

Folate Receptor Alpha and Caveolae Are Not Required for Ebola Virus Glycoprotein-Mediated Viral Infection

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Folate receptor alpha (FR α) has been described as a factor involved in mediating Ebola virus entry into cells (6). Furthermore, it was suggested that interaction with FR α results in internalization and subsequent viral ingress into the cytoplasm via caveolae (9). Descriptions of cellular receptors for Ebola virus and its entry mechanisms are of fundamental importance, particularly with the advent of vectors bearing Ebola virus glycoprotein (GP) being utilized for gene transfer into cell types such as airway epithelial cells. Thus, the ability of FR α to mediate efficient entry of viral pseudotypes carrying GP was investigated. We identified cell lines and primary cell types such as macrophages that were readily infected by GP pseudotypes despite lacking detectable surface FR α , indicating that this receptor is not essential for Ebola virus infection. Furthermore, we find that T-cell lines stably expressing FR α are not infectible, suggesting that FR α is also not sufficient to mediate entry. T-cell lines lack caveolae, the predominant route of FR α -mediated folate metabolism. However, the coexpression of FR α with caveolin-1, the major structural protein of caveolae, was not able to rescue infectivity in a T-cell line. In addition, other cell types lacking caveolae are fully infectible by GP pseudotypes. Finally, a panel of ligands to and soluble analogues of FR α were unable to inhibit infection on a range of cell lines, questioning the role of FR α as an important factor for Ebola virus entry.

The filoviruses, Ebola virus and Marburg virus, are emerging human pathogens responsible for outbreaks of severe hemorrhagic fever with mortality rates approaching 90%. Filoviruses are negative-stranded RNA viruses consisting of a ribonucleoprotein core surrounded by a matrix of viral proteins VP40 and VP24 and enveloped by a host cell-derived lipid bilayer. Entry into target cells is mediated by lipid bilayer-embedded viral glycoproteins (GP) via binding to cell surface receptors. The distribution of these cellular receptors is likely a major determinant in the cellular and tissue tropism of filoviruses. We and others have shown that retrovirus and rhabdovirus pseudotypes bearing Ebola virus GP have a very broad cell tropism; they infect many cell lines from multiple mammalian and avian species (7, 14, 35, 37). Immortalized B- and T-cell lines, as well as primary lymphocytes (31, 37), represent the only cell lines consistently nontransducible by Ebola virus pseudotypes. Animal studies have revealed that a wide range of cell types are infected by filoviruses, particularly during the later stages of disease (1, 28–30), again with the notable exception of lymphocytes (13, 29). Such a broad cell tropism suggests that filoviruses utilize either a widely expressed and highly conserved surface molecule or a number of alternative molecules for entry into cells.

Chan and colleagues recently used a retrovirus-based selection protocol to identify cDNAs able to confer susceptibility to Jurkat T-cells by pseudotypes bearing Ebola virus or Marburg virus GP (6). Using this method, a number of cDNAs were isolated from a HeLa cell cDNA library with identity to folate receptor alpha (FR α) were identified. FR α is an attractive candidate as a filovirus receptor as it is widely expressed but is not detectable on lymphocytes (12) and is highly conserved between different species. The initial cDNA isolated from the transduced Jurkat cells, however, lacked the 5' coding sequence of FR α and initiated translation from an internal in-frame ATG (Met92) (6). This clone of FR α thus lacks an N-terminal signal peptide sequence to target it to the lumen of the endoplasmic reticulum, making it highly unlikely that it is expressed at the cell surface. This inconsistency, together with the fact that a number of cell lines readily infectible by Ebola virus pseudotypes appear to lack FR α , led us to reexamine the role of FR α in Ebola virus infection.

