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The proteoglycan bamacan is a host cellular ligand of vaccinia virus neurovirulence factor N1L

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Neurovirulence is one of the pathological complications associated with vaccinia virus (VV) infection/vaccination. Although the viral N1L protein has been identified as the neurovirulence factor, none of the host N1L-interacting factors have been identified so far. In the present study, we identified N1L-interacting proteins by screening a human brain cDNA expression library with N1L as a bait protein in a yeast two-hybrid analysis. The analysis revealed that N1L interacts with human brain-originated cellular basement membrane–associated chondroitin sulfate proteoglycan (bamacan). The N1L-binding domain of bamacan was mapped to its C-terminal 227 amino acids. The N1L-bamacan interaction was further confirmed in both VV-infected and N1L-transfected mammalian cells. Following the confirmation of the protein interactions by coimmunoprecipitation experiments, confocal microscopic analysis revealed that N1L colocalizes with bamacan both in VV-infected B-SC-1 cells as well as in mice neuronal tissue. Furthermore, a human neural cell line, which expresses bamacan to moderately elevated levels relative to a non-neural cell line, supported enhanced viral growth. Overall, these studies clearly suggest that bamacan interacts with the VV-N1L and such interactions seem to play a positive role in promoting the viral growth and perhaps contribute to the virulence of VV in neural cells. *Journal of NeuroVirology* (2009) **15,** 229–237.

Keywords: chondroitin sulfate; CSPG6; GFP; poxvirus; SMC3; WR strain

Introduction

The possible use of smallpox virus as a biowarfare agent has led to renewed interest in stockpiling of smallpox vaccines utilizing live attenuated virus (Smith and McFadden, 2002). Use of such vaccines has been associated with postvaccinal neurological complications, possibly due to direct viral infection of the brain (neurovirulence), or an overwhelming autoimmune response associated with demyelination (Cono *et al*, 2003; Romanov and Bereshkova, 1975). The neurovirulent potential of vaccinia virus (VV) in mice was attributed to a single viral protein, N1L (Abrahams *et al*, 2005; Kotwal *et al*, 1989; Zhang *et al*, 2005). The N1L protein is a 13.8-kDa intracellular homodimer, reported to be a multifunctional protein (Abrahams *et al*, 2005; Zhang *et al*, 2005; Bartlett *et al*, 2002; Billings *et al*, 2004). N1L has been shown to influence cytokine secretion and ATP levels *in vivo* (Abrahams *et al*, 2005; Zhang *et al*, 2005). Additionally, the protein targets the inhibitor of nuclear factor kappa B (IκB) kinase complex and inhibits signaling to nuclear factor (NF)-κB and Interferon Regulatory Factor (IRF3) downstream of tumor necrosis factor (TNF) and Toll-like receptors (TLRs) (DiPerna *et al*, 2004).

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More recently, the crystal structure of N1L was resolved by two independent groups and the analysis revealed structural and functional similarities to an antiapoptotic protein, a B-cell lymphoma-2 (Bcl-2), illustrating the significance of evolutionary conservation of structure rather than sequence in protein function (Aoyagi *et al*, 2007; Cooray *et al*, 2007).

As VV neurological complications arise due to specific virus-host interactions, we hypothesized that the N1L protein has the potential to interact with host protein(s), and more specifically with proteins of the central nervous system. Therefore, we utilized N1L as a bait protein and screened a commercially available adult human brain cDNA expression library in a yeast two-hybrid system in order to identify N1L-interacting cellular proteins. Our analysis identified brain-derived cellular chondroitin sulfate proteoglycan (bamacan) as N1L-interacting partner. Bamacan was initially identified from embryonic parietal yolk sac and termed based on its cellular localization (Wu and Couchman, 1997). The protein is also known as chondroitin sulfate proteoglycan 6 (CSPG6), structural maintenance of chromosomes 3 (SMC3), or human chromosome-associated protein (hCAP) (Wu and Couchman, 1997; Ghiselli and Iozzo, 2000; Ghiselli *et al*, 1999). Bamacan protein is ubiquitous, with subcellular localization in the nucleus, nucleoli, and cytoplasm, and is a secretory protein as well (Wu and Couchman, 1997; Ghiselli and Iozzo, 2000; Ghiselli *et al*, 1999; Eijpe *et al*, 2000). Interaction of cellular proteoglycans, such as chondroitin sulfate (CS) or heparan sulfate (HS), with other VV proteins (D8L, A27L, and H3L) has been previously documented (Chung *et al*, 1998; Hsiao *et al*, 1999; Lin *et al*, 2000).

