Ephrin receptor A2 is a functional entry receptor for Epstein-Barr virus

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Epstein–Barr virus (EBV) is an oncogenic virus that infects more than 90% of the world’s population. EBV predominantly infects human B cells and epithelial cells, which is initiated by fusion of the viral envelope with a host cellular membrane. The mechanism of EBV entry into B cells has been well characterized. However, the mechanism for epithelial cell entry remains elusive. Here, we show that the integrins αvβ5, αvβ6 and αvβ8 do not function as entry and fusion receptors for epithelial cells, whereas Ephrin receptor tyrosine kinase A2 (EphA2) functions well for both. EphA2 overexpression significantly increased EBV infection of HEK293 cells. Using a virus-free cell–cell fusion assay, we found that EphA2 dramatically promoted EBV but not herpes simplex virus (HSV) fusion with HEK293 cells. EphA2 silencing using small hairpin RNA (shRNA) or knockout by CRISPR–Cas9 blocked fusion with epithelial cells. This inhibitory effect was rescued by the expression of EphA2. Antibody against EphA2 blocked epithelial cell infection. Using label-free surface plasmon resonance expression of EphA2. Antibody against EphA2 blocked epithelial cell infection. Using label-free surface plasmon resonance binding studies, we confirmed that EphA2 but not EphA4 specifically bound to EBV gHgL and this interaction is through the EphA2 extracellular domain (ECD). The discovery of EphA2 as an EBV epithelial cell receptor has important implications for EBV pathogenesis and may uncover new potential targets that can be used for the development of novel intervention strategies.

Epstein–Barr virus (EBV) is a member of the gammaherpesvirus family, which was discovered in 1964 and was the first human virus associated with cancer. EBV is the causative agent of infectious mononucleosis and is associated with Burkitt lymphoma, Hodgkin disease, nasopharyngeal carcinoma and gastric carcinoma, indicating the EBV tropism for B cells and epithelial cells. EBV infects more than 90% of the world’s population, but there is still a lack of therapies and vaccines. EBV entry into target cells is an essential step for EBV to cause disease and requires the fusion of viral and host membranes mediated by viral glycoproteins and cellular receptors. The viral glycoproteins important for EBV entry include gp350, gHgL, gB and gp42 (ref. 2). Among these glycoproteins, gp350 is important for virus attachment by binding to complement receptor type 2 (CR2/CD21), which is abundantly expressed on B cells and expressed on tonsilar epithelial cells. gHgL and gB are the core fusion machinery and are both required for B cell and epithelial cell fusion. However, gp42 is the tropism determinant required only for B cell fusion and inhibits epithelial cell fusion, indicating different infection mechanisms for these two cell types. The mechanism for B cell infection is better understood than the mechanism of epithelial cell infection. The B cell receptor HLA-DR was identified to bind to gp42 by a gp42 ligand binding screen in 1996 (ref. 7). In 1997, it was found that HLA-DR functions as a cofactor for infection of B lymphocytes. Since then, we have worked extensively on EBV entry, determining the structures of unbound gp42, the gp42:HLA complex, the gHgL complex and gB in the post-fusion form (ref. 7). Recently, we assembled and analysed the reconstituted B cell entry complex composed of gHgL, gp42 and HLA class II, and the crystal structure of the gHgL/gp42 complex bound to an anti-gHgL antibody (E1D1), providing an overall structural foundation for understanding EBV host-cell tropism.

