Identification of Host Factors Involved in Borna Disease Virus Cell Entry through a Small Interfering RNA Functional Genetic Screen[▽]

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Borna disease virus (BDV), the prototypic member of the *Bornaviridae* family, within the order *Mononegavirales*, is highly neurotropic and constitutes an important model system for the study of viral persistence in the central nervous system (CNS) and associated disorders. The virus surface glycoprotein (G) has been shown to direct BDV cell entry via receptor-mediated endocytosis, but the mechanisms governing cell tropism and propagation of BDV within the CNS are unknown. We developed a small interfering RNA (siRNA)-based screening to identify cellular genes and pathways that specifically contribute to BDV G-mediated cell entry. Our screen relied on silencing-mediated increased survival of cells infected with rVSVΔG*/BDVG, a cytolitic recombinant vesicular stomatitis virus expressing BDV G that mimics the cell tropism and entry pathway of bona fide BDV. We identified 24 cellular genes involved in BDV G-mediated cell entry. Identified genes are known to participate in a broad range of distinct cellular functions, revealing a complex process associated with BDV cell entry. The siRNA-based screening strategy we have developed should be applicable to identify cellular genes contributing to cell entry mediated by surface G proteins of other viruses.

Borna disease virus (BDV) is an enveloped virus with a nonsegmented negative-strand (NNS) RNA genome whose organization (3'-N-p10/P-M-G-L-5') is characteristic of mononegaviruses (MNV) (12, 49, 51). However, based on its unique genetics and biological features, BDV is considered to be the prototypic member of a new virus family, *Bornaviridae*, within the order *Mononegavirales* (13, 49). BDV is highly neurotropic and causes central nervous system (CNS) disease in a variety of vertebrate species that is frequently manifested by behavioral abnormalities (25, 53). Both host and viral factors contribute to a variable period of incubation and heterogeneity in the symptoms and pathology associated with BDV infection (18, 20, 32, 44, 46). BDV provides an important model for the investigation of both immune-mediated pathological events associated with virus-induced neurological disease and mechanisms whereby viruses induce neurodevelopmental and behavioral disturbances in the absence of the hallmarks of cytolysis and inflammation (19, 24, 42).

Serological data and molecular epidemiological studies suggest that BDV, or a BDV-like virus, can infect humans, and it might be associated with certain neuropsychiatric disorders (23, 29). Moreover, the recent identification of an avian bornavirus (22, 45) underscores the prospect of finding other BDV-like emerging viruses of potential relevance to human health. The identification of host cell factors involved in BDV cell entry will contribute to a better understanding of BDV cell tropism and propagation within the CNS, a first and necessary step to elucidate the mechanisms by which BDV disrupts normal brain functions. Likewise, the development of effective

antiviral strategies to combat bornavirus infections may be facilitated by targeting host cell factors that participate in BDV cell entry.

As with other MNV, the BDV surface glycoprotein (G) plays a key role in virus cell entry, which uses a receptor-mediated endocytosis pathway (3, 18, 40). BDV G gene directs the synthesis of a precursor glycoprotein (GPC) with a predicted molecular mass of 56 kDa but, due to extensive glycosylation, GPC migrates with a molecular mass of 84 to 94 kDa. GPC is posttranslationally cleaved by the cellular protease furin into GP1 (GP_N) and GP2 (GP_C) that correspond to the N- and C-terminal regions, respectively, of GPC (17, 43). GP1 is sufficient for virus receptor recognition and cell entry (40), whereas GP2 has been implicated in the pH-dependent fusion event between viral and endosome membranes (17). This fusion event delivers the virus ribonucleoprotein (RNP) core to the cytoplasm, which is followed by its import into the nucleus, where both RNA replication and transcription take place (12). In addition to cell surface molecules that act as viral receptors and coreceptors, many other host cell proteins can influence virus cell entry (11, 21, 35). The emergence of RNA interference (RNAi) as a tool for gene expression inhibition has enabled a forward genetic approach to facilitate the identification of host cell factors contributing to different steps of virus multiplication. This approach has been successfully used to identify components of the kinome involved in VSV cell entry (38), as well as host proteins required for virus infection, including HIV-1 (6, 36) and West Nile virus (WNV) (28). The use of RNAi-based genetics approaches to study BDV-host cell interactions involved in virus cell entry are complicated by the inability to grow cell-free infectious BDV to high titers and the absence of cytopathic effect (CPE) associated with BDV infection. To overcome these limitations we took advantage of a replication-competent recombinant vesicular stomatitis virus (VSV) expressing the BDV G (rVSVΔG*/BDVG), which was

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TABLE 1. Sequences of primers used for mRNA detection by RT-PCR

Primer ^a	Oligonucleotide sequence (5'-3')	RT-PCR fragment size (bp)	Reference
ABC5 F	GACATCGGCTGGTTTGATAGCT	167	54
ABC5 R	GAGTTTCAGCCCTTCACCA		
ADAM17 F	TTGGTGGTAGCAGATCATCG	389	55
ADAM17 R	CTGGGAGAGCCAACATAAGC		
CTSL2 F	TACGGCTTTGAAGGAGCAAATTCG	378	56
CTSL2 R	GAATTAAGCAATGAGTCTTTG		
DNCL2B F	ATGGCAGAGGTGGAGGAAAC	355	
DNCL2B R	TACTCATGAGAGAGCCAAGTG		
FURIN F	ACAACTATGGGACGCTGACC	163	
FURIN R	TGGACACAGCTCTTCTGGTG		
GABRA3 F	AAGATCTGGACACCGGACACC	176	
GABRA3 R	GCACATCCATGGGAAAATCTTCC		
HRMT1L1 F	TTGGGACGCTATGGAACCTCT	255	
HRMT1L1 R	GCTGGTACACGGTGATGATG		
LDLR F	ACTGGTGTGAGAGGACCACC	440	
LDLR R	CAAAGGAAGACGAGGAGCAC		
MGC11134 F	CACTGGCCATCCATCCTACT	205	
MGC11134 R	TCCCTGGAGTCAGAATCACC		
MTND4L F	TAACCCTCAACCCCACTCC	121	
MTND4L R	GGCCATATGTGTTGGAGATTG		
NCOA2 F	TCTGATGGCACTCTTGTTCG	136	
NCOA2 R	TCAGATCCGGATTTCATCACA		
PCOLN3 F	TAAACAACCTGGGTGCCTTCC	183	
PCOLN3 R	CAGCAAGTCCCTCCCAAGAG		
GAPDH F	TGGTATCGTGGAAAGGACTCATGAC	185	
GAPDH R	CCAGTGAGCTTCCCGTTCAGC		