In this report, we present evidence that bamacan is a cellular ligand for VV-N1L protein and demonstrate that N1L interacts with bamacan both in vaccinia virus–infected and N1L-transfected cells. As known earlier, bamacan is overexpressed in neural cells and thus the VV-N1L and the elevated levels of bamacan expression in a neural cell imply a possible enhanced role of this interaction in the neurovirulent outcome of the infection.

Results

Cellular bamacan C-terminal 227 amino acids interact with vaccinia virus N1L protein

Using N1L as a bait protein, we screened a human brain cDNA library. The screening and sequencing of the N1L-interacting clones revealed one of the clones to be coding for the C-terminus of a cellular basement membrane–associated chondroitin sulfate proteoglycān, a.k.a. bāmacan. In order to establish and confirm this interaction with the full-length bamacan, we amplified the bamacan coding sequences representing the open reading frame from a human cDNA library by polymerase chain reaction (PCR) and cloned it in to a yeast two-hybrid vector (Table 1). A series of deletion-constructs of bamacan

Table 1 Primers used in the study

No.	Gene/clone	Vector	Sequence	Restriction sites
1	N1L	pAS2-1 and pGAD424	5′-cgcgaattcatgaggactctac 3′-cgcctgcagttatttttcacc	EcoRI PstI
2	N1L	pEGFP-C2	5′-cgcgaattcatgaggactctac 3′-cgcgggcccttatttttcacc	EcoRI ApaI
3	Bam-FL	pAS2-1 and pACT-2	5′-cgcccatggagatgtacataaagcagg 3′-ggcggaattcttaaccatgtgtggt	NcoI EcoRI
4	Bam537–1217	pAS2-1 and pACT-2	5′-gcggccatggcgatgaataactttgaatg 3′-ggcggaattcttaaccatgtgtggt	NcoI EcoRI
5	Bam537–763	pACT-2	5′-gcggccatggcgatgaataactttgaatg 3′-gcggaattcctacaaactctgtaagct	NcoI EcoRI
6	Bam764–990	pACT-2	5′-gcgccatggaggcaagcttgcat 3′-gcggaattcctaaaactgatccaa	NcoI EcoRI
7	Bam991–1217	pACT-2	5′-gcgccatggtaaatttctccgag 3′-3′-ggcggaattcttaaccatgtgtggt	NcoI EcoRI
8	Bam537–990	pAS2-1 and pACT-2	5′-gcggccatggcgatgaataactttgaatg 3′-gcggaattcctaaaactgatccaa	NcoI EcoRI
9	Bam764–1217	pAS2-1 and pACT-2	5′-gcgccatggaggcaagcttgcat 3′-ggcggaattcttaaccatgtgtggt	NcoI EcoRI
10	N537Bam	pAS2-1 and pACT-2	5′-cgcccatggagatgtacataaagcagg 3′-gcggaattcctatacaataccatgatagcc	NcoI EcoRI
11	Bam269–537	pACT-2	5′-cgcccatggaggatatcgaacgccaag 3′-gcggaattcctatacaataccatgatagcc	NcoI EcoRI
12	N268Bam	pACT-2	5′-cgcccatggagatgtacataaagcagg 3′-cgcgaattcctactccattttatctct	NcoI EcoRI
13	Bam269–990	pACT-2	5′-cgcccatggaggatatcgaacgccaag 3′-gcggaattcctaaaactgatccaa	NcoI EcoRI

were also made and these were tested one-on-one with the N1L clone (Table 1). In order to confirm the true interaction between N1L and bamacan, N1L was cloned into both pAS2-1 as well as pGAD424 vectors and, likewise, bamacan was cloned into both pAS2-1 and pACT-2 vectors. N1L-pAS2-1 was tested against bamacan cloned into pACT-2, whereas N1L-pGAD424 was screened against bamacan cloned in the pAS2-1 vector (Table 1). The interaction was monitored by β-GAL assay and the results were expressed as +++, ++, +/− based on the time taken (within 1, 2, or 3 h respectively) for the colonies to turn blue (Figure 1). Clear demarcation could be made between "+++" and "++" reactive results, as all the strongly reactive binders (+++) turned blue within the first 15 to 20 min, whereas the "++" reactive ones took almost 2 h to turn blue. N1L reacted with the full-length bamacan and the interaction was localized predominantly to the C-terminal 227 amino acids (Figure 1).

