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Human and Host Species Transferrin Receptor 1 Use by North American Arenaviruses

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ABSTRACT

At least five New World (NW) arenaviruses cause hemorrhagic fevers in South America. These pathogenic clade B viruses, as well as nonpathogenic arenaviruses of the same clade, use transferrin receptor 1 (TfR1) of their host species to enter cells. Pathogenic viruses are distinguished from closely related nonpathogenic ones by their additional ability to utilize human TfR1 (hTfR1). Here, we investigate the receptor usage of North American arenaviruses, whose entry proteins share greatest similarity with those of the clade B viruses. We show that all six North American arenaviruses investigated utilize host species TfR1 orthologs and present evidence consistent with arenavirus-mediated selection pressure on the TfR1 of the North American arenavirus host species. Notably, one of these viruses, AV96010151, closely related to the prototype Whitewater Arroyo virus (WWAV), entered cells using hTfR1, consistent with a role for a WWAV-like virus in three fatal human infections whose causative agent has not been identified. In addition, modest changes were sufficient to convert hTfR1 into a functional receptor for most of these viruses, suggesting that a minor alteration in virus entry protein may allow these viruses to use hTfR1. Our data establish TfR1 as a cellular receptor for North American arenaviruses, highlight an "arms race" between these viruses and their host species, support the association of North American arenavirus with fatal human infections, and suggest that these viruses have a higher potential to emerge and cause human diseases than has previously been appreciated.

IMPORTANCE

hTfR1 use is a key determinant for a NW arenavirus to cause hemorrhagic fevers in humans. All known pathogenic NW arenaviruses are transmitted in South America by their host rodents. North American arenaviruses are generally considered nonpathogenic, but some of these viruses have been tentatively implicated in human fatalities. We show that these North American arenaviruses use the TfR1 orthologs of their rodent host species and identify TfR1 polymorphisms suggesting an ongoing "arms race" between these viruses and their hosts. We also show that a close relative of a North American arenavirus suggested to have caused human fatalities, the Whitewater Arroyo species complex virus AV96010151, uses human TfR1. Moreover, we present data that imply that modest changes in other North American arenaviruses might allow these viruses to infect humans. Collectively, our data suggest that North American arenaviruses have a higher potential to cause human disease than previously assumed.

Arenaviruses are one of the major causes of hemorrhagic fever in humans worldwide. Based on their antigenic properties and their geographic distribution, arenaviruses are divided into two distinct groups: the Lassa-lymphocytic choriomeningitis serocomplex (Old World) and the Tacaribe serocomplex (New World [NW]) viruses (Fig. 1A) (1–4). NW arenaviruses are further divided into four clades: A, B, C, and A/B (5). All NW arenaviruses that have been shown to cause hemorrhagic fevers in humans so far belong to clade B: Junín virus (JUNV), Machupo virus (MACV), Guanarito virus (GTOV), Sabia virus (SABV), and Chapare virus (CHAV). These viruses are responsible for hemorrhagic fever outbreaks in South America with case fatality rates of 10 to 35% (6–12). In addition to these five pathogenic viruses, there are three more viruses in clade B: Tacaribe virus (TCRV), Amapari virus (AMAV), and Cupixi virus (CPXV) (Fig. 1A). With the exception of a single TCRV infection of a laboratory worker (13), which resulted in mild neurological symptoms, these viruses do not typically infect humans.

While clades A, B, and C arenaviruses are restricted to South America, clade A/B viruses are found only in North America (14–17). These viruses are most closely related to clade B in their entry glycoproteins (GPC) (5), whereas their nucleoproteins (NP) are more closely related to those of clade A viruses. The first recorded North American arenavirus is Tamiami virus (TAMV), which was isolated from hispid cottons rats (*Sigmodon hispidus*) in southern Florida in 1970 (18, 19). Subsequently, Whitewater Arroyo virus (WWAV) was isolated in 1993 in northwestern New Mexico from white-throated wood rats (*Neotoma albigula*) (20). Numerous clade A/B viruses were isolated since then in North America from wild rodents. These include, *inter alia*, the WWAV-like virus AV96010151 isolated in New Mexico, Bear Canyon virus (BCNV) in California, as well as Big Brushy Tank virus (BBTV) and Skinner Tank virus (SKTV), both isolated in Arizona (Fig. 1A and B) (15, 21, 22). Although generally considered nonpathogenic viruses, clade A/B arenaviruses have drawn increasing interest since 2000; WWAV-like viruses were implicated in the deaths of three people in California (23, 24), although this association had never

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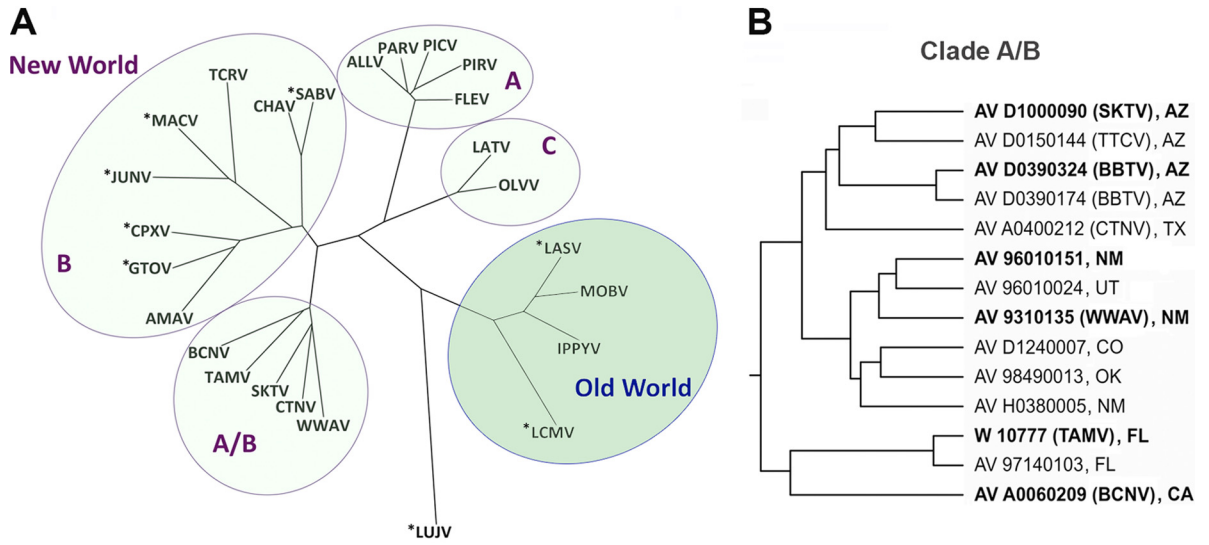

FIG 1 Phylogeny of arenaviruses based on GPC sequences. (A) Unrooted phylogenetic tree of representative arenaviruses based on GPC regions alone using ClustalW. Pathogenic arenaviruses are indicated by asterisks. (B) Rooted phylogenetic tree of North American Tacaribe serocomplex viruses, analyzed using ClustalW based on their GPC sequences. Branch labels include virus strain, virus abbreviation (when available), and the state where the virus-harboring animals were captured (14). The sequences used in these analyses are described in Materials and Methods.

been verified. In addition, antibodies directed against WWAV or TAMV have been detected in humans (25, 26). These observations suggest that arenaviruses native to North America have the potential to cause human diseases.

Viral entry is the first step of virus-host cell interactions leading to productive infection. Old World and NW clade C arenaviruses use α-dystroglycan as their cellular receptor to enter target cells (27, 28), while human transferrin receptor 1 (hTfR1) was identified as a receptor for pathogenic NW arenaviruses (29, 30). Importantly, the ability to use hTfR1 is a characteristic feature of NW arenaviruses that cause hemorrhagic fevers in humans (29–33). These viruses also efficiently use the TfR1 orthologs of their respective natural host species (34), highlighting the role of TfR1 in efficient zoonotic transmission of pathogenic viruses. To date, little is known about the entry mechanisms of North American clade A/B arenaviruses, and their receptor remains unknown. Deciphering this process may help to understand whether North American arenaviruses have the potential to become a threat for humans.

