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Neurovirulent strains of *Alphavirus* induce apoptosis in *bcl-2*-expressing cells: Role of a single amino acid change in the E2 glycoprotein

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ABSTRACT **The isolation and sequence comparison of avirulent and neurovirulent strains of polio virus, alpha virus, herpes virus, immunodeficiency virus, and other viruses have identified genetic changes that are required to cause disease in the nervous system. The molecular mechanisms by which these genetic changes result in neurovirulence are unknown. An avirulent laboratory strain of the *Alphavirus* Sindbis kills most cultured cell lines not by lethal parasitism, but by inducing apoptosis or programmed cell death. Transfection of cultured cells with the human *bcl-2* oncogene can block Sindbis virus-induced apoptosis, resulting in a persistent viral infection resembling that observed in brains of immunodeficient mice. We investigated the possibility that neurovirulent strains of Sindbis virus could overcome the protective effects of *bcl-2*—a potential mechanism to explain the ability of these strains to cause fatal disease. Strains of Sindbis virus that were lethal for 2- to 4-week-old mice induced apoptotic death in cultured cells despite the presence of *bcl-2*. Using recombinant viruses, we show that a single amino acid change in the E2 glycoprotein of Sindbis virus confers both neurovirulence and the ability to kill cells expressing *bcl-2*.**

Sindbis virus (SV) strain AR339 was originally isolated from *Culex* mosquitoes (1) and is closely related to several human pathogens including Eastern, Western, and Venezuelan equine encephalitis viruses. The Sindbis virion is composed primarily of three viral-encoded proteins. Two glycoproteins, E1 and E2, form heterodimers that span the lipid membrane surrounding the nucleocapsid. The nucleocapsid consists primarily of the capsid protein and a single-stranded, message-sense, genomic RNA of 11.7 kilobases. The outcome of SV infection is dependent on the cell type. SV infects and replicates in many cell lines, including baby hamster kidney (BHK-21), mouse neuroblastoma (N18), and rat prostate carcinoma cells (AT-3), which are susceptible to SV-induced apoptosis or cell suicide (2). However, SV can establish a persistent infection in mosquitoes that transmit the virus (3), brains of mature mice (4–6), and cultured rat dorsal root ganglia (7). These results suggest that cell factors modulate the outcome of SV infection. A candidate cellular factor is the *bcl-2* oncogene. *bcl-2* encodes a 26-kilodalton intracellular membrane protein that blocks programmed cell death induced by many different insults in several cell types (8–11). Expression of *bcl-2* in neurons prevents cell death induced by withdrawal of growth factor (12, 13). Similarly, transfection of AT-3 cells with *bcl-2* protects against killing by the avirulent AR339 strain of SV and allows the establishment of a persistently infected cell line (2). *bcl-2* or another member of the *bcl-2* family may protect the nervous system from viral-induced cell death (6, 14). We sought to identify a molecular mechanism for neurovirulence by comparing neurovirulent and avirulent SV strains for their effect on cells expressing the *bcl-2* oncogene.

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MATERIALS AND METHODS

Recombinant Viruses. Restriction enzyme fragments containing the E1 and/or E2 genes of AR339 (avirulent strain of SV) or NSV (neuroadapted strain of SV) were inserted into a full-length clone of SV (Toto1101) to generate recombinant viruses TE (formerly E2-H-55G-172) and TE12 as described (15). Recombinant virus 633 (formerly E2-Q-55G-172) was generated by inserting a Stu I–Nco I fragment containing an engineered glutamine codon at position 55 of E2 into recombinant virus TE (16). Recombinant virus 661 was generated by inserting a 6509-base-pair *Cla* I–*Sna*BI fragment from TE into strain SVB4 (P.C.T., R. J. Kuhn, E. G. Strauss, J. H. Strauss, and D.E.G., unpublished data). Full-length RNA was transcribed *in vitro* from the SP6 promoter and transfected into BHK-21 cells to obtain virus stocks (16).

