Amino Acid Changes in the Sindbis Virus E2 Glycoprotein That Increase Neurovirulence Improve Entry into Neuroblastoma Cells

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Sindbis virus (SV) is an alphavirus that causes encephalitis in mice and results in age-dependent mortality. The outcome is dependent on the virus strain. Residues at 55 and 172 in the E2 glycoprotein determine the neurovirulence for mice of different ages and the efficiency of replication in the nervous system and neuronal cells. To determine the effects of these two residues on the initial steps in replication, we studied viruses with a histidine or glutamine at E2 position 55 and a glycine or an arginine at position 172, E2[Q55G172], E2[H55G172], and E2[Q55R172]. The production of virus was detected earlier for viruses with a histidine at E2 position 55 in BHK-21 cells (4 to 6 versus 6 to 8 h) and for E2[H55G172] in N18 cells (6 versus 8 to 10 h). As shown previously, viruses with a glycine at E2 position 172 bound more efficiently to N18 cells and a histidine at E2 position 55 further improved binding only slightly. Viruses with E2[H55] exhibited more rapid internalization and degradation of viral proteins in both BHK-21 and N18 cells. Incubation of E2[H55G172] and E2[Q55G172] at various pHs and temperatures did not reveal differences in virion stability. These data suggest that the amino acids at E2 positions 172 and 55 affect both adsorption and penetration of SV and that these early steps in the replicative pathway contribute to increased neurovirulence.

Alphaviruses, a group of viruses in the family Togaviridae, cause a range of diseases, the most important of which is encephalitis. Sindbis virus (SV) is the prototype alphavirus and provides an excellent mouse model for studying the pathogenesis of alphavirus encephalitis.

The structure of SV is relatively simple. The genomic RNA is single-stranded and message sense. The 5′ two-thirds of the genome encodes the nonstructural proteins that function in viral replication. Replication is dependent on the virus strain. Residues at 55 and 172 in the E2 glycoprotein determine the neurovirulence for mice of different ages and the efficiency of replication in the nervous system and neuronal cells. To determine the effects of these two residues on the initial steps in replication, we studied viruses with a histidine or glutamine at E2 position 55 and a glycine or an arginine at position 172, E2[Q55G172], E2[H55G172], and E2[Q55R172]. The production of virus was detected earlier for viruses with a histidine at E2 position 55 in BHK-21 cells (4 to 6 versus 6 to 8 h) and for E2[H55G172] in N18 cells (6 versus 8 to 10 h). As shown previously, viruses with a glycine at E2 position 172 bound more efficiently to N18 cells and a histidine at E2 position 55 further improved binding only slightly. Viruses with E2[H55] exhibited more rapid internalization and degradation of viral proteins in both BHK-21 and N18 cells. Incubation of E2[H55G172] and E2[Q55G172] at various pHs and temperatures did not reveal differences in virion stability. These data suggest that the amino acids at E2 positions 172 and 55 affect both adsorption and penetration of SV and that these early steps in the replicative pathway contribute to increased neurovirulence.

The outcome of alphavirus encephalitis is determined by the age of the host and the virus strain used for infection. The AR339 strain of SV causes fatal encephalitis in newborn mice, but adult mice recover from infection even after intracerebral inoculation. AR339 differs from NSV, a neurovirulent strain that causes fatal encephalitis after intracerebral inoculation of adult mice, at only four amino acid positions in the structural proteins, positions 55 and 209 in the E2 glycoprotein and positions 72 and 313 in the E1 glycoprotein (9). A particularly important determinant of age-dependent virulence is the amino acid residue at E2 position 55. NSV has a histidine at this position, and AR339 has a glutamine. Recombinant viruses that contain a histidine at position 55 cause high rates of mortality in 1- and 2-week-old mice, whereas viruses with a glutamine at E2 position 55 cause little mortality in 1-week-old mice and are avirulent in 2-week-old mice (21).

