The Essential Role of Clathrin-mediated Endocytosis in the Infectious Entry of Human Enterovirus 71*

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Little is currently known about the infectious entry process of human enterovirus 71 (HEV71) into host cells, which may represent potential anti-viral targeting sites. In this study a targeted small-interfering RNA (siRNA) screening platform assay was established and validated to identify and profile key cellular genes involved in processes of endocytosis, cytoskeletal dynamics, and endosomal trafficking essential for HEV71 infection. Screen evaluation was conducted via the expression of well characterized dominant-negative mutants, bioimaging studies (double-labeled immunofluorescence assays, transmission electron microscopy analysis), secondary siRNA-based dosage dependence studies, and drug inhibition assays. The infectious entry of HEV71 into rhabdomyosarcoma cells was shown to be significantly inhibited by siRNAs targeting genes associated with clathrin-mediated endocytosis (CME) that include AP2A1, ARRB1, CLTC, CLTCL1, SYNJ1, ARPC5, PAK1, ROCK1, and WASF1. The functional role of CME was verified by the observation of strong co-localization between HEV71 particles and clathrin as well as dose-dependent inhibition of HEV71 infection upon siRNA knockdown of CME-associated genes. HEV71 entry by CME was further confirmed via inhibition by dominant-negative EPS15 mutants and treatment of CME drug inhibitors, with more than 80% inhibition observed at 20 μM chlorpromazine. Furthermore, HEV71 infection was shown to be sensitive to the disruption of human genes in regulating early to late endosomal trafficking as well as endosomal acidic pH. The identification of clathrin-mediated endocytosis as the entry pathway for HEV71 infection of susceptible host cells contributes to a better understanding of HEV71 pathogenesis and enables future development of anti-viral strategies against HEV71 infection.

Numerous animal viruses utilize various endocytic mechanisms available in mammalian cells for productive infection. Host endocytic pathways, such as clathrin-mediated endocytosis, caveolae-dependent uptake, macropinocytosis and cholesterol-dependent endocytosis, are commonly employed to mediate the infectious entry of virus particles into host cells (1). With the aid of these membrane trafficking processes, internalized viruses are able to fuse with cellular membranes for subsequent genome release as well as localize within the cell for effective replication (2). Many viruses require the induction of conformational changes by low pH levels (a result of acidification within the endosomal pathway) to drive essential infective steps of viral entry, such as fusion, penetration, and uncoating (3). In addition, viral transport within host cells may also be mediated by the host cytoskeleton network, particularly the actin filaments (4). With viral entry regarded as a major determinant of viral tropism and pathogenesis (5), understanding these initial events will enable future development of anti-viral strategies against HEV71 infection.

Human enterovirus 71 (HEV71)2 is a single-stranded, positive-sense RNA virus belonging to the human Enterovirus A (HEV-A) subspecies of the Enterovirus genus in the Picornaviridae family (6). First identified and characterized in 1969 in California from a stool specimen isolated from an infant with encephalitis (7), ensuing outbreaks of HEV71 have since been reported in various regions of the world, including Australia, Sweden, and Japan. In the past decade, HEV71-induced hand, foot, and, mouth disease outbreaks have mainly affected children within the Asia-Pacific region, including Hong Kong, China, Singapore, and Australia (8). The global control of poliovirus has also resulted in HEV71 becoming one of the most clinically significant etiological agents of acute neurological diseases such as polio-like acute flaccid paralysis, cerebellar ataxia, and brainstem encephalitis (9). No antiviral treatment has yet been developed to treat HEV71 infections; similarly, effective vaccines are currently unavailable, although several vaccine trials are being undertaken to develop effective therapeutic strategies to combat severe HEV71 infections. Emphasis is, therefore, being placed on understanding its virology, epidemiology, diagnosis, and management.

HEV71 infection begins with the attachment of virus particles onto host surface receptors followed by subsequent entry into the cells. Although it has been widely accepted that HEV71 enters permissive cells via receptor-mediated endocytosis, only several cell-specific candidate receptors have been identified to date. These cellular receptors include: scavenger receptor B2 (10), human P-selectin glycoprotein ligand-1 (11), and sialylated glycans (SA-a2,6Gal and SA-a2,3Gal) (12). Further...
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thermore, subsequent steps of HEV71 infection, such as the entry process and the uncoating of its RNA genome as well as the assembly pathway have not been clearly defined.

Although previous studies have attempted to decipher the entry processes of related enteroviruses, such as poliovirus (13–16) and echovirus (17–19), little is currently known about the specific cellular genes or host factors involved in mediating the infectious entry of HEV71 into human cells. With the recent development of small interfering RNA (siRNA) technology, high throughput screening surveys of mammalian genes and their functions has been made feasible (20).

In this study we assessed an array of siRNA libraries that specifically target human genes important for endocytosis processes, trafficking of membrane vesicles, actin polymerization, and cytoskeleton rearrangement to determine the cellular genes or factors that facilitate the infectious entry pathway of HEV71. Interestingly, we were able to show for the first time that the knockdown of human genes associated with clathrin-mediated endocytosis efficiently blocked HEV71 infection. The essential involvement of clathrin-mediated endocytosis in HEV71 entry into cells was confirmed by the expression of dominant-negative mutants and drug inhibitors to perturb this uptake pathway. In addition, we also identified cellular factors responsible for vesicle trafficking and maturation, signal transduction, and actin polymerization that are essential for the infectious entry process of HEV71.

EXPERIMENTAL PROCEDURES

Cells and Viruses—Human rhabdomyosarcoma (RD) cells (American Type Culture Collection, ATCC CCL-136) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma) supplemented with 10% inactivated fetal calf serum (FCS) (Invitrogen) and sodium bicarbonate (Merck). HEV71 strain H (VR-1432™) was obtained from ATCC and propagated in RD cells. The virus titer was quantitated via viral infective plaque assays using RD cells.