Cell Lines. AT3Bcl2 and AT3Neo cell lines were generated by transfecting AT-3 rat prostate carcinoma cells with pZIPbcl-2/Neo and pZIPNeo, respectively (2). Stable lines expressing *bcl-2* or the neomycin-resistance gene alone were selected in the presence of G418 and analyzed by immunoblotting to confirm appropriate expression of bcl-2 protein as described (2).

Viability Assays and Virus Production. AT-3 cells and their derivatives were plated in 35-mm wells at 5×10^5 cells per well and infected 4–18 hr later at a multiplicity of 5 plaque-forming units of virus per cell. Supernatants were clarified of floating cells and assayed for plaque formation on BHK-21 cells to determine production of progeny virus. Pelleted and adherent cells were collected and washed, and their viability was determined by trypan blue exclusion.

RESULTS

NSV Strain Kills Cells Expressing *bcl-2*. *In vivo*, SV replicates predominantly in neurons and causes encephalomyelitis with an age-dependent mortality. Intracranial inoculation of newborn mice with SV results in fatal disease, whereas weanling 3- to 4-week-old mice recover following a mild encephalitis (1, 17). When SV strain AR339 was serially passaged in mouse brain, NSV was recovered, which caused more severe encephalitis and death in 3- to 4-week-old animals (18). Here we confirm the earlier mortality data and show that mice as young as 2 weeks recover from AR339 but not from NSV infection (Table 1). The ability of neurovirulent NSV to kill older mice is not facilitated by access to new neural cell types, since neurons remain the primary target cell in the brain for both viruses (19). Therefore, other mechanisms for virulence must be involved. We tested the possi-

Abbreviations: SV, Sindbis virus; NSV, neuroadapted strain of SV.
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Table 1. SV-induced mortality in 2-week-old mice

SV strain	Amino acid variability: E2 positions 55	E2 positions 209	E1 positions 72	E1 positions 313	*n*	% mortality
Isolates						
NSV	His	Gly	Ala	Asp	33	100
AR339	Gln	Arg	Val	Gly	33	9
Recombinants						
TE12	His	Gly	Ala	Asp	33	91
661	His	Arg	Val	Gly	36	94
TE	His	Gly	Val	Gly	33	97
633	Gln	Gly	Val	Gly	33	0

Percent mortality was determined by inoculating 14-day-old Charles River Breeding Laboratories CD-1 mice intracerebrally with 1000 plaque-forming units of virus and observing for 21 days.

bility that NSV might be able to overcome the protective effects of *bcl-2* expression. AT-3 cells stably transfected with the human *bcl-2* cDNA (2) were infected with NSV or AR339, and cell viability was monitored. In contrast to AR339, which induced no significant loss in viability, NSV killed the *bcl-2*-expressing cells (Fig. 1 *Upper*). As expected, both AR339 and NSV killed wild-type AT-3 cells and control transfected cells expressing the neomycin-resistance gene without *bcl-2*.

Genetic Determinants of Neurovirulence. Previous work indicated that the SV glycoproteins were important determinants of neurovirulence (15). Sequence determination of AR339 and NSV structural genes showed that the two viruses differed by two amino acids in the E2 glycoprotein (positions 55 and 209) and two amino acids in the E1 glycoprotein (positions 72 and 313) (15) (Table 1). Because SV genomic RNA is infectious, it is possible to generate recombinant viruses to examine the effect of these individual amino acid changes on neurovirulence (20). When all four of the amino acid changes found in NSV were introduced, the resulting recombinant virus, TE12, killed 2-week-old mice. A similar mortality rate with the recombinant virus 661 demonstrated that changing only the glutamine at position 55 of E2 to a histidine was sufficient to confer neurovirulence. Consistent with previously reported mortality data for recombinant viruses TE and 633, which differ from each other only at amino acid 55 of E2 (16), we observed high mortality with histidine-containing TE and no mortality when the histidine was replaced by glutamine in 633 (Table 1). Work from several laboratories has identified many amino acid substitutions in the E2 and E1 glycoproteins that attenuate virus virulence for newborn mice and impair virus replication in culture (15, 21–24), but only residue 55 of E2 correlates with age-dependent neurovirulence. Although recombinant virus 633 failed to kill 2-week-old mice, it still induced 100% mortality in 1- to 2-day-old mice (16). Further evidence for the importance of histidine-55 comes from a recent study in which isolates of SV recovered from the brains of infected *scid* mice were sequenced and reinoculated into 4- to 6-week-old mice (25). All of those isolates that killed mice had a histidine at E2 position 55, while those that failed to kill mice had the parental glutamine.