^a F, forward; R, reverse.

shown to recreate the cell tropism and entry pathway of bona fide BDV (39), but retained the cytolytic phenotype of VSV and produced high titers (10^7 PFU/ml) of cell-free infectious progeny. The use of rVSVΔG*/BDVG allowed us to develop a small interfering RNA (siRNA)-based high-throughput screen (HTS) to identify cellular genes required for BDV G-mediated cell entry. We identified 24 host cell genes involved in BDV G-mediated cell entry. Identified genes are expressed in different subcellular locations and known to be involved in a wide range of different cellular physiological processes. These findings indicate that cell entry of BDV involves a complex array of interactions between the virus G and cellular proteins. This study provides also a novel and general approach to identifying host cellular genes involved in cell entry mediated by the surface glycoproteins of other viruses for which the corresponding rVSVΔG*/G could be generated.

MATERIALS AND METHODS

Cells and viruses. Ol, an established human oligodendroglial cell line, was maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 2 mM glutamine, 10 mM HEPES, and 10% heat-inactivated fetal bovine serum (FBS). The recombinant VSVs rVSVΔG*/BDVG and rVSVΔG*/VSVG have been described (39). The BDV, VSV, and lymphocytic choriomeningitis virus (LCMV) strains utilized were He80 (50), Indiana (26), and Armstrong (14), respectively.

siRNA library and HTS. The screen was performed essentially as described previously (2). Ambion Silencer Human Druggable Genome siRNA Library V2 was delivered into Ol cells by reverse transfection by mixing 1 μl of individual siRNA (0.5 μM) and 10 μl of diluted (1:100 in Optimem) siRNA MAX (Invitrogen) per well of a 384-well plate, followed by incubation for 15 min at room temperature. Ol cells (4,000 per well) diluted in 40 μl of DMEM supplemented with 10% FBS were added to the transfection mix and incubated for 36 h at 37°C 5% CO₂, followed by the addition of 10 μl of medium containing 2.5×10^4 PFU of either rVSVΔG*/BDVG or rVSVΔG*/VSVG. Infected cells were incubated

at 37°C for 48 h for rVSVΔG*/BDVG or 24 h in case of rVSVΔG*/VSVG, and cell viability was determined by addition of 10 μl of ATPlite (Perkin-Elmer) and measuring the levels of ATP by luminometry using an Envision 2103 Multilabel plate reader (Perkin-Elmer).

Reverse transcription-PCR (RT-PCR). Ol cells were reverse transfected with different siRNA, and 36 h later the total cellular RNA was isolated by using TRI Reagent (Sigma Aldrich). Equal amounts of RNA were DNase treated and subjected to RT using random hexamers (Invitrogen) and SuperScript III reverse transcriptase (Invitrogen). Samples were treated with RNase and cDNAs amplified by PCR using the indicated primers (Table 1). PCR was done using *Taq* polymerase (5 Prime, Germany) and the following conditions: 94°C for 5 min; 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and 72°C for 10 min and 4°C for infinity. Equal volumes of PCRs were subjected to 2% agarose gel electrophoresis analysis, and PCR products were visualized by ethidium bromide staining.

Pharmacological inhibition of cellular proteases. Ol cells were pretreated for 1 h prior infection with the indicated concentrations of the following inhibitors: the furin inhibitor hexa-D-arginine (Calbiochem), the ADAM17-selective inhibitor SP26 (Schering Plough), and the cathepsin L inhibitor Z-Phe-Tyr(tBu)-diazomethylketone (CatL; Axxora LLC). The cells were infected with 200 PFU of either rVSVΔG*/BDVG or rVSVΔG*/VSVG, BDV (multiplicity of infection [MOI] = 0.1), or LCMV (MOI = 0.01) in the presence of the drugs. Infected cells were fixed (4% paraformaldehyde in phosphate-buffered saline) at 7 h postinfection (p.i.) (infections with rVSV) or 48 h p.i. (infections with BDV and LCMV), permeabilized (0.1% saponin), and examined by immunofluorescence assay (IFA). Cells infected with rVSV were identified by using a mouse monoclonal antibody to VSVN (10G4; Ab-VSV N). Cells infected with LCMV or BDV were identified by using mouse monoclonal antibody 1.1.3 to LCMV NP or a rabbit antiserum to BDV N, respectively.