Antibodies and Reagents—Mouse monoclonal antibodies against HEV71 were purchased from Chemicon for immunofluorescent detection of HEV71 infection. Rabbit polyclonal antibodies to clathrin (CLTC, Chemicon), early endosomal antigen 1 (EEA1; Novus Biologicals), and lysosomal-associated membrane protein 1 (LAMP1; Santa Cruz Biotechnology) were purchased from Chemicon for immunofluorescence assays. The secondary antibodies conjugated to fluorescein isothiocyanate (FITC) and Texas Red (TR) were purchased from Invitrogen. 4′,6′-diamidino-2-phenylindole (DAPI) fluorescent dye was purchased from Invitrogen. Mouse monoclonal antibodies against AP2A1 (Santa Cruz Biotechnology), ARPC5 (Novus Biologicals), and RAB3A (BD Biosciences), SYNJ1 (Abnova Corp.), and WASF1 (Novus Biologicals) were used for the Western detection of the respective proteins. All drugs used in this study (chlorpromazine, filipin, bafilomycin A1, cytochalasin B, methyl-β-cyclodextrin, 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), concanamycin A, and nystatin) were purchased from Sigma and prepared according to the manufacturer’s instructions under sterile conditions.

siRNA Library—The human genome siRNA subset library targeting the endocytic and membrane trafficking genes (Dharmacon, RTF H-005500) was used in this study. A smart pool approach of incorporating four siRNAs targeting each gene was utilized. The advantages of this pooled approach as well as the issues of gene compensation of specific isotype of genes were discussed in Reynolds et al. (21). The list of 119 targeted human genes and isoforms (excluding the control set) is presented in supplemental Table 1.

RNA Transfection of siRNA Delivery into Cells—All transfections were performed in a 384-well plate format. A 1.2% (v/v) stock solution of the transfection reagent (DharmaFECT 1) was prepared in DCCR cell culture buffer (Dharmacon) and incubated at 25 °C for 10 min. From this stock, 8 μl was added to the lyophilized siRNA in each well of the 384-well plate and incubated at 25 °C for 30 min to allow the siRNAs to rehydrate and form siRNA-lipid complexes. Subsequently, 5 × 10³ RD cells in 42 μl of complete DMEM supplemented with 10% FCS were added. The siRNA cell mixture was then incubated at 37 °C for 48 h before HEV71 infection. The final concentration of pooled siRNAs was 50 nM per well. Individual siRNA duplexes were used at 6.25 pmol per well.

Screening of the siRNA Library—A high throughput platform for the specific detection of HEV71 infection in the 384-well plate format via immunofluorescence staining was employed for the screening assay, as described in Chu and Yang (22). Briefly, the siRNA-transfected RD cells were incubated at 37 °C for 48 h to ensure effective gene knockdown by the siRNAs before being subjected to HEV71 infection at a multiplicity of infection of 1. After 12 post-infection (p.i.), the cells were then fixed with cold absolute methanol (Sigma) for 15 min at −20 °C. Subsequent cell washing steps were performed using an automated 384-well format plate washer (EMBLA, Molecular Devices). The cells were then subjected to immunofluorescence staining using primary anti-HEV71 antibodies (Chemicon) followed by FITC-conjugated secondary antibodies (Invitrogen). Cell nuclei were counterstained with DAPI (100 nM; Invitrogen) before collation of image data by the ArrayScan VTI HCS automated fluorescence microscope Reader system (Cellomics) with appropriate excitation and emission wavelengths for FITC (495 and 520 nm, respectively) and DAPI (358 and 461 nm, respectively). Data collection and auto-focusing parameters were pre-determined using Cellomics Target Activation Bioapplication (Cellomics). A generic segmentation tool function was used to identify the two different stains (DAPI and FITC) with intensities above background staining, and data collection was obtained by logging the measurements. Data analysis after image acquisition was carried out using Cellomics Target Activation Bioapplication (Cellomics). DAPI-stained nuclei were counted to determine the number of virus-infected cells. Images with less than 500 cells were excluded from data analysis by the cell sorting module. Three independent screening assays were performed.

Controls included in individual sets of experiments were the use of transfection reagent (DharmaFECT 1) alone, a non-targeting siRNA (Dharmacon), a green fluorescent nonspecific siRNA (siGLO, Dharmacon), RISC-free™ siRNA (Dharmacon), and siRNA smart pools targeting cyclophilin B.
duplex, glyceraldehyde-3-phosphate dehydrogenase, and lamin A/C (Dharmacon). These siRNAs served as negative controls for nonspecific effects of siRNA and/or transfection reagents on cell viability and virus infection. In addition, cell viability was observed and monitored by visual inspection via the use of phase-contrast microscopy.

Transfection and Infection of Dominant Negative Mutants of Eps15—Plasmid constructs of dominant-negative Eps15 (pEGFP-Eps15Δ95/295, a component of the AP2 clathrin adaptor complex; the dominant-negative form inhibits clathrin-coated pit budding) and pEGFP-Eps15ΔIIIΔ2 (this construct lacks the AP2-binding sites and was used as a control that did not inhibit clathrin-mediated endocytosis) was kindly provided by A. Benmerah, Pasteur Institute, Paris, France. Briefly, RD cells were seeded onto 24-well tissue culture plates and grown overnight until 75% confluency was reached. 0.8 μg of the plasmid construct was then complexed with 50 μl of Opti-MEM medium (Invitrogen) for 5 min at room temperature. The mixture was then added to 48 μl of Opti-MEM containing 2 μl of Lipofectamine 2000™ (Invitrogen) that had undergone similar incubation conditions. After a further incubation period of 20 min, the DNA-liposome complexes were added to the cells, which had been starved in Opti-MEM medium for 4 h before transfection. After incubation for 6 h at 37 °C, 1 ml of maintenance medium was added and incubated for a further 48 h before virus infection.

To synchronize HEV71 entry, virus binding was first performed at 4 °C for 1 h before the temperature was shifted rapidly to 37 °C for internalization. Infected RD cells were subsequently processed for immunofluorescence assay and microscopic imaging. Supernatants were harvested from transfected cells, and plaque assays were performed.

Bioimaging Assay and Indirect Immunofluorescence—RD cells seeded on coverslips were incubated at 4 °C for 30 min and then washed with ice-cold PBS. The cells were subsequently infected with HEV71 at a multiplicity of infection of 1 for 30 min at 4 °C to allow viral attachment to cell surface without entry. The cells were then shifted to 37 °C for a further 10 min to allow the cells to return to their physiological temperature (designated as time 0). Cells were fixed in ice-cold absolute methanol at different time points, 0, 5, 10, 15, 20, and 30 min, followed by three 5-min washes in cold PBS before immunofluorescence processing.