To characterize the EBV epithelial cell entry complex, in a manner similar to that carried out for the B cell entry complex, we first wanted to verify the receptor used for epithelial cell entry. We chose the AGS cell line (a human gastric adenocarcinoma cell line), which has been used extensively as a model of EBV epithelial cell entry, and human embryonic kidney 293 (HEK293) cells, which we used in our cell-based fusion assay. Previous studies have indicated that the integrins (αvβ5, αvβ6 and αvβ8; but not αvβ3) function as receptors for epithelial cell entry. It was also found that blocking antibodies to integrins and siRNA targeting of integrin αv did not completely abolish epithelial cell fusion or infection. In addition, three anti-gHgL monoclonal antibodies (CL40, CL59 and E1D1) targeting different epitopes can all inhibit epithelial cell infection, indicating that multiple regions on gHgL may participate in EBV infection. To determine if integrins are the primary epithelial cell receptor, we chose to knock out the integrin αv gene using the CRISPR–Cas9 system in HEK293-T41 cells. Integrins αvβ5, αvβ6 and αvβ8 are heterodimeric complexes composed of the αv subunit and a β subunit. The crystal structure of EBV gHgL with an exposed KGD motif (RGD motif mimic) within gH domain II (D-II) suggested it might bind to the αv subunit of the heterodimeric integrins reported to function as entry receptors. By knocking out αv, we would block expression of the integrins previously shown to mediate EBV entry into epithelial cells. Integrin αv knockout clonal cell lines were readily obtained and were first analysed by flow cytometry, verifying the absence of αv (Fig. 1a). We next used our cell–cell fusion assay to monitor syncytia formation. We found that cells lacking integrin αv still fused with Chinese hamster ovary (CHO-K1) cells that expressed gHgL and gB (Fig. 1b), indicating that αvβ5, αvβ6 and αvβ8 integrins are not the primary receptor(s) in HEK293 cells.

