JOURNAL OF VIROLOGY, Aug. 1997, p. 6100–6105 Vol. 71, No. 8
0022-538X/97/$04.00+0

A Single Amino Acid Change in the E2 Glycoprotein of Sindbis Virus Confers Neurovirulence by Altering an Early Step of Virus Replication

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Received 11 October 1996/Accepted 18 March 1997

Amino acid changes in the envelope glycoproteins of Sindbis virus have been linked to neurovirulence; however, the molecular mechanisms by which these amino acid changes alter neurovirulence are not known. Recombinant-virus studies have mapped an important determinant of neurovirulence in adult mice to a single amino acid change, glutamine to histidine, at position 55 of the E2 glycoprotein (P. C. Tucker, E. G. Strauss, R. J. Kuhn, J. H. Strauss, and D. E. Griffin, J. Virol. 67:4605–4610, 1993). To investigate how histidine confers neurovirulence, we examined the various stages of the virus life cycle in neural (N18) and nonneural (BHK) cells. In BHK cells, recombinant viruses 633 (E255Q) and TE (E255H) replicated similarly. In contrast, in N18 neuroblastoma cells, TE established infection more efficiently, replicated faster, and achieved higher rates of virus release than did 633. Viral structural protein synthesis was similar in 633- and TE-infected BHK cells, while in N18 cells, structural protein synthesis was detected only in TE-infected cells at 6 h and remained higher for at least 16 h postinfection. Viral RNA synthesis was initiated more rapidly and was up to fivefold greater in TE- versus 633-infected N18 cells. Taken together with other data demonstrating minimal effects on virus binding and entry (P. C. Tucker, S. H. Lee, N. Bui, D. Martinie, and D. E. Griffin, J. Virol. 71:6106–6112, 1997), these data suggest that E2 position 55 plays an important role at early stages of infection of neural cells, thereby facilitating neurovirulence.

Sindbis virus (SV), a single-stranded, positive-sense RNA virus, belongs to the alphavirus group of arthropod-borne viruses. Eastern, Western, and Venezuelan equine encephalitis viruses cause encephalitis in humans, but SV causes a syndrome characterized by fever, rash, and arthritis (5). However, SV infection of mice results in encephalitis, providing a useful model for the study of the pathogenesis of acute viral encephalitis and of neurovirulence (5). As with other animal models of viral encephalitis, SV infection of mice results in age-dependent mortality (8, 17). AR339, a wild-type strain of SV, and NSV, a neuroadapted strain obtained after serial passages of AR339 in the mouse brain, cause fatal encephalitis in newborn mice but differ in their ability to cause fatal disease in 2-week-old mice, which recover from encephalitis caused by AR339 but die from NSV infection (4).

Neurovirulence is determined by the neuroinvasive potential of a virus, the target cell(s) infected, the efficiency of viral replication, and the extent of virus-induced cellular damage (27). Most strains of SV are not neuroinvasive when they are injected subcutaneously into adult mice; therefore, for studies of neurovirulence in adult-mouse models of encephalitis, SV is injected intracerebrally (5). The neuron is the primary neural cell infected by all strains of SV (6). Jackson et al. demonstrated that the neurovirulent strain NSV infects more neurons, replicates to a higher titer, and causes more cellular damage than does AR339 (7). Hence, for these strains of SV, neurovirulence correlates with the efficiency of virus replication in the brain (5, 7, 25).

The envelope glycoproteins of various viruses have been linked to neurovirulence (13, 14, 19, 23). Since glycoproteins are involved in cellular receptor recognition, virus attachment, and membrane fusion and penetration, it is not surprising that they are important molecular determinants of neurovirulence. The SV genome encodes three structural proteins that are translated from a subgenomic mRNA as a polyprotein precursor in the order capsid, pE2 (the precursor of E2), and E1. The capsid protein possesses serine protease activity that acts in *cis* to release itself from the polyprotein. The glycoproteins, pE2 and E1, are translocated into the endoplasmic reticulum, where they are folded and glycosylated. pE2 and E1 are then transported as trimerized heterodimers through the secretory pathway to the plasma membrane, eventually forming the glycoprotein spikes of the virion. pE2 is cleaved to E2 in the trans-Golgi network. The cellular receptor-binding domain of SV resides in E2, and the fusion domain resides in E1 (reviewed in reference 21).

