Molecular modeling, organ culture and reverse genetics for a newly identified human rhinovirus C

Yury A Bochkov1, Ann C Palmenberg2, Wai-Ming Lee1, Jennifer A Rathe3, Svetlana P Amineva1, Xin Sun4, Thomas R Pasti5, Nizar N Jarjour6, Stephen B Liggett3 & James E Gern1,6

A recently recognized human rhinovirus species C (HRV-C) is associated with up to half of HRV infections in young children. Here we propagated two HRV-C isolates ex vivo in organ culture of nasal epithelial cells, sequenced a new C15 isolate and developed the first, to our knowledge, reverse genetics system for HRV-C. Using contact points for the known HRV receptors, intercellular adhesion molecule-1 (ICAM-1) and low-density lipoprotein receptor (LDLR), inter- and intraspecies footprint analyses predicted a unique cell attachment site for HRV-Cs. Antibodies directed to binding sites for HRV-A and -B failed to inhibit HRV-C attachment, consistent with the alternative receptor footprint. HRV-A and HRV-B infected HeLa and WisL cells but HRV-C did not. However, HRV-C RNA synthesized in vitro and transfected into both cell types resulted in cytopathic effect and recovery of functional virus, indicating that the viral attachment mechanism is a primary distinguishing feature of HRV-C.

HRVs are positive-strand RNA viruses in the family Picornaviridae. They share a common genome organization, and isolates are typically classified into one of three species (A, B and C) according to phylogenetic sequence criteria1. HRV is the most frequent cause of the common cold, but recent data have shown that HRV infection has pathogenic potential beyond rhinosinusitis. It is now recognized that 50–85% of asthma exacerbations are due to HRV infections2–4, and wheezy illnesses in infancy caused by HRV are associated with a high risk of developing childhood asthma5. HRV infections of all types are major contributors to morbidity associated with exacerbations of other chronic lung diseases such as cystic fibrosis and chronic obstructive pulmonary disease6,7.

Recently, partial or full genome sequencing has been used to place clinical isolates within the 99 types of HRV-A and HRV-B. However, some cases, often associated with children hospitalized with lower respiratory tract disease, began to appear where the identified HRV sequences were not consistent with those of known HRV types8–14. Full-genome sequencing has shown the distinct nature of these HRVs, which are now designated as HRV-C1,15,16. The pathogenesis and antigenic variability associated with HRV-C, the mechanism of viral attachment to host cells and potential treatments have been elusive because, unlike HRV-A and HRV-B, HRV-C has not been successfully propagated in any cell type in vitro17.

To address these fundamental questions about HRV-C biology, it was first necessary to develop a functional culture system. We now report the characterization and complete genome sequencing of an HRV-C isolate (HRV-C15 type) that can be grown in mucosal organ culture. Bioinformatic comparisons of this new genome and other HRV-C sequences showed unique composition profiles in the putative ICAM-1 and LDLR footprint locales, inconsistent with all known major and minor group virus–receptor interactions. Full-length HRV-C15 RNA synthesized in vitro from a cDNA clone was infectious when transfected into cultured cell lines. We have used this methodology to initiate descriptions of the virus’s biology, tissue tropism, the replication cycle and response to antiviral and antireceptor compounds.

RESULTS

HRV-C amplification in sinus mucosal organ culture

We introduced several specimens of nasal lavage fluid (NLF) with high copy number of HRV-C RNA into multiple human fibroblast and epithelial cell lines (WI–38, WisL, BEAS-2B, A549 and HeLa) susceptible to HRV-A and HRV-B infection and primary cultures of human lung fibroblasts and bronchial, sinus and adenoidal epithelial cells from several donors. Primary epithelial cultures included cell monolayers, differentiated cells (air-liquid interface method) and primary bronchial epithelial (PBE) cells treated with interleukin-13 to induce goblet cell metaplasia. Assigning an increase in the quantitative RT-PCR (qRT-PCR) signal as an indicator of HRV-C replication, we were successful in none of these attempts, including those where multiple serial blind passages were undertaken.