FR α expression on susceptible and nonsusceptible cells. Widespread expression in different cell lines appears to support the hypothesis that FR α acts as a cell surface receptor for Ebola virus. However, the published literature indicates that a number of cell types are FR α negative. For example, A549 cells (a human lung carcinoma cell line) lack the ability to bind and internalize folate (36), due to a lack of FR α (21). This was confirmed by both flow cytometry using a mouse monoclonal antibody (LK26) to human FR α (Fig. 1A) and reverse transcription (RT)-PCR (data not shown). A549 cells were 3- to 10-fold more infectible by LacZ-encoding human immunodeficiency virus (HIV) pseudotypes bearing Ebola virus GP (Zaire Mayinga strain) than HeLa or Cos-7 cells, which express substantial levels of FR α (Fig. 1), (7.3×10^5 compared to 5.6

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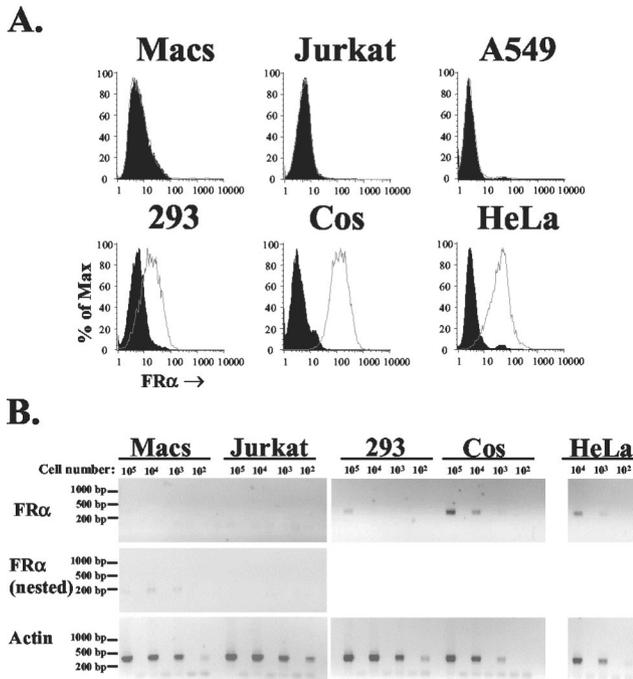


FIG. 1. FR α expression on common cell lines and primary macrophages. (A) Surface levels of FR α on five cell lines and day 6 primary macrophages were determined by standard flow cytometry (33) with a murine monoclonal antibody directed against human FR α (LK26; Signet) (open histograms) and an isotype control (immunoglobulin G2a; Sigma, St. Louis, Mo.) (filled histograms). Macrophages were isolated from human leukocytes by plastic adherence, as previously described (32). (B) Levels of cellular mRNA for FR α and human β -actin were evaluated by RT-PCR, using an Express direct mRNA capture and RT system (Pierce, Rockford, Ill.). mRNA was extracted and cDNA synthesis was performed in the same well containing serial 10-fold dilutions of each cell type. PCR was performed with 16.5 μ l of cDNA by using the conditions 95°C for 30 s followed by 30 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 40 s for FR α (forward primer, TGGGTGGCTGTAGTAGGGGAG; reverse primer, CAGGGGCA CGTTCAGTACC) and β -actin (forward primer, CTGGCACCCAC CTTCTACAATG; reverse primer, AATGTCACGCACGATTTCCC GC), resulting in 359- and 381-bp products, respectively. Primers were designed so that products spanned intron and exon boundaries, such that the predicted products derived from genomic DNAs were 3,440 and 822 bp for FR α and β -actin, respectively. For cell types negative for FR α in the first round of PCR (macrophages [Macs] and the T-cell line, Jurkat), 5 μ l was transferred to new tubes and nested PCR was performed with the same conditions as those described above and the following set of nested primers: forward primer GCCAAGCACCAC AAGGAAAAG and reverse primer CCTGGATGAAATGCCGT TTG. This resulted in a 189-bp product with a 3,118-bp predicted genomic DNA product. Both first-round and nested primers recognized all seven isoforms of FR α (8). Gaps between each sample represent controls for which the whole mRNA extraction and RT-PCR procedure was carried out on empty wells (a total of seven controls for the nested PCR). Control experiments with the same reaction mixtures performed on mRNA from each cell line in the absence of reverse transcriptase were all negative (data not shown).