Several studies have demonstrated that amino acid changes in the envelope glycoproteins of SV are associated with changes in neurovirulence (2, 15, 16, 25). Davis et al. (2) and Polo and Johnston (16) have identified mutations in E1 and E2 which result in attenuated infection in neonatal mice. Studies with recombinant viruses have demonstrated that the E1 and E2 glycoproteins are of paramount importance for neurovirulence in adult mice (15). The glycoproteins of AR339 and NSV differ by only four amino acids, two in E2 and two in E1 (15). Studies with recombinant viruses revealed that a histidine at position 55 of the E2 glycoprotein (present in NSV) rather than a glutamine (present in AR339) was a critical determinant of virulence in 2-week-old mice (25, 28). Site-directed mutagenesis of E2 position 55 to generate a lysine, arginine, glycine, or glutamic acid revealed that none of these amino acids resulted in a virus that was virulent for 2-week-old mice (25). The recombinant virus TE (Fig. 1) contains a histidine at E2 position 55 and causes 97% mortality, but infection with the

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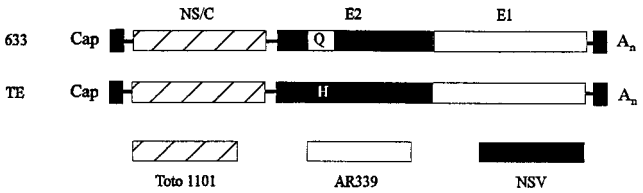

FIG. 1. Schematic diagrams of recombinant SV clones 633 and TE, showing the derivation of the nonstructural and capsid (NS/C), E2, and E1 regions. Toto1101 is an avirulent laboratory strain. Q, glutamine; H, histidine (at position 55 of E2). Genomic RNA is capped (Cap) and polyadenylated (A_n).

isogenic glutamine-containing virus 633 causes no mortality (25, 28). TE replicates more rapidly and to higher titers than does 633 in the brains of 7- and 14-day-old mice (12, 25), revealing that a single amino acid change from glutamine to histidine at E2 position 55 is a critical determinant of neurovirulence in older mice and acts by increasing the efficiency of virus replication in the nervous system.

Subsequent studies have confirmed the importance of E2 position 55 in SV neurovirulence. Persistent infection of *scid*/CB17 weanling mice with AR339 resulted in the emergence of a virus containing a histidine at position 55 in four of nine mice 30 or more days after infection independent of treatment with anti-E2 monoclonal antibody (10). These results suggest that histidine is important for neuroadaptation rather than a consequence of selective pressure in the presence of antibody. The significance of a histidine at E2 position 55 has also been tested in models of virus-induced apoptosis. SV kills mammalian cells by inducing apoptosis (11). Infection of *bcl2*-expressing rat prostate carcinoma (AT3) cells with avirulent strains of SV results in persistent infection, whereas infection with histidine-containing strains results in apoptosis (28). Furthermore, the ability of histidine-containing strains to induce apoptosis of neurons in vivo correlates with neurovirulence (12). The current studies were designed to investigate how the single amino acid change from glutamine to histidine in the E2 glycoprotein affects the various stages of the virus life cycle in neural and nonneural cells and to begin to determine the molecular mechanisms by which E2 affects neurovirulence.