Reasoning that organ culture might best mimic natural replication circumstances, we cultured surgery-derived byproduct tissues (from multiple anonymous donors) from sinus mucosa, adenoids, tonsils and nasal polyps. After 1–3 d, viable tissues (continuous ciliary activity) negative for common respiratory viral pathogens were inoculated with representative laboratory HRV-A strains that use ICAM-1 (A16)

1Department of Pediatrics, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin, USA. 2Institute for Molecular Virology, University of Wisconsin–Madison, Madison, Wisconsin, USA. 3Cardiopulmonary Genomics Program, University of Maryland School of Medicine, Baltimore, Maryland, USA. 4Laboratory of Genetics, University of Wisconsin–Madison, Madison, Wisconsin, USA. 5Department of Surgery, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin, USA. 6Department of Medicine, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin, USA. Correspondence should be addressed to Y.A.B. (yabochkov@wisc.edu).

Received 6 July 2010; accepted 1 December 2010; published online 10 April 2011; doi:10.1038/nm.2358
images showed a patchy distribution of HRV-C15–positive cells with probe specific for the HRV-C15 plus-strand RNA. We observed position using whole-mount procedures and a digoxigenin-labeled RNA effects. When we used similar doses (1 × 10^9 vRNA copies per ml) increased viral RNA by up to 100 fold, even at low (1 × 10^8 vRNA copies per ml) dose (Fig. 1a). Repeated tests with tonsils and nasal polyps (data not shown) and adenoid tissues (Fig. 1b) showed lower (fivefold or less) increase in vRNA. Furthermore, a NLF specimen containing 2 × 10^9 vRNA copies per ml of a field HRV-A isolate (A78) responded similarly to laboratory strains and we passaged it serially into three tissue specimens (Fig. 1c).

Using this approach, we inoculated an HRV-C15 isolate from a specimen of NLF (2.6 × 10^8 vRNA copies per ml) into sinus organ culture, and high-level viral replication was maintained through seven serial passages (Fig. 1d). During each passage, we observed partial inhibition of ciliary beating at 48–72 h, coinciding with the peak of virus replication, but we noted no other specific cytopathic effects. When we used similar doses (1 × 10^9 vRNA copies per ml) of HRV-C15 (Fig. 1e) and HRV-A16 (Fig. 1a) for infection, the growth kinetics were equivalent to each other in high-yield cultures. Serial passage of a second HRV-C isolate (W23) was also accomplished by a similar approach (data not shown). Our attempts to adapt these isolates grown in organ culture to multiple cell lines (listed above) were not successful.

**HRV-C localization in sinus epithelium**

We analyzed HRV-C15 replication in sinus tissues by *in situ* hybridization using whole-mount procedures and a digoxigenin-labeled RNA probe specific for the HRV-C15 plus-strand RNA. We observed positive hybridization in both of the inoculated sinus organ cultures, but it was absent in the mock-infected cultures (Fig. 2a). Higher-resolution images showed a patchy distribution of HRV-C15–positive cells with the highest signal intensities at the edges of tissues (Fig. 2b), suggesting that mechanical injury may increase susceptibility to infection, perhaps by exposing subapical epithelial layers expressing virus receptors.

The two tissue specimens inoculated with HRV-C15 differed in signal intensity and corresponding virus yield. In the tissue (dn17) with the lower yield (1 × 10^7 vRNA copies per ml) the RNA signal was limited to small area at the fragment edge, whereas the higher-yield tissue (dn13, 2 × 10^8 vRNA copies per ml) gave many foci, with a greater total density of infected cells (Fig. 2b). In tissue cross-sections, the positive signals originated mainly from middle and upper epithelial layers (Fig. 2c). An outermost sloughing of ciliated cells (Fig. 2c) was particularly apparent in the higher-titer tissues. There was no evidence for infection of cells deeper than the epithelial layer in either sample.

