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Folate Receptor-α Is a Cofactor for Cellular Entry by Marburg and Ebola Viruses

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Summary

Human infections by Marburg (MBG) and Ebola (EBO) viruses result in lethal hemorrhagic fever. To identify cellular entry factors employed by MBG virus, noninfectible cells transduced with an expression library were challenged with a selectable pseudotype virus packaged by MBG glycoproteins (GP). A cDNA encoding the folate receptor-α (FR-α) was recovered from cells exhibiting reconstitution of viral entry. A FR-α cDNA was recovered in a similar strategy employing EBO pseudotypes. FR-α expression in Jurkat cells facilitated MBG or EBO entry, and FR-blocking reagents inhibited infection by MBG or EBO. Finally, FR-α bound cells expressing MBG or EBO GP and mediated syncytia formation triggered by MBG GP. Thus, FR-α is a significant cofactor for cellular entry for MBG and EBO viruses.

Introduction

Marburg (MBG) and Ebola (EBO) filoviruses cause fatal hemorrhagic fevers characterized by widespread tissue infection and destruction (Peters et al., 1996). They are filamentous RNA viruses, packaged by lipid membrane envelopes. Despite primary sequence variation, all filoviruses have substantial similarity in genomic and virion structure (Peters et al., 1996) and infect an extensive and comparable range of mammalian cell types and species (Yang et al., 1998; Wool-Lewis and Bates, 1998; Chan et al., 2000b).

Filovirus entry into target cells is mediated by the binding of transmembrane virus envelope glycoproteins (GP) to an unknown cell surface factor(s) (Peters et al., 1996). Both MBG GP (Volchkov et al., 2000) and EBO GP (Volchkov et al., 1998) are expressed as precursor polyproteins that are cleaved intracellularly into an extracellular protein (GP1) and a membrane-anchored protein (GP2). GP1 presumably initiates infection by binding the target cell surface. While MBG and EBO viruses have common features in GP structure, tropism, and pathogenesis, significant differences among them also exist. First, due to pretranslational processing of EBO GP but not MBG GP, an additional EBO GP product, sGP, is produced along with the longer membrane-bound form (Volchkov et al., 1995; Sanchez et al., 1996). Second, membrane-bound GP expressed by EBO, but not MBG, induces cellular dysregulation that may affect endothelial cells and contribute to hemorrhage observed after infections (Yang et al., 2000; Chan et al., 2000a). Third, prevention of N-glycosylation of target cell proteins specifically inhibits entry of virions mediated by EBO GP but not MBG GP (Takada et al., 1997; Chan et al., 2000b).

Little is known about the identity of cellular factors that mediate entry of filoviruses. Previously, it was proposed that the asialoglycoprotein receptor (ASGP-R) may facilitate MBG infection of liver cells (Becker et al., 1995). However, since both MBG and EBO viruses infect an extensive range of mammalian cell types (Yang et al., 1998; Wool-Lewis and Bates, 1998; Chan et al., 2000b) that do not express ASGP-R, other relevant cellular factors must exist that likely are widely expressed and highly conserved.

Previous studies using pseudotype viruses packaged by MBG or EBO GP demonstrated that T cells are nonpermissive for entry (Wool-Lewis and Bates, 1998; Yang et al., 1998; Chan et al., 2000b). We utilized this information to design a genetic complementation protocol to identify cellular factors mediating entry by MBG or EBO viruses. From functionally reconstituted cells permissive for infection by MBG and the Zaire subtype of EBO (EBO-Z), we identified the folate receptor-α (FR-α) as a mediator of entry utilized by both viruses. This identification offers direct evidence that MBG and EBO viruses share a common pathway for infection and may provide a basis for developing new antiviral strategies directed against these lethal pathogens.

Results

Genetic Reconstitution of Entry into Target Cells by MBG Virus

To develop a quantitative, single-cycle infection system for studying MBG virus entry, MBG GP was incorporated into pseudotype viruses carrying an HIV-1 genome lacking *env* but containing a luciferase reporter gene (pNL-Luc-E$^-$R$^-$) as previously described (Chan et al., 2000b). The vesicular stomatitis virus-G (VSV) GP was packaged into similar pseudovirions. Challenge of human HeLa cells with either pseudotype resulted in significant virus entry (Figure 1A). In contrast, while permissive for VSV entry, human Jurkat T cells were not susceptible to MBG entry. Therefore, HeLa cells, but not Jurkat cells, express significant levels of a relevant cellular factor(s) that controls entry mediated by MBG GP.