Here, we demonstrate that clade A/B viruses, like South American clade B arenaviruses, use host species TfR1 orthologs. Sequence variation in one of the host species TfR1 orthologs and its differential usage by these viruses suggest an ongoing "arms race" between clade A/B viruses and their rodent hosts. Importantly, one virus, AV96010151, used hTfR1, consistent with the previous report that implicated WWAV-like viruses in the three human fatalities. Also, hTfR1 variants with modest changes were efficiently used by a few of these viruses, suggesting that modest changes in the entry glycoproteins may be sufficient for the viruses to gain use of hTfR1 and emerge as human pathogens.

MATERIALS AND METHODS

Phylogeny. Analysis of phylogenetic relationships based on GPC sequences among representative Old World and NW arenaviruses was done using ClustalW (35). The GPC sequences used in the analysis are those of Allpahuayo virus (ALLV) strain CHLP 2472 (GenBank accession no. AY012687); Amapari virus (AMAV) strain BeAn 70563 (AF512834); Big Brushy Tank virus (BBTV) strain AV D0390324 (EF619036) and strain AV D0390174 (EF619035); Bear Canyon virus (BCNV) strain A0060209 (AF512833); Catarina virus (CTNV) strain AV A0400212 (DQ865245); Chapare virus (CHAV) strain 810419 (EU260463); Cupixi virus (CPXV) strain BeAn 119303 (AF512832); Flexal virus (FLEV) strain BeAn 293022 (AF512831); Guanarito virus (GTOV) strain INH 95551 (NC_005077); Ippy virus (IPPYV) strain Dak An B188 d (NC_007905); Junín virus (JUNV) strain MC2 (D10072); Lassa virus (LASV) strain Josiah (NC_006573); Latino virus (LATV) strain MARU 10924 (AF512830); Lujo virus (LUJV) (NC_012776); lymphocytic choriomeningitis virus (LCMV) strain Armstrong (NC_004294); Machupo virus (MACV) strain Carvallo (NC_005078); Mobala virus (MOBV) strain ACAR 3080 MRC5 P2 (NC_007903); Oliveros virus (OLVV) strain 3229-1 (U34248); Paraná virus (PARV) strain 12056 (AF512829); Pichindé virus (PICV) strain AN3739 (NC_006447); Pirital virus (PIRV) strain VAV-488 (AF277659); Sabiá virus (SABV) strain SPH114202 (NC_006317); Skinner Tank virus (SKTV) strain AV D1000090 (EU123328); Tacaribe virus (TCRV) strain TRVL 11573 (NC_004293); Tamiami virus (TAMV) strain W 10777 (AF512828) and strain AV 97140103 (EU486821); Tonto Creek virus (TTCV) strain AV D0150144 (EF619033); Whitewater Arroyo virus (WWAV) strain AV 9310135 (AF228063); and Whitewater Arroyo virus species complex strain AV96010151 (EU123330), strain AV 96010024 (EU123331), strain AV D1240007 (EU123329), strain AV 98490013 (FJ032026), and strain AV H0380005 (EU910959).

Cells and glycoprotein plasmids. HEK293T cells (human embryonic kidney epithelial, CRL-11268; ATCC) and NIH 3T3 cells (mouse embryo fibroblast, CCL-92; ATCC) were cultured in Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum (Atlanta Biologicals). Plasmids encoding the full-length GPC of AMAV and TCRV have been previously described (36). Codon optimized forms of WWAV, AV96010151, BBTV, BCNV, SKTV, and TAMV GPC genes have been chemically synthesized (Genscript) according to their GenBank sequences, as follows: WWAV strain AV 9310135 (GenBank accession no. AF228063), AV96010151 strain AV96010151 (EU123330), BCNV strain AV A0060209 (AF512833), BBTV strain AV D0390324 (EF619036), SKTV strain AV D1000090 (EU123328), and TAMV strain W 10777 (AF512828). These synthesized GPC genes were cloned into the pCAGGS expression vector.

TABLE 1 Tacaribe serocomplex arenaviruses used in this study[a]

Virus name	Abbreviation	GenBank accession no.	Host species	Animal trapping location for museum tissue samples
Clade A/B				
Whitewater Arroyo virus	WWAV	AF228063	*Neotoma albigula*	Sandoval County, New Mexico, USA
AV96010151	AV96010151	EU123330	*Neotoma mexicana*	Yavapai County, Arizona, USA
Big Brushy Tank virus	BBTV	EF619036	*Neotoma albigula*	Sandoval County, New Mexico, USA
Bear Canyon virus	BCNV	AF512833	*Peromyscus californicus*	San Diego County, California, USA
Skinner Tank virus	SKTV	EU123328	*Neotoma mexicana*	Yavapai County, Arizona, USA
Tamiami virus	TAMV	AF512828	*Sigmodon hispidus*	Dade County, Florida, USA; Otero County, New Mexico, USA
Clade B				
Amapari virus	AMAV	AF512834	*Neacomys spinosus*	El Palmar, Cochabamba, Bolivia
Tacaribe virus	TCRV	NC_004293[b]	*Artibeus jamaicensis*	Camp Bay, Roatan, Honduras
Junín virus	JUNV	D10072	*Calomys musculinus*	Chubut, Argentina

[a] Clades, full names of the viruses, abbreviations, their host species and the locations of the trapped animals from which museum tissue samples were derived are indicated.
[b] The GPC gene of TCRV used in this paper has a deletion of amino acids 121 to 132 and three substitutions, I134A, G418S, and E458R, compared to GenBank accession no. NC_004293.

Cloning and expression of host species TfR1 orthologs. Frozen tissues of *Peromyscus californicus* (California mouse) (from museum catalog no. MSB 47723), *Neotoma albigula* (white-throated wood rat) (MSB 10002707), *Neotoma mexicana* (Mexican wood rat) (MSB 78145), and *Sigmodon hispidus* (cotton rat) (MSB 83340 and MSB 84908) were obtained from the Museum of Southwestern Biology, University of New Mexico. To clone the respective TfR1 genes, total RNA was isolated from these tissues using RNAqueous (Life Technologies, Inc.), and cDNA was generated using PrimeScript RTase (TaKaRa), according to the manufacturer's instructions. PCRs were performed using Phusion high-fidelity DNA polymerase (New England BioLabs). PCR primers used to amplify the genes for PcTfR1, NaTfR1, NmTfR1, and ShTfR1 are as follows: sense, 5′-AACTATGAGGATCCGGCACCATGATGRATCAAGCCAG-3′; and anti-sense, 5′-AACTATGACTCGAGAAACTCATTGTCAATATYCCAA ATGTCACC-3′. The respective TfR1 genes were fused with a FLAG tag sequence at their C terminus and cloned into the pQCXIX retroviral vector (Clontech Laboratories). Amino acid substitutions or deletions in human TfR1 were performed using the QuikChange method. Presence of the intended mutations in these variants was confirmed by sequencing. Plasmids encoding human, mouse, *Artibeus jamaicensis* (Mexican fruit bat) and *Neacomys spinosus* (bristly mouse) TfR1 have been described previously (36). These TfR1 orthologs and variants were expressed in 293T or NIH 3T3 cells through transduction. The transduction vectors were produced by transfection of 293T at a 3:2:1 ratio with the plasmid expressing gag and polymerase proteins of murine leukemia virus (MLV), a retroviral vector (pQCXIX) expressing the respective TfR1 molecules, and the plasmid expressing vesicular stomatitis virus (VSV) G protein. Transfected cell supernatants were harvested 48 h later and filtered through a 0.45-μm polyethersulfone (PES) filter (Celltreat Scientific Products). 293T and NIH 3T3 cells were then spinoculated with these supernatants carrying TfR1 ortholog-expressing transduction vectors at 2,500 × *g* for 30 min at 10°C. TfR1 expression levels were assessed by flow cytometry (Accuri, BD Biosciences) 48 h later, using 5 μg/ml anti-FLAG M2 antibody (Sigma) followed by 5 μg/ml PE-conjugated anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) (34).