MATERIALS AND METHODS

Virus production and sequencing. Full-length cDNA clones of 633 and TE were used for the production of viral RNA (18, 25, 28). Viral RNA was produced by in vitro transcription with SP6 polymerase (Promega, Madison, Wis.) (18). Viral RNA was transfected into BHK-21 (BHK) cells with Lipofectin (Gibco/BRL, Grand Island, N.Y.), and virus-containing supernatant fluids were collected at 24 or 48 h posttransfection. The virus titer was determined by plaque formation on BHK cells. By using posttransfection supernatant fluids, BHK cells were infected at a multiplicity of infection (MOI) of 0.01 to produce 633 and TE virus stocks. Total RNA was extracted from 633- and TE-infected BHK cells with TRIZOL (Gibco/BRL). cDNA was made with avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim, Indianapolis, Ind.) and oligonucleotide 5′-GCGAATTCGTCCTAGTGTGGC-3′ (nucleotides 8833 to 8852) to prime first-strand DNA synthesis. The sequence spanning amino acid position 55 of E2 was amplified by PCR with oligonucleotide 5′-TACTGTACCGTGCTTCAGC C-3′ (nucleotides 8693 to 8712) and the oligonucleotide mentioned above as primers. The PCR product was used as a template for sequencing via the dideoxy-mediated chain termination reaction (Sequenase; U.S. Biochemicals, Cleveland, Ohio). The sequences confirmed a single nucleotide difference at nucleotide 8795 (T or A), corresponding to a glutamine or histidine at position 55 of E2. First-passage virus was used in all experiments.

Virus growth curves. BHK and N18 neuroblastoma (N18 clone of C1300 line [1]; from Marshall Nirenberg, National Institutes of Health) cells were infected with 633 and TE at an MOI of 10 PFU/cell for 1 h at 37°C. Supernatant fluids were removed at hourly intervals and replaced with fresh medium. The PFU per milliliter per hour were determined by plaque formation on BHK cells.

Analysis of viral structural protein synthesis. BHK and N18 cells were infected at an MOI of 50 PFU/cell. At 5 or 15 h postinfection, cells were incubated for 1 h in methionine- and cysteine-free Dulbecco modified Eagle medium (DMEM; ICN, Costa Mesa, Calif.). Cells were pulsed for 2 min with methionine- and cysteine-free DMEM containing 200 μCi of Tran^{35}Slabel (ICN) per ml and subsequently chased by adding medium containing excess (5 mM) methionine and cysteine and 75 μg of cycloheximide (Sigma, St. Louis, Mo.) per ml. At various times during the chase, cells were placed on ice, washed three times with ice-cold phosphate-buffered saline, and lysed with radioimmunoprecipitation assay buffer (150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris Cl [pH 8.0]). For analysis of total protein, equal volumes of cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography. For immunoprecipitation of pE2 and E2, monoclonal antibody 202 (1:100 dilution) (20) was added to 10^6 cpm of cell lysate and incubated overnight at 4°C. Protein A-Sepharose beads (Pharmacia, Piscataway, N.J.) (1:5 dilution) were added for the precipitation of immune complexes, and the reaction mixture was incubated for 3 h at 4°C. The beads were washed three times with buffer A (0.01 M Tris Cl [pH 7.5], 0.15 M NaCl, 0.002 M EDTA, 0.2% Nonidet P-40), two times with buffer B (0.01 M Tris Cl [pH 7.5], 0.50 M NaCl, 0.002 M EDTA, 0.2% Nonidet P-40), and one time with buffer C (0.01 M Tris Cl [pH 7.5]). The beads were resuspended in SDS sample buffer containing β-mercaptoethanol and were boiled for 5 min, and the proteins were separated by electrophoresis through an SDS–15% polyacrylamide gel. The gels were processed for autoradiography, and the radioactivities in protein bands were quantified by densitometry (Image Quant; Molecular Dynamics).

Analysis of viral RNA synthesis. N18 cells were infected with 633 and TE at an MOI of 50 PFU/cell for 1 h at 37°C. At hourly intervals after infection, cells were labeled for 1 h in DMEM containing [5,6-^{3}H]uridine (50 μCi/ml; NEN, Boston, Mass.) and 20 μg of actinomycin D (Calbiochem, San Diego, Calif.) per ml. The cell monolayers were washed and then lysed with 5% lithium dodecyl sulfate containing 200 μg of proteinase K per ml. The proportion of [5,6-^{3}H]uridine incorporated into viral RNA was determined by scintillation counting of trichloroacetic acid (10%)-precipitated and nonprecipitated samples. The proportion of [5,6-^{3}H]uridine incorporated into the cellular RNA of mock-infected, actinomycin D-treated cell cultures ranged from 0.9 to 1.6%, which was less than that incorporated into the viral RNA of virus-infected cultures at times later than 1 h after infection.

