Biochemical and Biophysical Research Communications 437 (2013) 452–456

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Validation-based insertional mutagenesis for identification of Nup214 as a host factor for EV71 replication in RD cells

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ARTICLE INFO

Article history:
Received 8 June 2013
Available online 3 July 2013

Keywords:
VBIM
EV71
Host factor
Nup214

ABSTRACT

Lentiviral validation-based insertional mutagenesis (VBIM) is a sophisticated, forward genetic approach that is used for the investigation of signal transduction in mammalian cells. Using VBIM, we conducted function-based genetic screening for host genes that affect enterovirus 71 (EV71) viral replication. This included host factors that are required for the life cycle of EV71 and host restriction factors that inhibit EV71 replication. Several cell clones, resistant to EV71, were produced using EV71 infection as a selection pressure and the nuclear pore protein 214 (Nup214) was identified as a host factor required for EV71 replication. In SD2-2, the corresponding VBIM lentivirus transformed clone, the expression of endogenous Nup214 was significantly down-regulated by the reverse inserted VBIM promoter. After Cre recombinase-mediated excision of the VBIM promoter, the expression of Nup214 recovered and the clone regained sensitivity to the EV71 infection. Furthermore, over-expression of Nup214 in the cells suggested that Nup214 was promoting EV71 replication. Results of this study indicate that a successful mutagenesis strategy has been established for screening host genes related to viral replication.

1. Introduction

Enterovirus 71 (EV71) is one of the major causative agents of hand, foot and mouth disease (HFMD) and poses a significant threat to public health, particularly in Asia. In many cases, acute EV71 infection causes neurological disease resulting in a high number of fatalities. Currently, there are no specific antiviral medications for the treatment of HFMD partially due to the fact that the mechanism of action of EV71 is unclear. Like other viruses, EV71 depends on host cell machinery to support replication. The interplay between the invading viruses and the host cells is a continuous process, ultimately determining viral pathogenesis and infection outcome. Therefore, detecting the host genes associated with viral replication in EV71 could further enhance the understanding of EV71 viral pathogenesis and the development of antiviral therapies.

The host genes associated with viral replication fall into two categories depending on their roles. Host factors are required for viral replication and host restriction factors inhibit viral replication. The most common strategy used to identify these genes has been to create cell mutants from diverse libraries, including cDNA, siRNA and shRNA libraries. These routine methods have been successfully used to identify complex host–pathogen interactions in a number of viruses such as HIV, influenza and HCV [1]. However, there are some disadvantages with using these methods. For instance, some of the screens use easily transfected cells, but not the physiological substrate of the viral infection. Additionally, screens are prone to producing false positives resulting from spontaneous mutants and/or the off-target effects of siRNA and shRNA. In the current study, we used a novel mutagenesis method known as validation-based insertional mutagenesis (VBIM). VBIM is an improved, reversible promoter insertional technique that can be applied to most types of mammalian cells that have high titers of VBIM lentivirus, including cells that are not dividing. By using Cre recombinase-mediated removal of the VBIM vector, the mutant phenotype can be reversed so that the VBIM inserted mutants can be distinguished from spontaneous mutants [2]. The VBIM mutagenesis strategy has been successfully used for identifying the negative regulator of the NF-κB signaling pathway [2] and in the identification of the putative oncogene in immortalized human mammary epithelial cells [3]. In the current study, VBIM was used to perform function-based genetic screening to locate host genes that affect EV71 replication and to identify Nup214 as a host factor in the EV71 viral life cycle. In the VBIM transformed cell clone, endogenous Nup214 was dramatically down-regulated and the mutant phenotype was resistant to EV71. However, following Cre recombinase-mediated excision of VBIM, the cell clone regained sensitivity to EV71 demonstrating that the mutant phenotype was VBIM insertion specific. Moreover, over-expression of Nup214 in the cells promoted EV71 replication. Therefore, the

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0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.06.101

current study not only identifies a host factor for EV71 replication, but also establishes an effective screening strategy to identify genes that are associated with viral replication.

2. Materials and methods

2.1. Cells and viruses

Rhabdomyosarcoma (RD) cells and 293T cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in MEM or DMEM, supplemented with 10% FBS and penicillin/streptomycin. EV71, a Fuyang strain (GenBank accession No. FJ439769.1), was propagated in RD cells.

2.2. Plasmids and antibodies

The VBIM vector (SD 1, 2, and 3), pCMV-dR8.74, pMD2G and pBabe-Cre were generous gifts from Dr. Tao Lv and have been described previously [2]. PCMV-dR8.2 and pCMV-VSVG constructs were purchased from Clontech (Mountain View, CA, USA). PCMV6-XL5-NUP214-HA was constructed from PCMV6-XL5-NUP214 (OriGene, Rockville, MD, USA) by adding an HA tag epitope to the 5′ end of NUP214 ORF. To construct pMSCV-Cre, the Cre DNA fragment was amplified from pBabe-Cre and cloned into a pMSCV-puro vector.