Genetic Determinants for Induction of Apoptosis. To identify the viral amino acid changes important for the ability to kill cells expressing *bcl-2*, these recombinant viruses were used to infect AT3Bcl2 and control cells. Only those viruses with a histidine at E2 position 55 were able to kill the cultures expressing *bcl-2* (Fig. 1 *Lower*). Thus far, we have not been able to establish long-term cell lines expressing any of the histidine-containing viruses because all cells eventually died. In contrast, recombinant virus 633 with a glutamine at position 55 of E2, failed to kill AT3Bcl2 cells, resulting in a persistently infected cell line that continued to produce

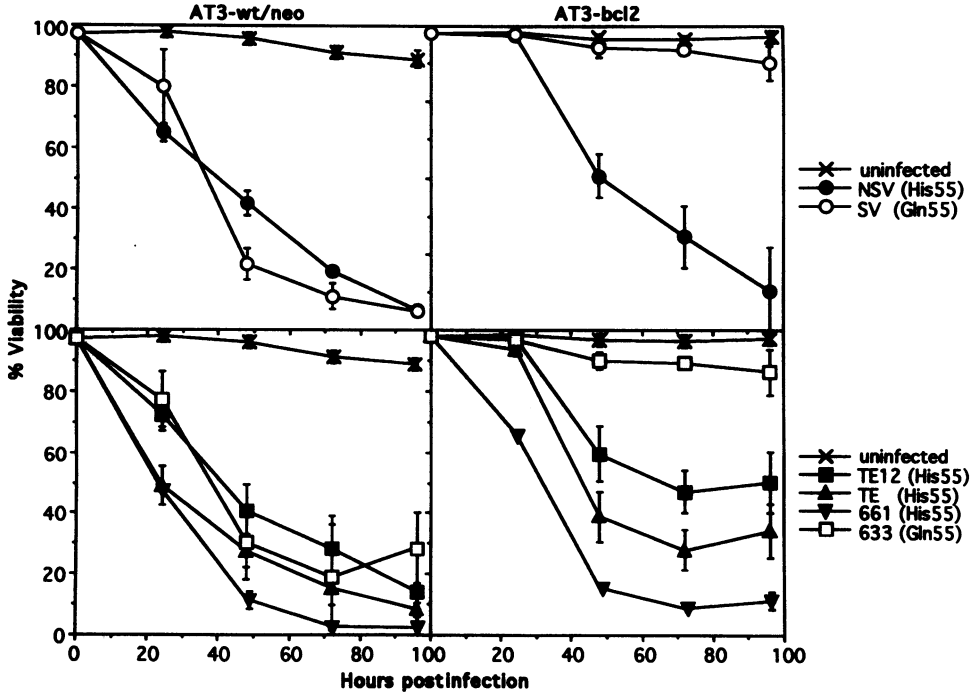

FIG. 1. Effect of SV infection on cell viability. AT3Bcl2, AT3Neo, and AT3 wild-type cells were either mock-infected (×), or infected at a multiplicity of 5 plaque-forming units per cell with the indicated strains of SV. SV (Gln55) indicates AR339. Solid symbols represent virulent strains containing a histidine at E2 position 55 and open symbols represent avirulent viruses with a glutamine at position 55. Cell viability was determined at 24-hr intervals by trypan blue exclusion. Each data point represents the mean of three to seven independent experiments, and bars indicate the standard error. No differences were observed between the AT-3 wild type and AT3Neo cell lines; therefore, the data from these cells were pooled.