Statistical analysis. The viability of siRNA-transfected and rVSVΔG*/BDVG-infected cell populations was normalized to positive and negative controls on each plate during the primary screen. The average score of duplicates for each siRNA was treated as the siRNA score. A "survival score" was determined based on the median score of the six wells with siRNA targeting each gene. Quantile-quantile plots of each siRNA score and each "survival score" against normal distributions of the same size show a good fit ($R = 0.992$). Genes with two or more siRNAs inducing 2-fold activation or greater were considered hits. *P* values

FIG. 1. OI as cell substrate for siRNA-based HTS to identify cellular genes involved in BDV cell entry. (A) OI cells exhibited high silencing efficiency. HeLa, known to be highly susceptible to siRNA silencing in the absence of significant toxicity, and OI cells were subjected to reverse transfection with a specific siRNA to CD29, and 48 h later the cells were fixed and CD29 expression detected by IFA. Nuclei were stained with DAPI (4',6'-diamidino-2-phenylindole). (B) OI cells transfected with control siRNAs remained highly susceptible to rVSVΔG*/BDVG-mediated CPE. OI cells were reverse transfected with control siRNAs and subsequently infected (MOI = 5) with either rVSVΔG*/BDVG or rVSVΔG*/VSVG or else left uninfected. At 48 h p.i., cell survival was determined by using the ATPlite assay.

were calculated for each gene by using a Student *t* test on all six well scores per gene versus scores for all nonspecific control wells.

RESULTS

Development of a cell-based assay to identify cellular genes contributing to BDV cell entry. To identify candidate host cell genes involved in BDV entry, we designed a siRNA-based HTS that used as readout the survival of OI cells after infection with rVSVΔG*/BDVG. We selected the OI cell line for this HTS because of its high susceptibility to BDV infection (7, 37) and great siRNA-mediated silencing efficiency in the absence of significant toxicity (Fig. 1A). Moreover, OI cells transfected with control siRNAs remained highly susceptible to both VSV and rVSVΔG*/BDVG, as determined by cell survival measurements using an ATPlite assay (41). This assay had an excellent

dynamic range when mock and virus infection conditions were compared (Fig. 1B).

The siRNA-based HTS consisted of three distinct steps (Fig. 2). The first step involved a reverse transfection protocol to deliver the Ambion Silencer Human Druggable Genome siRNA Library V2 into OI cells, followed 36 h later by infection of transfected cells with rVSVΔG*/BDVG. Initial hits were selected based on increased cell resistance to CPE associated with rVSVΔG*/BDVG infection. In a second step we compared the susceptibility to rVSVΔG*/BDVG and rVSVΔG*/VSVG of cells transfected with hits selected in the primary screen of the screen, which allowed us to identify siRNAs that specifically interfered with BDV G-mediated cell entry. The third and final step involved the validation of selected hits in the context of infection with bona fide BDV.

FIG. 2. Flow chart of the siRNA-based HTS and subsequent confirmation and validation steps to identify cellular genes involved in BDV entry. The screen consisted of three distinct steps. In the first step, initial candidates were selected based on the results of siRNA-mediated increased cell survival upon rVSVΔG*/BDVG infection. In the second step, siRNAs selected in the first step were examined with respect to their specific effects on BDV G-mediated cell entry. For this, OI cells were reverse transfected with siRNAs corresponding to the selected siRNA candidates and subsequently infected with either rVSVΔG*/BDVG or rVSVΔG*/VSVG. At 48 h p.i. the cell survival was determined to identify siRNAs that specifically reduced the rVSVΔG*/BDVG-mediated CPE. In the third step, candidates selected in the second step were examined for their effect on OI cells susceptibility to infection with bona fide BDV.

Primary screening. The siRNA library used in our screening targeted 5,516 different genes, with each gene being covered by three independent siRNAs. OI cells were reverse transfected in duplicate with each siRNA, and 36 h later the cells were infected with rVSVΔG*/BDVG (MOI = 5). To assess the inhibitory effect of transfected siRNAs on rVSVΔG*/BDVG infection, we measured cell viability at 48 h p.i. using a lumino-metric assay based on the determination of cellular concentrations of ATP (41). The data collected were inspected for plate patterns and other artifacts for quality assessment, and suspected wells were eliminated prior to subsequent statistical analysis (Table 2).

Curated data were normalized, and duplicate measurements for each siRNA were averaged to a single value to be used as siRNA score. Using this information, we calculated *P* values for each siRNA as described in Materials and Methods. We next calculated the median of the scores of the three siRNAs directed against each gene target (“survival score”), and ranked genes represented in the library according to this value.

TABLE 2. Quality parameters of the primary screen^a

Parameter	Value
Total no. of wells screened	36,864
No. (%) of faulty wells	699 (1.9)
Mean % CV	20
Total no. of siRNAs.....	16,560
No. of siRNAs, two wells/siRNA.....	15,909
No. of siRNAs, one well/siRNA	617
No. of siRNAs, no information.....	34

^a Total wells refers to the number of screened wells. It is equivalent to 96 384-well plates or two sets of 45 384-well plates. Faulty wells refers to the number of wells eliminated after primary data inspection due to faulty plate readings or edge, row, or column effects. The mean % CV is the mean of the standard deviations observed among the duplicate wells for all siRNAs across the screen. The total number of siRNAs contained in the library is indicated. It is equivalent to three siRNAs against each of 5,520 genes covered in the library. The numbers of siRNAs (two wells/siRNA and one well/siRNA) indicate the siRNAs for which results were obtained from two wells or one well after the removal of faulty wells. The “number of siRNAs, no information” refers to siRNAs for which both wells were faulty and thus removed from the analysis.

FIG. 3. Frequency distribution of screening results. A survival score was calculated for each target as described in the text. The histogram shows the distribution of binned survival scores obtained for all targets assayed in the screen. Quantile-quantile plot analysis revealed a good fit with a normal distribution ($R^2 = 0.992$, not shown). Arrows indicate the area from which hits were selected. From the initial list, we considered hits targets with two or more siRNAs inducing twofold activation or greater. The lower limit (survival score = 2.38) was determined based on TRIM11, the 57th and last ranked selected hit (see Table 3).