**Genome analysis of HRV-C15**

From the organ culture supernatant fluid (passage 4), we sequenced the complete HRV-C15 genome consisting of 7,111 bases. The single open reading frame encodes a polypeptide of 2,153 amino acids (the longest among the reported HRV-C strains), flanked by 5′ and 3′ untranslated regions (UTR) of 607 and 45 nucleotides, respectively. The RNA and deduced polypeptide sequences share characteristic HRV-C features such as a putative internal *cis*-acting replication element (*cre*) located within viral protein 2 (VP2) (1B), a Met67-Ser68 cleavage site at the VP4–VP2 junction, species-specific insertions and deletions in the VP1 region, and an isoleucine at the termination of the 3D polymerase. The full sequence itself shares only 67–70% aligned nucleotide identity with other members of the HRV-C species and less than 60% with HRV-A and HRV-B strains.

After our recent complete genome sequencing of the known HRV’s, we performed an alignment based on crystal structure superimposition and optimal energy RNA configurations, which we...
HRV-C relative to ICAM-1 and LDLR footprints

Differences in overall capsid sequence together with the failure of HRV-C15 to replicate in standard cell lines suggest that HRV-C viruses have unique growth requirements, possibly including binding to distinct cellular receptors. Notably, HRVs have great plasticity and rapid evolution, as demonstrated by the adaptation of major-group HRV (A89) to use another receptor (heparan sulfate) for cell entry20. We were therefore interested in determining whether HRV-C has sequence consistency in the same sites as receptor footprints mapped for HRV-A and HRV-B21–24.

For each alignment position harboring known contacts for ICAM-1 (HRV-B14, HRV-A16 or human coxsackievirus A21) or LDLR (HRV-A2), we recorded the group amino acid populations and then compared them (as groups or internally and pairwise, Supplementary Tables 1 and 2). When we compared the ICAM-1 binders among themselves, about 40% of identified footprint positions showed strong (r > 0.6) conservation composition (for example, Thr180 in VP3 of HRV-B14 (14-T-3-180)). Some of these positions were conserved among all HRV’s (for example, B14-P-1-155 and A16-G-1-148). In contrast, other sites showed a compositional bias between receptor groups (for example, B14-P-3-178). Figure 4a depicts the most abundant residues at each footprint position when the HRV-A and HRV-B sequences were subdivided by ICAM-1 and LDLR receptor groups and compared to the HRV-C sequences. There was little commonality between HRV-C and either the ICAM-1 or the LDLR contact points. For example, six of seven LDLR footprint positions and 47 of 50 ICAM-1 footprint locations were rejected by both statistical methods as compositional matches to the HRV-C (Supplementary Table 2). Overall, the analysis predicts the HRV-C viruses use a different receptor with a distinct compositional footprint from HRV-A and HRV-B viruses.

Inhibition of HRV-C15 attachment

We then tested the distinct HRV-C receptor footprint prediction in cell culture, using antibodies to block attachment of HRV to ICAM-1 or LDLR. In the absence of these antibodies, binding of HRV-A1 and HRV-A16 to both HeLa and PBE cells was two to three logs higher compared to HRV-C15 (Fig. 4b,c). Preincubation of HeLa cells with an ICAM-1–specific antibody reduced binding of major-group HRV-A16 but not minor-group HRV-A1 (Fig. 4b). In cultures of PBE cells, only HRV-A1 was inhibited by preincubation with an LDLR-specific antibody (Fig. 4c). When the experiment was carried out with matched tissue snippets from sinus organ cultures, binding of HRV-C15 was similar to that of the other two viruses. Receptor-specific antibodies inhibited attachment of the respective major (80% inhibition) and minor (58% inhibition) viruses, but there was no effect on HRV-C15 binding (Fig. 4d).

HRVs have been divided into two groups (A and B) on the basis of their sensitivity to different antiviral compounds known as capsid-binding agents25. These compounds (for example, WIN56291 and WIN52084) intercalate into a hydrophobic pocket within the VP1 protein and prevent viral attachment or uncoating by deforming the receptor binding site or inhibiting a requisite conformational change26–28; thus the primary sequence dictating shape and charge of the pocket determine the response to specific antivirals. Preincubation of virus inoculums with WIN56291 inhibited propagation of HRV-A16 (group B) and HRV-C15, but not HRV-B14 (group A), in sinus organ culture (Fig. 4e).