progeny virus throughout its passage history, similar to that described for SV strain AR339 (2). Thus, a single amino acid change was sufficient to confer the ability to kill *bcl2*-expressing cells as evidenced by comparing viruses TE with 633, and 661 with AR339, and this genetic change was the same as that required for neurovirulence. The histidine-containing viruses TE12, TE, and 661 killed AT3Bcl2 cells with different efficiencies, suggesting that other amino acid changes may influence the rate of cell death. All viruses tested were able to kill AT-3 wild-type cells and AT3Neo control cells.

The observation that neurovirulent SVs kill cells despite the presence of *bcl-2* raises the question of whether these viruses overcome the effect of *bcl-2* and induce apoptosis or kill cells by another mechanism. To address this issue, TE-infected AT3Bcl2 cell cultures were analyzed by electron microscopy. Cells infected with TE, but not those infected with 633, displayed severely condensed chromatin characteristic of apoptosis (Fig. 2). Both TE and 633 induced chromatin condensation in control AT3Neo cells, which have no detectable endogenous *bcl-2* mRNA when assayed by reverse transcriptase/polymerase chain reaction (2). Infected AT3Bcl2 cells were also analyzed for internucleosomal DNA fragmentation characteristic of apoptosis. NSV and TE viruses which have a histidine at E2 position 55 induced endonucleolytic cleavage of chromatin leading to the formation of 180- to 200-base-pair DNA ladders despite the presence of *bcl-2* (Fig. 3*B*, lanes 3 and 4). AR339 and 633 viruses containing a glutamine at position 55 did not induce DNA fragmentation (Fig. 3*B*, lanes 5 and 6). All viruses tested induced fragmentation in control AT3Neo cells (Fig. 3*A*, lanes 3–6). We concluded that the histidine-containing viruses are able to overpower the protective effect of *bcl-2*.

bcl-2 Protein Suppresses Growth of Avirulent Strains. To determine if viral replication was impaired by expression of bcl-2 protein, production of viral progeny was monitored over time. The neurovirulent histidine-containing viruses (NSV and TE) and their glutamine-containing counterparts (AR339 and 633) replicated to similar titers in the AT-3 wild-type cells and neomycin-resistant control cells (Fig. 4, solid symbols). Virus titers decreased as the infected cells died. In contrast, the glutamine-containing virus titers were

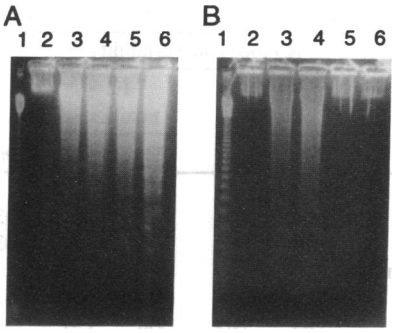

FIG. 3. DNA fragmentation in SV-infected cells. AT3Neo (*A*) and AT3Bcl2 (*B*) cells were uninfected (lane 2) or infected with strains NSV (lane 3), TE (lane 4), AR339 (lane 5), or 633 (lane 6). Lane 1 shows a 123-bp DNA ladder of size markers. Cellular DNA was extracted *in situ* and resolved by agarose gel electrophoresis as described (26).