Pseudovirus production, infection, and inhibition. Viruses pseudotyped with the indicated arenavirus GPC molecules or the G protein of VSV were produced as previously described (29, 36). Briefly, they were generated by transfection of 293T cells at equal ratios with the plasmid expressing the gag and polymerase proteins of MLV, a retroviral vector (pQCXIX) expressing enhanced green fluorescent protein (EGFP), and the plasmid encoding the indicated GPC protein of each virus. Transfected cell supernatants were harvested 36 to 48 h later, and filtered through a 0.45-μm PES filter. To study viral entry, these pseudoviruses were incubated for 30 min in a 37°C CO_2 incubator with 293T or NIH 3T3 cells and transduced to express TfR1 orthologs or their variants. Viral entry levels were determined 24 h (for 293T cells) or 48 h (for NIH 3T3 cells) later by measuring EGFP fluorescence; cells were trypsinized, washed twice with phosphate-buffered saline (PBS), fixed with 2% paraformaldehyde, and read with an Accuri flow cytometer. To assess viral entry inhibition, 293T or hTfR1-293T cells were preincubated with a humanized anti-hTfR1 antibody (ch128.1) (30) or a control antibody (1D4) for 30 min at 37°C. Pseudoviruses were added, and incubation was continued for 2 more hours in a 37°C CO_2 incubator. Infection levels were assessed 24 h later as described above.

Statistical analysis. Data are presented as the means ± standard deviations (SD) from one of 2 to 5 independent experiments performed in duplicate. Statistical analyses were performed using a Mann-Whitney test; *P* values of <0.001 were considered statistically significant.

RESULTS

North American arenaviruses use TfR1 to enter cells. We have previously shown that nonpathogenic clade B arenaviruses, AMAV and TCRV, were able to use their respective host species TfR1 to infect cells (36). Some of these TfR1 molecules, such as *Neacomys spinosus* (host species of AMAV) TfR1 (NsTfR1), are promiscuously used by all of the clade B arenaviruses we tested (36). To investigate whether clade A/B arenaviruses also use TfR1, we first assessed their entry into 293T cells expressing NsTfR1. We also used *Artibeus jamaicensis* (host species of TCRV) TfR1 (AjTfR1) that is used by a subset of NW arenaviruses (36). *Mus musculus* TfR1 (mTfR1) was included as a negative control since it is not used by any arenavirus (34, 36). Expression of each TfR1 ortholog was assessed by flow cytometry using an antibody directed against the FLAG tag at its C terminus. Cells overexpressing the indicated TfR1 were then infected with murine leukemia viruses pseudotyped with GPC proteins of clade A/B arenaviruses; for this study, we selected six viruses that are widely distributed in a phylogenetic tree based only on GPC sequences of clade A/B viruses (Fig. 1B and Table 1) (14). They are WWAV and BBTV isolated from white-throated wood rats (*Neotoma albigula*) in New Mexico in 1993 and in Arizona, respectively (15, 20); AV96010151 and SKTV isolated from Mexican wood rats (*Neotoma mexicana*) in New Mexico and in Arizona, respectively (22, 37); BCNV isolated from California mice (*Peromyscus californi-*

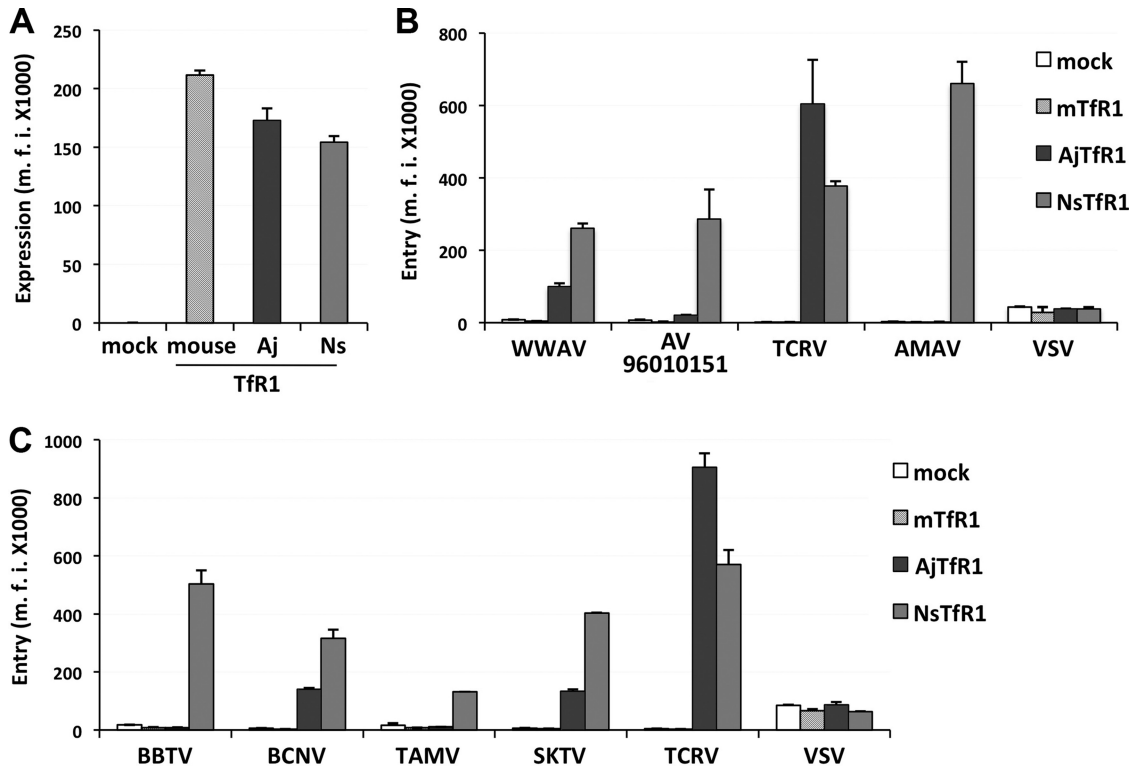

FIG 2 North American arenaviruses use TfR1 to enter cells. (A) TfR1 expression in 293T cells transduced with mouse (m), *Artibeus jamaicensis* (Aj), or *Neacomys spinosus* (Ns) TfR1. Their expression was determined by flow cytometry using anti-FLAG M2 antibody. (B and C) The same cells as in panel A were infected for 30 min at 37°C with pseudoviruses carrying the GPC of (B) WWAV or AV96010151 or (C) BBTV, BCNV, SKTV, or TAMV and produced by 293T cell transfection. Clade B TCRV and AMAV pseudoviruses were used as positive controls, and VSV pseudovirus was used as a negative control. Viral entry levels were determined 24 h later by measuring EGFP expression by flow cytometry of the trypsinized cells. m.f.i., mean fluorescence intensity; mock, mock infection.

cus) in California in 2002 (21); and TAMV isolated from hispid cotton rats (*Sigmodon hispidus*) in Florida in 1970 (18, 19). Pseudoviruses containing GPC molecules from clade B viruses TCRV and AMAV were included as positive controls, and the one harboring the G protein of vesicular stomatitis virus (VSV) was used as a negative control for the ability to use TfR1. While TfR1 expression levels are comparable (Fig. 2A), introduction of NsTfR1, but not mTfR1, into 293T cells dramatically enhanced entry of all clade A/B pseudoviruses tested (Fig. 2B and C). Similar to clade B pseudoviruses (36), introduction of AjTfR1 into 293T cells enhanced the entry of some, but not all, pseudoviruses: WWAV, BCNV, and SKTV. Entry of VSV pseudovirus was not affected. These results show that clade A/B arenaviruses also use TfR1 as their entry receptor.

