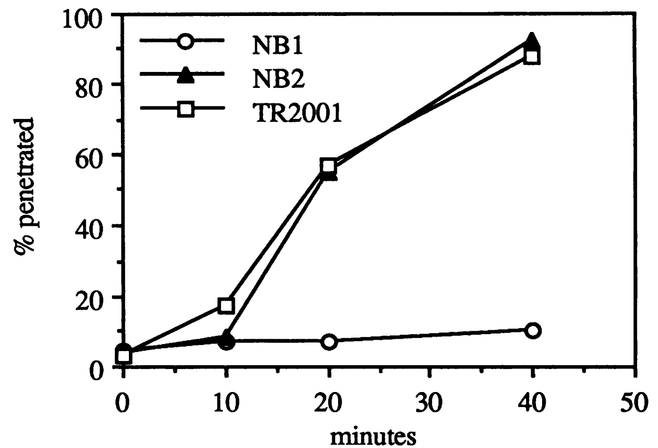

FIG. 8. Penetration kinetics of parental and mutant viruses. The CEF penetration rates of the parental virus (TR2001) and mutants NB1 and NB2 were compared. Penetration was measured as the appearance of proteinase K-resistant infectious centers as described in Materials and Methods. The standard deviations are not shown, since for most of the data the values were too small to be seen in the figure.

compared with that of NB2, the similar binding values (Fig. 7) suggested that NB1 is defective in an additional entry step. The kinetics of virus penetration were examined by adsorbing virus to CEF monolayers at 4°C and then incubating them for different periods at 37°C. Penetration was assayed by the appearance of proteinase K-resistant infectious centers (11). As shown in Fig. 8, the kinetics of penetration were significantly slower for NB1 than for NB2, which penetrated at a rate similar to that of the parental virus. These results suggest that the difference in infectivity observed between NB1 and NB2 in the CEF plaque assay was due to a difference in penetration rate.

PE2 cleavage and binding to CEF. Previous work has shown that mutant particles of SFV with uncleaved PE2 were essentially noninfectious for BHK-21 cells and were defective for binding (44). Infectivity could be restored by limited trypsin digestion of isolated virus, which cleaved the mutant PE2 near the normal E3-E2 cleavage site. Since the NB1 insertion mutation at the E3-E2 junction (or the deleted residues at the C terminus of E3) resulted in blocked PE2 cleavage (Fig. 5a), we examined the effect of trypsin treatment on NB1 PE2 cleavage and CEF binding. Mild trypsinization cleaved the PE2 glycoprotein on the surface of purified NB1 virions to produce an E2-specific species migrating slightly slower than parental E2 (Fig. 5b). Trypsin cleavage may have occurred in the inserted segment, since two arginine residues are present in this sequence (legend to Fig. 2). This trypsin treatment restored CEF binding to 74% of the level observed for the parental virus (Fig. 7a). These results suggest that the insertion mutation, per se, did not irreversibly inactivate the glycoprotein domain(s) important for binding. Rather, cleavage of PE2 may result in an altered conformation of E2 or E3 (or perhaps removal of E3 from the virion surface) such that NB1 attachment to CEF is no longer blocked. Mutant NB12, which also contains the inserted sequence at the E3-E2 junction, expressed significant quantities of unprocessed PE2 on its surface, which was converted to E2 after trypsin digestion. An obvious effect on CEF binding was not observed, since untreated NB12 showed only a slight reduction compared with the parental virus (see Discussion).

An unexpected finding was the increase in CEF binding observed for mutant NB2 after trypsin treatment. This mutant contains the inserted sequence after residue 69 of E2, and uncleaved PE2 was not detected on purified virions (Fig. 5a). Upon treatment with trypsin, the NB2 E2 protein was digested to produce a 41-kDa protein (Fig. 5b) and one or more additional proteolytic fragments (which were not detected). The susceptibility of NB2 E2 cleavage suggests that either the inserted sequence was exposed on the surface of the virion and accessible to trypsin or the insertion modifies the structure of E2 to expose a novel trypsin cleavage site which is otherwise buried in the parental E2 protein. The estimated molecular mass of the cleavage product, the location of the insert, and the presence of potential trypsin cleavage sites in the inserted sequence suggest that the former hypothesis is more likely. Such a cleavage might have removed a portion of E2 from the virus which had inhibited attachment or, more likely, induced a conformational change in E2 which directly affected interaction with

TABLE 2. Indices of anti-E2 MAb binding to NB1, NB2, and NB12[a]

MAb	Mean binding index ± SEM		
	NB1	NB2	NB12
49	1.60 ± 0.10	0.71 ± 0.07	1.06 ± 0.06
50	0.37 ± 0.01	0.16 ± 0.01	0.28 ± 0.01
R6	0.05 ± 0.01	0.50 ± 0.01	0.19 ± 0.06
R13	0.04 ± 0.00	0.62 ± 0.10	0.14 ± 0.01

[a] Binding indices, relative to that of the parental virus (1.00), were measured by ELISA and calculated as described in Materials and Methods. It should be noted that these values were normalized to El-specific MAb 33 binding, which did not differ by more that twofold between virus preparations. This point is relevant since previous work has shown that the E2ab antigenic domain and the El domain recognized by neutralizing MAb 33 are in close physical proximity (51). Hence, insertions in E2 could conceivably reduce MAb 33 binding, making the values given in the table minimum estimates. Samples were analyzed in triplicate, and the experiment was repeated with similar results.

the cell surface receptor or exposed the fusogenic region on the viral spike.