To identify essential epithelial cell receptors in HEK293 cell fusion, we compared RNA-seq data of human B cells and HEK293 cells. We chose this comparison because it has been shown
**Fig. 1** | Identification of EphA2 as the potential EBV epithelial cell receptor. **a**, Integrin αv cell surface expression in integrin αv wild type (WT) and knock out (KO) HEK293 cells, assessed by flow cytometry. **b**, CHO-K1 cells transfected with GFP plasmid plus either control plasmid or EBV gHgL and gB were overlaid with integrin αv WT and KO HEK293 cells. Syncytia formation (indicated by the arrows) was visualized and captured with an EVOS fluorescence microscope (representative data from three independent experiments). **c**, Heatmap of the normalized expression of potential epithelial cell receptors compared to B cells for HEK293 and AGS cells (left), and heatmap of ephrin receptors for B cells, HEK293 cells and AGS cells (right). **d**, Comparison of EphA2 expression in different cell types. Total RNA from each cell type was assayed for EphA2 and GAPDH mRNA levels by qRT-PCR. Bars represent relative EphA2 mRNA expression normalized to GAPDH. Data are means plus standard errors of the mean (s.e.) for three independent experiments. **e**, Flow cytometry histograms of EphA2 in Daudi B, HEK293 and AGS cells, with the dotted line as a negative control. Representative flow cytometry data for at least two independent experiments are shown in **a** and **e**. *P < 0.05; **P < 0.01 (ANOVA followed by post-hoc Tukey’s multiple comparison test).
previously that EBV containing only gB and gHgL, but lacking gp42, is unable to infect B cells\(^1\). In addition, previous data have shown that CHO-K1 cells that express gHgL and gB, but not gp42, can fuse with HEK293 cells but not B cells, further indicating that B cells do not express epithelial cell receptors\(^1\). To identify candidate epithelial cell receptors, we screened B cell and HEK293 cell RNA-seq data sets. In our analysis, we screened the data sets such that the mRNA of HEK293 candidates versus the mRNA of B cell candidates was tenfold greater and that the HEK293 cell gene reads were over the 5 FPKM (fragment per kilobase per million mapped reads) threshold as the lower boundary for reliable detection of gene expression. From this analysis, we selected 2,039 genes out of 18,400 genes that were compared. We then selected membrane proteins and excluded genes that had B cell reads of more than 5 FPKM. This analysis resulted in 245 genes (Supplementary Table 1). To further reduce the number of potential genes, we used RNA-seq data from AGS cells. AGS cells are a human gastric adenocarcinoma and have been used extensively as a model of EBV epithelial cell entry\(^1,4,9\). This cell line forms larger syncytia and cells are more readily infected than HEK293 cells (data not shown), and gastric epithelial cells are a target of EBV infection\(^1\). When comparing the expression of the 245 genes (EphA2 ranked no. 14, fold difference 864, Supplementary Table 1) identified in the HEK293 cell RNA-seq data and selecting only those genes that are expressed twofold greater in AGS cells, we identified 39 genes. After ranking these genes according to the ratio of AGS to B cell expression from high to low, EphA2 was ranked as the no. 1 candidate (Fig. 1c and Supplementary Table 2). Interestingly, if we first compared AGS cells with B cells using similar parameters (B cell expression <5 FPKM, AGS cell expression >5 FPKM, AGS cell/B cell >10, membrane protein), this resulted in 278 genes (Supplementary Table 3) and EphA2 was ranked no. 9 (fold difference of 6,798). If we selected only those genes that are expressed twofold greater in AGS cells, we identified 65 genes (Supplementary Fig. 1 and Supplementary Table 4). If we compared the first 15 genes of membrane proteins selected out by two methods (Supplementary Tables 1 and 3), EphA2 and PAQR5 are the only genes shared by the two methods. EphA2 is also the receptor for Kaposi’s sarcoma-associated herpesvirus (KSHV), another human gammaherpesvirus\(^1,5,21\). Previous data have shown that KSHV gHgL binds to the ligand-binding domain of EphA2 (ref. 22). To examine whether the EphA2 functions through the ligand-binding domain in EBV fusion, we swapped the ligand-binding domains of EphA2 and EphA4 to generate EphA2A4 or EphA4A2 chimaeras. We found that replacing the EphA4 ligand-binding domain with the EphA2 ligand-binding domain (EphA2A4 chimaera) increased fusion activity threefold, suggesting that, like KSHV, EBV gHgL also binds the ligand-binding domain of EphA2 (Fig. 4a). To examine whether EphA2 kinase activity is important for fusion, we also tested three EphA2 kinase dead mutants that were mutated in the EphA2 kinase domain and found that the kinase activity of EphA2 is not important for EBV fusion (Fig. 4b). It has been shown that the kinase activity of EphA2 is important for KSHV infection\(^21\). Depending upon cell type, EBV can enter cells by fusion at the plasma membrane (epithelial cells) or fusion with an endocytic membrane after endocytosis (B cells)\(^1\). KSHV enters human B cells, fibroblast, epithelial and endothelial cells by endocytosis, and EphA2 regulates clathrin-mediated KSHV endocytosis\(^1,21,22\). The different route of entry may explain the differing requirements of EphA2 kinase function in EBV and KSHV infection. To explore the region of gHgL that binds EphA2, we took advantage of previous observations indicating that the gHgL domain for gp42 binding and epithelial cell binding overlap\(^23\). When an expression vector expressing gp42 or purified soluble gp42 is included in our fusion assay, the level of fusion is dramatically reduced (Fig. 4c), indicating that gp42 binding to gHgL blocks EphA2-mediated membrane fusion, in line with previous observations. To further confirm the direct binding of the EphA2-EC2 domain and EBV gHgL, we used label-free surface plasmon resonance (SPR) binding studies and found that purified EBV gHgL bound to EphA2 with a dissociation constant \((K_d)\) of 5 \(\mu\)M in three independently repeated binding kinetics experiments, but EBV gHgL did not bind to EphA4 under the same conditions (Fig. 4d,e and Supplementary Table S). The 200 nM concentration of EBV gHgL was injected in duplicate each time, with randomized order of injections, with the second 200 nM injection...
occurring after the 1.8 μM EBV gHgL injection to serve as internal control. Figure 4d shows that both injections of the same concentration (200 nM) overlap each other. Previous data from a flow cytometry binding assay showed that KSHV gHgL can bind tightly with EphA2 in particular, but only weakly with other ephrin receptors including EphA4 (ref. 34). This suggests that gammaherpesviruses share a common entry receptor. Taken together, we identify EphA2 as an important epithelial cell entry receptor for EBV infection. This result provides important insight into the mechanism EBV uses to infect epithelial cells, and may provide a better understanding of the development of epithelial pathologies and cancers associated with EBV infection, as well as strategies to lessen EBV-associated epithelial cancers. Finally, our studies using bioinformatics to implicate EphA2 as an EBV epithelial receptor highlight the benefit of using readily obtainable sequencing data to aid in the discovery of pathogen receptors.