≈10-fold lower in AT3Bcl2 cells compared with the histidine-containing viruses (Fig. 4, open symbols). These results suggest that a glutamine at position 55 of E2 may impair viral replication in the presence of *bcl-2*, thus sparing cells from viral-induced cell death. A role for E2 in viral replication has been implicated in other studies (27–29). Because the E2 glycoprotein also mediates viral attachment to cellular receptors (30, 31), it is possible that virulent viruses simply infect bcl-2-expressing cells more efficiently. However, virus binding assays and infectious center assays indicate that SV and NSV bind and infect AT3Bcl2 cells equally well (J. Lewis, P.C.T., and J.M.H., unpublished data). A similar reduction in progeny virus production by AR339 compared with NSV is observed in brains of 2- to 4-week-old mice (19). We suggest that a restriction of SV replication *in vivo* may be due to inhibitors of apoptosis in neurons.

DISCUSSION

bcl-2-mediated protection against viral-induced apoptosis is not unique to SV. MDCK cells transfected with *bcl-2* are protected from apoptosis induced by type A and B influenza viruses (32). Furthermore, *bcl-2* expression suppresses in-

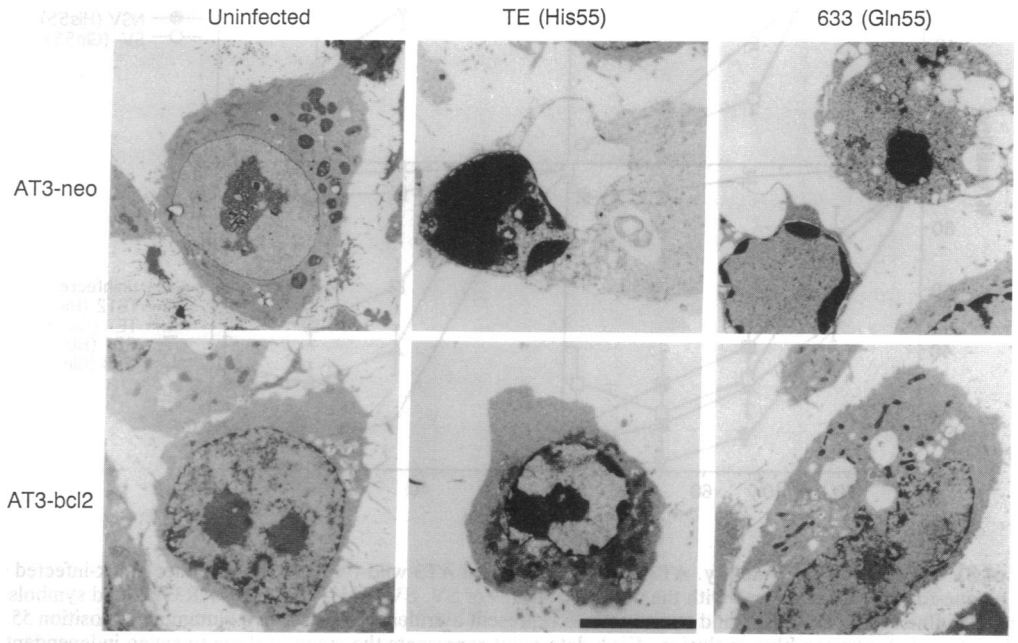

FIG. 2. Electron microscopy of SV-infected AT-3-derived cell lines. AT3Neo (*Upper*) cells or AT3Bcl2 (*Lower*) cells were infected with recombinant SV strain TE or 633 and harvested 18–24 hr later. (Bar = 5 μm.)