Infectious-center assay. N18 cells in 75-cm^2 flasks were infected with 633 and TE at an MOI of 10 or 50 PFU/cell for 1 h at 37°C. Two hours after the 1-h infection period, cells were harvested by gentle trypsinization, washed five times with cold PBS, and resuspended in phosphate-buffered saline at a concentration of 2×10^6 cells/ml. Infected cells were serially diluted 10-fold, and 200 μl was placed onto confluent monolayers of BHK cells. To control for free virus, a 200-μl aliquot of supernatant fluids from the original cell suspension was serially diluted 10-fold and plated onto BHK monolayers. After incubation for 1 h at 37°C, BHK cells were overlaid with 0.6% agar in modified Eagle medium (Gibco/BRL). After 48 h of incubation at 37°C, plaques were counted and the number of infectious centers per cell was calculated as follows: number of plaques/number of N18 cells plated per well.

RESULTS

Virus growth in BHK and N18 cells. Previous studies suggested that cumulative virus growth of 633 was similar to that of TE in BHK cells, but not in N18 neuroblastoma cells (25). To determine more accurately the differences in production of infectious virus in nonneural and neural cells, BHK and N18 cells were infected with 633 and TE at an MOI of 10 PFU/cell and the rates of virus release were determined by collecting supernatant fluids at hourly intervals after replacement of the overlying medium. In BHK cells, 633 and TE exhibited similar rates of replication and release (Fig. 2A). In N18 cells, TE grew more rapidly than did 633, with significantly higher titers and rates of virus release between 4 and 8 h after infection (Fig. 2B). The latent period was longer for 633, with new virus production detected 4 h after TE infection and 5 h after 633 infection. From 4 to 8 h after infection, TE-infected N18 cells released at least 10-fold-more PFU per milliliter per hour than did 633-infected cells (Fig. 2B). At later times postinfection, the rate of 633 release approached that of TE.

Analysis of viral structural protein synthesis. To begin to determine the stage of virus replication affected by the amino acid change at position 55 of the E2 glycoprotein, we investigated the synthesis of viral structural proteins. Pulse-chase experiments were performed at 6 h after infection, when a difference in virus release was present in N18 cells (Fig. 2B). The levels of synthesis of viral structural proteins in 633- and

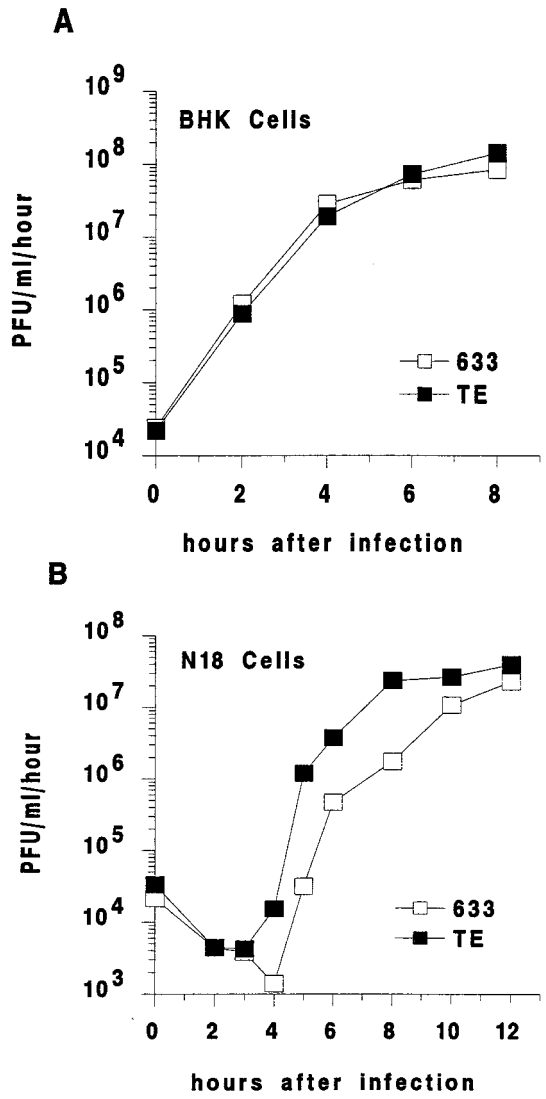

FIG. 2. Rate of virus release from BHK (A) and N18 (B) cells. Supernatant fluids were removed at hourly intervals after the addition of fresh medium, and virus titers were determined by plaque formation on BHK cells. Plotted data are the average amounts of virus produced per hour from three determinations. Error bars representing standard deviations have been incorporated into the graph but are not visible.