N1L interacts with bamacan in VV-infected and N1L-transfected cells

In order to establish N1L-bamacan binding as a true interaction, we further tested the status of N1L-bamacan binding in VV-infected B-SC-1 cells and N1L-transfected COS-7L cells by immunoprecipitation analysis using anti-N1L and anti-bamacan antibodies. The anti-N1L antibody was able to bind and pull down the N1L-bamacan complex only in the WR-infected lysate and did not pull down bamacan in the mock-infected lysate. Conversely, the anti-bamacan antibody was able to bind and pull down bamacan in both mock and VV-infected lysates and, as predicted, N1L was detected only in the WR-infected lane, suggesting a true interaction of N1L with bamacan in WR vaccinia-infected cells (Figure 2A).

In N1L-GFP–transfected cells, the interaction was analyzed by using anti-GFP and anti-bamacan antibodies. Here again, the anti-GFP antibody was able to pull down bamacan only in the N1L-GFP–transfected lysate and the anti-bamacan antibody pulled down only the N1L-GFP–fused protein and not the GFP-alone lysate, suggesting a true interaction (Figure 2B).

N1L colocalizes with bamacan both in cell culture and mice brain cells

Because bamacan is known to be a multifunctional protein and has varied cellular distribution, we analyzed the localization of N1L in WR-infected B-SC-1 cells. Immunostaining, followed by confocal microscopy analysis of the infected cells, revealed that N1L is predominantly localized to the cytoplasm and colocalizes with the intracellular bamacan (Figure 3A to F). N1L-bamacan localization analysis was also performed in brain sections derived from 3-day-old neonatal mice infected with WR virus. N1L was localized to the virus-infected foci within the brain cortex region and bamacan colocalized with N1L protein in these neural cells as well (Figure 3G to L).

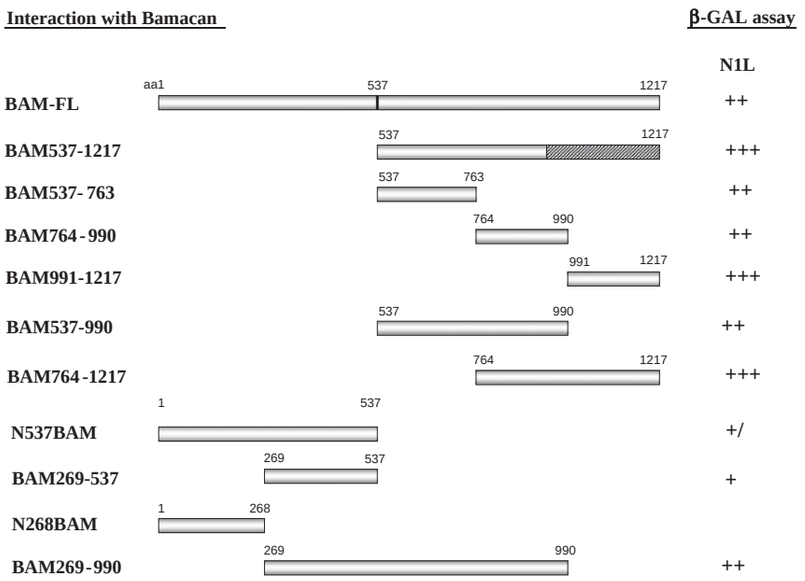

Figure 1 Identification of N1L-binding domain of bamacan. Full-length and deletion constructs of bamacan were utilized for mapping N1L-binding domain of bamacan by the yeast two-hybrid β-gal assay. Results are expressed as +++, ++, +/− based on the time taken (within 1, 2, or 3 h, respectively) for the colonies to turn blue. As indicated, the C-terminal 227 amino acids of N1L exhibit the strongest interaction with bamacan. Shaded area in Bam537–1217 represents region of bamacan that was identified in the original brain cDNA library screening.