Differences in SV neurovirulence are not due to changes in virus tropism for neural cells (10, 20). For AR339 and NSV, as well as for the very avirulent tissue-culture-adapted strain HRSP, neurons are the primary cells infected in the brain. However, an important determinant of neurovirulence is the efficiency of replication in neurons. Several steps are involved in viral entry to allow for productive alphavirus infection; one or more of these steps may be altered by amino acid changes in a surface glycoprotein. Previously, we have shown that a glycine-to-arginine change at E2 position 172 decreases binding to neuronal cells (20). After attachment to the target cell receptor, alphaviruses are internalized through receptor-mediated endocytosis. In endosomes, attached virions are exposed to acid pH, triggering a conformational change, and the virus envelope fuses with the endosomal membrane. Nucleocapsid is released into the cytoplasm, and remaining viral structural proteins presumably are degraded. In this study, we sought to define further the molecular determinants of virus...
ence by examining whether the amino acid change at E2 position 55 alters SV entry into neural and nonneural cells. We found that attachment is not affected but that entry into neural cells is more rapid when the amino acid at E2 position 55 is a histidine.

MATERIALS AND METHODS

Cell lines. Baby hamster kidney (BHK-21) cells and the N18 clone (2) of C1300 mouse neuroblastoma cells were grown in Dulbecco's minimal essential medium (DMEM; GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal bovine serum (FBS) and 50 μg of gentamicin per ml.

Viruses. Construction of the four viruses used has been fully described previously (13, 21). BRIEFLY, E2[H55G172] (TE) and E2[H55R172] (TES) were constructed by replacing the genes for E1 and E2 in full-length clone Toto110 with the E1 gene from AR339 and the E2 gene from either NSV (E2[H55G172]) or NSV/HRSP (E2[H55R172]) (13). These recombinant viruses differ only at E2 position 172. E2 position 55 was changed from histidine to glutamine in these recombinant viruses by site-directed mutagenesis as described previously (21). Therefore, four viruses, E2[H55G172] (TE), E2[Q55G172] (633), E2[H55R172] (TES), and E2[Q55R172] (628), were generated (21); this allowed us to compare the effects of a change at E2 position 55 (histidine—glutamine) on viral entry into cells in the presence of either glycine or arginine at E2 position 172.

Radiolabeling and purification of virus. BHK-21 cells were inoculated with each virus at a multiplicity of infection (MOI) of 1.0 in DMEM–1% FBS, incubated at 37°C for 1 h, and fed with methionine- and cysteine-free minimal essential medium containing 2.5 μg of [35S]methionine and 2.5 μg of [35S]cysteine (NEN-Dupont, Wilmington, Del.) per ml. When a >90% cytopathic effect was evident, supernatant fluids were harvested and clarified, and viruses were precipitated in 10% (wt/vol) polyethylene glycol 8000 in 0.5 M NaCl for 2 to 3 h at 4°C. Each virus was pelleted, resuspended in NET buffer (10 mM Tris, 3 mM EDTA, 150 mM NaCl [pH 7.3]), and banded on a continuous gradient of 15 to 30% (wt/vol) glycerol in the presence of either glycine or arginine at E2 position 172.

Replication in vitro. BHK-21 cells were treated with proteinase K (10 μg/ml) for 30 min at 4°C, then washed twice with cold BM. The fluids from both washes were collected in 1.5-ml microcentrifuge tubes, incubated on ice with 10% trichloroacetic acid for 1 h, and then centrifuged for 15 min to separate whole precipitated virus from degraded acid-soluble viral proteins released from cells. Cells from each well were treated with proteinase K (10 μg/ml) for BHK-21 cells and 15 μg/ml for N18 cells) and 0.2% sodium azide and collected in 1.5-ml microcentrifuge tubes. Cells containing internalized virus were pelleted at low speed for 3 min, and supernatant fluids containing bound virus that had not been internalized were collected. SDS (1%) was added to all samples, and the counts per minute in each fraction were determined in a liquid scintillation counter and expressed as a percentage of total counts per minute of virus bound.

Treatment of virus at various temperatures. To determine the stability of virions at elevated temperatures, 10^7 PFU of E2[H55G172] or E2[Q55G172] were added to 1 ml of DMEM–1% FBS and then incubated for 5 or 30 min in a water bath heated to 37, 45, or 57°C. Each virus was put on ice and frozen at –70°C. Virus infectivity was determined by plaque formation on N18 cells.

Statistical analysis. All results were analyzed for statistical significance with StatView software. Student’s unpaired, two-tailed test was used to compare differences in binding and entry.