TE-infected BHK cells were similar (Fig. 3A). However, in N18 cells, structural protein synthesis was detected in TE-infected cells but not in 633-infected cells (Fig. 3B). A difference in shutoff of host cell protein synthesis, which occurs with SV infection (21), was also evident at this time postinfection. Infection with 633 or TE completely inhibited host protein synthesis in BHK cells by 6 h (Fig. 3A), whereas in N18 cells, shutoff of host cell protein synthesis was evident only in TE-infected cells (Fig. 3B).

To determine whether the inability to detect structural protein synthesis at 6 h postinfection in 633-infected N18 cells was due solely to a delay in replication, followed by replication similar to that of TE at later times postinfection, N18 cells were examined at 16 h postinfection. At that time, viral protein synthesis was detected in 633-infected cells; however, the amount in TE-infected cells was much greater (Fig. 3C). Thus, the difference in structural protein synthesis between 633- and TE-infected N18 cells continued, indicating that this difference was not simply a result of delayed 633 replication at early times postinfection and suggesting an inherent decreased ability of 633 to replicate in N18 cells. At 16 h, complete shutoff of host cell protein synthesis occurred in TE-infected N18 cells but was still not complete in 633-infected cells (Fig. 3C). At 20 h, the levels of viral protein synthesis were more comparable; however, shutoff of host protein synthesis was still not complete in 633-infected N18 cells (data not shown).

To ensure that the structural proteins of TE did not accumulate intracellularly and to determine the amount of virus released from infected cells, the proteins in the supernatant fluids from the 16-h postinfection pulse-chase experiment were precipitated with ethanol and analyzed by SDS-PAGE and autoradiography (Fig. 3D). The amount of released TE virus was greater than that of 633 at 30- and 45-min chase times. Thus, the reduced level of 633 virus proteins in supernatant fluids correlated with the reduced production of infectious virions (Fig. 2). To determine whether the single amino acid change at E2 position 55 altered the processing of the E2 precursor, pE2, to E2, lysates from ^{35}S-labeled 633- and TE-infected N18 cells were immunoprecipitated with an anti-E2 monoclonal antibody and analyzed by SDS-PAGE and autoradiography at various times after labeling. E2 was detected after a 20-min chase for both viruses (Fig. 4A). The relative amount of pE2 processed to E2 was greater in 633-infected cells than in TE-infected cells (Fig. 4B). This may be due to saturation of furin, the cellular serine proteinase that cleaves pE2 (21), by the abundance of precursor protein present in TE-infected cells. Taken together, these data suggested that once structural proteins were made, they were efficiently processed, assembled into virions, and released from cells.

Analysis of viral RNA synthesis. The structural protein synthesis results indicated that the difference in replication between 633 and TE could be attributed to a step prior to the synthesis of structural proteins. Viral RNA synthesis was quantitated by infecting N18 cells in the presence of [^{3}H]uridine and actinomycin D and determining the amount of [^{3}H]uridine incorporated into viral RNA. Viral RNA synthesis in TE-infected N18 cells rapidly increased beginning 2 h after infection, whereas viral RNA synthesis in 633-infected cells increased only slightly above background during the 8-h course of the experiment (Fig. 5). At 6 h postinfection, the level of TE RNA synthesis peaked at fivefold more than the level of 633 RNA synthesis.