Reverse genetics system for HRV-C

The results of the viral binding studies suggest that HRV-C15 does not replicate in HeLa and PBE cells, which are readily infected with HRV-A and HRV-B, because these cells do not express a cell surface receptor for the virus. Alternatively, these cells could lack an intracellular protein that is required for HRV-C replication. To test this hypothesis, we developed a plasmid (pC15) for cell transfection (as opposed to viral infection) by cloning a full-length cDNA copy of the HRV-C15 genome into the plasmid vector pMJS (ref. 29) directly downstream of the T7 RNA polymerase promoter (Supplementary Fig. 2). The cloned HRV-C15 sequence was identical to the complete genome sequence of passage four HRV-C15 (Fig. 1d) and contained a 3′-end poly(A59) tail.

Supplementary Materials

Figure 3 Neighbor-joining phylogenetic tree based on full-length nucleotide sequences of HRV-A, HRV-B and HRV-C. Complete 5′ and 3′ UTR sequences and the first and second codon positions of the open reading frames were considered (MEGA 4.1 software). All major nodes are labeled with bootstrap values (% of 1,000 replicates). HRV-A and HRV-B reference strain accession numbers correspond to those published previously1. The HRV-C15 (W10) genome sequence in this study is shown in bold type. Branch lengths are proportional to nucleotide similarity (ρ distance). Human enteroviruses (HEV) are included as an outgroup. HRV-C types (followed by strain designations and accession numbers) correspond to the recent classification proposal31.

Figure 4a–d Depicts the most abundant residues at each footprint position when the HRV-A and HRV-B sequences were subdivided by ICAM-1 and LDLR receptor groups and compared to the HRV-C sequences. There was little commonality between HRV-C and either the ICAM-1 or the LDLR contact points. For example, six of seven LDLR footprint positions and 47 of 50 ICAM-1 footprint locations were rejected by both statistical methods as compositional matches to the HRV-C (Supplementary Table 2). Overall, the analysis predicts the HRV-C viruses use a different receptor with a distinct compositional footprint from HRV-A and HRV-B viruses.

Inhibition of HRV-C15 attachment

We then tested the distinct HRV-C receptor footprint prediction in cell culture, using antibodies to block attachment of HRV to ICAM-1 or LDLR. In the absence of these antibodies, binding of HRV-A1 and HRV-A16 to both HeLa and PBE cells was two to three logs higher compared to HRV-C15 (Fig. 4b,c). Preincubation of HeLa cells with an ICAM-1–specific antibody reduced binding of major-group HRV-A16 but not minor-group HRV-A1 (Fig. 4b). In cultures of PBE cells, only HRV-A1 was inhibited by preincubation with an LDLR-specific antibody (Fig. 4c). When the experiment was carried out with matched tissue snippets from sinus organ cultures, binding of HRV-C15 was similar to that of the other two viruses. Receptor-specific antibodies inhibited attachment of the respective major (80% inhibition) and minor (58% inhibition) viruses, but there was no effect on HRV-C15 binding (Fig. 4d).

HRVs have been divided into two groups (A and B) on the basis of their sensitivity to different antiviral compounds known as capsid-binding agents25. These compounds (for example, WIN56291 and WIN52084) intercalate into a hydrophobic pocket within the VP1 protein and prevent viral attachment or uncoating by deforming the receptor binding site or inhibiting a requisite conformational change26–28; thus the primary sequence dictating shape and charge of the pocket determine the response to specific antivirals. Preincubation of virus inoculums with WIN56291 inhibited propagation of HRV-A16 (group B) and HRV-C15, but not HRV-B14 (group A), in sinus organ culture (Fig. 4e).
RNA transcripts synthesized in vitro from pC15 induced cytopathic effects when transfected into both HeLa and WisL (fetal lung fibroblast) cell monolayers, and these effects were similar to those induced by parallel transfection of HRV-A16 RNA from pR16.11 (ref. 30) (Fig. 5a). After transfection of lower doses of RNA, only HRV-A16 RNA caused CPE progression over time, confirming that the newly synthesized HRV-C15 virus cannot spread from transfected cells to neighboring (not transfected) cells (Supplementary Fig. 3). We inoculated HRV-C15 virus recovered 24 h after transfection (1 × 10^5 vRNA copies per ml) into mucosal organ cultures, and the growth kinetics of progeny virus (Fig. 5b) was similar to those of the parental HRV-C15 (Fig. 1e). Examination of concentrated cell lysates by electron microscopy revealed intact HRV virions (about 30 nm in diameter) as well as the expected empty capsids (Fig. 5c).