Viral entry assay. Viral entry assay (Fig. 1) was performed essentially by the method of Marsh and Helenius (14). N18 and BHK-21 cells were grown to confluen ce on 12-well tissue culture plates. Cells were cooled to 4°C and washed twice in cold BM. Virus was added to each well at an MOI of 5 to 10 PFU per cell (at least 1,000 cpm per well) in 0.6 ml of cold BM and incubated at 4°C for 1.5 to 2 h on a rocking platform to allow attachment but not penetration of radiolabeled virus. Virus was removed, and cells were washed twice with cold BM and refed with 0.6 ml of cold BM. Time zero samples were taken at 4°C, and then plates were moved to 37°C. This shift in temperature allowed attachment to be endocytosed and enter cells. Three samples were taken at each time point. Supernatant fluids were collected in 1.5-ml microcentrifuge tubes, incubated on ice with 10% trichloroacetic acid for 1 h, and then centrifuged for 15 min to separate whole precipitated virus from degraded acid-soluble viral proteins released from cells. Cells from each well were treated with proteinase K (10 μg/ml) for BHK-21 cells and 15 μg/ml for N18 cells) and 0.2% sodium azide and collected in 1.5-ml microcentrifuge tubes. Cells containing internalized virus were pelleted at low speed for 3 min, and supernatant fluids containing bound virus that had not been internalized were collected. SDS (1%) was added to pelleted, proteinase K-treated cells (internalized virus). Liquiscint was added to all samples, and the counts per minute in each fraction were determined in a liquid scintillation counter and expressed as a percentage of total counts per minute of virus bound.

FIG. 1. Steps leading to identification of bound, internalized virus, penetrated virus, degraded virus, and unpenetrated virus. TCA, trichloroacetic acid.

FIG. 2. Cumulative release of infectious virus from BHK-21 and N18 cells infected at an MOI of 0.1 with recombinant SVs differing at E2 positions 55 (histidine or glutamine) and 172 (glycine or arginine).
RESULTS

Replication of virus in cultured cells. In BHK-21 cells infected at a low MOI, the production of new virus was detected at between 4 and 6 h after infection for E2[H55] viruses and at between 6 and 8 h after infection for E2[Q55] viruses (Fig. 2). At 24 h after infection, there was an approximately 5- to 10-fold difference in the amount of virus released. For N18 cells, an Arg at position 172 is known to decrease binding by SV and to affect the time course of virus replication (20). This is also seen in Fig. 2. The effect of the amino acid at position 172 is less apparent when the amino acid at position 55 is a glutamine. For the recombinant virus E2[H55G172], new virus was produced by 6 h after infection, but for the other three recombinant viruses, new virus was not detected until 8 to 10 h after infection.

Virus adsorption. Assays of viral binding to BHK and N18 cells were performed to determine whether attachment to cellular receptors could explain differences in the time course of virus replication. A comparison of the binding of the four viruses assayed showed that the rates and extents of binding to BHK-21 cells were similar (Fig. 3A and B).

The previously reported ability of viruses with a glycine at position 172 to bind more rapidly and more extensively to N18 neuroblastoma cells was confirmed (Fig. 3C and D). In addition, recombinant viruses with a histidine at E2 position 55 bound better than did viruses with a glutamine at E2 position 55, although this difference was less dramatic than was the effect of the change at E2 position 172.

Virus entry. Viral surface proteins are important for cell attachment and for internalization. A change in an amino acid in the E2 glycoprotein may be important for the ability of the virus to be internalized by receptor-mediated endocytosis and to enter the replicative pathway. We examined the release of bound virus, the rate of internalization of bound virus, and the degradation of radiolabeled viral proteins in both BHK-21 and N18 cells. Each virus was allowed to bind to the cell surface at 4°C, and then the temperature was shifted to 37°C to allow entry into cells (Fig. 1). We presume that virus appearing as acid-precipitable counts per minute in supernatant fluids within 30 to 45 min after the shift to 37°C (Fig. 4) is virus released from the cell surface, although we cannot rule out the possibility that it was endocytosed before release. For BHK cells, this release was more masked for E2[Q55] viruses than for E2[H55] viruses and the amino acid at E2 position 172 was irrelevant. However, for N18 cells, release was more marked for E2[R172] viruses than for E2[G172] viruses, with no detectable contribution from the amino acid at E2 position 55. These observations further suggest differences in initial virus interaction with receptors on the surfaces of neural and nonneural cells that are influenced by amino acid changes in the E2 glycoprotein.