To construct a system of genetic complementation (Figure 1B), a retroviral cDNA library (pLIB-HeLa) derived from HeLa was incorporated into pseudotype virions. As target cells for this study, we used a highly transducible derivative of Jurkat cells, Jurkat-EctR, which stably ex-

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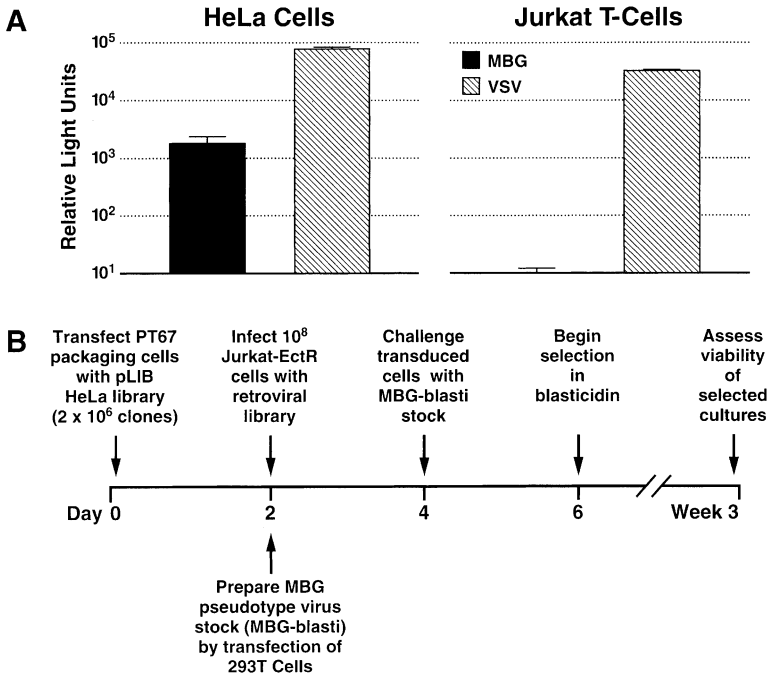


Figure 1. A Genetic Complementation Strategy Based on Jurkat Cells and a Retroviral Expression Library

(A) HeLa cells, but not Jurkat cells, are permissive for entry mediated by MBG GP. Pseudotype virus infection was quantitated by measuring luciferase activity. Data represent the means derived from three separate infections (± SEM).

(B) Selection strategy to recover cDNA inserts encoding cellular factors that reconstitute permissivity for MBG virus entry in Jurkat cells expressing the ecotropic murine leukemia virus receptor (EctR).

press the ecotropic murine leukemia virus (MLV) receptor yet are nonpermissive for entry by MBG pseudotypes (data not shown). Jurkat-EctR cells were transduced with the retroviral library or with pseudovirions carrying a GFP reporter gene (pLIB-GFP) to allow for monitoring of transduction efficiency (30%–40%). Library-transduced cells were then challenged with a selectable MBG pseudotype virus (MBG-blasti) in which MBG GP was used to package an *env*-negative HIV-1 provirus (pHIV-blasti) containing the blasticidin S deaminase gene. Selection in blasticidin S was begun 2 days later. After selection, cell viability was quantitated by Trypan Blue exclusion. In cultures of parental Jurkat-EctR cells challenged with no pseudovirions, library-transduced cells challenged with no pseudovirions, or parental cells challenged with MBG-blasti pseudotypes, all cells died within 8 days, and no viable cells were recovered over a 3-week interval. In contrast, in the library-transduced samples challenged with MBG-blasti, viable cells were readily detected after 8 days of selection and were expanded.

Verification of Permissivity for MBG Virus Entry in Selected Jurkat-EctR Cells

To confirm that these viable cells were permissive for MBG virus entry, they were rechallenged with MBG luciferase virus. Unlike untransduced parental Jurkat-EctR cells that yielded negligible luciferase signals, all blasticidin-selected bulk cultures (such as 2-11, 2-14, 2-23, 2-24) allowed for significant MBG infection (Figure 2A). Importantly, these recovered cells remained nonpermissive for entry by a negative control pseudotype packaged by the HIV-1 JR-FL envelope GP (data not shown). Therefore, MBG entry was not facilitated by a factor specific for HIV components expressed in the pseudotype viruses, nor was it the result of increases in endocytic activity or general alterations in cell surface permeability that might nonspecifically increase entry of diverse viruses.