DISCUSSION

HRV-C viruses do not grow in standard cell culture used for virus isolation and have only been detected by molecular assays. Since 2006, over 60 types of this species have been implicated in both upper and lower respiratory tract infections, particularly in children and in individuals with chronic respiratory diseases. We employed human organ culture of sinus mucosa to propagate in vitro and study the basic biologic properties of an HRV-C clinical isolate (C15 type). Virus replication was demonstrated by an increase in viral RNA, which localized to focal areas in epithelial cell layers. The high-titer virus obtained after serial passage in sinus organ culture allowed complete genome sequencing and cloning of the full-length cDNA copy of the HRV-C15 genome into a plasmid vector. RNA transcripts synthesized in vitro from this clone were infectious when transfected into cell lines resistant to HRV-C15 infection, providing an efficient system for production of recombinant HRV-C.
Our experimental findings and bioinformatic predictions indicate unique HRV-C receptor-binding specificity.

Mucosal organ culture is the only model to date that supports HRV-C infection in vitro. We and others have tried to grow these newly discovered rhinoviruses from numerous NLF samples in multiple cell cultures without success, indicating unique growth requirements. Moreover, our attempts to adapt high-titer HRV-C15 virus grown in sinus cultures or recombinant virus from transfected cells to a cell line were not successful. HRV-C replication in sinus tissue seems to be limited to the epithelium, and hybridization signals were associated with both nonciliated and ciliated cells. The degree of virus amplification (high or low virus yields) showed considerable variability, which could be related to the condition of the epithelium (all specimens were from individuals with sinusitis), other underlying diagnoses (for example, allergy or asthma) or perhaps the status of local innate immune elements. Furthermore, the expression of HRV-C–specific receptors on epithelial cells may depend on factors found in vivo such as microbial products or interactions with other types of cells (for example, dendritic cells or lymphocytes) present in human airways.

Footprints of virus capsid residues that make contact with the cellular receptors have been determined for both major and minor HRV groups. Sequence comparisons within the mapped receptor footprints show variability and a tendency toward a species-specific compositional bias among ICAM-1 binders. However, amino acid residues corresponding to the documented ICAM-1 footprint have been shown to clearly classify the two receptor groups. The LDLR binders are harder to define by sequence. They always have a conserved central lysine (for example, in HRV-A2 VP1 it is Lys224), but this residue is not unique to LDLR binders, and predictive modeling instead suggests that the overall binding specificity primarily relies on a favorable combination of charge complementarities and hydrophobic interactions. Comparisons of the group amino acid populations of the all known ICAM-1 or LDLR contact residues using two correlation metrics (Spearman and Pearson) revealed that the majority of footprint locations were rejected by both methods as compositional matches to the HRV-C, suggesting that an alternate receptor is used by these viruses. In agreement with this analysis, very low amounts of HRV-C15 attached to HeLa and PBE cells, and incubation of mucosal organ cultures with blocking antibodies to major and minor HRV receptors showed no effects on HRV-C15 binding.

The development of sinus organ culture and a reverse genetics system have enabled studies of HRV-C15 growth in vitro and have provided new insights into its unique replication cycle. HRV-C15 binds to an unknown receptor that is expressed on epithelial cells in differentiated tissues but is either absent or underexpressed in many cell lines and is clearly distinct from receptors used by other HRV species. It is possible that the lack of knowledge about the HRV-C receptor and the virus’s biology has impeded the development of effective antivirals for HRV, as previous candidate medications were tested only against HRV-A and HRV-B viruses before moving into clinical trials. Even though not all enterovirus species have VP1 motifs that confer susceptibility to capsid binding agents, our results show that this approach can be used to block HRV-C binding and replication. Thus, these studies provide evidence that there are at least two feasible approaches to the treatment or prevention of HRV infections; refinement of capsid binding agents that target multiple species and development of competitive antagonists for the HRV receptors. The availability of organ culture and reverse genetics system should greatly facilitate further studies on HRV-C biology and its receptor identification.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