$\times 10^4$ and 2.7×10^5 focus-forming units [FFU] per ml, respectively). In confirmation of previous findings (7, 37), HeLa cells were also approximately 20-fold-less infectible than 293 cells (1.2×10^6 FFU/ml), which express low levels of FR α (Fig. 1). Thus, FR α is not absolutely required for efficient viral entry mediated by Ebola virus GP, and levels of FR α expression do

not appear to correlate with infectivity. Importantly, primary macrophages, the major initial *in vivo* target for Ebola virus replication in primate animal models (13, 16, 29, 34), were found to express a very small amount of FR α mRNA, with a set of internal nested primers required for detection by RT-PCR (Fig. 1B). No detectable surface expression of FR α on primary macrophages was seen by flow cytometry following 3 to 8 days of culture (Fig. 1A and data not shown). Low levels of FR α message in macrophages may potentially be sufficient to mediate efficient Ebola virus infection, though the lack of detectable surface expression suggests that receptors other than FR α are responsible for infection of this important cell type.

Expression of FR α on FR α -negative cells does not enhance infection. Lentiviral vectors expressing full-length FR α or the human chemokine receptor CCR5 as a control were used to transduce A549 cells, leading to FR α expression on these otherwise FR α -negative cells (Fig. 2A). No discernible differences in levels of either Ebola virus GP or vesicular stomatitis virus G protein (VSV-G) pseudotype infection was seen in mock-transduced or FR α - or CCR5-expressing A549 cells (Fig. 2C). Thus, FR α is not required for and does not enhance infection of A549 cells, suggesting that alternative receptors are sufficient to give maximal infectivity of this cell type.

To address directly whether FR α expression confers Ebola virus susceptibility to lymphoid cells, a stable population of CEMss cells, a human CD4⁺-T-cell line, expressing high levels of full-length FR α was generated by transfection and drug selection (termed CEM/FR α). Expression of FR α was demonstrated by fluorescence-activated cell sorting analysis (Fig. 2A), and these cells were challenged with lentiviral vector pseudotypes carrying either Ebola virus GP or, as a control, HIV Env. By both a sensitive chemiluminescent assay for soluble β -galactosidase (Tropix, Bedford, Mass.) (Fig. 2C) and single-cell quantification by β -galactosidase staining, both the FR α -expressing cells and the parental CEMss were resistant to infection by pseudotypes bearing Ebola virus GP (titers of <12 FFU/ml for both parental CEMss and CEM/FR α cells were found by using a viral stock with a titer of 5.0×10^6 FFU/ml on 293 cells). Levels of infectivity were unchanged regardless of whether the infections were carried out in RPMI or M199 medium, which lacks folic acid (see below). Thus, FR α expression on a nonpermissive lymphoid cell line does not confer susceptibility to Ebola virus GP pseudotypes. A small but reproducible increase in infection mediated by HIV Env derived from the CXCR4-tropic strain HXB2 was observed. It is likely that this increase was due merely to the random selection of a slightly more susceptible subpopulation during the cloning process. Importantly, the Jurkat T-cell lines F10 and A9, which contain FR α cDNA as described by Chan et al. (6), were also found not to be infectible by either *lacZ* or luciferase reporter pseudotypes bearing Ebola virus GP (data not shown).

Although extensive analysis (7, 37, 38) has yet to identify any differences in viral entry between infectious Ebola virus and retroviral pseudotypes bearing Ebola virus GP, it has been suggested that the lack of similarity between long, filamentous Ebola virus particles and small, spherical retroviral particles may give rise to artifactual results. To address this concern, a unique assay to determine the entry of filamentous Ebola virus-like particle (VLP) has been developed (G. Simmons and