Folate Receptor-α Reconstitutes Permissivity for MBG Pseudotype Entry

To identify the relevant library cDNA insert, PCR amplification of DNA from reconstituted cells (bulk culture 2-23) was performed using primers based on sequences flanking the cDNA inserts. Four inserts were found to contain small open reading frames (ORF). One carried perfect identity with the 3′ two-thirds of the known cDNA sequence encoding for the human folate receptor-α (FR-α), beginning with an in-frame internal methionine codon (Met-#92) and carrying an intact signal sequence for attachment of the membrane anchor. Flow cytometry verified expression of FR-α on HeLa cells (Figure 2B), on the F10 subclone derived from reconstituted Jurkat-EctR culture 2-23 (Figure 2C), and on other permissive cell types such as Vero E6 (Figure 2D), typically used to passage filoviruses in cell culture (Peters et al., 1996). Northern blot analysis confirmed FR expression in these as well as additional permissive cell types such as human (HOS) and dog osteosarcoma cells (data not shown).

To seek independent confirmation that FR-α reconstitutes permissivity, equivalent numbers of Jurkat-EctR cells expressing either FR-α or GFP were challenged with blasti pseudotypes, selected in blasticidin S, and monitored for viability. Cells expressing either GFP or FR-α were equally permissive for entry by VSV-blasti pseudotypes, as demonstrated by comparable growth of viable cells after 3 days of selection (Figure 3A). A small but detectable number of cells was periodically amplified under selection in the FR-α-negative control sample after challenge with MBG-blasti pseudotypes, perhaps due to the high sensitivity of the viral entry assay. Nonetheless, after 15 days of selection, the culture expressing FR-α exhibited a more than 8-fold greater

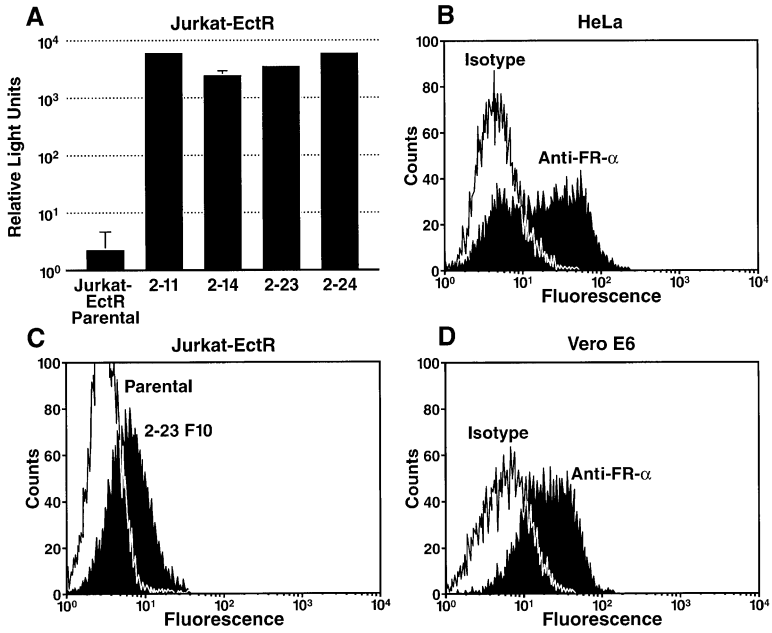


Figure 2. Reconstitution of Permissivity for MBG Entry by Library Transduction Correlates with FR-α Expression

(A) Library-transduced Jurkat-EctR cells challenged with MBG-blasti virus and selected for survival (bulk cultures 2-11, 2-14, 2-23, and 2-23) are permissive for entry by MBG luciferase virus. Displayed values represent the means derived from three separate infections (± SEM).

(B) Flow cytometry confirms that HeLa cells express FR-α. Cells were stained with monoclonal antibody raised against soluble bovine FR-α (filled curve) or isotype control (outlined curve).

(C) Anti-bovine FR-α staining reveals FR-α expression on Jurkat-EctR F10 cells (filled curve), but not parental cells (outlined curve).

(D) Vero E6 cells express FR-α. To detect epitopes on simian FR-α, cells were stained with anti-human FR-α (filled curve) or isotype control (outlined curve).

amplification of viable cells after challenge with MBG-blasti virus compared to the low levels of background permissivity in cells expressing GFP. In contrast, independent expression of other recovered library inserts encoding a start codon and an ORF did not allow for restoration of permissivity (data not shown). Therefore, these results provide direct genetic evidence that FR-α can serve as a specific mediator for infection by MBG virus.