Fig. 2 | EphA2 can promote both EBV infection and virus-free cell–cell fusion. a, b, HEK293 cells were transfected with pcDNA 3.1, EphA2 or EphA4. At 24 h post-transfection, 5 x 10^4 cells were seeded onto a 48-well plate. After 24 h, the cells were infected with 100 μl EBfaV-GFP virus concentrated from 1 ml virus-containing supernatant. After 72 h, the infected GFP cells were visualized and captured with an EVOS fluorescence microscope (a) or analysed by flow cytometry (b). Data are representative data from three independent experiments. c, Quantification of flow cytometry data from three independent experiments. Bars represent the percentage of infection, with infection of pcDNA 3.1 transfected HEK293 cells set to 100%. d, CHO-K1 cells transfected with T7 luciferase plasmid together with either control plasmid, EBV gHgL and gB, or HSV gHgL, gB and gD were overlaid with HEK293 cells transfected with pcDNA 3.1, EphA2 or EphA4. EBV fusion with HEK293 cells transfected with pcDNA 3.1 was set to 100%. HSV fusion was normalized to EBV fusion and standardized with pcDNA 3.1 transfected cells set to 100%. T7 pol., T7 RNA polymerase; T7 luc., T7 luciferase. Data are presented as mean plus s.e. for three independent experiments for c and d. ***P < 0.001 (ANOVA followed by post-hoc Tukey’s multiple comparison test).
Fig. 3 | EphA2 is essential for EBV infection and virus-free cell–cell fusion. a, EphA2 cell surface expression in EphA2 WT and KO HEK293-T14 cells by flow cytometry. Representative flow cytometry data of three independent experiments are shown. b, Virus-free EBV or HSV fusion with EphA2 WT and KO HEK293-T14 cells or EphA2 KO cells that overexpress EphA2 or EphA4 (pcDNA 3.1 used as control). Bars represent fusion activity, and data are mean plus s.e. for three independent experiments. c,d, EphA2 WT and KO HEK293-T14 cells or EphA2 KO cells that overexpress EphA2 or EphA4 were infected with EBFaV-GFP virus. At 72 h post-infection, EBFaV-GFP-infected cells were visualized and captured with an EVOS fluorescence microscope (d) or GFP-positive cells were analysed by flow cytometry (c). Data are representative of three independent experiments. e, Quantification of flow cytometry data from three independent experiments. Bars represent percentage of infection, with infection of WT HEK293-T14 cells set to 100%. Data are means plus s.e. *P < 0.05; ***P < 0.001 versus WT HEK293-T14 (ANOVA followed by post-hoc Tukey’s multiple comparison test).
**Methods**

**Cell culture.** CHO-K1 cells (ATCC CCL-61 or CRL-9618) were grown in Ham’s F-12 medium (Corning) containing 10% heat-inactivated fetal bovine serum complex (FBS) (Corning) and 1% penicillin–streptomycin (100 U penicillin ml⁻¹, 100 µg streptomycin ml⁻¹; Sigma). CHO-K1, HEK293, HEK293T, AGS and Detroit 562 cells were obtained from ATCC and routinely tested for mycoplasma using a LookOut Mycoplasma PCR Detection Kit (Sigma). Cell lines were authenticated by ATCC and not authenticated in our laboratory.

**Constructs.** The EphA2, EphA2 kinase-dead mutants and EphA4 constructs were a gift from S. Getsios. Construction of the EphA2 and EphA4 chimaeras (EphA2A4 or EphA4A2) was performed using overlapping PCR with the following primers: EphA4A2 HindIII F: 5-TTAAGCTTATGGCTGGGATTTTCTATTTC-3, EphA4A2 HindIII R: 5-CGAGGATCCCATGCCTTCAGCCG-3. HEK293 cells transfected with T7 polymerase were used to transfect CHO-K1, HEK293T, AGS and Detroit 562 cells expressing T7 RNA polymerase, respectively containing 10% heat-inactivated FBS (Corning) and 1% penicillin–streptomycin (100 U penicillin ml⁻¹, 100 µg streptomycin ml⁻¹; Sigma). CHO-K1, HEK293, HEK293T, AGS and Detroit 562 cells were obtained from ATCC and routinely tested for mycoplasma using a LookOut Mycoplasma PCR Detection Kit (Sigma). Cell lines were authenticated by ATCC and not authenticated in our laboratory.