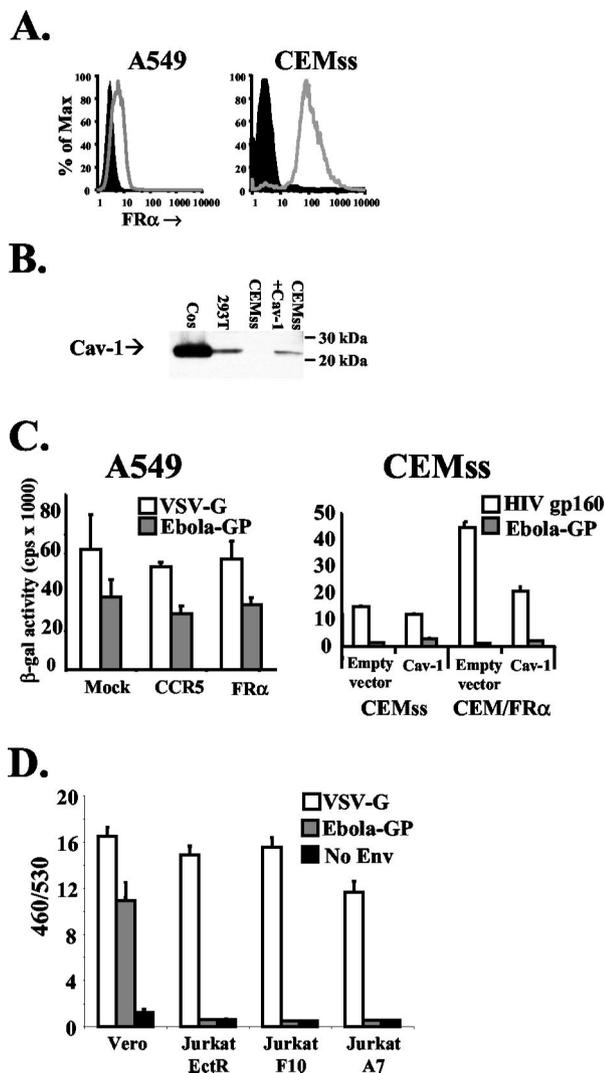


FIG. 2. Expression of FR α and Cav-1 in cell lines does not enhance Ebola virus pseudotype infection. (A) Surface levels of FR α were assessed as described in the legend to Fig. 1 for A549 cells (left panel) transduced with lentiviral vectors (for protocols, see reference 31) expressing a control of CCR5 (filled histogram) or FR α (open histogram) and for CEMss cells (right panel, filled histogram) or CEM cells stably expressing FR α (open histogram). (B) Total protein levels of Cav-1 were assessed by Western blotting using a directly horseradish peroxidase-conjugated polyclonal rabbit antibody raised against the N terminus of Cav-1 (N-20; Santa Cruz Biotechnology, Santa Cruz, Calif.). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on cell lysates from 10^6 cells. CEMss cells were transduced with MLV amphotropic envelope pseudotyped MLV particles carrying the MIGR1 packaging vector encoding human CD8 (27) or MLV MIGR1 encoding human Cav-1. Cos and 293 cells were also tested to determine endogenous levels of Cav-1. (C) Mock-transduced and CCR5- or FR α -transduced A549 cells (left panel) or CEMss and CEM/FR α cells transduced with control or Cav-1-encoding MLV pseudovirions (right panel) were challenged with HIV-based pseudovirions encoding LacZ (18) pseudotyped with Ebola virus GP, VSV-G, or HIV gp160 (HXB2 strain). Two days postchallenge, cells were lysed and soluble β -galactosidase (β -gal) levels were measured with a chemiluminescence kit (Galactostar; Tropix) as per the manufacturer's instructions. Similar results were gained by using pseudovirions encoding luciferase as a reporter gene (data not shown). (D) Vero cells, but not Jurkat cells stably expressing ecotropic MLV receptor or previously described derivatives expressing FR α (6), were transducible with VLPs bearing Ebola virus GP. VLPs were made by cotransfecting