To verify reconstitution of infectibility for wild-type MBG virus in FR-α-positive cells, F10 cells were challenged with wild-type MBG virus, stained with anti-MBG antisera, and visualized by immunofluorescence. Although high levels of reconstitution were not observed, positive staining was nonetheless detected in F10 cells, but not parental cells, after MBG inoculation (Figures 3B and 3C). Taken together with the fact that FR-α can directly reconstitute MBG pseudotype entry, these data indicate that expression of FR-α successfully complemented a key step in cellular entry important for both pseudotype and wild-type MBG viruses.

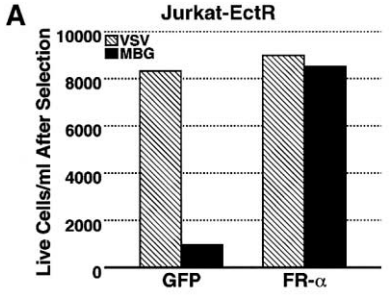
FR-α-Specific Antagonists Inhibit MBG Entry

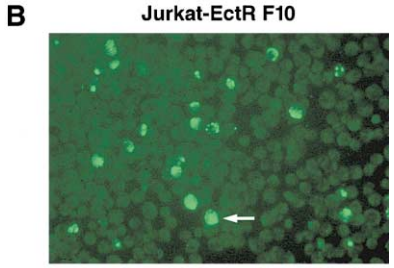
FR-α is a glycosyl-phosphatidylinositol-linked (GPI-linked) protein that binds folic acid for transport into the cytoplasm (Antony, 1996). GPI-linked proteins are sensitive to cleavage by phospholipase C (PLC). Because HeLa cells tolerate the cytotoxic effects of PLC well (data not shown), these cells were pretreated with PLC followed by challenge with pseudotype luciferase viruses. MBG pseudotype infection decreased markedly and in a dose-dependent manner under these conditions, unlike amphotropic (Ampho) MLV pseudotype virus (Figure 4A). A specific, dose-dependent effect on MBG entry was also seen upon treatment of F10 cells (Figure 4B) at noncytotoxic doses confirmed to cleave FR-α from the surface of FR-α-positive Jurkat cells (data not shown). Specific inhibition in two different target cell types provides further support for a role for a GPI-linked protein such as FR-α in MBG entry.

To determine if more specific FR-blocking reagents could inhibit MBG entry, Jurkat-EctR F10 cells were treated with a monoclonal anti-human FR-α antibody (Franklin et al., 1994). MBG infection was specifically and potently reduced by anti-FR-α (Figure 4C). Similarly, the natural ligand of FR-α, folic acid, was tested as a specific inhibitor of MBG entry. Naturally permissive human osteosarcoma (HOS) cells were preincubated in folic acid-containing media followed by pseudotype virus challenge. HOS cells were chosen as target cells since they are more highly permissive for pseudotype filovirus entry than F10, HeLa, or Vero E6 cells (Chan et al., 2000b) and thus could be challenged with smaller viral inocula. While VSV entry was minimally inhibited, MBG entry was significantly reduced in a dose-dependent manner (Figure 4D). Additionally, a cleaved, soluble form of bovine FR-α (folate binding protein, FBP) was used to compete for the binding of MBG GP expressed on the virion envelope. Luciferase viruses were preincubated with FBP. HOS cells were then inoculated with these mixtures, and infection level was compared with that of uncomplexed virus. MBG entry was specifically inhibited by more than 50% by FBP (Figure 4E). Therefore, specific inhibition by anti-FR-α, folic acid, and soluble FBP indicates not only that FR-α facilitates MBG virus entry in both genetically reconstituted cells and naturally permissive cells, but also that this function likely relies on it directly binding MBG virions.

FR-α Mediates Membrane Fusion Triggered by MBG GP

The role of FR-α in cell-cell fusion driven by MBG GP was also assessed. 293T cells overexpressing MBG GP were cocultivated with cells overexpressing either CD4 or FR-α. Because fusion mediated by MBG GP may be pH-dependent (Chan et al., 2000b), syncytia formation (≥3 nuclei) was quantitated after pulsing cells at pH 5 or pH 7 and allowing for recovery. As described previously (Vey et al., 1992), expression of the influenza hemagluti-





C

Positive Cells by IFA (Approximate % of Total Cells)			
Cell Line	MOI = 10	MOI = 1	MOI = 0.1
Vero E6	100	60	10
Jurkat-EctR Parental	0	0	0
Jurkat-EctR F10	10	1	0

Figure 3. FR-α Reconstitutes Permissivity for MBG Entry in Jurkat-EctR Cells

(A) Jurkat-EctR cells expressing FR-α are permissive for entry by MBG-blasti virus. GFP- or FR-α-positive cells were inoculated with blasti viruses, selected in blasticidin S, and monitored for cell viability. Displayed values are representative of two separate reconstitution procedures.