ACKNOWLEDGMENTS

We thank D. Healey, G. Hartig and J.S. McMurray (University of Wisconsin–Madison) for providing surgical samples and R. Brockman-Schneider, R. Vetis, T. Pappas, G. Crisafi, M. Hill, J. Bork, R. Massey, A. Lashua and E. Domyan for technical assistance. This work was supported by US National Institutes of Health grants U19 AI070503, U19 AI070503-0481, R01 HL080412 and R01 HL091490 and the National Institute of Allergy and Infectious Diseases–funded University of Maryland School of Medicine Genome Sequencing Center for Infectious Disease.

AUTHOR CONTRIBUTIONS

Y.A.B. designed and performed experiments, analyzed data and was the principal author of the paper; A.C.P. performed sequence alignments, statistical receptor footprint analysis and contributed to writing; W.-M.L. performed partial sequencing of clinical isolates, constructed pW10-2R, designed antiviral compound experiments and provided purified HRVs; J.A.R. determined the complete genome sequence of HRV-C15; S.P.A. assisted with virus inhibition experiments; X.S. designed and assisted with in situ hybridization experiments; T.R.P. assisted with establishment of the sinus organ culture; N.N.J. and S.B.L. analyzed data and contributed to writing; J.E.G. designed the project, analyzed data and contributed to writing.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemedicine/.

Published online at http://www.nature.com/naturemedicine/. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Palmenberg, A.C. et al. Sequencing and analyses of all known human rhinovirus genomes reveal structure and evolution. Science 324, 55–59 (2009).
2. Bern, J.E. & Busse, W.W. Association of rhinovirus infections with asthma. Clin. Microbiol. Rev. 12, 9–18 (1999).
3. Hayden, F.G. Rhinovirus and the lower respiratory tract. Rev. Med. Virol. 14, 17–31 (2004).
4. Dougherty, R.H. & Fahy, J.V. Acute exacerbations of asthma: epidemiology, biology and the exacerbation-prone phenotype. Clin. Exp. Allergy 39, 193–202 (2009).
5. Jackson, D.J. et al. Wheezeing rhinovirus illnesses in early life predict asthma development in high-risk children. Am. J. Respir. Crit. Care Med. 178, 667–672 (2008).
6. Wat, D. et al. The role of respiratory viruses in cystic fibrosis. J. Cyst. Fibros. 7, 320–328 (2008).
7. McManus, T.E. et al. Respiratory viral infection in exacerbations of COPD. Respir. Med. 102, 1573–1580 (2008).
8. Lamson, D. et al. MassTag polymerase-chain-reaction detection of respiratory pathogens, including a new rhinovirus genotype, that caused influenza-like illness in New York State during 2004–2005. J. Infect. Dis. 194, 1398–1402 (2006).
9. McErlean, P. et al. Characterisation of a newly identified human rhinovirus, HRV-QPM, discovered in infants with bronchiolitis. J. Clin. Virol. 39, 67–75 (2007).
10. Lee, W.M. et al. A diverse group of previously unrecognized human rhinoviruses are common causes of respiratory illnesses in infants. PLoS ONE 2, e966 (2007).
11. Briese, T. et al. Global distribution of novel rhinovirus genotype. Emerg. Infect. Dis. 14, 944–947 (2008).
12. Mackay, I.M. et al. Prior evidence of putative novel rhinovirus species, Australia. Emerg. Infect. Dis. 14, 1823–1824 (2008).
13. Tapparel, C. et al. Pneumonia and pericarditis in a child with HRV-C infection: a case report. J. Clin. Virol. 45, 157–160 (2009).
14. Bizzintino, J. et al. Association between human rhinovirus C and severity of acute asthma in children. Eur. Respir. J. published online, doi:10.1183/09031936.00092410 (6 August 2010).
15. Lau, S.K. et al. Clinical features and complete genome characterization of a distinct human rhinovirus (HRV) genetic cluster, probably representing a previously undetected HRV species, HRV-C, associated with acute respiratory illness in children. J. Clin. Microbiol. 45, 3655–3664 (2007).
16. McErlean, P. et al. Distinguishing molecular features and clinical characteristics of a putative new rhinovirus species, human rhinovirus C (HRV C). PLoS One 3, e1847 (2008).
17. Arden, K.E. & Mackay, I.M. Newly identified human rhinoviruses: molecular methods heat up the cold viruses. Rev. Med. Virol. 20, 156–176 (2010).
18. Huang, T. et al. Evidence of recombination and genetic diversity in human rhinoviruses in children with acute respiratory infection. *PloS ONE* 4, e6355 (2009).