We next confirmed that these viruses indeed efficiently utilized the TfR1 orthologs of their own host species. We obtained from the Museum of Southwestern Biology (Joseph Cook and Cheryl Parmenter, University of New Mexico, NM) frozen tissues of the host species for these arenaviruses. These species included *Peromyscus californicus* (a host species of BCNV; for the PcTfR1 ortholog), *Neotoma albigula* (a host species of WWAV and BBTV; NaTfR1), and *Neotoma mexicana* (a host species of AV96010151 and SKTV; NmTfR1). We amplified the respective TfR1 genes by reverse transcription-PCR (RT-PCR) using degenerate primers and cloned them into the pQCXIX retroviral vector. 293T cells were transduced to express the respective orthologs of TfR1 (mouse [mTfR1], NsTfR1, PcTfR1, NaTfR1, or NmTfR1) and infected with clade A/B pseudoviruses. Expression of each TfR1 was assessed by flow cytometry using an anti-FLAG tag antibody (Fig. 3A). As shown in Fig. 3B, NaTfR1 and NmTfR1 were efficiently used by all clade A/B and clade B pseudoviruses tested, with the exception of TAMV for NaTfR1, while PcTfR1 was used only by a subset of clade A/B and none of the clade B pseudoviruses. These results confirm that North American arenaviruses use TfR1 as an entry receptor. Furthermore, our data also show that receptor usage by clade A/B viruses lacks specificity, since no virus preferentially uses the TfR1 ortholog of its own species.

ShTfR1 sequence variation contributes to host species specificity. Among the samples we obtained from the Museum of Southwestern Biology are two different *Sigmodon hispidus* tissues. Hispid cotton rats, *S. hispidus*, are the principal host of TAMV in Florida (18, 19). TfR1 genes cloned from these two samples exhibited several amino acid variations (GenBank accession no. KJ158051 and KJ158052). Of these, two residues are located within the region important for arenavirus entry (34, 36); one sequence from the tissue sample, MSB 84908, contains a lysine and a leucine at positions 214 and 216, respectively, whereas the other one, from tissue sample MSB 83340, contains a glutamic acid and a proline at the same positions (Fig. 4A). These two clones are named Sh(KL)TfR1 and Sh(EP)TfR1, respectively. The animal for the former tissue was captured in Dade County, Florida, and the latter in Otero County, New Mexico. To address whether the residue difference affects viral entry, we assessed the entry of clade A/B pseudoviruses in 293T cells expressing mTfR1,

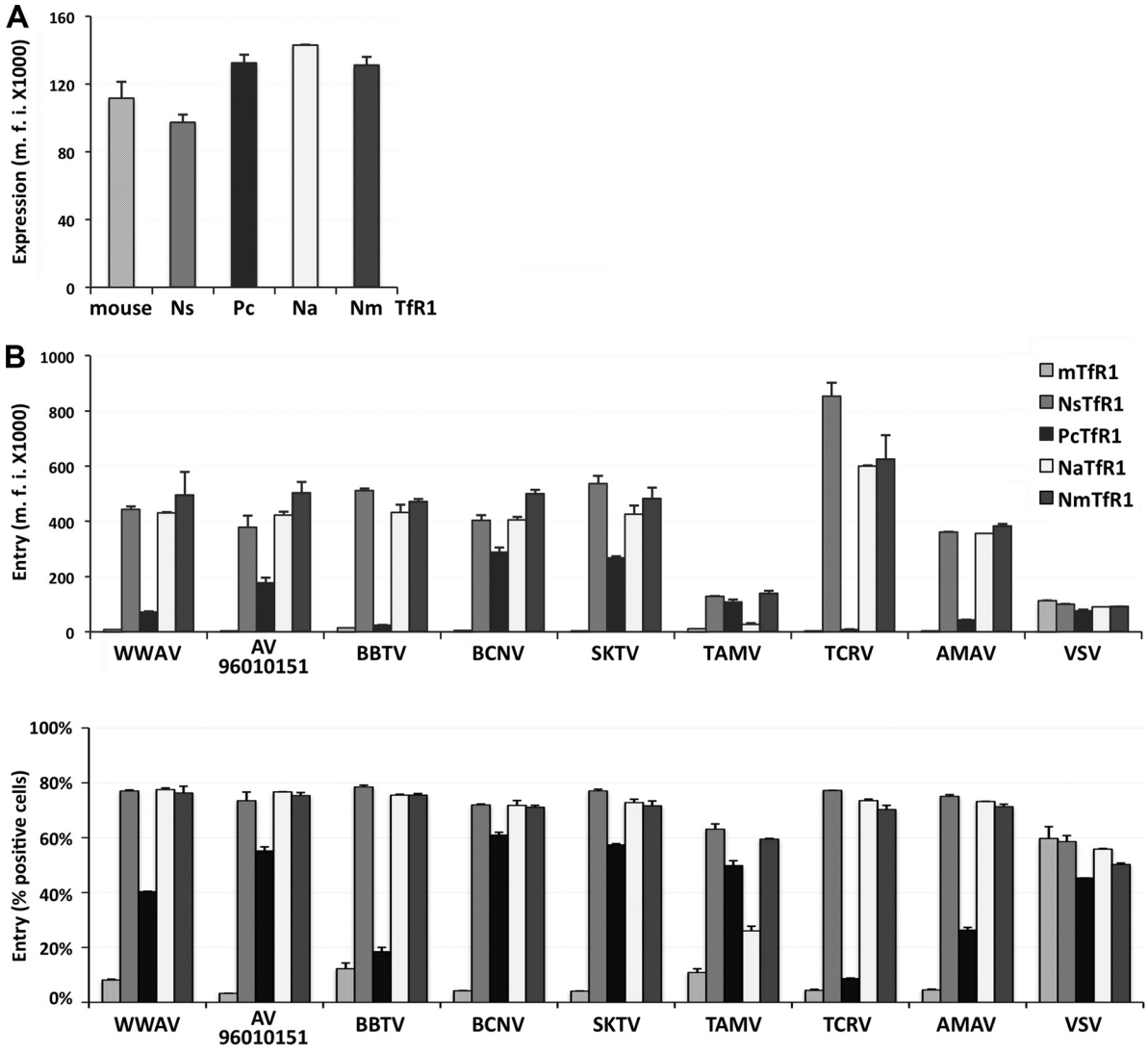

FIG 3 North American arenaviruses use their own host species TfR1 orthologs. (A) TfR1 expression in 293T cells transduced with *Neotoma albigula* (Na; WWAV and BBTV host), *Peromyscus californicus* (Pc; BCNV host), or *Neotoma mexicana* (Nm; AV96010151 and SKTV host) TfR1 as well as mTfR1 and NsTfR1. Their expression was determined by flow cytometry using an anti-FLAG M2 antibody. (B) Infection of these cells was performed with the indicated pseudoviruses, viral entry levels were assessed as described in Fig. 2, and data are presented as mean fluorescence intensities (upper panel) and percent positive cells (lower panel). m.f.i., mean fluorescence intensity.

NsTfR1, Sh(KL)TfR1, or Sh(EP)TfR1 (Fig. 4B). As shown in Fig. 4C, only TAMV used Sh(KL)TfR1, while all clade A/B and clade B pseudoviruses used Sh(EP)TfR1, suggesting that residues at 214 and 216 may play an important role in the host species specificity of clade A/B arenaviruses.

To identify the residue responsible for this host species specificity, we individually introduced residues E214 and P216 of Sh(EP)TfR1 into Sh(KL)TfR1 (Fig. 5A). Transduction of ShTfR1 variants resulted in comparable expression levels in 293T cells (Fig. 5B). As shown in Fig. 5C, introduction of a proline at position 216 rendered the resulting variant Sh(KP)TfR1 susceptible to infection only by BCNV and clade B TCRV, in addition to TAMV. In contrast, introduction of a glutamate at position 214 [Sh(EL)TfR1] resulted in entry by all clade A/B and clade B pseudoviruses tested, except BBTV. These data identify the residue E214 of ShTfR1 (Fig. 6B) as a polymorphism that contributes to host species specificity.