Reactivity with MAbs. A collection of neutralizing MAbs has been used to define antigenic domains on the Sindbis virus E2 glycoprotein (called E2a, E2b, and E2c) (see Discussion). Epitopes present in the E2a and E2b domains are very close or overlap and are recognized by representative MAbs 49 and 50 (49, 51). The E2c antigenic domain, defined by MAbs R6 and R13 (30), does not overlap the E2ab domain. To examine the effects of these insertion mutations on the structure of E2 on the virion surface, an ELISA was performed to measure the relative binding of these neutralizing MAbs. The binding indices are given in Table 2. Similar patterns of reactivity were found for mutants NB1 and NB12, which contained the inserted sequence in the E3-E2 junction. Binding to MAb 49 was either enhanced (NB1) or similar to that of the parental virus (NB12). Both mutants exhibited reduced binding to MAb 50 and dramatically reduced binding to E2c MAbs R13 and R6. For NB2, MAb 50 showed the most pronounced reduction in binding, with only about a twofold reduction for binding to the E2c MAbs.

Construction and characterization of a double mutant. Given the diverse tissues which can be infected by alphaviruses, one goal of this work was to isolate mutants with broad defects in binding to a variety of cell types. Such mutants, with a highly restricted host range, might then be useful for targeting infection to specific cell types via engineering of the expression of appropriate peptide ligands on the virion surface. To reduce Sindbis virus binding to vertebrate cells further, a double mutant (NB1+2) which contained the insertions from the two mutants which had shown diminished binding to both CEF and BHK-21 cells (NB1 and NB2) was constructed. Plaques produced by NB1+2 on CEF monolayers were at the threshold of visibility, even when the immunostaining technique was used (Fig. 3). After electroporation of RNA transcripts, the kinetics of NB1+2 virus production were significantly slower than those of the parent (Fig. 4d). Since the titration was done by end-point dilution using P388D1 cells in the presence of MAbs 49 and 33, the difference observed in the NB1+2 $TCID_{50}$ titer might have been due to less efficient recognition by at least the E2-specific MAb as a consequence of the two insertions in E2. However, similar results were obtained when titration was performed in the presence of polyclonal hyperimmune serum specific for El and E2 (data not shown). The slower virus release and lower yields did not appear to result from a defect in accumulation of the structural proteins, since levels similar to those of the parent were present, as determined by ELISA (data not shown). These results suggest that NB1+2 contains defects in virion assembly and/or release. Compared with NB1, NB1+2 showed a reduction in the number of PFU produced in the entry assay (Fig. 6), but the mutants bound to CEF similarly when radiolabeled virus was used (data not shown). Taken together, these results suggest that the combination of the two insertion mutations does not result in an additive or synergistic reduction in binding to CEF.

DISCUSSION

In this study, random mutagenesis was used to identify insertion sites in a Sindbis virus glycoprotein which impair virus binding to CEF and BHK-21 cells. Two such insertion mutations were identified in PE2, one located near the PE2 cleavage site (NB1) and a second between E2 residues 69 and 74 (NB2). No mutant was recovered from the El insertion library, which suggests that either El is not directly involved in attachment (see below) or insertions in El domains critical for binding also disrupt other steps in virion assembly, release, or penetration. In any case, characterization of the PE2 insertion mutants has provided additional insight into the importance of PE2 cleavage for virus attachment and penetration, the role of E2 in receptor binding, and the structure of the E2 glycoprotein.

Several lines of evidence suggest that PE2 cleavage, rather than being required for particle assembly and release (37, 42, 44), is important for alphavirus receptor binding and penetration. Impaired binding and slow penetration were observed for NB1, in which the inserted sequence resulted in blocked PE2 cleavage. Mild trypsin treatment cleaved NB1 PE2 and partially restored binding to CEF monolayers. Similar results have been obtained for a mutant of SFV in which PE2 cleavage was blocked by substituting a Leu residue for the *P1* Arg residue at the cleavage site (44). As for NB1, particle assembly and release occurred normally, but the SFV mutant was essentially noninfectious and exhibited impaired attachment to BHK-21 cells. The infectivity of the SFV mutant was restored by trypsin treatment, which correlated with PE2 cleavage (44). Only partial cleavage of PE2 was necessary to restore the infectivity of the mutant SFV particles, indicating that only a fraction of the mature spikes were necessary for efficient entry. This finding may explain the efficient entry of mutant NB12 (this work) or Sindbis virus grown in the presence of monensin (37), where PE2 cleavage was only partially blocked and virions contained both PE2 and E2.