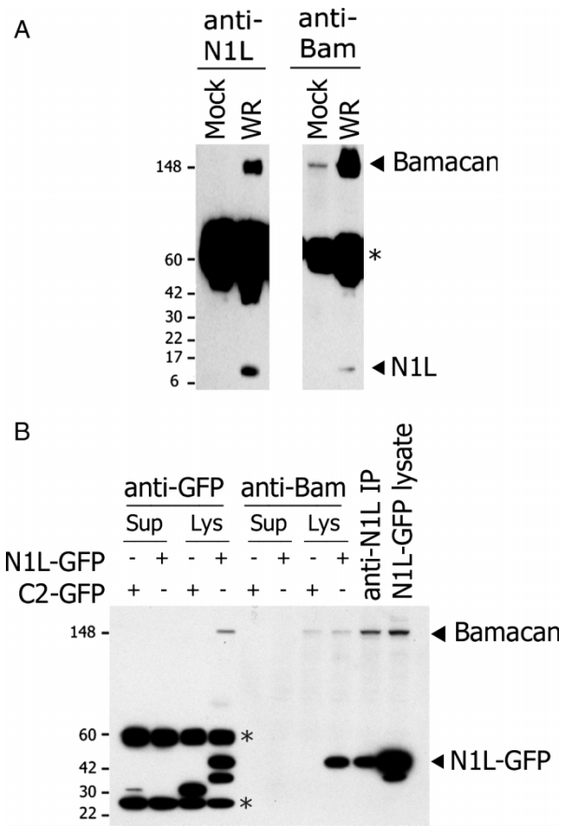

Figure 2 N1L-bamacan interaction in VV-infected and N1L-transfected cells. (**A**) VV infection. B-SC-1 cells were infected with VV WR and lysates were immunoprecipiated (IP) with anti-N1L or anti-bamacan antibody. Following separation of proteins on a polyacrylamide gel, N1L and bamacan were detected by an immunoblot analysis. In the anti-N1L IP lysates, N1L and bamacan were detected only in the WR-infected lane, indicating a true interaction. Anti-bamacan IP analysis resulted in detection of bamacan in both lysates and N1L detection only in the WR-infected lane. (**B**) N1L-transfected cells. COS-7L cells were transfected either with C2-GFP (vector control) or N1L-GFP. At 24 h post transfection, cell supernatants and cytolysates were immunoprecipitated with a mouse anti-GFP or a goat anti-bamacan or an anti-N1L antibody. N1L-GFP and bamacan were detected by immunoblot analysis using mouse anti-GFP antibody and a rabbit anti-bamacan antibody. As evident from the analysis, bamacan was detected only in the N1L-GFP–transfected lysate and anti-bamacan antibody pulled down only the N1L-GFP and not the GFP (vector control). IgG fraction (both heavy and light) of the antibody are represented by asterisk (*).

A human neural cell line with higher bamacan level provides growth advantage to the virus

Because VV-N1L interacts with bamacan, we rationalized that if this interaction is crucial to the virus survival, then the virus should demonstrate a selective advantage in cells that express this protein to relatively high levels. To test this hypothesis, we selected two cell lines that differ in bamacan expression levels. The two cell types were infected with a wildtype VV strain (WR) and viral production was determined by a standard plaque assay on B-SC-1 cells (Kotwal et al, 1999; Agnes et al, 2005). A previous study reported that mammalian brain, testes, and muscle tissues express higher levels of bamacan compared to other organs and tissues (Ghiselli et al, 1999). This prompted us to first compare the bamacan expression levels between a human brain-derived neural cell line, DBTRG-05MG, and Vero 76 cells by immunoblot analysis using anti-bamacan antibodies. The analysis revealed that bamacan was indeed expressed significantly higher in the neural cell line (Figure 4A). We then compared the growth of vaccinia virus WR in the neural and non-neural cell lines by first infecting the cells with VV-WR and then harvesting the virus 72 h post infection (h.p.i.). Our analysis of results from a set of five repeat experiments indicated that in fact VV-WR infection in DBTRG-05MG cells yields relatively higher viral titer ($P < .005$) compared to infection in Vero 76 cells (Figure 4B and C).

Discussion

Neurovirulent complications, associated with the use of vaccinia virus–based current smallpox vaccine, are a major public health concern (Smith and McFadden, 2002; Cono *et al*, 2003; Guharoy *et al*, 2004). Such complications could be attributed either to viral or host factors or a combination of these factors. Although the neurovirulence potential of VV has been attributed to a single viral gene, N1L, there are no reports of potential host factors contributing to vaccinia neurovirulence (Kotwal *et al*, 1989; Bartlett *et al*, 2002; Billings *et al*, 2004). We sought to identify brain-derived cellular partners of the viral neurovirulence factor N1L as a first step in elucidating the virus neurovirulence mechanisms. Our analysis thus revealed bamacan as a N1L-interacting cellular protein and that the N1L-interacting domain on bamacan was localized within its C-terminal 227 amino acids. Similar VV-proteoglycan interactions have been documented earlier, with VV proteins D8L, A27L, and H3L interacting with either cell surface chondroitin sulfate (CS) or with heparan sulfate (HS), respectively, in promoting cellular entry of the virus (Chung *et al*, 1998; Hsiao *et al*, 1999; Lin *et al*, 2000). It is to be noted that in all the above studies the role of either HS or CS was unambiguously towards promoting cellular entry of VV as these proteoglycans are localized to the cellular membranes and also that the VV proteins D8L, A27L, and H3L are all structural components on the infectious virion. Additionally, because it is already known that the VV D8L protein interacts with a proteoglycan, also a chondroitin sulfate, we analyzed whether D8L would interact with bamacan as well in a yeast two-hybrid assay (Hsiao *et al*, 1999). Our results revealed that the D8L did not bind to any of the bamacan clones (data not shown), suggesting that