**Fig. 4 | EphA2 binds to EBV gHgL with low affinity.** 

(a) Virus-free EBV fusion with HEK293 cells transfected with pcDNA 3.1, EphA2, EphA4, or EphA2 and EphA4 chimaeras as indicated. b, Virus-free EBV fusion with HEK293 cells transfected with control, EphA2, EphA4 or EphA2 kinase-dead mutants as indicated. Fusion activity of HEK293 cells transfected with pcDNA 3.1 was set to 100 in a and b. c, HEK293 cells transfected with T7 polymerase together with pcDNA 3.1 or EphA4 were overlaid with CHO-K1 cells transfected with T7 luciferase plasmid together with EBV gB, gHgL in the presence or absence of gp42 plasmid or 100 nM soluble gp42. Fusion activity of HEK293 cells transfected with pcDNA 3.1 was set to 100. Bars in a–c represent fusion activity and data are mean plus s.e. for three independent experiments. d, EphA2 and EphA4 were immobilized on two separate channels of a CMD biosensor chip surface. Label-free SPR binding kinetics of EBV gHgL with EphA2 (d) or EphA4 (e) are depicted, with model fits represented by dashed lines. Global curve fitting with a 1:1 interaction model (dashed lines) closely matches the experimental data for EphA2 binding to EBV gHgL. EphA4 does not bind to EBV gHgL under identical conditions (representative data from three independent experiments). RU, relative units. ***P < 0.001 versus EBV gHgL, pcDNA 3.1 in a and versus gB gHgL, EphA2 in c (ANOVA followed by post-hoc Tukey’s multiple comparison test). NS, not significant.
The cells were colonized and expanded for 2–3 weeks. Cas9 expression in CHO-K1 cells grown to ~80% confluency were transiently transfected with 5 μg of the pCDNA3.1 plasmid. After puromycin selection for 2 days, the cells were examined by flow cytometry. The cells were collected, counted and seeded at a concentration of 0.5 × 10⁵ cells ml⁻¹ in 1 ml and frozen at −80 °C. The supernatant was collected after 4 days by centrifugation and passed through a 0.1 mm cell strainer. The cells were counted and seeded at a concentration of 0.5 × 10⁵ cells ml⁻¹, Life Technologies, 371600) for 1 h and infected with EBV virus 10⁴ HEK293 cells per well were changed to fresh medium with 5 μg ml⁻¹ blasticidin for selection. After one week, the cells were harvested and expanded for 2–3 weeks. Cas9 expression in these single-cell colonies was analysed using western blotting against Flag. 2.5 × 10⁵ HEK293-T14-Cas9 cells per well in a 12-well plate were infected with lentivirus supermix from BioRad. We used a two-step amplification (40 cycles of 95 °C, 15 s; 60 °C, 30 s; followed by melting temperature determination stage) and quantified relative changes in gene expression using the ΔΔCt method according to the manufacturer's instructions (Applied Biosciences).