In this way high medians (and low P values) would correspond to genes whose knockdown via siRNA led to increased cell survival upon virus infection. The distribution of survival scores fitted well a normal distribution as assessed by quantile-quantile plot analysis (not shown). The histogram representing the distribution of survival scores calculated for each gene (Fig. 3) was used to identify genes with two or more siRNAs inducing twofold activation or greater. Subsequent P value calculations were made under the assumption of a normal distribution (Table 3).

Since our assay was based on the cytolytic effect of rVSVΔG*/BDVG, high scoring targets were likely to represent cellular genes necessary for either viral infection or viral induced cell death. Accordingly, several top ranked targets were well known proapoptotic genes likely involved in the cytopathic activity of VSV (data not shown), and were no longer pursued. A caveat associated with siRNA-based screens relates to the frequency of false positives resulting from off-target effects. This concern is minimized when different siRNAs targeting the same mRNA species produce the same phenotype. Consequently, for further analysis we selected only candidate genes that had a score higher than two and at least two siRNA with a P value of <0.05 . Based on these criteria we selected 123 distinct siRNAs ($P \leq 0.05$) representing 58 different candidate gene targets (Table 3).

Validation of initially identified hits. We next examined which of the 123 siRNA hits selected by the primary screen inhibited consistently and specifically infection with rVSVΔG*/BDVG. We first assessed the effect of the transfected gene-

specific siRNAs on cell viability. For this, we compared the magnitudes of the ATP signals generated by cells transfected with each one of the 123 gene-specific siRNA candidates and those obtained from cells transfected with each of the 48 different nonspecific (NS) control siRNAs. We used the data from this comparison to normalize readouts from cells that were siRNA transfected and subsequently infected with either rVSVΔG*/BDVG or rVSVΔG*/VSVG.

We next compared the 123 gene-specific selected siRNAs with NS control siRNAs with respect to their ability to increase cell survival upon infection with rVSVΔG*/BDVG. The data for this analysis were obtained from four independent replicates for each siRNA. This allowed us to select 47 siRNAs, among the 123 gene-specific siRNAs, that consistently inhibited infection with rVSVΔG*/BDVG. To determine which of these selected 47 siRNAs exhibited their effects by specifically targeting BDV G-mediated cell entry, we compared their effects on cell survival upon infection with either rVSVΔG*/BDVG or rVSVΔG*/VSVG (Fig. 4). This approach led us to classify the 58 initially identified candidate genes into three groups. The first two groups included (i) genes ($n = 21$) whose siRNA-mediated knockdown resulted in specific increased resistance to rVSVΔG*/BDVG compared to rVSVΔG*/VSVG infection and (ii) genes ($n = 15$) whose siRNA-mediated knockdown conferred similar levels of resistance to both rVSVΔG*/BDVG and rVSVΔG*/VSVG. Genes belonging to this latter group likely represented genes that played a role in any of the multiple steps involved in RNA replication or gene expression of VSV. However, it should be noted that some