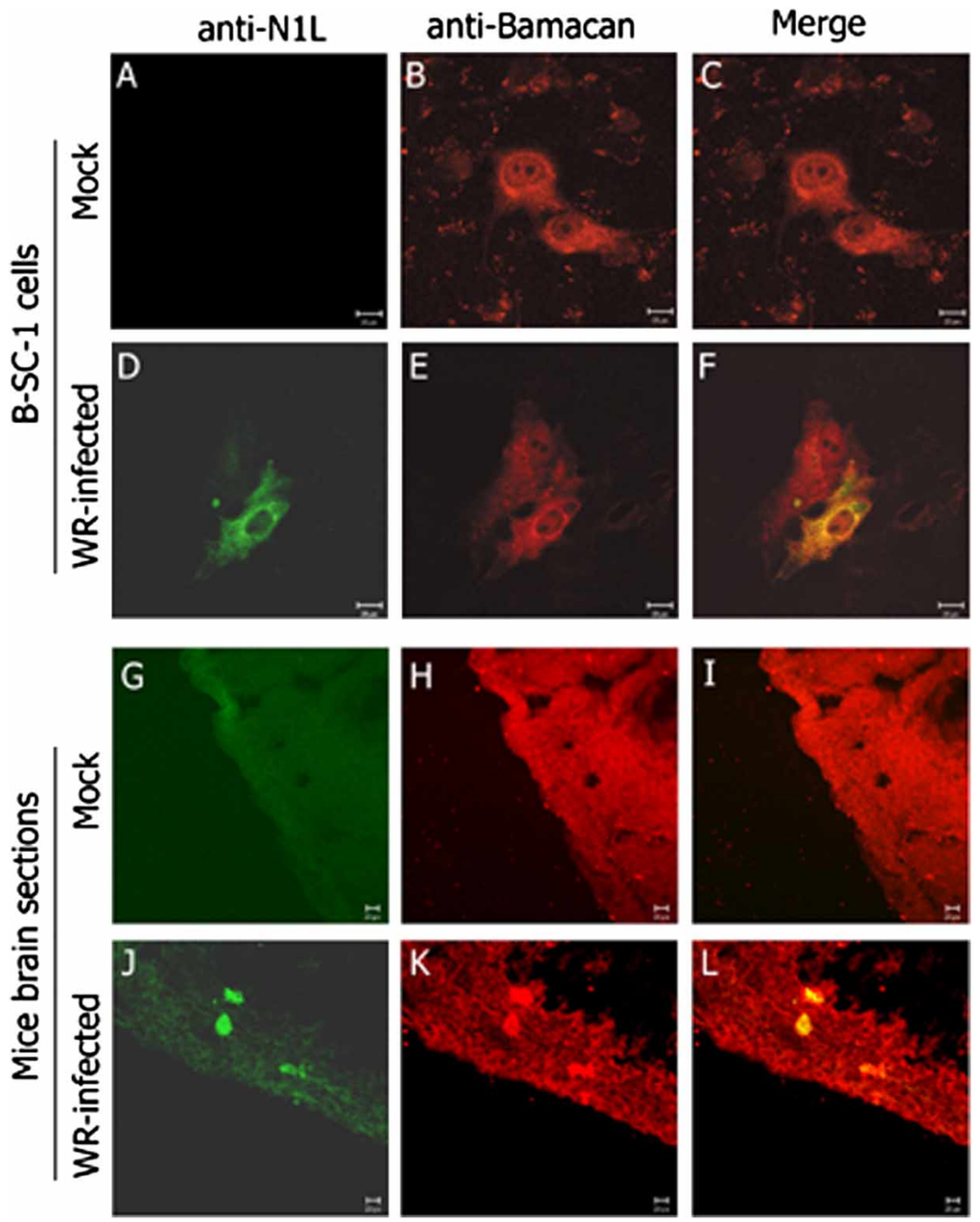

Figure 3 Colocalization of N1L with bamacan. Mock-infected (**A–C** and **G–I)** or WR-infected (**D–F** and **J–L**) cells were fixed and immunostained with rabbit anti-N1L and goat anti-bamacan antibodies. Secondary incubation with an anti-rabbit-FITC and an anti-goat-Texas Red was performed for detection of N1L and bamacan, respectively. Laser scanning confocal microscopy reveals that N1L is detected in WR-infected B-SC-1 cells (**D**) and colocalizes with bamacan in cytoplasm (**F**). Similarly, immunofluorescence analysis of 3-day postinfection brain sections derived from VV-infected 3-day-old neonatal mice reveal N1L localized in infected cells as foci (**J**) and bamacan colocalizes with N1L (**L**) in neural cells as well. Magnification bar 20 μm.

previously reported CS and bamacan identified in this report are two different proteoglycans.