Cells were incubated with the appropriate primary antibodies (with a 1:500 dilution for anti-HEV71 (Chemicon), a 1:300 dilution for anti-clathrin (Chemicon), a 1:200 dilution for anti-EEA1 (Novus Biologicals), and 1:500 dilution for anti-LAMP1 antibodies (Santa Cruz Biotechnology) in a humidity chamber for 1 h at 37 °C. After three 5-min washes with PBS, cells were further incubated with appropriate FITC- or TR-conjugated secondary antibodies (Invitrogen) before washing again with PBS. Nuclei staining were performed by incubating the cells with DAPI (Invitrogen) before mounting the processed coverslips onto ethanol-cleansed glass slides using Dabco mountant. The specimens were viewed with an inverted fluorescence microscope (IX81 Olympus) with excitation wavelengths of 480 and 543 nm for FITC and TR, respectively, using oil immersion objectives.

Drug Inhibitory Treatments—To determine the effects of the drugs used to inhibit the entry of HEV71, serum-starved RD cells were pretreated with drugs at different concentrations (Table 2) for 2 h at 37 °C followed by virus infection as described above. At 12 h p.i., HEV71-infected cells were processed for immunofluorescence assay. Three independent experiments were carried out for each set of drugs used. The inhibition of virus entry was determined by the number of virus antigen-positive cells in relation to the total number of cells (virus antigen-positive and -negative) and was expressed as the percentage virus antigen-positive cells.

Cell Viability Assay—Cell viability upon siRNA transfection and drug treatments was assessed by employing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Chemicon International), according to the manufacturer’s recommendations. Briefly, RD cells were seeded in 96-well cell culture plates and subsequently treated with individual siRNAs and drugs for 48 and 2 h, respectively, before incubation with AB solution for 4 h at 37 °C. After this, Solution C containing isopropyl alcohol/HCl was added, and the plates were subjected to absorbance reading by an ELISA plate reader (Bio-Rad) at test wavelength of 570 nm and reference wavelength of 630 nm.

RESULTS

Optimization of siRNA Screening Platform for HEV71 Infection—siRNA profiling using the human endocytic/membrane trafficking library from Dharmacon was conducted as a preliminary assay to identify key endocytic and membrane trafficking genes essential in HEV71 entry into host cells. The immunofluorescence screening assay was based on the detection of HEV71 capsid (VP1) protein in HEV71-infected RD cell monolayers. To ensure minimal signal variation and consistently high signal-to-background ratio, the Z’ factor of the screening assay (23), a measure of quality of high throughput screening, was determined. An assay with a Z’ factor value of between 0.5 and 1 would be considered to be an excellent assay for screening, with minimal well-well variation in the 384-well plate (23). A Z’ factor of 0.562 was consistently measured (data not shown), demonstrating the reliability and robustness of the assay. With the optimization of the siRNA screening platform for HEV71 infection, it was employed as a tool for rapid discovery of cellular factors essential for mediating the infectious entry process of HEV71 into cells.

siRNA Profiling of Human Endocytic and Membrane Trafficking Genes—An array of 119 siRNA smart pool (Dharmacon) targeting genes known to be directly or indirectly involved in regulating the different endocytic pathways (clathrin, caveolae, macropinocytosis, etc.), polymerization of actin, and cytoskeleton rearrangement and vesicle/cargo trafficking was used to identify host genes necessary for the infectious entry of HEV71. A list of the targeted human genes and a brief description of the reported functional role for each of the genes are provided in supplemental Table 1.

Effects of siRNA knockdown of different endocytic genes as determined by the decrease in the percentage of viral antigen-positive cells on HEV71 infection are shown in Fig. 1. Em-
ploying a 50% reduction in the number of fluorescently stained HEV71-infected cells as the siRNA-induced effect and criterion that suppressed HEV71 infection, the list of human genes that have an inhibitory effect on HEV71 infection were obtained using the screening platform, and the results are shown in Fig. 1 and further classified based on their functional roles (Table 1). A substantial number of genes resulting in the decrease in HEV71 infection have been known to be involved in clathrin-mediated endocytosis (Table 1); these genes include the subunit of the clathrin-associated adaptor protein complex 2 (AP2A1) and clathrin heavy chains (CLTCL and CLTCL1). AP2A1 is a subunit of the clathrin-associated adaptor protein complex 2 that regulates the formation of clathrin-coated pits as well as links clathrin to cellular receptors in endocytic vesicles (2). The clathrin heavy and light chains are intricately braided together to form the triskelion.
Genes required for HEV71 infection of RD cells

The descriptions of the function(s) of the individual genes are summarized from the Online Mendelian Inheritance in Man as of July 2010.