Clade A/B arenaviruses use human TfR1 with modest alteration. The ability of pathogenic clade B arenaviruses to cause hemorrhagic fevers in humans strictly correlates with their usage of hTfR1 (29–31, 36). For example, nonpathogenic clade B viruses, such as TCRV and AMAV, infect human cells, but this infection is mediated through a hTfR1-independent route, and they do not cause diseases in humans (31, 36). One way to assess the likelihood that clade A/B viruses might evolve to use hTfR1 is to introduce variations in the GPC molecules and measure their use of hTfR1. However, due to low sequence homology among arenavirus GPC molecules, engineering such gain-of-function variants is exceedingly complex. Thus, we have introduced changes in hTfR1 instead and have shown that nonpathogenic clade B arenaviruses efficiently used hTfR1 variants that contain only modest changes (36). We extended this approach here to clade A/B viruses and investigated whether similar alterations in hTfR1 also allow these

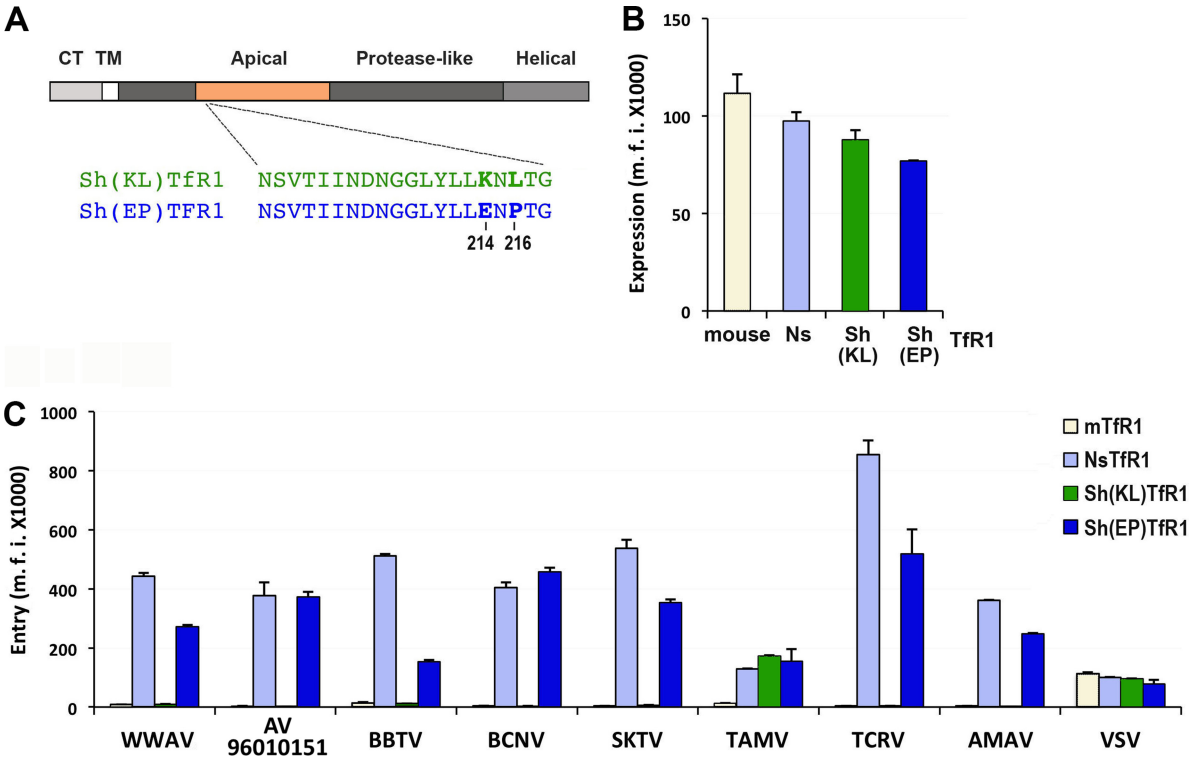

FIG 4 ShTfR1 sequence variation contributes to host species specificity. (A) A diagram of TfR1 gene is shown in the top panel, and a sequence alignment of the arenavirus-binding region (residues 199 through 218 by hTfR1 numbering) of Sh(KL)TfR1 and Sh(EP)TfR1 is shown at the bottom. Residues 214 and 216, where sequence variation is detected, are in boldface type. CT, cytoplasmic domain; TM, transmembrane domain. (B) Expression levels of NsTfR1 and ShTfR1 variants in transduced 293T cells, determined by flow cytometry using anti-FLAG M2 antibody. (C) The same cells were infected with the indicated pseudoviruses, and virus entry levels were measured as described for Fig. 2. m.f.i., mean fluorescence intensity.

viruses to use hTfR1 variants. Since NmTfR1 is the most promiscuously used by all clade A/B viruses (Fig. 3B), we introduced one to four NmTfR1 residues into hTfR1 in the region that is critical for arenavirus entry (Fig. 6A and B). Cells were transduced to express wild-type hTfR1 or these variants (Fig. 6C) and infected with clade A/B or VSV pseudoviruses. Mouse TfR1 was used as a negative control. As shown in Fig. 6D, variant Nmh1 containing two residue alterations, K205E and R208G, turned hTfR1 into an efficient receptor for AV96010151. Deletion of V210 in Nmh1 (Nmh2; K205E, R208G, and ΔV210) led to efficient entry of AV96010151 and BCNV. Introducing another mutation at position 204 (Nmh3; D204N, K205E, R208G, and ΔV210) converted hTfR1 into an efficient receptor for BCNV, SKTV, and TAMV, while it was less efficient for WWAV and AV96010151. Variant Nmh4 containing three alterations, D204N, R208G, and ΔV210, served as an efficient receptor for WWAV, BCNV, and SKTV, while it was a less efficient receptor for AV96010151. Finally, introduction of a single mutation at position 208 (Nmh5; R208G) made hTfR1 a functional receptor for AV96010151 and BCNV. These results demonstrate that like nonpathogenic clade B viruses (35), the majority of clade A/B arenaviruses are able to use hTfR1 when modest changes are introduced, and they imply that similar modest changes in the entry glycoproteins may be sufficient for these viruses to use hTfR1.

AV96010151 uses human TfR1. As can be seen in Fig. 6D, AV96010151 was capable of using wild-type hTfR1 without any alteration. In contrast, the prototype WWAV did not use hTfR1, consistent with the previous report by Reignier and colleagues (38). No other clade A/B pseudovirus used hTfR1, while all of them efficiently used NmTfR1. When normalized to its entry level in the mTfR1-expressing cells, AV96010151 pseudovirus entry enhancement in hTfR1-293T cells was approximately 9-fold ($P < 0.001$). To confirm that this entry increase was indeed due to hTfR1 use, we performed entry inhibition assays using an anti-hTfR1 antibody (30). Cells were preincubated with anti-hTfR1 or a control antibody prior to infection by AV96010151 pseudovirus. Other clade A/B viruses, WWAV and BBTV, which do not use hTfR1, and a pathogenic clade B virus, JUNV, which uses hTfR1 (29, 31, 39), were included as controls. As shown in Fig. 7A, the anti-hTfR1 antibody specifically and efficiently inhibited the entry of AV96010151 into hTfR1-293T cells in a dose-dependent manner, while that of WWAV and BBTV was not affected at any concentration of the antibody; entry of AV96010151 was inhibited by 85% at a 20 nM concentration of anti-hTfR1 antibody. As expected, the anti-hTfR1 antibody inhibited the entry of JUNV pseudovirus, and the control antibody had no effect on any virus tested.