Because N1L is synthesized exclusively following cellular infection, the N1L-bamacan interaction is obviously a late event in the life cycle of VV that might provide either a growth advantage or result in a neurovirulent outcome. This phenomenon has been observed with other viruses and protozoans as well (Argyris *et al*, 2003; Bobardt *et al*, 2004; Misinzo *et al*, 2006; Pouvelle *et al*, 2000). The involvement of cellular proteoglycans in up-regulating infectivity, virulence, or pathogenecity has been previously documented in the case of human immunodeficiency virus (HIV)-1, porcine

circovirus-2, and plasmodium parasite and several other microbial infections (Argyris *et al*, 2003; Bobardt *et al*, 2004; Misinzo *et al*, 2006; Pouvelle *et al*, 2000). More recently, the role of cellular proteoglycans in HIV-1 invasion of brain has been documented as well (Bobardt *et al*, 2004).

More significantly, the neurovirulent potential of N1L as observed in previous studies with VV demonstrating a significant decrease in LD_{50} and VV replication of the N1L-deletion mutant in mouse brain is noteworthy (Smith and McFadden, 2002; Bartlett *et al*, 2002; Billings *et al*, 2004). Within this context, it is interesting to note that bamacan is expressed to relatively higher levels in mammalian brain and testes (Ghiselli *et al*, 1999). Though our analysis demonstrating N1L-bamacan interaction was performed in non-neural cells such as B-SC-1, Vero 76 and COS-7L cells (all simian kidney epithelial cell lines), it was based upon previously established standard procedures for analyzing vaccinia infection in cell culture and primed towards achieving optimal results (e.g., superior transfection efficiency in COS-7L cells than in neural cells). However, because it is also critical to demonstrate the N1L-bamacan interaction or its effects in neural cells, especially human brain-derived neural cells, our analysis included assays in mice brain and human neural cells. The neonatal mouse model is a well-established and a very amenable model preferred to study the pathogenesis of vaccinia infection because these mice are more sensitive to produce vaccinia-induced neurological disease than the adults (Li *et al*, 2004). The experimental observations from the current study demonstrating colocalization of bamacan and VV-N1L in VV-infected mice neuronal tissue further proves that this interaction does occur in neural tissue as well. A time-course analysis of N1L-bamacan interaction may provide additional insights into viral pathogenesis, which did not fall within the scope of this study as the main emphasis of the present study was to demonstrate only the N1L-bamacan binding in brain tissue. The enhanced VV growth in a human neuronal cell line, wherein bamacan is expressed to relatively higher levels, compared to Vero 76 illustrates the possibility that N1L-bamacan interaction promotes VV growth and possibly play a role in VV neurovirulence.