| GENES | ACCESSION NUMBER | NAME AND FUNCTION OF GENES | TARGET LOCI OF siRNA (Dₖ Duplex Number) |
|-------|-------------------|----------------------------|----------------------------------------|
| AP2A1 | NM_014203         | Adaptor related protein complex 2, subunit is part of the so-called AP2 coat assembly protein complex which links clathrin to receptors in the coated vesicles | Dₖ: GCAGUAUGUCUCUCCAAUAAG<br>D₅: CCGAGAUCUGCCGUAGCAAG<br>D₄: UUGAAGGCUCCAGUAAAG<br>D₃: CCGUGCGAGUUGCCAAUAG |
| ARFIP2| NM_012402         | ADP-Ribosylation factor-interacting protein 2, mediator cross-talk between RAC and ARF small GTPases; ARFs are implicated in vesicle transport between endoplasmic reticulum and the Golgi complex | D₅: CAAGACAGUGCCUUAAG<br>D₄: GGAAGAAUGCCGAGCAAG<br>D₃: UUGAAGGCUCCAGUAAAG<br>D₂: GAGCACCACUCUCAAGAA |
| ARPC5 | NM_005717         | Actin related protein 2/3 complex subunit 5 | D₅: GCAGUGAGGCUCCAGUAAAG<br>D₄: GAGGAGACGGCCGUAGCAAG<br>D₃: GAGGAGACGGCCGUAGCAAG<br>D₂: CCAUACUGCAAGGAAG |
| ARRB1 | NM_004041         | Arrestin beta 1, inhibits the signaling function of BARK-phosphorylated beta-adrenergic receptors. Beta-arrestin-1 mutants, are impaired either in SRC binding or in the ability to target receptors to clathrin-coated pits. | D₅: GCAUGCCUCUCCACUAA<br>D₄: UUGAAGGCUCCAGUAAAG<br>D₃: CCAUACUGCAAGGAAG<br>D₂: GAGGAGACGGCCGUAGCAAG |
| CLTC  | NM_004859         | Clathrin, heavy polypeptide; Clathrin molecules have a triskelion structure composed of 3 non-covalently bound heavy chains (CLTC) and 3 light chains (CLTL) | D₅: CCAUACUGCAAGGAAG<br>D₄: UUGAAGGCUCCAGUAAAG<br>D₃: GAGGAGACGGCCGUAGCAAG<br>D₂: GAAUAGGCUCCUAAAG |
| CLTL1 | NM_001835         | Clathrin, heavy polypeptide-like 1; High degree of homology to the amino acid level to human, rat and Drosophila clathrin heavy chain. | D₅: CCAUACUGCAAGGAAG<br>D₄: UUGAAGGCUCCAGUAAAG<br>D₃: GAGGAGACGGCCGUAGCAAG<br>D₂: GGGAGAGCGGUCCUAAAG |
| ELKS  | NM_015064         | RAB6-interacting protein 2; essential regulatory subunit of the IKK complex; Silencing ELKS expression by RNA interference blocked induced expression of NF-kappa-B target genes | D₅: GAGGAGACGCUCCGUAGCAAG<br>D₄: CCAUACUGCAAGGAAG<br>D₃: GAGGAGACGCUCCGUAGCAAG<br>D₂: GGGAGAGCGGUCCUAAAG |
| SYNJ1 | NM_003895         | Synaptopjanin 1; phosphatidylinositol (4,5)-biphosphate (Pip4,5P2) that has a role in clathrin-coated pit dynamics, can be recruited to clathrin-coated pits via a multiplicity of interactions | D₅: CCAUACUGCAAGGAAG<br>D₄: UUGAAGGCUCCAGUAAAG<br>D₃: GAGGAGACGCUCCGUAGCAAG<br>D₂: GGGAGAGCGGUCCUAAAG |
| MAP4K2| NM_004579         | Mitogen activated protein kinase 2; Specifically activates the SAPK pathway; SAPK pathway may be active in the differentiation and selection of B cells in the germinal centre | D₅: GCAUACUGCAAGGAAG<br>D₄: UUGAAGGCUCCAGUAAAG<br>D₃: GAGGAGACGCUCCGUAGCAAG<br>D₂: GGGAGAGCGGUCCUAAAG |
| PAK1  | NM_002576         | p21/CDC42/RAC1-activated Kinase 1; regulates actin through Rac1 | D₅: CCAUACUGCAAGGAAG<br>D₄: UUGAAGGCUCCAGUAAAG<br>D₃: GAGGAGACGCUCCGUAGCAAG<br>D₂: GGGAGAGCGGUCCUAAAG |
| PIK3CG| NM_002649         | Phosphoinositide 3 kinase; PI3K plays a pivotal role in the regulation of cytotoxicity in NK cells; | D₅: GCAGCAAGCUCGUCCGAAG<br>D₄: CCAUACUGCAAGGAAG<br>D₃: GAGGAGACGCUCCGUAGCAAG<br>D₂: GGGAGAGCGGUCCUAAAG |
| PIK3C2G| NM_004570        | Phosphotyidylinositol-3-kinase, class D; regulates diverse cellular responses, such as cell proliferation, oncogenic transformation, cell migration, intracellular protein trafficking, and cell survival, by phosphorylating the hydroxyl group at the D-3 position of the inositol ring of phosphoinositides | D₅: GCAGCAAGCUCGUCCGAAG<br>D₄: CCAUACUGCAAGGAAG<br>D₃: GAGGAGACGCUCCGUAGCAAG<br>D₂: GGGAGAGCGGUCCUAAAG |
| RAB3A | NM_002666         | Ras associated protein 3A Rab3a inhibited synapsin I binding to actin and synapsin I induced synaptic vesicle clustering | D₅: GCAGCAAGCUCGUCCGAAG<br>D₄: CCAUACUGCAAGGAAG<br>D₃: GAGGAGACGCUCCGUAGCAAG<br>D₂: GGGAGAGCGGUCCUAAAG |
| RAB6B | NM_016577         | Ras associated protein 6; a role in retrograde membrane traffic at the level of the Golgi complex | D₅: GCAGCAAGCUCGUCCGAAG<br>D₄: CCAUACUGCAAGGAAG<br>D₃: GAGGAGACGCUCCGUAGCAAG<br>D₂: GGGAGAGCGGUCCUAAAG |
| ROCK1 | NM_005406         | Rho associated coiled-coil-containing protein kinase; protein serine/threonine kinase that is activated when bound to the GTP-bound form of Rho | D₅: GCAGCAAGCUCGUCCGAAG<br>D₄: CCAUACUGCAAGGAAG<br>D₃: GAGGAGACGCUCCGUAGCAAG<br>D₂: GGGAGAGCGGUCCUAAAG |
| WASF1 | NM_003931         | Wiskott-Aldrich syndrome protein family; downstream effector molecule involved in the transmission of signals from tyrosine kinase receptors and small GTPases to the actin cytoskeleton. | D₅: GCAGCAAGCUCGUCCGAAG<br>D₄: CCAUACUGCAAGGAAG<br>D₃: GAGGAGACGCUCCGUAGCAAG<br>D₂: GGGAGAGCGGUCCUAAAG |
In addition, siRNA dosage knockdown of the selected targeted genes (AP2A1, ARPC5, CLTC, RAB3A, SYNJ1, and WASF1) with varying concentrations of siRNAs (0, 5, 25, and 50 nM) on RD cells and subjected to HEV71 infection. The data showed obvious dose-dependent inhibition of HEV71 infection with increasing concentrations of the respective siRNAs targeting AP2A1, ARPC5, CLTC, RAB3A, SYNJ1, and WASF1 upon siRNA transfection into cells (Fig. 3). Western blot detection was also carried out to ensure efficient knockdown of specific protein expression (Fig. 3). Indeed, the transfection of cells with siRNAs targeting AP2A1, ARPC5, CLTC, RAB3A, SYNJ1, and WASF1 showed dose-dependent reductions in the levels of the respective proteins when compared with the levels in the mock-transfected cells (Fig. 3). At the concentration of 25 nM of the transfected siRNA for the respective proteins, more than 60% reduction (as measured by densitometry) can be observed when compared with the mock-transfected samples (Fig. 3). Furthermore, the different concentrations of siRNA transfected into cells have minimal cellular cytotoxicity as revealed by the cell viability assays (Fig. 3). Indeed, the data obtained from the siRNA pool deconvolution and dose-dependent knockdown experiments may support a functional relationship between these identified genes and their inhibitory roles in the infectious entry of HEV71 into cells.