Infectious-center assay for analysis of early steps in the virus life cycle. The viral structural protein synthesis data combined with the RNA synthesis data further localized the difference between 633 and TE replication in N18 cells to a step prior to transcription. Therefore, we examined the efficiency of infection of N18 cells by 633 and TE by an infectious-center assay which assesses the ability of a virus to establish productive infection in cells. With 1 h of incubation at an MOI of 10 PFU/ml, 28% of N18 cells were scored as infected by 633, whereas 100% of cells were scored as infected by TE. At an MOI of 50 PFU/ml, 39% of cells were scored as infected by 633 and 100% of cells were scored as infected by TE (Fig. 6). The control supernatant fluids for the experiment at an MOI of 10 resulted in no plaques. For the experiment at an MOI of 50, the 633 control supernatant fluids resulted in no plaques and the TE supernatant fluids yielded 1 plaque, corresponding to 1.2% of plaques seen on the corresponding infected-cell dilution. For BHK cells, 100% of cells were scored as infected by 633 and TE at an MOI of 10 (data not shown). Hence, TE was more efficient than was 633 in productively infecting N18 cells. The difference in the efficiency of infectious-center formation,

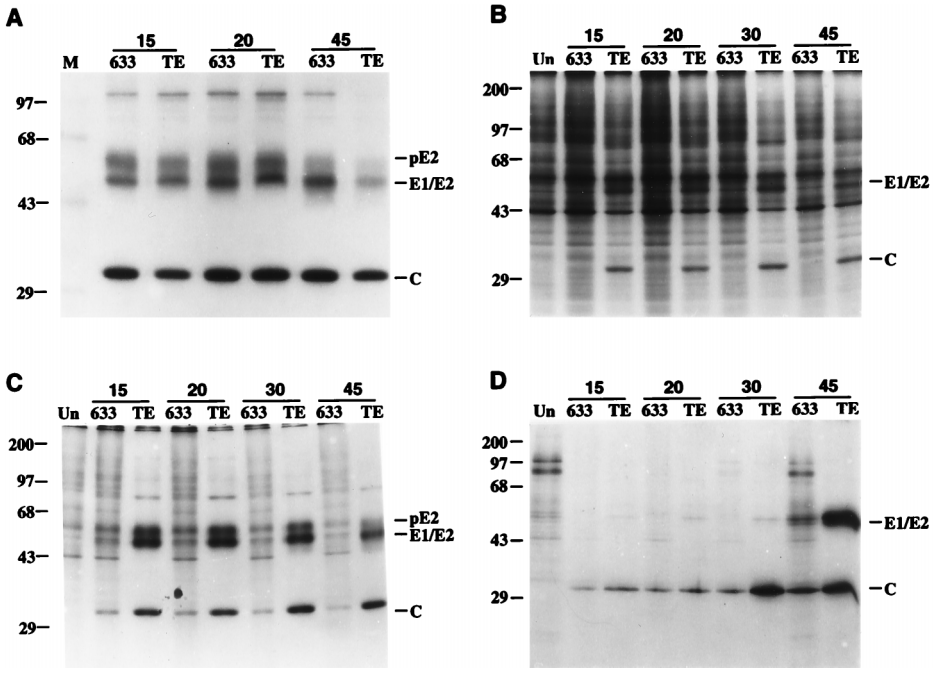

FIG. 3. Viral structural protein synthesis in BHK and N18 cells. (A through C) ^{35}S-labeled cell lysates from the indicated chase times (in minutes) were analyzed on an SDS–15% polyacrylamide gel at 6 h after infection of BHK cells, at 6 h after infection of N18 cells, and at 16 h after infection of N18 cells, respectively. (D) Virion proteins, ethanol precipitated from supernatant fluids collected 16 h postinfection of N18 cells, were analyzed on an SDS–15% polyacrylamide gel. M, molecular weight markers; pE2, precursor of the E2 glycoprotein; C, capsid; Un, uninfected.

along with the protein and RNA synthesis data, indicates that an early step in virus replication is affected by the glutamine-to-histidine change.