Quantitative RT–PCR. Expression of EphA2 and EphA4 mRNA in HEK293 cells was examined by quantitative RT–PCR (qRT–PCR) analysis, using the following primers: EphA2 F 5′-AAGGAAGTGGTACTGCTGGA-3′ and R: 5′-TTCAATCTTTGAGACAGCTAC-3′; EphA4 F 5′-CTGCTCCTCTAACAAGTGTCCTAC-3′ and R: 5′-CATGCCATGGGCATATGCAAGTG-3′. Integrin αv KO cells, 5 μg of the pCDNA 3.1 plasmid. After puromycin selection for 2 days, the cells were harvested and expanded for 2–3 weeks. Cas9 expression in these single-cell colonies was analysed using western blotting against Flag. 2.5 × 10⁵ HEK293-T14-Cas9 cells per well in a 12-well plate were infected with lentivirus supermix from BioRad. We used a two-step amplification (40 cycles of 95 °C, 15 s; 60 °C, 30 s; followed by melting temperature determination stage) and quantified relative changes in gene expression using the ΔΔCt method according to the manufacturer's instructions (Applied Biosciences).
Pierce Gentle Ag/Ab elution buffer pH 6.6 (Thermo Fisher Scientific) and purified by a final Superdex 200 gel filtration column (GE Life Sciences) in 1× PBS pH 7.4. Gp42 was expressed and purified as previously described.

SPR binding kinetics. The binding kinetics assay to determine on rate (k1) off rate (k2) and affinity (Kd) between EBV gHgL and EphA2 or EphA4 was performed using a Bioptec 404pi biosensor instrument (BioOptix, CO). 1× PBS pH 7.4 with 0.05% (vol/vol) Tween-20 was used as the running buffer. EphA2 (R&D systems 3035-A2-100) or EphA4 (R&D systems, 6827-A-050) was immobilized onto separate channels on a carboxy-methyl dextrin (CMD-200m, BioOptix) biosensor chip by the amine-coupling method using EDC/NHS (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride/N-Hydroxysuccinimide) chemicals (Sigma). Deactivated surface using ethanolamine was used as the reference channel. Sensorgrams with different serial dilutions, that is, the concentration series of gp42/gL as the mobile analyte, were flown over the ligand and the reference subtracted sensorgram data fit globally to a 1:1 interaction model using GraphPad Prism 7. Kinetic parameters from the model fit are presented in Supplementary Table 5. The low analyte concentration range used for KD measurements here is due to sample limitations. Sensorgram traces with the model fit overlaid on the data are shown in Fig. 4d,e.

Statistical analysis. Data were collected from three independent experiments. Statistical differences between multiple groups were determined by one-way analysis of variance (ANOVA) with post-hoc Tukey’s multiple comparison test. Two-group comparisons were analysed by the two-tailed unpaired Student’s t-test. P < 0.05 denotes the presence of a statistically significant difference. Data are expressed as mean ± s.e. The analysis was performed using GraphPad Prism, version 6.0c, for Mac (GraphPad Software). Flow cytometry histograms and microscopy images are representative of at least two independent experiments.

Life Sciences Reporting Summary. Further information on experimental design is available in the Life Sciences Reporting Summary.

Data availability. The data that support the findings of this study are available within this letter and its Supplementary Information files or upon request from the corresponding author.

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experiments. X.Z. performed the RNA-seq analysis and helped with the sgRNA constructs and statistical analysis. S.S. helped with cell cultures. B.E.P.W. contributed key reagents. J.C. and R.L. wrote the manuscript. X.Z., K.S. and T.S.J. contributed expertise and helped write the paper. All authors analysed the results, read and approved the manuscript for submission.

Competing interests
The authors declare no competing financial interests.

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There are no restrictions on availability of unique materials

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

PE-conjugated EphA2 Antibody (SHM16, detecting human EphA2) were purchased from Biolegend. PE-conjugated Integrin aV (FAB1219P, detecting human integrin aV) and anti-EphA2 (AF3035) were purchased from R&D systems. anti-EphA4 (371600) was purchased from Life technologies. All have validation statements on their websites.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

Chinese Hamster Ovary (CHO-K1) cells (ATCC CCL-61 or CRL-9618), (HEK293) cells (ATCC CRL-1573), HEK293-T14 cells (HEK293T cells stably expressing T7 RNA polymerase, ATCC CRL-3216), and Detroit 562 cells (ATCC CCL138) were from ATCC

b. Describe the method of cell line authentication used.

N/A

c. Report whether the cell lines were tested for mycoplasma contamination.

The cell lines were tested for mycoplasma contamination and there were no contamination

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

N/A

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

N/A

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

N/A