Infectious Entry of HEV71 into RD Cells Involved Clathrin-mediated Endocytosis—To further characterize the involvement of clathrin during the initial stages of HEV71 infection, a double-labeled immunofluorescence assay was performed to track the entry process and cellular localization of HEV71 within infected cells at fixed time points (0, 5, 10, 15, 20, and 30 min post-infection). At time 0 min p.i., HEV71 particles were observed predominantly attached to the plasma membrane of the cells with little or no co-localization of virus particles with clathrin (Fig. 4a). Strong co-localization of HEV71 particles and clathrin (arrows) within the cytoplasm was subsequently observed from 5 to 30 min p.i. (Fig. 4). These data may suggest the involvement of clathrin in the endocytosis of the HEV71 particles.

Transmission Electron Microscopy Analysis of HEV71 Entry Process—To visualize synchronized entry of HEV71 at the ultrastructural level, RD cells were first incubated with HEV71 (multiplicity of infection of 10) at 4 °C for 1 h. Low temperature treatment allows binding of HEV71 to the cell surface receptors but prevents the internalization of virus particles into the cells. Subsequently, the RD cells were warmed to 37 °C, and the virus-infected cells were processed for transmission electron microscopy analysis at appropriate times after warming. At 0 min of warming to 37 °C, attachment of HEV71 particles (gray arrows) along the cell surface of RD cells was observed (Fig. 5a). At 5 min after warm-up, HEV71 particles (gray arrows) were observed within invaginations of the plasma membrane (Fig. 5b). These invaginations are heavily decorated with clathrin molecules and resembled those of the clathrin-coated pits (white arrows) (35). After 10 min at 37 °C, the HEV71 virus particles (gray arrows) were observed within structures typical of clathrin-coated vesicles (white arrows; Fig. 5c) (35). Single virus particles were contained...
within each of these vesicles. By 15–20 min of warm-up, vesicles containing numerous HEV71 virus particles (Fig. 5d, gray arrows) were observed. These virus-containing vesicles are predominantly localized to the perinuclear region in close association with the endoplasmic reticulum. Thus far, it has been a challenge to visualize the uncoating process of HEV71 in endocytic vesicles. It is believed that the acidification of the endocytic vesicles would probably cause the rearrangement of the viral capsid proteins and trigger the release of the viral RNA into the cytoplasm for the initiation of HEV71 RNA replication.

**Dominant Negative EPS15 Mutants Inhibit HEV71 Entry into Cells**—Molecular inhibitors in the form of dominant-negative mutants were also used to further confirm the role of clathrin-mediated endocytosis in the infectious entry of HEV71. The use of dominant-negative mutants may provide an alternative way to analyze the specific function of defined pathways within the cells. Eps15, a protein that binds to AP-2 (adaptor protein 2), has been shown to be necessary for internalization through clathrin-coated pits (36). However, the deletion of the EH domain of Eps15 produced a dominant-negative mutant protein that disrupts the formation of clathrin-coated pits and inhibits the uptake of transferrin, a cellular marker of clathrin-mediated endocytosis (37).

In this study, RD cells were first transfected with either pEGFP-Eps15Δ95/295 plasmid (dominant-negative mutant of Eps15 protein), pEGFP-Eps15ΔIIΔ Δ2 plasmid (this construct lacks the AP2-binding sites of Eps15 and was used as a control that did not inhibit clathrin-mediated endocytosis), or pEGFP-C2 plasmid (coding for green fluorescent protein (GFP) as an internal control) for 48 h (37) before being infected with HEV71 at 4 °C for 1 h (to enable virus binding, after which the temperature was shifted rapidly to 37 °C for internalization) and subsequently processed for immunofluorescence staining and microscopic imaging. As shown in Fig. 6a, HEV71 particles (stained by anti-HEV71 antibodies conju-
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FIGURE 4. Bio-imaging analysis of the interaction of clathrin molecules with HEV71 particles. A double-labeled immunofluorescence assay was performed to track the entry process and cellular localization of HEV71 within infected cells at fixed time points. a, no co-localization between HEV71 viral particles (arrows) and clathrin molecules was observed at 0-min p.i. b, HEV71 viral particles began co-localizing with clathrin molecules (indicated by light gray arrows) at 5 min p.i. c, more co-localization occurred at 10-min p.i. d, co-localization was observed forming around the perinuclear region at 15 min p.i. e, enhanced co-localization was seen at 20 min p.i. f, co-localization between HEV71 viral particles and clathrin molecules was completed at 30 min p.i. Cell nuclei were stained blue with DAPI.

FIGURE 5. Ultrastructural analysis of HEV71 infectious entry. To visualize synchronized HEV71 entry, RD cells were incubated with HEV71 at 4 °C for 1 h, after which they were warmed to 37 °C before processing for transmission electron microscopy analysis. a, at 0-min of warming to 37 °C, HEV71 particles (indicated by gray arrows) were seen attached to the cell surface. b, at 5 min after warm-up, HEV71 particles were observed within invaginations of the plasma membrane (clathrin pits indicated by white arrows). c, at 10 min of warm-up, HEV71 particles were seen enclosed within clathrin-coated vesicles. d, at 15–20 min of warm-up, vesicles containing numerous HEV71 virus particles were observed.

gated to TR (arrows)) were observed to attach onto the plasma membrane without visible signs of viral entry, with punctuated staining of the pEGFP-Eps15Δ95/295 plasmid within the cells. In contrast, internalization of HEV71 (arrows, speckled staining) occurred within the cytoplasm of the pEGFP-Eps15ΔIIIΔ2 or GFP-expressing cells (Fig. 6, b and c, respectively). These differences were further proven upon enumerating the percentage of viral entry into cells. Although the percentage of viral antigen-positive cells in the pEGFP control and pEGFP-Eps15ΔIIIΔ2 was similar to that of the mock-transfected cells, only ~40% of cells transfected with the dominant negative mutant plasmid (pEGFP-Eps15Δ95/295) were viral antigen-positive (Fig. 6d). These results provide further evidence that HEV71 entry into cells takes place through clathrin-mediated endocytosis.