DISCUSSION

SV neurovirulence has been linked to the amino acid sequence of the envelope glycoproteins, but the molecular mechanisms by which these amino acid changes alter virulence have not been elucidated. We have begun to examine the molecular effects of the amino acid change from glutamine to histidine at position 55 of the E2 glycoprotein on SV neurovirulence and have found that this single amino acid change alters the ability of SV to replicate in neural N18 cells but not in nonneural BHK cells. The ability of the histidine-containing virus TE to replicate more efficiently in N18 cells than does the glutamine-containing virus 633 correlates with its more efficient replication in the brains of infected mice (12, 25). Since both viruses replicated efficiently in BHK cells, a glutamine at position 55 appears to selectively impair replication in neural cells. An examination of viral protein and RNA synthesis revealed that the less efficient replication of 633 in N18 cells was attributable to a step prior to RNA transcription. Infectious-center assays showed that 633 infected N18 cells less efficiently than did TE. These data indicated that the single amino acid change affects an early step of virus replication.

Single amino acid changes in the E2 glycoprotein can affect SV virulence by altering virus binding or entry into neural cells (2, 24). However, studies by Tucker et al. have demonstrated minimal differences in the binding of 633 and TE to N18 cells; therefore, it is unlikely that this entirely explains the dramatic difference in replication between 633 and TE in N18 cells (26). In the same studies, TE was internalized more efficiently than was 633 in both BHK and N18 cells; therefore, a difference in internalization also does not entirely explain the difference in replication in N18 cells (26). Therefore, it is possible that other early events of replication in neural cells are affected by this single amino acid change. Several studies have suggested that alphavirus structural glycoproteins play a role in RNA synthesis. Chimeric Ross River-SVs containing heterologous structural proteins exhibited less efficient RNA synthesis compared to those of parental viruses (9). The investigators postulated that the structural proteins or sequence elements within the structural coding regions interact with the nonstructural proteins or sequence elements within the nonstructural coding regions and that these interactions may be required for efficient RNA replication. The capsid protein of SV bound specifically to viral RNA, and deletion of particular portions of the RNA sequence to which the capsid bound resulted in less efficient RNA synthesis, indicating that the capsid protein may regulate RNA synthesis (31). The E2 glycoprotein also may regulate RNA synthesis since a deletion of 7 amino acids around residue 55 of E2 in SV and Ross River virus affects RNA synthesis after infection of some but not all cells (29, 30). Interestingly, deletion of this E2 region in Ross River virus was associated with loss of virulence for older mice (29).

Given the dramatic difference in RNA synthesis between 633 and TE in N18 cells, the minimal difference in binding, and the non-cell-type-specific effects on virus entry, we propose that E2 residue 55 may also affect RNA synthesis in neural cells. Because the differences in replication were observed at early times after infection, position 55 of E2 may affect early steps of RNA replication, such as establishment of replicase complexes. After infection, RNA replicase complexes consisting of viral nonstructural and cellular proteins are assembled on modified endosomal and lysosomal membranes for transcription of viral RNA (3). Glycoproteins have not been recognized as participants in viral replicase complexes. However, Suomalainen and Garoff postulated that the anti-idiotypic antibody F13, made to an antibody for the E2 cytoplasmic do-

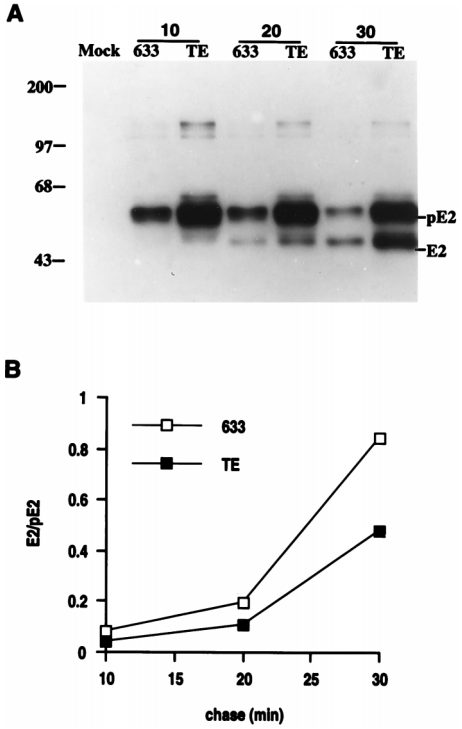

FIG. 4. Processing of the precursor of E2 glycoprotein, pE2. (A) ^{35}S-labeled cell lysates from 16 h after infection with 633 or TE were immunoprecipitated with the anti-E2 monoclonal antibody 202. Mock, mock infected. The positions of molecular weight markers are given on the left. (B) The relative amount of pE2 processed to E2 was determined by quantifying the radioactivities in protein bands at the indicated chase times by densitometry and calculating the pE2/E2 ratio, shown on the *y* axis.