TABLE 3. Gene characteristics and fold increases^a

Rank	Gene no.	Symbol	Description	Fold increase			P
				siRNA1	siRNA2	siRNA3	
1	5119	PCOLN3	Chromatin-modifying protein 1A	4.13	-0.23	4.18	0.00055
2	135152	B3GAT2	β -1,3-Glucuronyltransferase 2 (glucuronosyltransferase S)	5.69	2.23	3.57	1.51E-06
3	8511	MMP23A	Matrix metalloproteinase 23A (pseudogene)	2.92	4.98	3.11	0.000439
4	1357	CPA1	Carboxypeptidase A1 (pancreatic)	4.24	3.87	0.27	9.35E-08
5	25797	QPCT	Glutaminyl-peptide cyclotransferase	-0.72	3.43	3.85	2.01E-05
6	2356	FPGS	Folypolyglutamate synthase	3.13	1.61	5.09	2.69E-11
7	1515	CTSL2	Cathepsin L2	0.8	6.45	2.98	6.64E-06
8	3275	HRMT1L1	Protein arginine methyltransferase 2	-0.58	3.24	3.12	0.003443
9	54858	PGPEP1	Pyroglutamyl-peptidase I	0.48	3.97	2.86	1.92E-10
10	205564	SEN5	SUMO1/sentrin specific peptidase 5	2.76	-0.54	3.47	0.000374
11	5139	PDE3A	Phosphodiesterase 3A, cGMP-inhibited	2.72	3.31	-0.75	0.001013
12	2952	GSTT1	Glutathione S-transferase theta 1	-0.37	5.07	2.69	3.53E-06
13	6327	SCN2B	Sodium channel, voltage-gated, type II, beta	4.37	2.68	-0.43	0.000485
14	3355	HTR1F	5-Hydroxytryptamine (serotonin) receptor 1F	-0.91	3.25	3.04	0.001414
15	9791	PTDSS1	Phosphatidylserine synthase 1	3.18	3.91	1.85	0.001215
16	1910	EDNRB	Endothelin receptor type B	0.77	3.83	2.59	0.000587
17	340273	ABCB5	ATP-binding cassette, subfamily B (MDR/TAP), member 5	2.56	4.53	-0.86	0.001589
18	79054	TRPM8	Transient receptor potential cation channel, subfamily M, member 8	3.05	2.55	0.02	0.000579
19	51056	LAP3	Leucine aminopeptidase 3	2.57	4.12	-0.48	0.000212
20	9127	P2RX1	Purinergic receptor P2X, ligand-gated ion channel, 6	3.95	1.47	3.65	3E-07
21	3949	LDLR	Low-density lipoprotein receptor	2.5	0.31	2.89	0.001816
22	83707	TRPT1	tRNA phosphotransferase 1	0.61	3.28	3.04	0.000283
23	2556	GABRA3	γ -Aminobutyric acid (GABA) A receptor, alpha 3	2.46	3.29	0.13	0.029221
24	51146	A4GNT	α -1,4-N-Acetylglucosaminyltransferase	3.28	2.45	0.47	0.042799
25	4539	MTND4L	NADH dehydrogenase, subunit 4L (complex I)	2.45	-0.72	3.99	0.00038
26	89845	ABCC10	ATP-binding cassette, subfamily C (CFTR/MRP), member 10	0.68	2.98	2.69	4.54E-05
27	64699	TMPSR3	Transmembrane protease, serine 3	2.42	7.8	0.07	0.014565
28	64170	CARD9	Caspase recruitment domain family, member 9	-0.02	3.06	2.73	0.000433
29	1487	CTBP1	C-terminal binding protein 1	2.4	1.9	2.56	5.08E-13
30	339501	MPN2	Marapsin 2	3.38	2.91	0.21	5.27E-09
31	2224	FDPS	Farnesyl diphosphate synthase (farnesyl pyrophosphate synthetase, dimethylallyltransferase, geranyltransferase)	3.19	-0.59	2.39	3.66E-06
32	55699	FLJ10326	Isoleucyl-tRNA synthetase 2, mitochondrial	-1.08	5.18	2.35	9.24E-09
33	6272	SORT1	Sortilin 1	2.86	2.68	0.33	0.000425
34	118856	MMP21	Matrix metalloproteinase 21	-0.16	3.21	2.3	5.36E-05
35	218	ALDH3A1	Aldehyde dehydrogenase 3 family, member A1	0.66	2.48	2.59	1.42E-05
36	5301	PIN1L	Peptidylprolyl <i>cis/trans</i> -isomerase, NIMA-interacting 1-like (pseudogene)	2.29	4.52	-0.83	0.000151
37	81794	ADAMTS10	ADAM metalloproteinase with thrombospondin type 1 motif, 10	5.40E-03	2.42	2.9	0.000145
38	25947	SLC22A23	Solute carrier family 22, member 23	1	2.46	4.51	4.7E-06
39	1158	CKM	Creatine kinase, muscle	0.87	2.66	2.27	7.46E-05
40	9743	RICS	Rho GTPase-activating protein	2.32	1.99	3.07	0.000509
41	3816	KLK1	Kallikrein 1	2.37	0.28	2.76	2.04E-11
42	147746	HIPK4	Homeodomain interacting protein kinase 4	2.3	-0.33	3.69	0.000721
43	10499	NCOA2	Nuclear receptor coactivator 2	0.53	2.52	2.78	0.00091
44	221823	PRPS1L1	Phosphoribosyl pyrophosphate synthetase 1-like 1	0.9	2.84	3.13	0.002638
45	83451	WBSCR21	Abhydrolase domain containing 11	2.9	2.46	0.7	0.001543
46	6868	ADAM17	ADAM metalloproteinase domain 17	-1.01	2.6	2.73	0.000223
47	79071	ELOVL6	ELOVL family member 6, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)	2.71	-0.25	3.08	0.000198
48	7417	VDAC2	Voltage-dependent anion channel 2	2.4	2.98	0.44	1.59E-05
49	4296	MAP3K11	Mitogen-activated protein kinase kinase kinase 11	2.67	2.44	0.21	0.0001
50	558	AXL	AXL receptor tyrosine kinase	3.36	0.76	3.5	0.001477
51	5609	MAP2K7	Mitogen-activated protein kinase kinase 7	-0.85	2.29	2.29	4.08E-14
52	83657	DNCL2B	Dynein, light chain, roadblock-type 2	3.09	0.64	2.37	0.000688
53	153090	DAB2IP	DAB2 interacting protein	3.13	-0.5	2.29	0.003951
54	5409	PNMT	Phenylethanolamine N-methyltransferase	2.29	-1.01	2.67	0.000646
55	259249	MRGPRX1	MAS-related GPR, member X1	1.01	2.94	2.29	2.01E-05
56	2876	GPX1	Glutathione peroxidase 1	2.45	0.16	2.87	0.000133
57	81559	TRIM11	Tripartite motif-containing 11	2.87	2.32	-0.08	6.3E-05
58	5045	FURIN	Furin (paired basic amino acid cleaving enzyme)	1	3.64	1.45	0.000141

^a For determination of the fold changes, the raw values for each sample were normalized against the plate median, and duplicates for each siRNA were averaged. P values were obtained by using Student *t* test analysis wherein fold change values obtained for the six wells representing each target (duplicates of three siRNAs) were tested against the values of the whole siRNA collection.

FIG. 4. Identification of candidate genes involved in cell entry-mediated by BDV G. Ol cells were reverse transfected with the indicated siRNAs prespotted in triplicates onto M386 plates. After incubation for 36 h to induce gene silencing, the plates were infected with either rVSVΔG*/BDVG or rVSVΔG*/VSVG, and cell viability was determined at 48 or 24 h p.i. by using the ATPlite assay. Values obtained were normalized and corrected by readouts of cells transfected with each siRNA assayed.

genes within this group might play a common role in entry mediated by the G protein of both BDV and VSV. (iii) The third group included genes ($n = 22$) whose knockdown by any of the gene-specific siRNA selected for this analysis did not result in a statistically significant increase in cell survival upon infection with rVSVΔG*/BDVG compared to cells transfected with NS siRNA prior infection with rVSVΔG*/BDVG. Candidate genes within this group were considered to be false positives and thus were not examined further.