Drug Inhibition Analysis of Entry Pathways—The results presented above suggested the involvement of a clathrin-mediated endocytic pathway in HEV71 entry into RD cells. To affirm these results, RD cells were pretreated with drugs that selectively inhibit clathrin-dependent endocytosis (chlorpromazine and cytochalasin B), caveolae-dependent endocytosis (filipin, nystatin, and methyl-β-cyclodextrin), and macropinocytosis EIPA (Table 2). Possible drug-induced cytotoxic effects were assessed by MTT cell viability assays and observation of cellular morphological changes. Minimal cellular cytotoxicity was observed in drug-treated cells throughout the spectra of concentrations used in these experiments (Figs. 7 and 8).

Clathrin-mediated entry pathway can be inhibited by drugs such as chlorpromazine and cytochalasin B. Chlorpromazine is a known clathrin-dependent endocytosis inhibitor (38), whereas cytochalasin B induces depolymerization of actin filaments (39). F-actin dynamics have been shown to be necessary for various stages of clathrin-coated vesicle formation, including coated pit formation, constriction, and internalization (40). Both chlorpromazine and cytochalasin B are specific in inhibiting clathrin-mediated endocytosis (even at the highest concentrations used in this study) as illustrated by the inhibition of transferrin (a specific maker for clathrin-mediated endocytosis) uptake (supplemental Fig. 1). Pretreatment of RD cells with increasing concentrations of either chlorpromazine (Fig. 7a) or cytochalasin B (Fig. 7b) revealed significant dose-dependent inhibition of HEV71 infection. More than 80% inhibition at 20 μM chlorpromazine and 50% inhibition at 4 μM cytochalasin B can be observed, further confirming HEV71 entry via clathrin-mediated endocytosis.

To eliminate the involvement of other entry pathways during HEV71 infection, drugs known to inhibit caveola-mediated endocytosis and macropinocytosis were also evaluated on RD cells. Inhibitors of caveola-dependent endocytosis used in this study include filipin, which disrupts caveola-mediated endocytosis by binding specifically to cholesterol abundantly found in caveolae (41, 42), nystatin, which binds to sterols (42), and methyl-β-cyclodextrin, which depletes the cells of cholesterol, thus disrupting caveola formation (42). Treatment of filipin (Fig. 8a) and nystatin (Fig. 8b) did not exhibit inhibitory effects on HEV71 infection at all drug concentrations used, whereas cells treated with methyl-β-cyclodextrin displayed slight inhibition of ~30% at 7.5 mM (Fig. 8c). These results suggest minimal involvement of caveola-mediated endocytosis upon HEV71 infection in RD cells. Furthermore, an inhibitor of macropinocytosis, EIPA (43, 44), failed to show inhibitory
effects on HEV71 infection; instead, the blocking of the macropinocytotic pathway by EIPA seemed to enhance HEV71 infection (Fig. 8).

Endocytic Trafficking of Internalized HEV71 Particles within Cells—To further substantiate the role of clathrin-mediated endocytosis in HEV71 infection, the importance of endosomal trafficking of internalized HEV71 particles was tracked via microscopic analysis. Within 15 min p.i., a double-labeled immunofluorescence assay with anti-HEV71 VP1 protein and anti-EEA1 antibodies showed co-localization of HEV71 particles with early endosomes, suggesting that the virus particles were translocated to the early endosomes after clathrin-mediated endocytosis (Fig. 9, upper panels). By 30 min p.i., HEV71 particles were found mainly in vesicles that were stained with anti-LAMP1 antibodies (Fig. 9, upper panels), suggesting that HEV71 were localized to the late endosomes by this time point. The fluorescent staining was more intense at the perinuclear region. Conversely, minimal co-localization was observed between HEV71 viral particles and late endosomes at 15 min p.i. The majority of the HEV71 particles (arrows) are localized near the plasma membrane of the cell (Fig. 9a, lower panels). Similarly, minimal co-localization

### TABLE 2

Concentrations and functions of inhibitory drugs used in this study

| Drug                          | Concentrations used | Function                              |
|-------------------------------|---------------------|---------------------------------------|
| Chlorpromazine                | 2, 10, 20, 30 μM    | Inhibitor of clathrin-dependent endocytosis |
| Cytochalasin B                | 0.2, 1, 2, 3 μM     | Inhibitor of actin polymerization     |
| Filamin                       | 0.5, 1, 1.5, 2 μg/ml | Inhibitor of caveolin-dependent endocytosis |
| Nystatin                      | 5, 10, 20, 40 μg/ml | Increases permeability of cell membrane of sensitive fungi by sterol binding |
| Methyl cyclodextrin-β-cyclodextrin | 2.5, 5, 7.5, 10 mM | Inhibitor of lipid raft synthesis and caveolin-dependent endocytosis |
| 5-((N-Ethyl-N-isopropyl)amo)lride | 10, 25, 50, 100 μM | Selective blocker of Na⁺/H⁺ antiport |
| Bafilomycin A                 | 0.01, 0.05, 0.1 μM | Inhibitor of vacuolar H⁺-ATPase     |
| Methyl cyclodextrin-cyclodextrin | 2.5, 5, 7.5, 10 mM | Inhibitor of lipid raft synthesis and caveolin-dependent endocytosis |
| 5-((N-Ethyl-N-isopropyl)amo)lride | 10, 25, 50, 100 μM | Selective blocker of Na⁺/H⁺ antiport |
| Concanamycin A                | 20, 40, 60, 80, 100 nM | Inhibitor of acidification of organelles and perforin-mediated cytotoxicity |