main, bound to some component of the replicase complex, implying that the cytoplasmic domain of E2 interacts with the replicase complex (22). Perhaps other domains of E2 also interact with components of the replicase complex. A glutamine at E2 position 55 may cause altered interaction with a

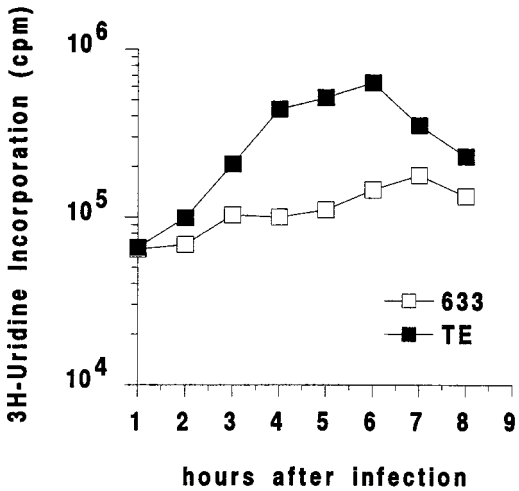

FIG. 5. Viral RNA synthesis in N18 cells. The incorporation of [^{3}H]uridine into viral RNA was determined by liquid scintillation counting of trichloroacetic acid-precipitated samples obtained at various times after infection. Data are averages of three determinations. Error bars representing standard deviations have been incorporated into the graph but are not visible.

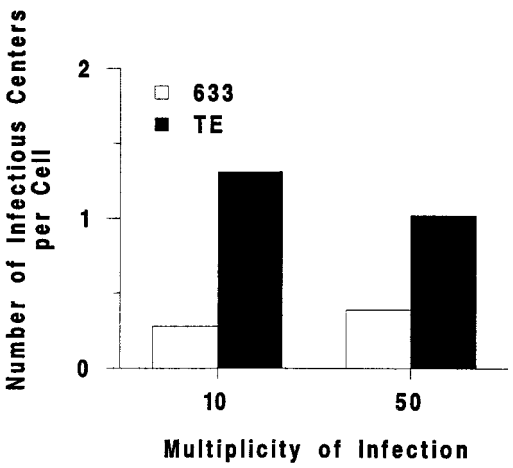

FIG. 6. Infectious-center assay of 633- and TE-infected N18 cells. Infected cells were harvested at 2 h after infection, serially diluted 10-fold, and plated onto BHK monolayers. The number of infectious centers per cell was determined by counting the number of plaques per well and dividing by the number of N18 cells plated per well.

cellular protein which is part of the replicase complex in N18 cells and result in less efficient RNA synthesis.

The SV glycoproteins may have multiple roles as determinants of neurovirulence. We have determined that a single amino acid change from glutamine to histidine at position 55 of the E2 glycoprotein confers neurovirulence by affecting virus replication at an early step. In addition to effects on binding and entry, we propose that the surface glycoproteins may exert their effects on virulence by other, less obvious mechanisms. The E2 envelope glycoprotein of SV may also influence virulence by affecting early stages of replication, such as the establishment or amplification of RNA replicase complexes. As suggested by previous studies, the region around position 55 may be particularly important for this function (29, 30) and a histidine at this position may facilitate RNA synthesis in neurons presumably by interacting with a cellular factor. Further investigation of these potential molecular mechanisms of neurovirulence may reveal new and intriguing roles for the envelope glycoproteins of alphaviruses.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants NS18596 (D.E.G.) and NS34175 (J.M.H.) and institutional training grant NS07000 (L.K.D.) from the National Institutes of Health.

We are grateful to Philippe Despres for helpful suggestions at the onset of this work.

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