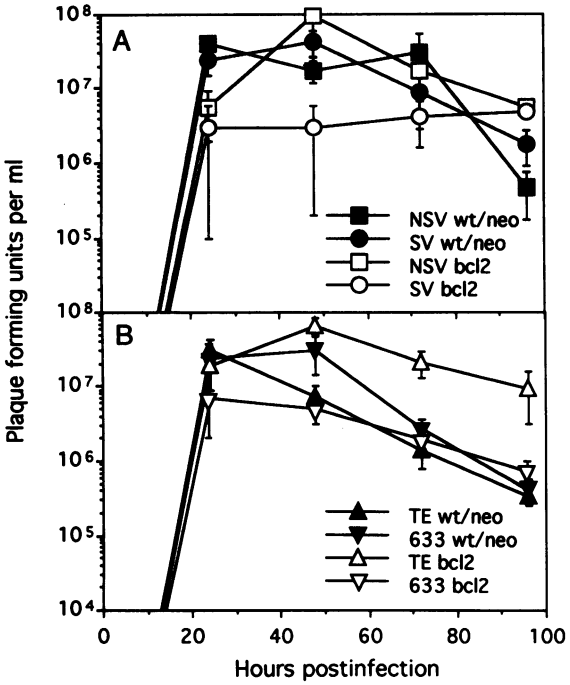

FIG. 4. Virus production in the presence and absence of *bcl-2*. Supernatants from infected AT3Bcl2, AT3Neo, or AT-3 wild-type cells in three to seven independent experiments were plaque-assayed in duplicate. The geometric mean was determined, and bars indicate standard error. No differences were observed between AT-3 wild-type cells and AT3Neo cells; therefore, the data from these cells were pooled. SV indicates strain AR339.

fluenza virus replication, suggesting that a general mechanism for suppressing viral growth may be essential to cell survival. In contrast to the neurovirulent strains of SV, the more virulent influenza virus strains were unable to kill cells expressing bcl-2.

It is not anticipated that bcl-2 will protect every cell type from viral-induced apoptosis. BHK21 cells, which are readily killed by SV, express *bcl-2* mRNA, although bcl-2 protein expression has not been confirmed (J.M.H., unpublished data). However, overexpression of bcl-2 does not protect BHK cell derivatives from apoptosis induced by other insults (33). The inability of bcl-2 to protect against apoptosis in other circumstances has been described and may in part be due to the presence of bcl-2-related proteins such as BAX and bcl-x_S which block the protective effects of bcl-2 (14, 34).

We have identified a single amino acid change in the SV E2 glycoprotein that confers both neurovirulence and the ability to kill AT3 cells expressing *bcl-2*. A single amino acid change in E2 could alter a direct interaction between E2 and bcl-2. bcl-2 is anchored to membranes via a C-terminal hydrophobic domain and has been localized to the endoplasmic reticulum, nuclear, and outer mitochondrial membranes (35, 36). Therefore, bcl-2 and E2 could colocalize in the endoplasmic reticulum. Alternatively, bcl-2 could alter the efficiency of E2 protein folding in an intracellular environment influenced by the expression of bcl-2 (37). Single amino acid changes that alter the glycosylation pattern of viral glycoproteins have been shown to modulate neurovirulence of both flavi and influenza viruses (38, 39). The mechanism proposed for influenza is that loss of a carbohydrate side chain facilitates neuraminidase activity, which enhances the spread of infection in brain. Replication in lung was unaffected by the mutation presumably because the requirement for neuraminidase activity was reduced (39). Although the mutation in Sindbis virus E2 does not alter either of the two N-linked glycosylation sites, the impairment of virus spread remains a possible mechanism to explain reduced virus titers in cells expressing *bcl-2*.

The E2 glycoprotein mediates another interesting biological phenomenon. Treatment of (*i*) persistently infected cultured neurons, (*ii*) persistently infected AT3Bcl2 cells, or (*iii*) persistently infected mice with anti-E2 monoclonal antibody results in an inhibition of viral replication and clearance of virus from infected cells (2, 7). Antibodies to other SV proteins have no effect. It is possible that E2 bound by antibody could transmit a signal to the cell, resulting in suppression of viral replication. Perhaps this antibody/E2-mediated suppression of viral gene expression can be mimicked by bcl-2. Regardless of the mechanisms, the results presented here suggest that the ability of Sindbis virus to kill cells expressing *bcl-2* is an important determinant of neurovirulence.

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