FIGURE 6. Inhibition of HEV71 entry into cells by dominant negative Eps15 mutants. RD cells were first transfected with pEGFP-Eps15Δ95/295 plasmid, pEGFP-Eps15DIIIΔ2, or pEGFP-C2 plasmid for 48 h before HEV71 infection at 4 °C for 1 h to allow for virus binding. The temperature was subsequently shifted rapidly to 37 °C for internalization, and the cells were then processed for immunofluorescence staining and microscopic imaging. a, HEV71 viral particles (arrows) were observed to attach on the surface of cell expressing Eps15 dominant negative mutant of pEGFP-Eps15Δ95/295. HEV71 viral particles were observed within the cytoplasm of pEGFP-Eps15DIIIΔ2 (b) and pEGFP-C2 (c)-expressing cells. The expression of GFP-tagged plasmids emitted the green color observed (indicated here as white dots). Cell nuclei were stained blue with DAPI (indicated here in dark gray). d, the histogram represents the inhibition of virus entry determined by the percentage virus antigen-positive cells (with S.E. bars) against pEGFP-Eps15Δ95/295 plasmid (dominant-negative mutant), pEGFP-Eps15DIIIΔ2, pEGFP-C2 plasmid (internal control), and mock-transfection control. The plots shown are representative of three independent experiments. The asterisk indicates p values of ≥0.05 by Student’s t test.
can be observed between HEV71 viral particles and early endosomes at 30 min p.i. Most of the HEV71 particles (arrows) were observed as green particles in the overlay micrograph, indicating a lack of co-localization with the early endosomes (Fig. 9b, lower panels). Thus, these data further indicated that HEV71 particles do transit from early to late endosomes during the 15–30 min infection period.

Some viruses require low endosomal pH, maintained by vacuolar proton-ATPase, to uncoat and release its viral genome for replication (45). Assays to examine pH-dependent entry of HEV71 in the presence of vacuolar proton-ATPase inhibitors, bafilomycin A1 and concanamycin A, were performed. Bafilomycin A1 is a potent and specific inhibitor of vacuolar proton-ATPase that inhibits endosome and lysosome acidification (46, 47), whereas concanamycin A inhibits acidification of organelles and perforin-mediated cytotoxicity (48, 49). Both bafilomycin A1 and concanamycin A are highly specific in inhibiting endosomal acidification (even at the highest concentrations used in this study) as illustrated by the absence of acridine orange (granular orange fluorescence) staining (a specific maker for acidification of endosomes) (supplemental Fig. 2). Minimal cellular cytotoxicity was observed in drug-treated cells throughout the spectra of concentrations used in these experiments (Fig. 10). Pretreatment of cells with bafilomycin A1 (Fig. 10a) and concanamycin A (Fig. 10b) showed dose-dependent inhibition of HEV71 infection, with more than 60% inhibition at 0.5 μM and 80 nM, respectively, thus strongly suggesting that low endosomal pH is required for the infectious entry of HEV71 into RD cells.

**DISCUSSION**

The application of RNA interference-based screens offers an alternative route to conventional means in identifying cellular proteins or components of endocytic pathways that mediate the infectious entry of HEV71. Despite its recent discovery, the application of RNA interference has already profoundly enhanced the study of large scale loss-of-functional gene analysis in a rapid and cost-effective manner. For this purpose we have established and validated an RNA interference screening platform assay that allows identification of host proteins involved in the endocytic and membrane trafficking process mediating the infectious entry of HEV71 into cells. A siRNA library screening using a similar subset of the human membrane trafficking library has previously been used successfully to screen for endocytic genes involved in respiratory syncytial virus infection (50).

In this study clathrin-mediated endocytosis has been identified as the main pathway of HEV71 internalization, with knockdown of clathrin heavy chains (CLTC and CLTCL1) and AP2A1, a subunit of the AP2 coat assembly protein complex linking clathrin to receptors in coated vesicles, significantly inhibiting virus uptake by up to 60% and more. In addition, knockdown of several other endocytic proteins including ARRB1, ARPC5, PAK1, RAB3A, SYNJ1, ROCK1, and WASF1 also significantly reduced the uptake of HEV71 into RD cells (Figs. 1–3 and Table 1).

The functional role of clathrin-mediated endocytosis in mediating HEV71 entry was independently verified by drug inhibition assays, ultrastructural analysis, and microscopic cellular localization analysis as well as transfection of cells with a well characterized dominant-negative mutant form of Eps15. Treatment with chlorpromazine, a known specific inhibitor of clathrin-mediated endocytosis, exhibited effective inhibition of HEV71 infection (Fig. 7a). Co-localization of HEV71 particles with clathrin molecules could be observed within the first 5 min post-infection (Fig. 6). Eps15 is an accessory factor that associates with AP2 complex and is essential for the formation of clathrin-coated pits at the plasma membrane (51). The dominant-negative form of Eps15 has been shown to effectively block clathrin-mediated endocytosis but not other endocytic processes by caveolae or macropinoscytosis (37). The lack of co-localization between GFP-tagged dominant negative EPS15 and HEV71 viral particles indicated that clathrin-mediated endocytosis was required for HEV71 viral entry. To further verify the effect of clathrin knockdown on HEV71 infection, a secondary assay was performed with increasing siRNA concentrations to knock down CLTC, a clathrin heavy chain gene. A dose-dependent decrease in HEV71 infection as observed, thus, indicated the involvement of clathrin in HEV71 infection (Fig. 3).

Furthermore, p-selectin glycoprotein ligand-1 was recently discovered as a functional receptor for HEV71 in leukocytes (11). Setiadi et al. (52–54) found P-selectin to be rapidly internalized in clathrin-coated pits, thus, enhancing the adhesive
function of endothelial cells. Similarly, another recently discovered cellular receptor for HEV71, scavenger receptor B2 (10), had been earlier found to undergo endocytosis by a clathrin-dependent mechanism (55). These findings further substantiate our present study that HEV71 viral particles enter via clathrin-mediated endocytosis.

The silencing of the genes PAK1, PIK3CG, PIK3C2G, and ELKS, involved in PI3K/Akt or JNK or NK-KB signaling pathways, also caused a reduction in viral infection. It had previously been reported that HEV71-induced VCAM-1 expression via PDGF receptor, PI3K/Akt, p38MAPK, JNK, and NK-KB in vascular smooth muscle cells (56). Silencing of other genes involved in vesicle transport such as ARFIP2, RAB3A, and RAB6B also led to a decrease in viral infection, indicating the importance of vesicle transport in HEV71 infection. These genes can be further analyzed to evaluate their potential roles in HEV71 infection.