Several possibilities exist for the inhibitory effect of uncleaved PE2 on alphavirus binding and penetration. Although required for heterodimer formation and transport to the plasma membrane (21), for the attachment step, the E3 portion of uncleaved PE2 may shield critical domains of the El-E2 oligomer from interaction with host receptors. Alternatively, cleavage of PE2, which is known to destabilize the E2-El interaction with respect to low pH (44, 59), may trigger a conformational change in E2 required for formation or accessibility of the receptor-binding sites on the spike. PE2 cleavage, however, is not required by all alphaviruses. Some mutants of the S.A.AR86 strain of Sindbis virus produce particles with uncleaved PE2 which are still capable of efficient entry (42). The mutation blocking cleavage of PE2 appears to be the substitution of Asn for Ser at position 1 of E2, which creates a novel site for N-linked glycosyla-

tion. It is possible that the additional sugar moiety, per se, allows PE2 to assume a conformation similar to that of E2 produced by PE2 cleavage. Alternatively, other changes in the S.A.AR86 glycoproteins may allow the PE2-E1 spike to function efficiently for binding and penetration.

The NB2 mutant, which contained the insertion between E2 residues 69 and 74, was also defective for attachment to CEF and BHK-21 cells but penetrated CEF cells with kinetics indistinguishable from those of the parental virus. Although both spike glycoproteins may be involved in receptor binding (31), this phenotype suggests a direct involvement for E2, a hypothesis which is consistent with several other lines of evidence. First, E2-specific antibodies often neutralize virus infectivity, while most antibodies directed against determinants on E1 are nonneutralizing (7, 41, 49), with the exception of the epitope recognized by E1-specific MAb 33 (49, 51; see below). Second, potential receptors have been isolated from CEF (61) and mouse neuronal cells (55) by using anti-idiotypic antibodies raised against E2-specific neutralizing MAbs. Finally, Sindbis virus strains which differ by only a single substitution at E2 residue 172 differ in the ability to bind to N18 neuroblastoma cells (54).

It is unclear whether single or multiple sites exist on the virus spike for binding to the apparently diverse receptors on BHK-21 (60), N18 neuroblastoma (55), and CEF cells (61). The finding by Tucker and Griffin (54) that a single substitution in E2 affects binding to N18 neuroblastoma but not BHK-21 cells suggests that distinct spike determinants are involved in modulation of virus binding to alternative host receptors. This finding can be accommodated by a model in which the spike contains at least two distinct receptor-binding sites or, alternatively, a single binding site where critical interactions with alternative receptors are determined by different glycoprotein residues. In either case, the diminished binding of NB2 to both CEF and BHK-21 cells indicates that mutations in E2 can affect the structure of the spike in such a way that attachment to a least two distinct receptors is impaired.

A collection of neutralizing MAbs has been used to define three E2 antigenic domains (called E2a, E2b, and E2c) (30, 49). Significant overlap between E2a and E2b is apparent (51), but E2c appears to be distinct (33). The mutations in NB1, NB2, and NB12 resulted in altered binding by some of the E2ab and E2c MAbs. Mutants with insertions at the PE2 cleavage site (NB1 and NB12) showed dramatically reduced binding to E2c MAbs, suggesting that either PE2 cleavage is necessary for formation of the E2c antigenic domain or the presence of uncleaved E3 (or the inserted sequence) shields the epitope from recognition by MAbs R6 and R13. A similar reduction in binding of E2c MAbs was found for the S.A.AR86 mutants containing the Asn at position 1 of E2 which blocked PE2 cleavage (42). Mutant NB2 showed only slightly reduced binding to E2ab MAb 49 and E2c MAbs R6 and R13 but dramatically reduced binding to E2ab MAb 50. This result is surprising, since anti-idiotypic antibodies to MAb 49 define a potential Sindbis virus receptor on CEF (61), yet NB2, which is still recognized by MAb 49, binds poorly to CEF. A possible explanation is that the NB2 insertion, while not destroying the E2ab antigenic domain or its ability to react with the E2ab MAbs, alters the spike conformation in such a way that the interaction of this binding site with the CEF receptor is blocked.

As mentioned earlier, one of the goals of this study was to identify mutations in the Sindbis virus glycoproteins which would allow normal particle assembly and release but lead to a block in virus entry at the level of attachment. Mutant NB2, with an insertion mutation in E2, exhibits defective binding to CEF and BHK-21 cells but is otherwise unimpaired at other steps in the replication cycle. NB2, or similar mutants, may ultimately be useful for restriction of Sindbis virus tissue tropism and targeting of infection to specific cell types.

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