The sum of the counts per minute of intracellular, proteinase K-resistant virus and the counts per minute of acid-soluble degraded virus is the total virus which has entered cells and is presented as a percentage of the counts per minute of bound virus to eliminate differences due to initial binding (Fig. 5). In BHK-21 cells, viruses with a histidine at E2 position 55 entered more rapidly. This difference in internalization was statistically significant at 15, 30, 45, 60, 75, and 90 min for viruses with a glycine at E2 position 172 (P < 0.005) and at 75 and 90 min for viruses with an arginine at E2 position 172 (P < 0.05). E2[Q55G172] was internalized most slowly. Similar results were found when we compared the internalization rates of these viruses bound to N18 cells, a neuronal cell line. E2[H55G172] was more rapidly and completely internalized by N18 cells than was E2[Q55G172] (P < 0.05 at 15, 30, 45, 60, 75, and 90 min). E2[H55R172] was also internalized better than was E2[Q55R172] (P < 0.05 at 30, 60, 75, and 90 min).

After bound virus is endocytosed, the E1 and E2 glycoproteins undergo low-pH-induced conformational changes which

FIG. 3. Time course of the binding of 35S-labeled viruses to nonneuronal (BHK-21; A and B) and neuronal (N18; C and D) cells at 4°C. *, P < 0.05.
dissociate E2 from newly stabilized E1 trimers, leading to fusion of the viral and cell membranes (7). The nucleocapsid is released into the cytoplasm. The proteolytic degradation of viral proteins may be important in allowing efficient viral disassembly and subsequent replication and transcription or may simply represent nonproductive infection. When internalization of virus and degradation of viral proteins were separately assessed, it was apparent that E2[H55] viruses were more rapidly internalized and more rapidly degraded after binding to both BHK (Fig. 6) and N18 (Fig. 7) cells. In fact, internalization of some bound E2[H55] virus unexpectedly occurred even at 4°C (Fig. 6 and 7) at time zero. Although degradation occurs intracellularly, degraded trichloroacetic acid-soluble viral proteins were located entirely in the medium. Degraded viral proteins appear to be rapidly released from cells since degradation products were not observed when extracts from proteinase K-treated cells were examined by SDS-polyacrylamide gel electrophoresis at each time point (data not shown). This is similar to what is seen after endocytosis of Semliki Forest virus (14).

Effects of temperature and pH changes on virus survival. To determine whether the amino acid at E2 position 55 altered the physical stability of the virion, we compared the infectivities of recombinant viruses after treatment with heat or acid (Table 1). E2[H55G172] had a greater loss of viability at 57°C than did E2[Q55G172], whereas these two viruses exhibited similar infectivities after exposure to a lower pH.

DISCUSSION

Amino acid changes in the surface glycoproteins are important determinants of SV neurovirulence in mice (3, 5, 13, 16, 20, 21) and of the neurovirulence of other viruses (1, 6). In the E2 glycoprotein of SV, the residues at positions 55 and 172 determine the neurovirulence for mice of different ages and the efficiency of replication in nervous system tissue (21). These alterations in in vivo replication are mirrored by changes in replication in vitro. Those amino acid changes have a limited effect on replication in BHK cells, but a histidine at position 55 and a glycine at position 172 synergize, leading to efficient replication in N18 cells and virulence for 1- to 2-week-old mice (21) and suggesting that replication in N18 cells may mirror in vitro aspects of neurovirulence in vivo. In these studies, we have confirmed that the amino acid at E2 position 172 is an important determinant of efficient attachment to neuronal N18 cells, but not nonneuronal BHK cells (20), and we have shown

FIG. 4. Time course of the appearance of previously bound acid-precipitable virus in the supernatant fluids of BHK-21 and N18 cells after the shift from 4 to 37°C.

FIG. 5. Time course of the internalization of recombinant SVs after binding to BHK-21 and N18 cells. Data are for internalized, proteinase K-resistant virus plus degraded virus at each time point.
that the amino acid at E2 position 55 has a limited additional effect on attachment to N18 cells. However, viruses with a histidine at E2 position 55 were less likely to be released from BHK cells and were more rapidly internalized and degraded by both N18 and BHK cells than were viruses with a glutamine at E2 position 55, suggesting that a histidine at E2 position 55 facilitates entry but that this entry step may not be the major determinant of improved replication of E2[H55]-containing viruses in neural cells.