Effects on infection with bona fide BDV of knockdown of genes identified as capable of inhibiting rVSVΔG*/BDVG infection. To assess the biological significance of our siRNA-based HTS results, we examined whether siRNA-mediated knockdown of genes that resulted in specific inhibition of rVSVΔG*/BDVG infection, resulted also in inhibition of infection with bona fide BDV. For this, we conducted six independent experiments where Ol cells were transfected with each of the 28 siRNAs identified as capable of inhibiting specifically

BDV G-mediated cell entry (Fig. 4) and 36 h later infected them with BDV (MOI = 2). At 48 h p.i., we determined the number of BDV-infected cells by IFA using a rabbit serum to BDV N. As a control, Ol cells transfected with nonspecific siRNA were also infected with BDV. All but two 28 siRNA assayed inhibited to different degrees BDV infection (Fig. 5). Statistical analysis based on P values obtained for each siRNA indicated that of the 21 initially selected candidate genes, 15 could be considered bona fide host cell factors specifically required for BDV G-mediated cell entry (Table 4). We used the same approach to test the set of 18 siRNAs, representing 15 different genes, that inhibited the infection of both rVSVΔG*/BDVG and rVSVΔG*/VSVG (Fig. 4). The results from this analysis identified nine additional candidate genes involved in BDV cell entry (Fig. 6).

To assess further the specificity of our findings and rule out possible nonspecific off target effects, we examined the effects of additional distinct specific siRNAs targeting a subset of five

FIG. 5. Validation of candidate genes identified by the screen as host factors involved in cell entry mediated by BDVG. OI cells were reverse transfected with the indicated siRNAs and 36 h later were infected with BDV (MOI = 2). At 48 h p.i. the cells were fixed and examined by IFA using a rabbit serum to BDV N to identify BDV-infected cells. To normalize numbers of BDV-infected cells in each sample, a value of 1 was assigned to the number of BDV N-positive cells observed in cells transfected with a nonspecific control siRNA and infected with BDV. *, $P \leq 0.05$ by Student's t test.

candidate genes among the 24 validated as contributing to BDV G-mediated cell entry. Cells transfected with these additional siRNAs exhibited levels of increased resistance to infection with rVSVΔG*/BDVG similar to that provided by the corresponding gene-specific siRNA used during the primary screen (compared Fig. 4 and Fig. 7A). We used RT-PCR assays (Fig. 7B) to confirm the expression in OI cells of all candidate genes that our HTS identified as involved in BDV G-mediated entry. Likewise, we used a selected group of candidate genes to confirm that transfection with gene-specific siRNAs resulted in decreased levels of the corresponding gene mRNA (Fig. 7C).

Role of cellular proteases on BDV cell entry. Our siRNA-based HTS identified furin, as well as metalloprotease ADAM17, and the lysosomal cysteine protease cathepsin L as host cell factors contributing to BDV cell entry. We have documented that furin plays a key role in the maturation of BDV G and the production of infectious progeny (3, 9, 17, 43). Therefore, our finding that furin was also playing a role in BDV cell entry was rather intriguing. To further validate this observation, we examined the effect of pharmacological inhibition of furin on BDV G-mediated cell entry. For this analysis, we treated OI cells with increasing concentrations of the furin inhibitor hexa-D-arginine (H-D-R)

TABLE 4. Cell host genes identified as factors involved in BDV cell entry^a

Gene name	Gene no.	Description
ABCB5	340273	ATP-binding cassette, subfamily B (MDR/TAP), member 5
ADAM17	6868	Disintegrin and metalloproteinase domain 17 (tumor necrosis factor, alpha, converting enzyme)
CTSL2	1515	Cathepsin L2
DAB2IP	153090	DAB2 interacting protein
DNCL2B	83657	Dynein, cytoplasmic, light polypeptide 2B
EDNRB	1910	Endothelin receptor type B
FURIN	5045	Furin (paired basic amino acid cleaving enzyme)
GABRA3	2556	γ -Aminobutyric acid (GABA) A receptor, alpha 3
HRMT1L1	3275	HMT1 hnRNP methyltransferase-like 1 (<i>S. cerevisiae</i>)
HTR1F	3355	5-Hydroxytryptamine (serotonin) receptor 1F
LDLR	3949	Low-density lipoprotein receptor (familial hypercholesterolemia)
MGC11134	83707	MGC11134
MTND4L	4539	NADH dehydrogenase 4L
NCOA2	10499	Nuclear receptor coactivator 2
PCOLN3	5119	Procollagen (type III) N-endopeptidase
A4GNT	51146	α -1,4-N-Acetylglucosaminyltransferase
ABCC10	89845	ATP-binding cassette, subfamily C (CFTR/MRP), member 10
B3GAT2	135152	β -1,3-Glucuronyltransferase 2 (glucuronosyltransferase S)
CPA1	1357	Carboxypeptidase A1 (pancreatic)
DKFZP434F011	25947	DKFZP434F011
FDPS	2224	Farnesyl diphosphate synthase (farnesyl pyrophosphate synthetase, dimethylallyltranstransferase, geranyltranstransferase)
MAP3K11	4296	Mitogen-activated protein kinase kinase kinase 11
MMP21	118856	Matrix metalloproteinase 21
TMPRSS3	64699	Transmembrane protease, serine 3

^a Genes that inhibit BDV G-mediated, but not VSV G-mediated, cell entry are indicated in boldface. The other genes listed in the table inhibited both BDV and VSV infection.

starting 1 h prior to infection (MOI = 0.1) with rVSVΔG*/BDVG, bona fide BDV, or (as a control) rVSVΔG*/VSVG. Treatment with the furin inhibitor exerted a dose-dependent inhibition of infection with rVSVΔG*/BDVG, but not with rVSVΔG*/VSVG (Fig. 8A). Treatment with H-D-R also inhibited infection with bona fide BDV, whereas infection with the prototypic arenavirus LCMV was not affected (Fig. 8B). We used LCMV, instead of VSV, as a control for this experiment because BDV antigen in infected cells can be readily detected only after 36 to 48 h p.i., a time at which infection with VSV had caused a massive CPE that impeded a quantitative assessment of a potential drug effect on virus multiplication. When treatment with H-D-R was initiated at 1 h p.i., we did not observe a significant reduction in the numbers of BDV-infected cells at 48 h p.i. (data not shown).