Drugs used to inhibit caveolae-mediated endocytosis are methyl-β-cyclodextrin, filipin, and nystatin, with treatment of the latter two failing to exhibit any inhibitory effects on HEV71 infection at all drug concentrations tested. In contrast, methyl-β-cyclodextrin-treated cells showed slight inhibition of ~30% at 7.5 mM. These results suggested that there was minimal involvement of caveolae-mediated endocytosis HEV71 infection in RD cells. The depletion but not binding of cholesterol inhibiting HEV71 infection could be due to the importance of cholesterol in the formation of endocytic vesicles. Grimmer et al. (57) found that cholesterol depletion inhibited the transport of endosomes to Golgi bodies, and cholesterol is required for the formation of endocytic vesicles. Inhibition of HEV71 infection could, thus, result from defective formation of endocytic vesicles. Furthermore, complete inhibition of infection is highly unlikely, as HEV71 may be capable of entering susceptible cells via alternative pathways in the event of their primary route of entry being inhibited. Many viruses are known to exhibit this feature during infectious entry into cells or are able to enter cells via multiple routes, such as Simian virus 40 (caveolar and cholesterol-dependent pathways), influenza (both clathrin-dependent and clathrin-independent routes), echovirus 1 (caveolar and dynamin-2 dependent pathways) (1), and adenovirus (clathrin-mediated endocytosis and macropinocytosis) (58). Nevertheless, as shown in this study, clathrin-mediated endocytosis is found to be the primary route of entry for HEV71 into cells.

EIPA was used to target the macropinocytotic pathway. EIPA was previously shown to inhibit propagation of human rhinovirus 2 and Coxsackie B3 virus in HeLa cells (59). In this study, EIPA failed to inhibit HEV71 infection. On the contrary, the blocking of macropinocytosis using EIPA seemed to enhance HEV71 infection, possibly due to the activation of reflex mechanisms in RD cells such that endocytic uptake was increased through other pathways. EIPA could also be sufficiently nonspecific to be able to trigger the activation of cellular mechanisms, thus, resulting in an increase in virus uptake.

In addition, the cytoskeleton, comprising of actin filaments and microtubules, also plays a dynamic role in endocytic trafficking, with both up-and down-regulation of actin or microtubule polymerization shown to affect endocytic kinetics (60).

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**FIGURE 8. Effects of treatment of caveolae-mediated endocytic inhibitors (a–c) and macropinocytic inhibitor (d) on HEV71-infected RD cells.**

RD cells were pretreated with varying concentrations of filipin (0.5, 1, 1.5, 2, and 3 μg/ml) (a), nystatin (5, 10, 20, and 40 μM) (b), methyl-β-cyclodextrin (2.5, 5, 7.5, and 10 mM) (c), and EIPA (10, 25, 50, and 100 μM) (d) for 2 h at 37 °C before HEV71 infection. At 12 h p.i., the infected cells were processed for immunofluorescence assay. Histograms represent the inhibition of virus entry determined by the percentage virus antigen-positive cells with S.E. bars against drug concentrations. Cell viability upon drug treatments was unaffected as represented by the line graphs. The plots shown are representative of three independent experiments. The asterisk indicates p values of ≤0.05 by Student’s t test. UT, untreated cells; SC, solvent control.
Actin filaments are required for the initial uptake of ligands via clathrin-coated pits and subsequent degradative pathway, whereas microtubules are involved in maintaining the endosomal traffic between peripheral early and late endosomes (60, 61). Actin cytoskeleton is shown to be closely associated with clathrin-coated pits, and actin polymerization may be involved in moving endocytic vesicles into cytosol after they are pinched off from the plasma membrane (62). The actin binding molecular motor, myosin VI, was also recently shown to mediate clathrin endocytosis (63). Disruption of actin filaments can have a dramatic effect on receptor-mediated endocytosis (60). In this study, the siRNA knockdown of ARPC5, ARRB1, and WASF1 genes that are important in actin polymerization have resulted in the reduction of HEV71 infection. Arrestin B1 (ARRB1) is involved in the desensitization of receptors by targeting them to clathrin-coated vesicles through a RhoA and actin-dependent mechanism (30). WASF1 is an important downstream effector molecule involved in the transmission of signals from tyrosine kinase receptors and small GTPases to the actin cytoskeleton. WASF family members also play important roles late in clathrin-coated pit formation by coupling to the ARP2/3 actin-regulating complex and may act to move the coated vesicles through the cell (31). Therefore, these results suggest a potential role of these genes in regulating HEV71 endocytosis upon binding to putative cellular receptors. The involvement of actin in mediating HEV71 entry was further confirmed by treatment with cytochalasin B. Cytochalasin B, an actin-disrupting drug, specifically affects the actin cytoskeleton by preventing its proper polymerization into microfilaments and promoting microfilament disassembly (64). Disruption of actin filaments was shown to inhibit HEV71 infection in a dose-dependent manner.

Some viruses such as Semliki Forest virus, vesicular stomatitis virus, vaccinia virus, adenovirus, and poliovirus utilize the pH gradient to promote entry into their host cells. Poliovirus permeabilizes human cells during virus entry through the uncoating of virus particles and the functioning of the vacuolar proton-ATPase (65, 66). Inhibition of vacuolar proton-ATPase would disrupt endosomal pH (45). In this study, cells treated with vacuolar proton-ATPase inhibitors bafilomycin A1 and concanamycin A showed dose-dependent reductions in infection, thus understating the importance of low endosomal pH in HEV71 infection.
Infectious Entry of Human Enterovirus 71

This current work has highlighted the power of using specific subset siRNA libraries to identify important cellular genes and pathways that mediate the process of endocytosis and endocytic trafficking of HEV71 infection. The study has provided much in-depth analysis of human genes that is essential for endocytosis as well as the endocytic trafficking of internalized HEV71 for productive infection. Understanding these processes may allow specific cellular pathways or molecular mechanisms to be targeted pharmacologically to inhibit the entry of HEV71 that uses the route for infection. Furthermore, the development of drugs, dominant negative mutants, and RNAi therapeutic approach targeting virus entry can be effective in disease intervention of HEV71 or other related enteroviruses. Indeed, the current screening assay can be adapted and optimized for the screening of putative antiviral agents and pharmacological compounds against HEV71 infection.

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