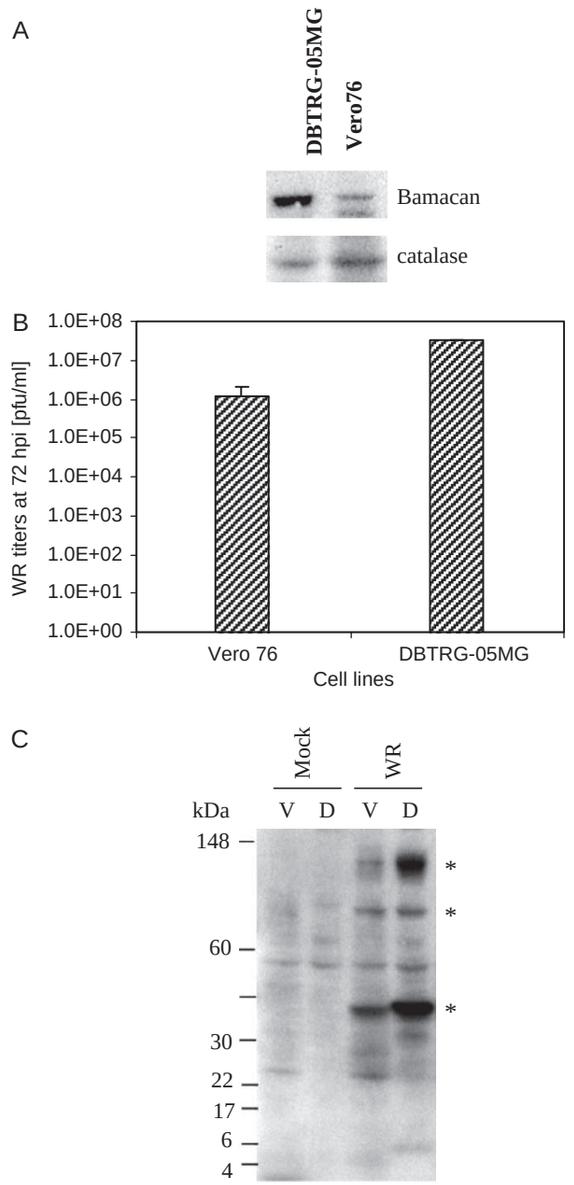

Figure 4 (*Continued*)

Materials and methods

Cells, virus, and reagents

Simian kidney epithelial cell lines Vero 76, BS-C-1 cells (ATCC, Manassas, VA), and COS-7L (Invitrogen, Gaithersberg, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Mediatech,

Figure 4 Effect of bamacan expression levels on WR titers. (**A**) Bamacan expression levels in two cell lines. Lysates were prepared from Vero 76 and DBTRG-05MG (human glioblastoma cell line) and analyzed for bamacan expression levels by an immunoblot analysis. The DBTRG-05MG reveals higher expression levels of bamacan than the Vero 76 cells. A housekeeping gene, catalase, was probed as a loading control. (**B**) Comparison of WR growth on Vero 76 and DBTRG-05MG cells. Vero 76 and DBTRG-05MG cells were infected with WR at a MOI of 0.001 and the progeny virus was harvested at 72 h.p.i. Virus titers from 5 individual experiments were determined by a plaque assay on B-SC-1 cells. WR titers were atleast a log higher when grown on DBTRG-05MG cells ($P < .005$). (**C**) Immunoblot analysis of WR infection on Vero 76 and DBTRG-05MG. Lysates were prepared from WR-infected Vero 76 and DBTRG-05MG cells and analyzed for viral protein levels as indicator of viral growth in these two cell lines. Human VIG (Vaccinia Immuno Globulin) was used for detecting viral proteins. Bands represented by an * are the most significant VV proteins that are detected by VIG. DBTRG-05MG indicates higher levels of viral proteins than Vero 76 cells.

Herndon, VA) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine (Mediatech, Herndon, VA), and penicillin-streptomycin (Invitrogen). Vaccinia virus (WR strain; kind gift from B. Moss) was propagated in Vero 76 cells as described previously (Kotwal *et al*, 1989; Agnes *et al*, 2005).

Yeast two-hybrid screening

Yeast Matchmaker pretransformed human adult brain cDNA library, cloned at EcoRI-XhoI sites of GAL4 activation domain (AD) plasmid (pACT2) and a GAL4 binding domain (BD) plasmid (pAS2-1) for cloning N1L-coding sequence, was used. Vaccinia virus DNA was extracted from WR-infected Vero 76 cells using a ViralXpress DNA extraction kit (Chemicon, Temecula, CA). Using this DNA, the open reading frame (ORF) of vaccinia virus N1L was polymerase chain reaction (PCR)-amplified with appropriate primers and cloned into pAS2-1 vector (Table 1). Using this plasmid as bait, a human brain cDNA library, cloned in pACT2 vector, was screened for N1L-interacting proteins essentially according to the manufacturer's instructions (Fields and Song, 1989). Appropriate yeast growth and selection media, yeast transformation reagents, positive and negative plasmid controls, and cloning vectors were included in the analysis (Clontech/ BD Biosciences, Emeryville, CA). Primers for amplifying full-length bamacan and deletion constructs were designed based on the published sequence (GenBank accession no. NM_005445) (Table 1). Full-length bamacan was amplified from a human cDNA library (Panomics, CA) using appropriate primers as per the manufacturer's instructions and the amplified ORF product cloned into both pAS2-1 and pACT-2 vectors (Table 1). Confirmation and localization of the interacting domain was performed by the one-on-one yeast two-hybrid analysis using both N1L-pAS2-1 and N1L-pGAD424 plasmids (Mohan *et al*, 2002).

Virus stock preparation and infection

VV WR seed stocks were prepared by infecting three $T75^2$ flasks of Vero 76 cells. Infected cells were freeze-thawed three times at 72 h post infection (h.p.i.), followed by low speed centrifugation, and the supernatant was collected, aliquoted, and stored at -80°C, until used (Kotwal *et al*, 1989; Agnes *et al*, 2005). VV infection of B-SC-1 (simian kidney epithelial) cells for immunoprecipitation experiments was performed in 6-well plates. Following virus binding, inoculum was aspirated and fresh medium was added. Cells were then incubated at 37°C for 72 h.