Since 293T cells endogenously express hTfR1, we then confirmed the ability of the AV96010151 virus to use hTfR1 in a murine cell line, NIH 3T3. A mutant hTfR1 (hTfR1-Y211T) was used as a negative control because mTfR1, which was used as a control in other experiments, is endogenously expressed in NIH 3T3 cells. hTfR1-Y211T does not support the entry of clade B arenaviruses (34). NIH 3T3 cells were transduced with hTfR1-Y211T or hTfR1

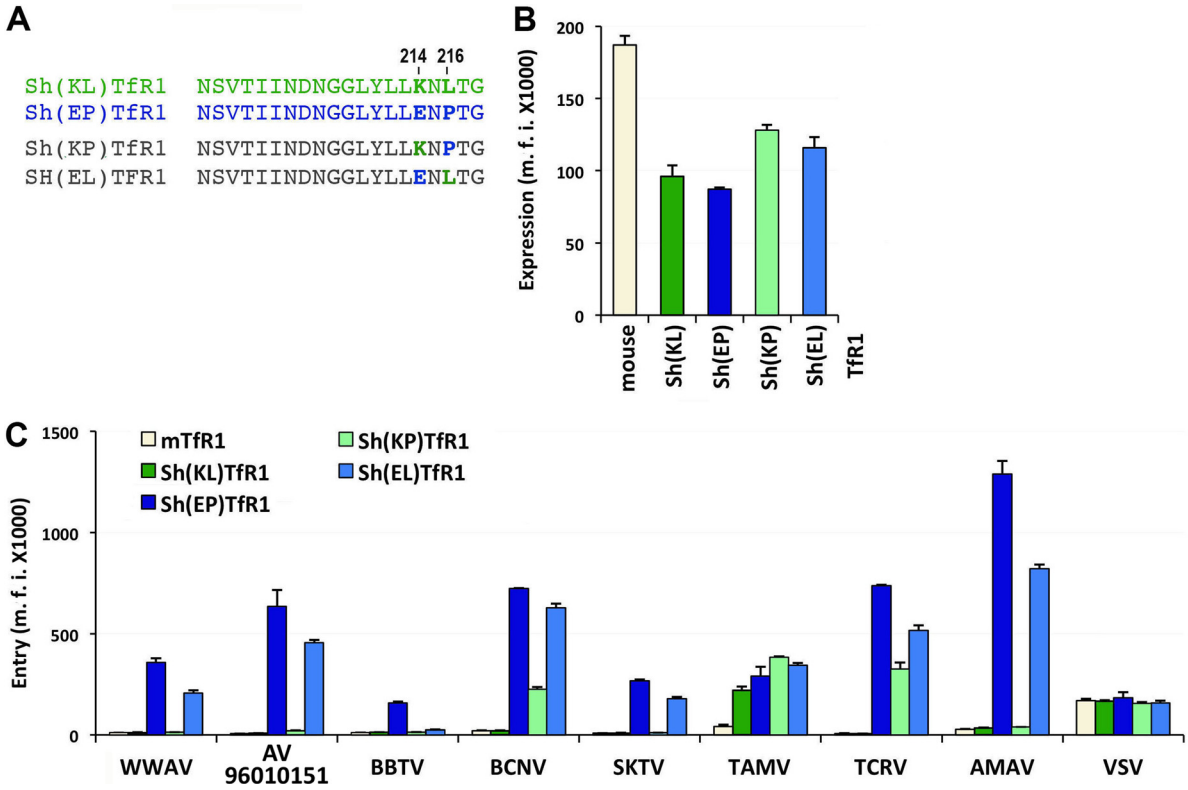

FIG 5 Residue E214 of ShTfR1 was under selection pressure. (A) A sequence alignment of the arenavirus-binding region (residues 199 through 218 by hTfR1 numbering) of the natural (KL and EP) and artificial (KP and EL) variants of ShTfR1 is shown. Residues 214 and 216, where sequence variation is detected, are in boldface type. (B) Expression of TfR1 variants in the transduced 293T cells was determined by flow cytometry using anti-FLAG M2 antibody. (C) Viral entry assay was performed in these cells with the indicated pseudoviruses as described for Fig. 2. m.f.i., mean fluorescence intensity.

(Fig. 7B) and infected with WWAV, AV96010151, BBTV, JUNV, or VSV pseudovirus. The low level of entry by arenaviruses in the mock-transduced NIH 3T3 cells might indicate their ability to use phosphatidylserine receptors, C-type lectins (40, 41), or other similar molecules that are expressed in these cells. As shown in Fig. 7C and D, hTfR1-Y211T did not support the entry of any of these pseudoviruses. On the other hand, hTfR1 expression in NIH 3T3 cells, compared to that of hTfR1-Y211T, enhanced the entry of AV96010151 approximately 5.5-fold ($P < 0.001$), confirming the results from 293T cells. JUNV pseudovirus entry was enhanced by hTfR1 as expected. Although this hTfR1 use by AV96010151 was observed when hTfR1 was overexpressed, no other clade A/B virus used hTfR1 under the same conditions. Taken together with the results from 293T cells, these data show that AV96010151 is able to use hTfR1 to enter cells.

DISCUSSION

Five NW arenaviruses belonging to clade B have been described to cause hemorrhagic fevers in humans. We and others have previously shown that all of these pathogenic NW arenaviruses use hTfR1 to enter cells (29–31, 33, 34). Closely related nonpathogenic viruses in the same clade do not use hTfR1 (31, 36) and do not cause human diseases, although they are able to enter human cells. Therefore, the ability to use hTfR1 is a key determinant of the pathogenicity of clade B arenaviruses. One possible explanation for this correlation could be the differential host immune responses to the infection by pathogenic and nonpathogenic viruses (42). This correlation could also be explained by the fact that TfR1 marks activated cells giving rise to more progeny viruses and marks cells that tackle virus infection. A third possibility is that TfR1 upregulation in cells and tissues in response to acute-phase iron sequestration creates a destructive feedback loop, resulting in amplified viral replication. Furthermore, TfR1 has been shown to play an essential role in the zoonotic transmission of clade B arenaviruses (34, 36). However, little is known about receptor use by clade A/B arenaviruses that are found in North America. As shown in Fig. 2 to 5, these North American arenaviruses also use host species TfR1 orthologs to enter cells.

Clade A/B viruses, however, do not exhibit host species preference in their receptor usage (Fig. 2 to 3). South American arenaviruses geographically codistribute with their own host species, which resulted from a long-term coevolution between a virus and a host species (3, 43, 44). This coevolution is indicated by the tendency of each of these viruses to prefer TfR1 orthologs of its own and closely related viruses' host species (34, 36). As shown in Fig. 3, however, most of the North American arenaviruses do not display a receptor preference, and they utilize TfR1 orthologs of other host species as efficiently as those of their own. Alternatively, North American arenavirus isolates and their designated host species may represent a snapshot of continuously evolving viral quasispecies spread through closely related rodent species. Regardless, the lack of host species specificity/boundary suggests a possible cross-species transmission and recombination among these viruses.