19. Arden, K.E. et al. Molecular characterization and distinguishing features of a novel human rhinovirus (HRV) C, HRVC-QCE, detected in children with fever, cough and wheeze during 2003. *J. Clin. Virol.* 47, 219–223 (2010).

20. Reischl, A. et al. Viral evolution toward change in receptor usage: adaptation of a major group human rhinovirus to grow in ICAM-1-negative cells. *J. Virol.* 75, 9312–9319 (2001).

21. Olson, N.H. et al. Structure of a human rhinovirus complexed with its receptor molecule. *Proc. Natl. Acad. Sci. USA* 90, 507–511 (1993).

22. Bella, J., Kolatkar, P.R., Marlor, C.W., Greve, J.M. & Rossmann, M.G. The structure of the two amino-terminal domains of human ICAM-1 suggests how it functions as a rhinovirus receptor and as an LFA-1 integrin ligand. *Proc. Natl. Acad. Sci. USA* 95, 4140–4145 (1998).

23. Verdaguer, N., Fita, I., Reithmayer, M., Moser, R. & Blaas, D. X-ray structure of a minor group human rhinovirus bound to a fragment of its cellular receptor protein. *Nat. Struct. Mol. Biol.* 11, 429–434 (2004).

24. Xiao, C. et al. Discrimination among rhinovirus serotypes for a variant ICAM-1 receptor molecule. *J. Virol.* 78, 10034–10044 (2004).

25. Andries, K. et al. Two groups of rhinoviruses revealed by a panel of antiviral compounds present sequence divergence and differential pathogenicity. *J. Virol.* 64, 1117–1123 (1990).

26. Oliveira, M.A. et al. The structure of human rhinovirus 16. *Structure* 1, 51–68 (1993).

27. Zhao, R. et al. Human rhinovirus 3 at 3.0 A resolution. *Structure* 4, 1205–1220 (1996).

28. Ledford, R.M. et al. VP1 sequencing of all human rhinovirus serotypes: insights into genus phylogeny and susceptibility to antiviral capsid-binding compounds. *J. Virol.* 78, 3663–3674 (2004).

29. Lee, W.M., Monroe, S.S. & Rueckert, R.R. Role of maturation cleavage in infectivity of picornaviruses: activation of an infectosome. *J. Virol.* 67, 2110–2122 (1993).

30. Lee, W.M. & Wang, W. Human rhinovirus type 16: mutant V1210A requires capsid-binding drug for assembly of pentamers to form virions during morphogenesis. *J. Virol.* 77, 6235–6244 (2003).

31. Simmonds, P. et al. Proposals for the classification of human rhinovirus species C into genotypically assigned types. *J. Gen. Virol.* 91, 2409–2419 (2010).

32. Rossmann, M.G. & Palmenberg, A.C. Conservation of the putative receptor attachment site in picornaviruses. *Virology* 164, 373–382 (1988).

33. Laine, P., Blomqvist, S., Savolainen, C., Andries, K. & Hovi, T. Alignment of capsid protein VP1 sequences of all human rhinovirus prototype strains: conserved motifs and functional domains. *J. Gen. Virol.* 87, 129–138 (2006).

34. Weber, C. et al. Predictive bioinformatic identification of minor receptor group human rhinoviruses. *FEBS Lett.* 583, 2547–2551 (2009).