P. Bates, unpublished data). This assay relies on the published observation that expression of Ebola virus VP40 matrix protein leads to budding of VLPs in the absence of any other viral proteins (15). Furthermore, these VLPs are filamentous and closely resemble infectious Ebola virus particles by electron microscopy (15). Coexpression of Ebola virus GP with VP40 gives rise to VLPs studded with GP on their surfaces (2, 25). To monitor the cellular entry of these VLPs, a chimeric β -lactamase (Bla)-VP40 protein was produced. We have found that N-terminal tagging of VP40 with Bla does not adversely effect budding but rather produces particles able to deliver Bla enzymatic activity to target cells, such as Vero cells, upon membrane fusion (Fig. 2D). Bla-VP40 particles budding from transfected 293T cells in the presence or absence of GP formed long, filamentous particles as judged by electron microscopy (data not shown) and closely resembled previously described particles (2, 25). GP-mediated transfer of functional Bla enzymatic activity to the cytoplasm of target cells is measured by labeling these cells with CCF2-AM, a substrate capable of fluorescing at a wavelength of 530 nm when excited at 409 nm. Hydrolysis of a β -lactam ring in CCF2 by Bla creates a product that, when excited at 409 nm, fluoresces at a lower emission wavelength of 460 nm (4, 19). Thus, the degree of Bla enzymatic activity can be ascertained by plotting a ratio of the fluorescence at 460 nm to that at 530 nm (Fig. 2D). Vero cells were efficiently transduced by Bla⁺ VLPs bearing either Ebola virus GP or VSV-G but not by particles containing no envelope (Fig. 2D). VLP transduction of Bla mediated by Ebola virus GP, as in wild-type Ebola virus infection, is pH dependent and can be inhibited by neutralizing anti-Ebola virus GP sera (Simmons and Bates, unpublished). In accordance with the results of the Ebola virus GP pseudovirion experiments described above, neither parental Jurkat cells expressing the ecotropic murine leukemia virus (MLV) receptor (Jurkat EctR cells) nor derivatives expressing FR α (Jurkat A7 and F10 cells) (6) were transduced above background levels by Bla⁺ VLPs bearing Ebola virus GP. For both the Vero and Jurkat cell lines, background levels of Bla⁺ VLPs with no envelope proteins were similar to those of CCF2-AM-labeled cells with no particles added (data not shown). In contrast to the Ebola virus GP results, VLPs bearing VSV-G exhibited levels of entry into all of the Jurkat cell lines that were similar to levels of entry into Vero cells (Fig. 2D). Overall, these experiments with filamentous particles confirm the pseudovirion results, demonstrating

293T cells with equimolar amounts of a Bla-VP40 fusion protein and GP, VSV-G, or empty vector. Forty-eight hours posttransfection, filtered supernatant was collected and diluted. Cells (5×10^5) were spin infected at 2,500 rpm for 2 h at 4°C and then incubated for 4 h at 37°C to allow entry. Cells were then labeled with CCF2-AM (Invitrogen, Carlsbad, Calif.) as per the manufacturer's instructions, washed, transferred to clear-bottomed, black-walled microtiter plates, and incubated overnight at room temperature to allow the enzymatic reaction to occur. The following morning, plates were read at 460 and 530 nm (Cytofluor 4000; Applied Biosystems, Foster City, Calif.). Background fluorescence from unlabeled cells was subtracted, and the ratio of fluorescence at 460 nm to that at 530 nm (460/530) was calculated for each well. Means and standard deviations shown are calculated from the results of four replicates, and the results presented are representative of three independent experiments.

that FR α does not affect T-cell susceptibility to Ebola virus GP-mediated entry.