We also sought to gain further evidence for a role of ADAM17 and cathepsin L in cell entry of BDV by examining the effects of pharmacological inhibition of these proteases. For this, we used SP26 and Z-Phe-Tyr(tBu)-diazomethylketone (CatL), which are well-established inhibitors of ADAM17 (30) and cathepsin L (47), respectively. Treatment with both SP26 and CatL resulted in a drug dose-dependent significant reduction of the numbers of BDV-infected but not LCMV-infected cells (Fig. 9).

DISCUSSION

We have reported that BDV enters cells via receptor-mediated endocytosis (17). This process requires the initial participation of lipid rafts (10) and is completed in the endosomal compartment of the cell via a pH-dependent fusion event between viral and cellular membranes required to liberate the

virus RNP core into the cell environment (17). As with other MNVs, the G surface glycoprotein of BDV mediates receptor recognition and directs the pH-dependent fusion event necessary for a productive infection (17, 40). However, many of the specific host cell factors and pathways involved in the BDV cell entry process remain unknown. To fill this gap of knowledge about cell entry of BDV, we utilized an siRNA-based screen aimed at identifying host cellular genes whose expression is required for virus cell entry mediated by BDV G.

BDV is characterized by its noncytolytic multiplication in all cell types thus far examined, which together with the inability to produce high titers of cell-free infectious either BDV or viral particles pseudotyped with BDV G, posed significant obstacles for the development of an HTS to identify host cell factors involved in BDV entry. We overcame this problem by using a previously described recombinant VSV (rVSVΔG*/BDVG) wherein VSVG was replaced by BDV G. rVSVΔG*/BDVG recreated the cell tropism and entry pathway of bona fide BDV (39) but retained the strong cytolytic phenotype characteristically associated with VSV infection, which facilitated the use of cell survival as a readout of the assay.

Our screen identified 24 candidate genes whose silencing resulted in the inhibition of BDV infection. It should be noted that several reasons precluded our screen to identify all possible candidate cellular genes that might contribute to BDV cell entry. Three key limitations associated with our siRNA-based HTS were as follows: (i) the library utilized for this HTS did not cover all of the transcriptome; (ii) in some cases, the siRNAs present in our library may not efficiently knock down their respective mRNA targets; and (iii) there was a potentially

FIG. 6. Validation as bona fide BDV cell entry factors of cellular genes whose siRNA induced knockdown conferred increased resistance to both rVSVΔG*/BDVG and rVSVΔG*/VSVG. OI cells were reverse transfected with the indicated siRNAs and 36 h later infected with BDV (MOI = 2). At 48 h p.i. the cells were fixed and examined by IFA using a rabbit serum to BDV N to identify BDV-infected cells. To normalize numbers of BDV-infected cells in each sample, a value of 1 was assigned to the number of BDV N-positive cells observed in cells transfected with a nonspecific control siRNA and infected with BDV. *, $P \leq 0.05$ by Student's t test.

long half-life stability for polypeptides encoded by mRNAs targeted via siRNA.

Previous reports have documented the use of siRNA-based functional genetic screens to examine the role of host cellular genes during infection with a variety of viruses, including HIV (6, 36), RSV (27), and WNV (28). However, a unique feature of our HTS design was that it allowed us to identify candidate genes that specifically contribute to BDV G-mediated cell entry rather than to any of the multiple steps involved in virus RNA replication and gene expression. This was achieved by comparing the susceptibility of siRNA-transfected cells to infection with either rVSVΔG*/BDVG or rVSVΔG*/VSVG. Likewise, this approach identified host candidate genes involved in cell entry steps common to both VSV and BDV. Among the 58 candidate genes identified by the initial screen, 24 of them were validated as host cell factors required for infection with bona fide BDV. Of these 24 genes, 15 corresponded to cellular factors that contributed specifically to

BDV G-mediated cell entry, whereas the other 9 appeared to contribute to cell entry or gene expression processes, or both, that were common to both VSV and BDV.

The host cell proteins identified by our HTS as required for BDV G-mediated cell entry are known to participate in a broad range of cellular functions and to display different subcellular localization patterns. Cellular receptors for BDV remain to be identified, and any candidate gene uncovered by our HTS that encodes a protein expressed at the cell surface could be considered a potential receptor, coreceptor, or attachment factor for BDV. Some of the targets identified are predominantly CNS molecules, including the subunit alpha-3 of the GABA_A receptor (GABRA3) or the serotonin receptor 1F (HTR1F), but whether these molecules contribute to the neurotropism or neuropathogenesis, or both, of BDV remains to be determined. Cortical shrinkage associated with neonatal BDV infection is associated with a decrease in parvalbumin-positive large neurons (a diameter of >100 μm), suggesting