35. Tamura, K., Dudley, J., Nei, M. & Kumar, S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24, 1596–1599 (2007).

36. Crooks, G.E., Hon, G., Chandonia, J.M. & Brenner, S.E. WebLogo: a sequence logo generator. *Genome Res.* 14, 1188–1190 (2004).
ONLINE METHODS

Viruses. The virus nomenclature follows current recommendations of the International Committee on Taxonomy of Viruses *Picornaviridae* Study Group, designating the HRV species (A, B, C), followed by the assigned virus type (for example, HRV-A16, HRV-C15 and so on). We propagated laboratory stocks of HRV-A1 strain A (minor receptor group), HRV-A16 and HRV-B14 (major receptor group) in H1–HeLa cells (American Type Culture Collection CRL1958), and purified and titered as previously described. HRV-A78 and HRV-C15 (previously designated W10) are clinical isolates that we identified by partial sequencing of the 5′ UTR as previously described.

Sinus mucosal organ culture. All protocols for these studies were reviewed and approved by the University of Wisconsin–Madison Health Sciences Institutional Review Board. We obtained residual airway tissue specimens from individuals with chronic sinusitis undergoing endoscopic surgery (informed consent was not required, as the specimens would otherwise have been discarded after surgery). We washed, sectioned (4 × 4 mm) and submersed snippets of epithelium along with underlying tissues individually in 24-well plates with 0.3 ml bronchial epithelial growth medium (BEGM, Lonza) and incubated them at 37 °C (5% CO2). Before use, we tested all specimens (on average, the tissue samples were tested a total of three to five per well) with PBS (three washes with 0.5 ml) before inoculation (2 × 108 vRNA copies per well containing either mucosal organ culture or PBE cell monolayers (six-well plate) or per tube containing 1 × 107 HeLa cells in suspension. We added virus into samples with the virus (1 h, 25 °C), washed three times and analyzed by qPCR. To test the efficacy of the capsid-binding compound WIN56291 (Sterling Winthrop), we incubated (30 min, 25 °C) virus samples (2 × 108 vRNA copies) in 0.1 ml BEGM containing the compound (0.5 µg ml⁻1, 0.1% DMSO), followed by inoculation (5 h, 34 °C) into sinus organ cultures. After aspirating unattached virus, we incubated tissues for 72 h and tested by qPCR.

Construction of the full-length cDNA copy of HRV-C15. We isolated total RNA (RNeasy Mini Kit, Qiagen) from 100 µl of organ culture supernatant (HRV-C15 passage 4). After reverse transcription with random primers (TaqMan, Applied Biosystems), we performed PCR with the Platinum PCR SuperMix HF (Invitrogen) and primers (Supplementary Table 3). The strategy for cloning the full-length cDNA copy of HRV-C15 is described in Supplementary Figure 2. We confirmed each viral cDNA insert by automated sequencing of both strands.

RNA transfection. We purified full-length RNA transcripts synthesized from the linearized (BstBI) pC15 DNA (RiboMax large-scale RNA production system T7, Promega) with an RNeasy Mini Kit (Qiagen) and analyzed by agarose gel electrophoresis. We transfected cells with RNA and Lipofectamine 2000 (Invitrogen) mixture (1 to 5 wt/vol ratio) using the manufacturer’s recommendations and incubated for 24–72 h. See the Supplementary Methods for a detailed protocol.

Statistical analysis. We used Student’s t test to analyze viral replication data (SigmaPlot 11.0, Systat Software). Significance was defined at P < 0.05. Statistical evaluation of HRV receptor footprints is described in the Supplementary Methods.

37. Lee, W.M., Wang, W. & Rueckert, R.R. Complete sequence of the RNA genome of human rhinovirus 16, a clinically useful common cold virus belonging to the ICAM-1 receptor group. *Virus Genes* 9, 177–181 (1995).
38. Lee, W.M. et al. High-throughput, sensitive, and accurate multiplex PCR-microsphere flow cytometry system for large-scale comprehensive detection of respiratory viruses. *J. Clin. Microbiol.* 45, 2626–2634 (2007).
39. Henrique, D. et al. Expression of a Delta homologue in prospective neurons in the chick. *Nature* 375, 787–790 (1995).
40. Neubüser, A., Peters, H., Baling, R. & Martin, G.R. Antagonistic interactions between FGF and BMP signaling pathways: a mechanism for positioning the sites of tooth formation. *Cell* 90, 247–255 (1997).