Caveolin-1 expression does not enhance lymphocyte infection. FR α either inhabits or is induced by folate or antibody binding to cluster into cholesterol-rich, flask-like invaginations in the plasma membrane termed caveolae (3). Upon binding with folate, FR α 's internalization of the caveolae occurs as part of a process termed potocytosis. An H⁺-pump-mediated decrease in vesicular pH then leads to folate release, and FR α is trafficked back to the cell surface (17). Ebola virus, a pH-dependent virus (37), likely requires endocytosis and trafficking to low-pH vesicles in order to efficiently infect cells. Therefore, if FR α does function as a receptor for Ebola virus, then a pathway similar to that utilized for folic acid metabolism may be employed by the virus for entry. This hypothesis is supported by the finding that caveolin-1, the key structural component of caveolae, appears to colocalize with internalized Ebola virus GP, but not VSV-G, pseudotypes following infection (9). Lymphocytes, including T-cell lines such as CEMss (Fig. 2B), lack expression of caveolin-1 (Cav-1) and hence fail to form caveolae (10, 11), although introduction of Cav-1 into lymphocytes establishes caveolae formation in these cells (10). Thus, Chan and colleagues argue that the failure of Ebola virus GP pseudotypes to efficiently infect T-cells expressing FR α may be due to the lack of caveolae in such cells (6, 9). Other cell types, such as a number of rat thyroid epithelial cell lines (including FRT, WRT, and PCC13 cells), however, are also devoid of caveolae (20, 23, 39; data not shown). To address whether caveolae are essential for infection, these cell lines were analyzed for infection by Ebola virus pseudotypes and found to be susceptible (titers of 1.5×10^5 , 2.8×10^5 , and 3.5×10^5 FFU/ml on FRT, WRT, and PCC13 cells, respectively). FRT cells stably expressing Cav-1 efficiently form caveolae (23). To further assess the role of caveolae in Ebola virus entry, parental FRT cells and cells expressing Cav-1 were challenged with Ebola virus GP pseudotypes. A roughly twofold increase in infection by Ebola virus GP pseudotypes was seen in the cells expressing Cav-1 (1.5×10^5 and 3.1×10^5 FFU/ml for FRT and FRT/Cav-1 cells, respectively). Pseudotypes bearing VSV-G, however, showed similar increases on these cells (1.3×10^7 and 2.7×10^7 FFU/ml for FRT and FRT/Cav-1 cells, respectively), suggesting that the observed increase may be due to selection of a subpopulation which is more permissive for expression of the HIV vector.

While caveolae are clearly not necessary for efficient infection in these cell types, it is possible that caveolae represent only one of a number of potential routes of cellular entry. Therefore, as Cav-1 expression in lymphocytes reconstitutes caveolae formation (10), CEMss and CEM/FR α cells were transduced with MLV pseudotypes encoding human Cav-1. SDS-PAGE analysis of transduced CEMss cells demonstrated Cav-1 expression at levels similar to endogenous Cav-1 seen in 293T cells (Fig. 2B and data not shown). Only a slight increase in Ebola virus-GP-mediated infection of both CEMss and CEM/FR α cells transduced with Cav-1 compared to cells transduced with empty vector was noted (Fig. 2C). CEM/FR α cells expressing Cav-1 were no more infectible by Ebola virus-GP pseudotypes than parental CEMss with Cav-1 (Fig. 2C). Thus, caveolae formation does not facilitate FR α -mediated infection of T-cell lines, suggesting that lack of caveolae is

not the reason for inefficient Ebola virus-GP pseudotype infection in T-cell lines expressing FR α , but rather either other missing components are required for viral entry or FR α does not function as an efficient mediator of entry for Ebola virus.

Viral pseudotypes expressing a membrane-bound form of protein A can bind to cell surface molecules via captured monoclonal antibodies (26). A mutant form of influenza virus hemagglutinin (HA) functional for membrane fusion but not sialic acid binding (22) can then be reincorporated into these viral pseudotypes and used to mediate viral entry (N. Chai and P. Bates, unpublished results). In this manner, lentiviral particles targeted to FR α with an anti-FR α monoclonal antibody (LK26; Signet, Dedham, Mass.) were able to infect the FR α -positive cell lines 293T and CEM/FR α (7.3×10^5 and 2.1×10^2 FFU/ml, respectively) but not CEMss cells (<12 FFU/ml). Thus, FR α on T cells is able to internalize and direct virus to suitable low-pH endosomes for triggering of HA. Complementation of Ebola virus GP with coincorporated HA, however, did not lead to either CEMss nor CEM/FR α cell infection (<12 FFU/ml for both cell lines compared to 5.6×10^6 on 293T cells), suggesting a lack of sufficient interactions between GP and FR α to allow viral internalization.