Interestingly, of the four host species we studied, only the TfR1

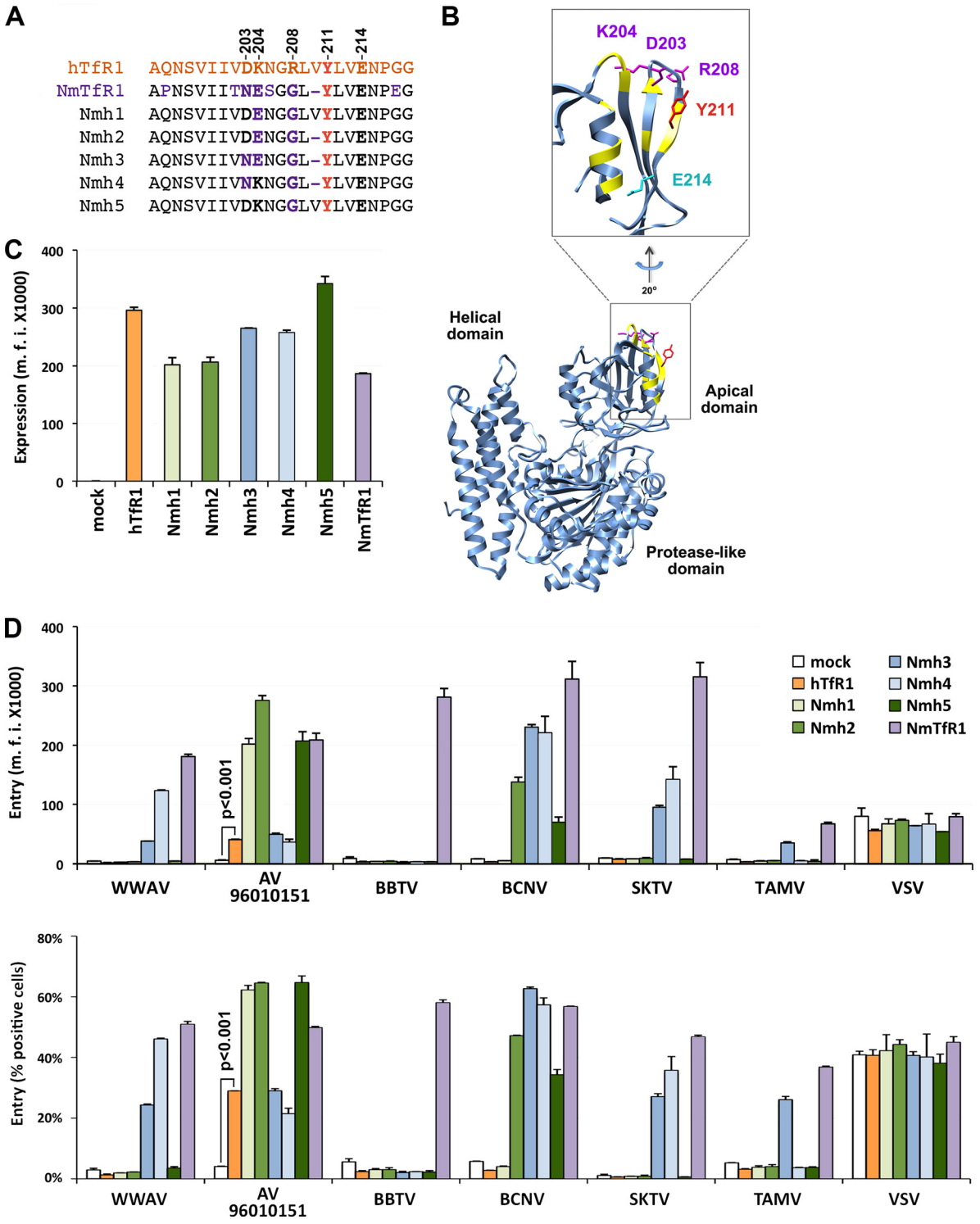

FIG 6 Modest changes in hTfR1 are sufficient to support infection of most North American arenaviruses. (A) Sequence alignment of residues 199 through 218 of hTfR1 and NmTfR1 is shown. Residue Y211, critical for all TfR1-using arenaviruses, is shown in red boldface. NmTfR1 residues that vary from those of hTfR1 in this region are shown in purple. These NmTfR1 residues introduced into hTfR1 to generate variants Nmh1 through Nmh5 are indicated by the same color. (B) The structure of the hTfR1 extracellular domain monomer is shown. Three major domains, protease-like, apical, and helical, are indicated. The inset shows a critical region of the apical domain, enlarged and rotated 20° with occluding residues removed. Residues important for arenavirus entry (34, 45) are highlighted in yellow on the backbone. The side chain of residue Y211 is shown in red. The side chain of residue E214, a host specificity determinant for North American arenaviruses (Fig. 5), is shown in cyan. The side chains of residues D302, K204, and R208 are shown in purple. The image was generated using Chimera from PBD 3KAS (45). (C) Expression of hTfR1, NmTfR1, or hTfR1 variants in the transduced 293T cells was assessed by flow cytometry using anti-FLAG M2 antibody. (D) Pseudovirus entry assays in these cells were performed as described for Fig. 2. Data are presented as mean fluorescence intensities (upper panel) and percent positive cells (lower panel). The indicated *P* value was calculated using nonparametric Mann-Whitney test using the values from five independent experiments performed in duplicate. m.f.i., mean fluorescence intensity. Virus entry levels in mock-transduced cells are 4,042 (in m.f.i.) for WWAV; 5,488 for AV96010151; 8,825 for BBTV; 8,037 for BCNV; 9,536 for SKTV; 6,977 for TAMV; and 79,805 for VSV, after autofluorescence of cells (2,723 in m.f.i.) is subtracted.