Mouse monoclonal antibodies directed against β-Actin (AC-15) and GFP (GSN24) were purchased from Sigma (St. Louis, MO, USA). Rabbit polyclonal antibody against HA was purchased from Bethyl Laboratories (Montgomery, TX, USA) and mouse anti-EV71-VP1 (3D7) was purchased from Abnova (Taipei, Taiwan). Rabbit anti-Nup214 was obtained from Abcam (Cambridge, MA, USA).

2.3. Virus production

The VBIM lentiviruses were packaged in 293T cells as described previously [2]. Retroviruses encoding Cre recombinase and the RFP control were also packaged in 293T cells, using constructs pCMV-dR8.2 and pCMV-VSVG. Supernatants that contained virus were collected 48 h after transfection and then supplemented with 4 μg/ml polybrene.

2.4. Cell infection and selection

RD cells were infected with VBIM lentivirus. After 48 h, the media was replaced and the cells infected with EV71. The media was again replaced 12 h later. After approximately 10 days, the surviving single cells had formed small clones and the clones were infected with EV71. The surviving cell clones that had GFP expression were collected and cultured independently for subsequent identification.

Cell clones were infected, as above, with retroviruses that encoded for Cre recombinase and RFP. After 48 h, the infected cells were treated for 5 days with 1 μg/ml puromycin.

2.5. Inverse PCR

Inverse PCR was performed as previously described [3]. Briefly, genomic DNA was isolated from corresponding cells and a 10 μg quantity was digested with EcoRI and MfeI. A 2 μg quantity of the double-digested DNA was then ligated using T4 ligase. The reaction mixture was purified and used as the template for the first PCR reaction, using primers 5 (5′-CCAGAGTCACACAACAGACG-3′) and 7 (5′-GTAAGACCACCGCACAGC-3′). A volume of 0.5 μl of the first PCR product was used as the template for the second PCR reaction using primers 4 (5′-CCAGAGAGACCCAGTACAAGC-3′) and 6 (5′-GATCTTCAGACCTGGAGGAG-3′). The second PCR product was separated by agarose gel electrophoresis and excised for the subsequent DNA sequencing. Mapping of the insertion site was performed using bioinformatics from NCBI.

2.6. Western blot

Whole-cell extracts were separated by 6–12% SDS–PAGE. After electrophoresis, proteins were transferred to a PVDF membrane (Bio-Rad, CA, USA). The membranes were blocked for 1 h, at room temperature, in 5% dried milk and then probed with the indicated primary antibodies at an appropriate dilution, at 4 °C, overnight. The following day, the membranes were incubated with the corresponding IRD Flour 680-labeled IgG secondary antibodies (Li-COR Biosciences, Lincoln, NE, USA) and were scanned using the Odyssey Infrared Imaging System (Li-COR Biosciences).

3. Results

3.1. VBIM genetic screen to obtain EV71 resistant mutant cell clones

To identify host genes that are capable of affecting EV71 replication, including host and restriction factors, we performed a VBIM forward genetic screen using human RD cells. Since EV71 is a lytic virus, it can cause significant cytopathic effects (CPE), eventually leading to death of infected cells. Mutations that silence the host factors, or that drive the expression of host restriction factors, allow cells that are infected with EV71 to survive. Based on this principle, RD cells were infected with VBIM lentivirus that had been generated from VBIM constructs (SD1, -2, -3) [2]. After 2 days, approximately 90% of the RD cells were positive for GFP expression (Fig. 1, upper panel). At this point, the cells were infected with EV71. Most cells died within 2 days, though a small number of cells survived. The surviving cells formed clones, which were then infected with EV71. Ultimately, the surviving cell clones with GFP expression (Fig. 1, middle and lower panels) were collected and cultured separately. Following two rounds of selection, we had obtained several cell clones that were resistant to infection with EV71.

3.2. SD2-2, a phenotype reversible cell clone resistant to EV71 infection, was identified

All EV71 resistant cell clones were infected with retrovirus that encoded Cre recombinase or RFP in order to investigate whether the mutated phenotype was reversible. Among the selected cell clones, SD2-2 exhibited good reversibility. GFP expression in the SD2-2 clones was abolished after infection with retrovirus encoding Cre recombinase, however GFP remained in the SD2-2 clones infected with retrovirus encoding RFP (Fig. 2A). This result was confirmed by Western blot (Fig. 2B). Sensitivity to EV71 returned in the SD2-2 cells infected with Cre recombinase retrovirus, but not in the SD2-2 cells infected with RFP retrovirus, as illustrated by the EV71-VP1 expression in infected cells, as detected by Western blot (Fig. 2C). Collectively, these results confirm that SD2-2 is a reversible phenotype, VBIM inserted mutant.

3.3. NUP214 was identified as a putative gene that affected EV71 infection

To identify the gene responsible for EV71 resistance in the SD2-2 cell clone, a two-step inverse PCR procedure was carried out. This was done in order to identify the nucleotide sequence flanking the VBIM insertion site (Fig. 3A). DNA sequencing identified the affected gene in this cell clone was Nup214 and the insertion site was mapped to the fourteenth intron of Nup214. Using a flag epi-