Other neurovirulent viruses also exhibit altered virulence patterns with single amino acid changes in the glycoproteins. For some, these changes alter tropism; for others, the efficiency of replication is affected. A single amino acid change at position 260 of the glycoprotein of the Armstrong strain of lymphocytic choriomeningitis virus changes tissue tropism in mice. Viruses with a phenylalanine predominate in the central nervous system, but viruses with a leucine predominate in lymphoid tissue (1). An arginine at position 333 of the rabies virus glycoprotein is necessary for lethal infection (6), and a single amino acid change at position 308 or 311 of the viral envelope protein alters the virulence of louping ill virus (11). For SV, E2 position 172 is most important for absorption to neural cells. Viruses with a histidine at E2 position 55 exhibited a slight increase in binding compared to that of viruses with a glutamine, suggesting that the amino acid at this position contributes somewhat to adsorption but even more so to subsequent steps in entry.

Changes at other positions in the N-terminal portion of SV E2 have also been shown to affect penetration. A single amino acid change from arginine to serine at E2 position 114 is sufficient to attenuate virulence in newborn mice and accel-
ate penetration of BHK cells (5). Other amino acid substitutions at E2 position 114 also result in altered virulence, possibly due to an alteration in structure (17). How any of these amino acid changes in E2 influence viral entry is not understood.

SV infects cells by a process of receptor-mediated endocytosis (12). Once bound to the vertebrate cellular receptor, the E1-E2 heterodimer undergoes a conformational change (7), the virus is endocytosed via coated vesicles, an acid-induced conformational change occurs, and fusion of the E1 glycoprotein to the vesicle membrane occurs. In both BHK-21 and N18 cells, viruses with a histidine at E2 position 55 were more rapidly internalized than were viruses with a glutamine. This increase was more marked in viruses with a glycine at E2 position 172; however, after 60 min, this increase was also evident in viruses with an arginine at E2 position 172. Overall, virus E2[Q55R172] was internalized most slowly. In vitro conformational changes representing transitional epitopes associated with virus-cell interaction have previously been induced by heat, reducing agents, and a low pH (15). If an amino acid change in the E2 glycoprotein could result in a virus that was less stable in an acid environment, differences in disassembly with subsequent differences in replication might result. Likewise, if a virus is temperature sensitive, it might exhibit altered disassembly and replication. Viruses differing only at E2 position 55 and exposed to lower pHs and increased temperatures did not exhibit a difference in the recovery of infectious virus, suggesting that a histidine or glutamine at E2 position 55 did not alter virion stability.

After fusion, the nucleocapsid is released into the cytoplasm, proteins are degraded in the lysosomal pathway, genomic RNA is uncoated, and replication events proceed. We also found that more virulent viruses with a histidine at position 55 exhibited more rapid degradation than did viruses with a glutamine at position 55. It is not clear whether the degraded viral proteins are the remnants of virions that have led to productive or nonproductive replication in cells. For Semliki Forest virus, E2 is more sensitive to degradation than is E1 or the capsid protein (14) and amino acid changes in E2 may alter the degradation of all structural proteins. Perhaps efficient degradation can lead to efficient replication by providing rapid disassembly and escape of viral proteins into the cytosol. For adenovirus type 2, the elimination of specific proteins at sequential stages of viral entry is necessary for efficient release of DNA into the nucleus (8). On the other hand, degraded particles may exert an inhibitory role by interacting with cellular proteins important for antiviral defense.

A change from histidine to glutamine may affect the trafficking of viral glycoproteins through the endocytic pathway. For instance, ras-like GTP-binding proteins bound on plasma membranes and early endosomes are important in the early endocytic pathway as are proteins responsible for vesicle trafficking (4, 18, 22). An altered structure for the E2 glycoprotein of SV might interact or interfere with trafficking proteins and alter the flow from entry into the endosomal compartment to establishment of membrane-bound replication complexes. Some of these proteins are expressed differently in cells of different origins and during development and may alter the interactions of virus through the entry pathway (19).

Our data suggest that E2[H55] improves internalization in
neuronal and nonneuronal cells, suggesting that this step in entry is not the major determinant of increased neurovirulence of E2[H55]-containing viruses.

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