Immunoprecipitation analysis

VV-infected cells

B-SC-1 cells were infected with WR strain at an multiplicity of infection (MOI) of 0.01 and incubated for 72 h. Cytolysates were prepared and immunoprecipitation was performed using a rabbit anti-N1L antibody and a goat anti-bamacan antibody as described previously (Mohan *et al*, 2003). The complexes were resolved on a sodium dodecyl sulfate–4% to 20% polyacrylamide gel and detection of the proteins was carried out by immunoblotting with anti-N1L and anti-bamacan antibody.

N1L-GFP–transfected cells

Transient transfections were performed on COS-7L cells maintained in 6-well plates. Two to five micrograms of N1L-GFP or GFP-C2 plasmid DNA (BD Biosciences/Clontech) mixed with lipofectamine plus reagent was used for each transfection as per the manufacturer's instructions (Invitrogen). Lysates were prepared 24 h post transfection and immunoprecipitated with an anti-green fluorescence protein (GFP) monoclonal antibody (BD Biosciences/Clontech) and an anti-bamacan goat polyclonal antibody (Santa Cruz Biotechology, Santa Cruz, CA) by incubating overnight at 4°C. Detection of the immunoprecipitated proteins was performed by immunoblotting with the anti-GFP antibody and a rabbit anti-bamacan antibody (Bethyl Labs, CA).

Confocal Immunofluorescence assay

Cell culture

B-SC-1 cells cultured in 8-well chamber slides (Labtek, USA) were infected with VV WR at MOI of 0.001 as mentioned above. Slides were fixed 24 h post infection in ice-cold methanol-acetone mixture, rinsed with 1× phosphate-buffered saline (PBS), permeabilized with 0.3% Triton X-100 PBS for 5 min at room temperature, and washed twice with 1 × PBS (Mohan *et al*, 2002). Cells were then incubated with rabbit anti-N1L antibody and goat anti-bamacan antibody (Santa Cruz) at 37°C for 90 min. After 3 washes with 1 × PBS, incubations were carried out with secondary anti-rabbit fluorescein isothiocyanate (FITC) – and anti-goat Texas Red–labeled antibodies (Vector Labs, CA) for 90 min at 37°C. Appropriate secondary antibody controls were included in the assay. The cells were then washed 3 times with 1 × PBS, air-dried, and mounted with Vectashield (Vector Labs). Visual analysis and photography was performed using a Carl Zeiss laser-scanning confocal microscope (model: LSM5 PASCAL). Images were transferred to PC version of Adobe Photoshope 5.0 for labeling and printing.

Mice brain

Three-day-old neonatal CD-1 mice (Harlan Sprague-Dawley, Indianapolis, IN) were inoculated intracranially with 10 μl of 10^2 plaque-forming units (PFU) dose of WR strain and an equivalent volume of

Earle's Minimum Essential Medium (EMEM) for the control group (Li *et al*, 2004). Infection was followed up by sacrificing mice after deeply anesthetizing them every 24 h on day 1 till day 5. The infected and control brains were removed and bisected and immediately flash frozen with Optimal Cutting Temperature (OCT) compound (Sakura Finetech, Torrance, CA). Frozen brains were then sagittally sectioned across the cortical region into 10 to 20 μm thick sections and stored at -20°C until processed. Sections were rinsed in $1\times$PBS and immunostained as described above with rabbit anti-N1L antibody and a goat anti-bamacan (a.k.a anti-SMC3) antibody (Santa Cruz Biotechnology). Secondary antibody staining was carried out as described above and analysis performed under a laser scanning confocal microscope.

Growth of VV on Vero 76 and a human neuronal cell culture

A human brain-derived glioblastoma cell line, DBTRG-05MG (ATCC, Manassas, VA), and Vero 76 cells were utilized to compare VV growth between the two cell lines. Each cell line was cultured in 6-well plates and infected with 10^3 PFU/ml of WR virus strain. Following virus binding, inoculum was aspirated, fresh medium added, and cells were incubated at 37°C. Virus was harvested at 72 h.p.i. from these cells and plaqued on B-SC-1 cells for quantification as previously described (Kotwal *et al*, 1989; Agnes *et al*, 2005). Simultaneously, cell lysates were prepared from a duplicate set of infected cells for immunoblot analysis. Cellular bamacan levels in both cells lines were detected using a rabbit anti-bamacan antibody and viral infection was detected using a human anti-vaccinia (total) antibody (VIG; kind gift from Dr. Dorothy Scott, CBER) (Agnes *et al*, 2005).

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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