FIG. 7. (A) Inhibition of BDV infection by additional siRNA targeting cellular genes identified by the screen as host factors involved in BDV G-mediated cell entry. OI cells were reverse transfected with the indicated siRNAs and 36 h later infected with BDV (MOI = 2). At 48 h p.i. cells were fixed and examined by IFA using a rabbit serum to BDV N to identify BDV-infected cells. To normalize the numbers of BDV-infected cells in each sample, a value of 1 was assigned to the number of BDV N-positive cells observed in cells transfected with a nonspecific (NS) control siRNA and infected with BDV. (B) OI cells express candidate genes identified by the screen and host cell factors involved in BDV cell entry. RNA from OI cells was first subjected to RT and then amplified by PCR using specific oligonucleotides to detect gene expression in OI cells. (C) Gene silencing by transfection with specific siRNAs targeting genes identified by the screen. OI cells were transfected with siRNAs targeting LDLR, furin, DNCL2B, and nonspecific siRNA. At 36 h posttransfection, cellular RNA was purified, and mRNAs from genes targeted were detected by RT-PCR. As a control, we also detected GAPDH mRNA from every sample analyzed. RT-PCRs were performed as follows. Total cellular RNA (5 μ g) was treated with DNase and then subjected to an RT reaction in a final volume of 50 μ l. In each case different amounts (0.1, 0.5, 2, and 10 μ l) of the RT reaction product (cDNA) were used for PCR (three cycles of 1 min at 55°C in a final volume of 50 μ l), and 10 μ l of each PCR product was analyzed by agarose gel electrophoresis. Amplified DNA fragments were detected by ethidium bromide staining. The amount of amplified DNA product followed a dose-dependent response with respect to the amount of cDNA used for the PCR. (C) Results of PCR assays with 2 μ l of cDNA. *, $P \leq 0.05$ by Student's t test.

that GABA-ergic neurons may be particularly susceptible to neonatal BDV infection (32, 42).

Several cell surface proteins identified by our HTS as host factors involved in BDVG-mediated entry, including ADAM17 (55), endothelin receptor B (EDNRB) (15), and GABA receptors bearing the subunit GABRA3 (31), have been shown to be located in lipid rafts, a finding potentially related with our observation that lipid rafts are involved in BDV cell entry (10).

Our HTS identified several cellular proteases as host factors contributing to BDV cell entry. One of these proteases, furin, has been shown to play a critical role in BDV G maturation and production of infectious BDV progeny (3, 9, 43), as well as in the processing of several other viral glycoproteins (4). Accordingly, furin inhibitors have been shown to block infection by influenza virus (52) and HIV (36). However, before this

work there was no evidence for a role of furin in BDV cell entry, which might be related to the role played by furin in the activation of ADAM17 and MMP21 (33), two different metalloproteases identified in our HTS as host factors involved in BDV cell entry. Notably, ADAM17 is located in lipid rafts (55) and may be part of the selected group of proteins recruited in this compartment to facilitate efficient BDV cell entry. Pharmacological inhibition of ADAM17 resulted in an ~60% reduction in the numbers of BDV-infected cells at 48 h p.i. In contrast, the numbers of LCMV-infected were minimally (<15% reduction) affected by treatment with SP26 and only at the highest drug concentration used, a finding which may indicate nonspecific effects on virus growth.

Our HTS identified also cathepsin L2, also known as cathepsin V, as a host cell factor involved in BDV cell entry. Notably,

FIG. 8. Effect of furin inhibitor on BDV G-mediated cell entry. OI cells were pretreated with increasing concentrations of the furin inhibitor hexa-D-arginine 1 h prior infection (MOI = 0.1) with rVSVΔG*/BDVG, bona fide BDV, or (as a control) rVSVΔG*/VSVG. (A) At 7 h p.i. cells infected with rVSVΔG*/BDVG or rVSVΔG*/VSVG were trypsin treated and fixed for green fluorescent protein detection by fluorescence-activated cell sorting analysis. (B) At 48 h p.i., BDV-infected cells were subjected to IFA to determine the numbers of cells expressing BDV N.

Ebola virus G protein requires the participation of two different endosomal cathepsins to generate the G conformation competent in fusion (8). Whether BDV requires a similar additional processing remains to be determined. It is, however, equally plausible that cathepsin L2-mediated processing of a host cell protein is required for cell entry of BDV. Cathepsin L2 was reported to be expressed at high levels in the thymus and testis, whereas CNS appears to express very low levels (48). However, RT-PCR of OI cells readily detected the presence of cathepsin L2 mRNA in these cells (Fig. 7).

Our HTS identified also LDLR and DNCL2B (Table 2) as cellular factors required for BDV cell entry. LDLR has been shown to function as a receptor for rhinoviruses (34, 56) and potentially also HCV (16); whether LDLR can serve a similar role for BDV remains to be investigated. In addition, LDLR can trigger the clathrin-mediated endocytotic pathway (5), which we have found to direct the entry of BDV into cells

(submitted for publication). We have also obtained evidence that microtubules play a key role in the trafficking of BDV-containing endosomes to the location where viral and cell membranes fuse (1, 54), a step required for the release of the viral RNP into the cell environment. This finding is consistent with the inhibition of BDV cell entry observed in cells subjected to siRNA-mediated knockdown of DNCL2B, a member of the complex dynactin that interacts with endosomes and shuttle these compartments through the cytoplasm using the microtubule network (21).

Further studies aimed at elucidating the roles of each of the BDV cell entry factors identified by our siRNA-based HTS would provide us with a detailed picture of this first and critical step in the virus life cycle. Moreover, it is worth noting that the design of our screen could be applied to identifying host cellular genes contributing to cell entry mediated by the surface glycoprotein of other viruses for which the corresponding rVSVΔG*/G can be generated.

FIG. 9. Effect of ADAM17 and cathepsin L inhibitors on BDV G-mediated cell entry. OI cells were pretreated with the indicated concentrations of each inhibitor at 1 h prior infection (MOI = 0.1) with BDV or (as a control) LCMV. At 48 h p.i. BDV- and LCMV-infected cells were subjected to IFA to determine the numbers of cells expressing BDV or LCMV nucleoprotein (N) antigen. The numbers of infected cells were normalized by considering as 100% the numbers of infected cells detected in the absence of inhibitor.

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