Folic acid fails to inhibit Ebola virus pseudotype infection. Folic acid, the natural ligand for FR α , was tested for the ability to inhibit Ebola virus GP bearing pseudotype infection of GHOST cells. GHOST cells are a derivative of HOS, a human osteosarcoma cell line, that contain green fluorescent protein (GFP) under the control of an HIV long terminal repeat (LTR). Thus, upon infection by HIV pseudotypes, GFP expression is activated by Tat protein (5). Many formulations of growth media, such as Dulbecco's modified Eagle's medium (DMEM), contain higher levels of folic acid than that reported by Chan et al. to give a 90% reduction in infection on HOS cells (6). Thus, M199 medium, together with charcoal-stripped fetal bovine serum, was used due to its low folic acid content. No differences in Ebola virus GP pseudotype titers were observed in GHOST cells, regardless of whether infections were carried out in M199 medium or in DMEM (data not shown), suggesting that the high levels of folate in DMEM do not affect basal levels of Ebola virus GP pseudotype infection. M199 medium supplemented with concentrations of folic acid up to 2.2 mM also had no effect on Ebola virus pseudotype infection (Fig. 3A). Due to the use of sodium hydroxide for complete solubility of folic acid, the medium-alone controls were adjusted to the same pH (8.5). This adjustment caused a small decrease in levels of infection for both Ebola virus GP and VSV-G pseudotypes compared to levels in neutral-pH M199 medium (data not shown). Thus, contrary to the findings published by Chan et al. (6), folic acid did not significantly reduce Ebola virus GP-mediated entry into a cell line derived from HOS cells. Similar findings with LacZ-encoding pseudotypes were also found in Vero cells (Fig. 3B). In addition, 5-methyltetrahydrofolic acid, an analogue of folic acid that is readily soluble in water and thus can be used at neutral pH, also showed no specific inhibition of Ebola virus GP pseudotype infection of GHOST or Vero cells at concentrations up to 5 mM (Fig. 3B and data not shown). Similarly, a number of polyclonal and monoclonal antibodies directed against FR α (LK26 [Signet], polyclonal rabbit anti-bovine folate binding protein [Biogenesis, Brentwood, N.H.], and goat anti-bovine