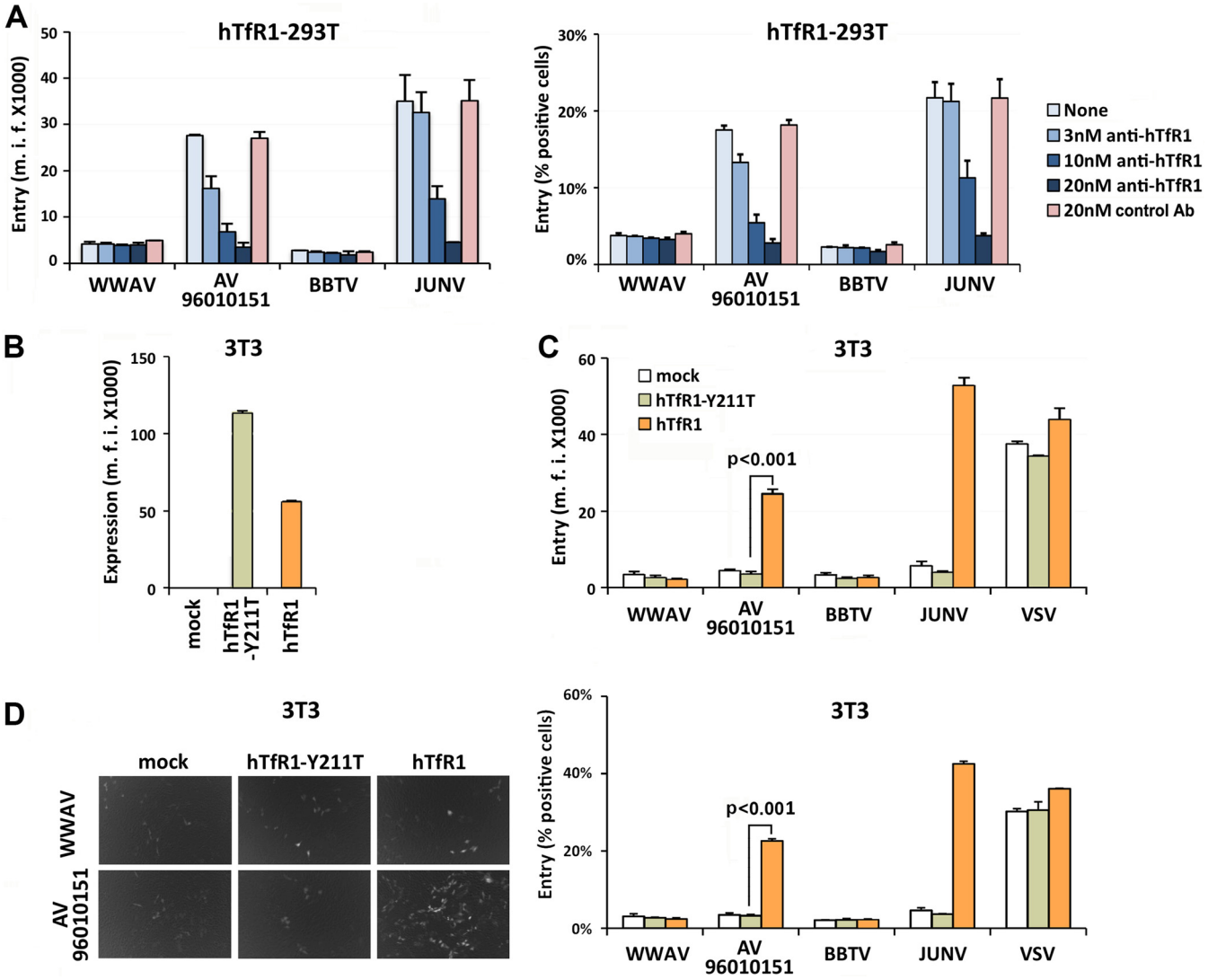

FIG 7 AV 96010150 uses human TfR1. (A) 293T cells transduced to express hTfR1 were preincubated for 30 min at 37°C with 3, 10, or 20 nM anti-hTfR1 antibody (ch128.1) or with 20 nM control antibody. The indicated pseudoviruses were added, incubation was continued for 2 h at 37°C, and viral entry was determined 24 h later. Data are presented as mean fluorescence intensities (left panel) and percent positive cells (right panel). (B) TfR1 expression in NIH 3T3 cells, transduced to express hTfR1 or an hTfR1 mutant (hTfR1-Y211T) that does not support arenavirus infection, was assessed by flow cytometry using anti-FLAG M2 antibody. (C) These cells were infected for 30 min at 37°C with the indicated pseudoviruses, and viral entry was assessed 48 h later by measuring EGFP expression by flow cytometry of the trypsinized cells. Data are presented as mean fluorescence intensities (upper panel) and percent positive cells (lower panel). The indicated *P* value was calculated using nonparametric Mann-Whitney test using the values from four independent experiments in duplicate. m.f.i., mean fluorescence intensity. Virus entry levels in mock-transduced cells are 3,383 (in m.f.i.) for WWAV; 4,121 for AV96010151; 3,315 for BBTV; 5,663 for JUNV; and 37,549 for VSV, after autofluorescence of the cells (3,279 in m.f.i.) is subtracted. (D) Fluorescent micrographs of the same cells as in panel C, infected with either WWAV or AV96010151.

gene of *Sigmodon hispidus*, the host of TAMV, carries a polymorphism that contributes to host species specificity. Of the five residues that vary in two ShTfR1 clones, obtained from two different museum tissue samples, residues 214 and 216 are located in close vicinity to the region in the TfR1 apical domain, which is critical for arenavirus entry (Fig. 6B) (33, 34, 45). Of these, glutamate at position 214 is critical for all clade A/B viruses for efficient use of Sh(EP)TfR1 (Fig. 5). Only TAMV can use the TfR1 clone Sh(KL)TfR1 with a lysine at this position. Notably, all other known TfR1 genes have a glutamate at this position (Table 2). These data imply that E214 of ShTfR1 was under selection pressure; in response to virus-driven selective pressure, cotton rats evolved to include variations in their TfR1 gene, and those with advantageous mutations survived pathogenic infection by an ancestral virus of TAMV. These data also imply that TAMV has since adapted to and is in equilibrium with the new TfR1, Sh(KL)TfR1, while other viruses have not yet adapted to it.

Clade A, B, and C arenaviruses are found in South America, while clade A/B viruses have been isolated in North America. None of the latter has so far been correlated to human diseases, with the exception of the WWAV-like viruses, which were suspected to have caused three fatalities in California in 1999-2000 (23, 24). The nucleotide sequences of the PCR fragments of the viruses isolated from two of these patients share 87% identity with

TABLE 2 Partial list of the NW arenavirus host species and their TfR1 sequences in the region critical for arenavirus infection[a]

Species	TfR1 abbreviation	Sequence, GPC binding region	GenBank accession no.	Virus (clade)
Supportive species				
Homo sapiens	hTfR1	NSVIIVDKNGRLVYLVENPGG	NM_003234	NA
Nonhuman primates				
Cercopithecus aethiops	CaTfR1	NSVIIVDKNGGLVYLVENPGG	JQ014203	NA
Callithrix jacchus	CjTfR1	NSVTITGTNSEFVYLVENPGG	JQ014205	NA
Rodents				
Calomys callosus	CcTfR1	NSVTIINASNGV-YLLESPAG	EU164540	JUNV (B)
Neacomys spinosus	NsTfR1	NSVTIINSSGGL-YLLESPEG	FJ154604	AMAV (B)
Neotoma albigula	NaTfR1	NSVTITNASGGL-YLVEYPEG	KF982058	WWAV, BBTV (A/B)
Neotoma mexicana	NmTfR1	NSVIITNESGGL-YLVENPEG	KF982059	WWAV2, SKTV (A/B)
Peromyscus californicus	PcTfR1	NSVTIINENGAL-YLLENPEG	KF982060	BCNV (A/B)
Sigmodon hispidus	Sh(KL)TfR1	NSVTIINDNGGL-YLLKNLTG	KJ158051	TAMV (A/B)
	Sh(EP)TfR1	NSVTIINDNGGL-YLLENPTG	KJ158052	TAMV (A/B)
Zygodontomys brevicauda	ZbTfR1	NSVTIINTSGGL-YLLENPVG	EU340259	GTOV (B)
Bat				
Artibeus jamaicensus	AjTfR1	NAVTIVAVSSGAGYLVENPAG	FJ154605	TCRV (B)
Nonsupportive species				
Mus musculus	mTfR1	NMVTIVQSNGNL-DPVESPEG	NM_011638	NA
Rattus norvegicus	RnTfR1	NLVTI-NSGSNI-DPVEAPEG	NM_022712	NA

[a] Names of the host species, abbreviations, GenBank accession numbers and partial sequences of their TfR1, and the viruses they host are listed. Tyrosine 211 of TfR1 (by hTfR1 numbering), a critical determinant of NW arenaviral entry, is shown in red. This residue is an aspartic acid (gray) in mouse and rat TfR1 orthologs that do not support arenavirus infection. Residues E214 and P216, major determinants of host species specificity of North American arenaviruses, are indicated in blue. Among all the known host species TfR1 orthologs, only Sh(KL)TfR1 encodes a lysine and a leucine at those positions (green). The right-most column summarizes the major NW arenaviruses isolated from these host species. NA, not applicable.

the prototype WWAV (23). However, no follow-up studies have appeared, the identity of the causative agent has not been confirmed, and no further human fatal cases have been reported since then. Thus, it still remains unclear whether clade A/B viruses are a threat for public health. Surprisingly, as shown in Fig. 6 and 7, one of the clade A/B viruses we tested, AV96010151, which is closely related to the prototype WWAV (Fig. 1B), utilized hTfR1, while none of the other clade A/B viruses could enter cells using hTfR1. Coincidentally, AV96010151 and the prototype WWAV share 86% of the nucleotide sequences in their GPC genes. This observation supports the implication of clade A/B arenaviruses in the three human cases. All three patients exhibited febrile illness and respiratory distress, while two of them developed liver failure and hemorrhagic symptoms. All three died 1 to 8 weeks after the onset of illness (23, 24). Possible human infection by North American arenaviruses has also been observed in additional studies. In one study, antibodies against TAMV were detected in 5 of 131 Seminole Native Americans (26). Another study reported elevated antibody titers against WWAV in 13 out of 1,185 patients who had acute central nervous system disease or undifferentiated febrile illnesses without any identifiable cause (25). Also, as we show in Fig. 6, hTfR1 variants with only modest changes allow efficient infection by most of the clade A/B viruses tested, implying that a small number of mutations in GPC may be sufficient for these viruses to gain use of hTfR1. Moreover, lack of host species specificity among clade A/B viruses may promote cross-species transmission and recombination, which can in turn increase the likelihood of producing hTfR1-using viruses. Thus, our study suggests that North American arenaviruses may infect humans more frequently than previously understood and highlights the potential of these viruses to emerge as human pathogens.

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