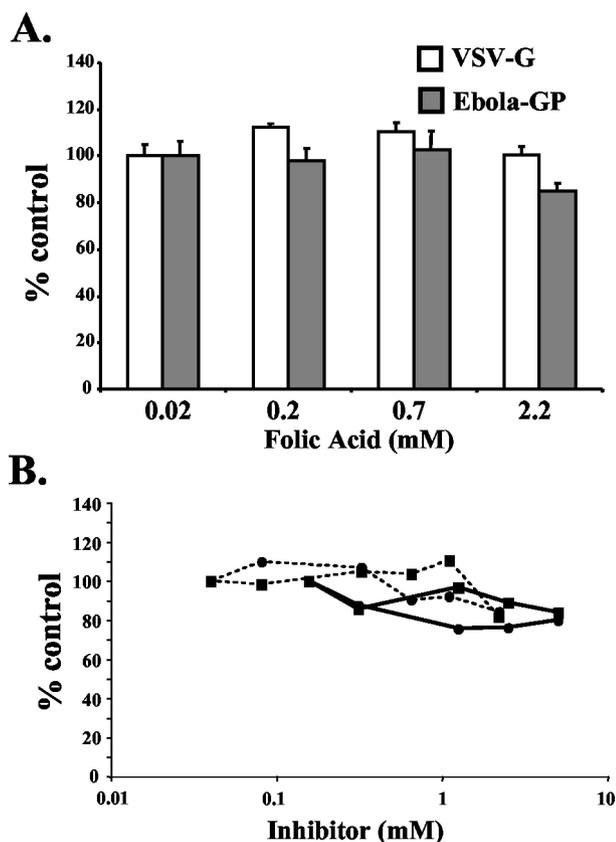


FIG. 3. Inhibition of Ebola virus pseudotype infection of GHOST cells by folic acid. (A) GHOST cells were incubated at 4°C for 30 min with M199 medium containing 5% charcoal-stripped fetal bovine serum alone (approximate basal folic acid concentration in M199 medium is 0.02 mM) or supplemented with various concentrations of folic acid dissolved in sodium hydroxide (1 M). The pH of all solutions was adjusted to 8.5. An equal volume of HIV-based pseudovirions encoding GFP pseudotyped with Ebola virus GP or VSV-G was then added to give final concentrations of folic acid of 0.02, 0.22, 0.73, and 2.2 mM. Cells were incubated for 6 h, the medium was replaced, and the cells were incubated for a further 48 h. GFP expression was then analyzed by flow cytometry, and results are displayed as percentages of the expression in the control (M199 medium alone [pH 8.5], i.e., 0.02 mM folic acid). The results of the experiment shown are representative of three independent experiments. Error bars represent standard deviations of results from three replicates. (B) Vero cells were incubated as described above with M199 medium supplemented with folic acid (broken lines) or 5-methyltetrafolate acid (solid lines). An equal volume of HIV-based pseudovirions encoding LacZ pseudotyped with Ebola virus GP (squares) or MLV amphotropic envelope (circles) was then added to give the plotted concentrations. Cells were incubated for 6 h, the medium was replaced, and the cells were incubated for a further 48 h. *lacZ* expression was then analyzed by quantification of X-galactosidase-stained cells as previously described (32), and results are displayed as percentages of the expression level in the control.

folate binding protein [Rockland, Gilbertsville, Pa.]), as well as soluble versions of folate binding protein (purified bovine folate binding protein [Biogenesis]), failed to specifically inhibit Ebola virus pseudotype infection in HOS and HeLa cells (data not shown). It is possible that neither folic acid nor the antibodies and other ligands tested utilize the same binding sites as Ebola virus GP on FR α ; however, some of these same reagents were reported previously to inhibit infection on HOS and Vero

cells (6). The inhibitory effects of cholesterol depletion on Ebola virus infection (2, 9) suggest a role for lipid rafts in viral entry. It is possible that in experiments by others, ligands to glycosylphosphatidylinositol-anchored proteins (such as FR α) that cluster in rafts may give weak, nonspecific inhibition of viral entry. Monoclonal antibodies to at least three separate GPI-anchored, lipid raft-specific proteins are able to relatively weakly inhibit human T-cell leukemia virus type 1 infection (24). It is unlikely that all of these proteins play a direct role in human T-cell leukemia virus type 1 entry, suggesting that, indeed, viral entry through lipid rafts may be adversely affected nonspecifically by ligands to raft-associated proteins.

In this report, we describe a lack of evidence for the role of FR α as a major component in Ebola virus GP-mediated cell entry. While it is clear that a number of cell types, including primary macrophages, lack FR α and thus must require other molecules for efficient entry, it is more difficult to definitively rule out a role for FR α in all cell types. However, a number of ligands to FR α , including folic acid and antibodies previously reported to inhibit Ebola virus GP pseudotype infection (6), were not found to significantly affect infection on the cell lines tested. As demonstrated with FR α -deficient A549 cells, receptors other than FR α that are capable of mediating efficient infection of Ebola virus exist. It is possible that these receptors are also present on the cell lines used to analyze FR α ligand inhibition and thus allow continued efficient infection even in the presence of complete FR α occlusion. The fact that expression of FR α , even in the presence of Cav-1, was unable to mediate infection of a T-cell line, however, questions the role that FR α plays in Ebola virus infection. Thus, we believe that the receptor(s) for filoviruses remains to be elucidated. Ebola virus GP pseudotyped retroviruses have been used to efficiently transduce cell types, such as airway epithelial cells, that are refractory to the more commonly used glycoprotein from VSV (18). Thus, Ebola virus GP may be utilized as an alternative for gene transfer protocols and highlights the need for a clearer understanding of the mechanisms of Ebola virus entry. In addition, the description of Ebola virus-specific receptors will facilitate the design and testing of inhibitors of Ebola virus infection as potential therapeutics.

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