(B) FR-α-positive F10 cells are infectible (white arrow) by wild-type MBG virus. F10 cells were challenged with MBG virus and stained using anti-MBG antisera for indirect immunofluorescence analysis (IFA).

(C) F10 cells, but not parental cells, are infectible by wild-type MBG virus. Vero E6, Jurkat-EctR parental, and F10 cells were challenged with wild-type MBG virus at increasing multiplicity of infection (MOI), and infected cells were quantitated by IFA.

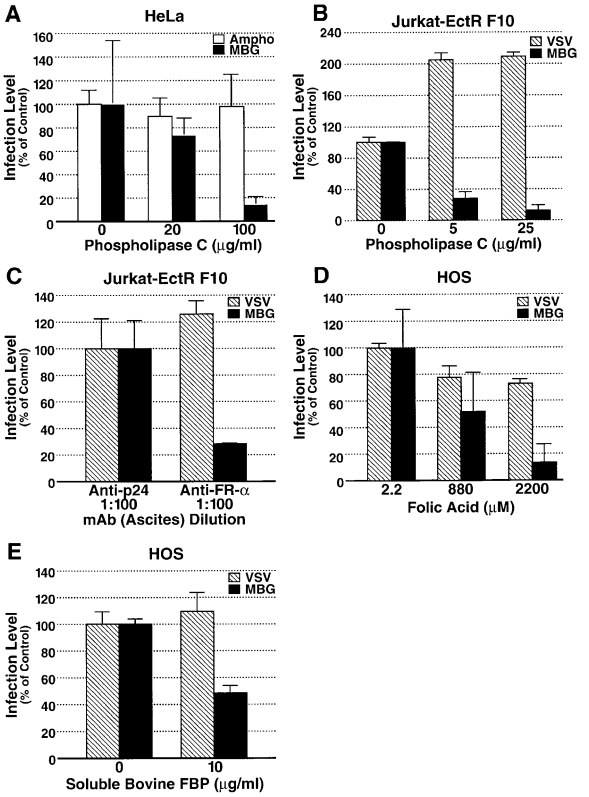


Figure 4. Inhibition of MBG Pseudotype Entry with FR-Specific Blocking Reagents

Pretreatment of (A) HeLa cells or (B) F10 cells with phospholipase C (PLC) specifically abrogates MBG pseudotype entry in a dose-dependent manner. (C) Entry by MBG luciferase virus into F10 cells is specifically abrogated by anti-human FR-α IgG1 compared with that seen with isotype control (anti-HIV p24 IgG1). (D) Dose-dependent inhibition of MBG pseudotype entry into human osteosarcoma (HOS) cells by folic acid. (E) MBG pseudotype infection of HOS cells specifically decreases in the presence of soluble bovine FBP. Displayed values in (A)–(E) are the means derived from three separate infections (± SEM).

nin protein (HA) allowed for robust formation of syncytia (>100 per well) when exposed to pH 5 but not pH 7 (Figure 5A). No significant fusion was detected at either pH in cultures expressing MBG GP with CD4 (Figure 5B), MBG GP alone, FR-α alone, or ecotropic MLV GP together with FR-α (data not shown). In contrast, exclusively after pH 5 exposure, a range of 1 to 6 unambiguous syncytia per well was consistently observed in cocultures expressing MBG GP with FR-α (Figure 5C). While less robust than that seen with HA, this reproducible level of MBG GP-induced syncytia formation facilitated by FR-α provides independent evidence that FR-α interacts with MBG GP for subsequent fusion.

FR-α Mediates Entry by EBO-Z Virus

The genetic complementation protocol described earlier was employed to identify factors that mediate entry by EBO-Z virus. HeLa, but not Jurkat, are permissive for EBO-Z entry (Yang et al., 1998; Chan et al., 2000b). Therefore, after HeLa library delivery, transduced Jurkat-EctR cells were challenged with a pseudotype virus packaged by EBO-Z GP (EBO-Z-blasti) and selected in blasticidin S. Only library-transduced, but not parental, cultures that had been challenged by EBO-Z-blasti virus yielded viable cells after selection. Individual cell clones surviving selection, such as A7-1, were indeed infectible by EBO-Z luciferase virus (Figure 6A) but not by the negative control HIV-1 JR-FL pseudotype (data not shown). Strikingly, A7-1 was also permissive for MBG entry, suggesting that a common factor(s) may complement deficiencies of permissivity for both MBG and EBO-Z virus entry. Correspondingly, A7-1 cells were found by PCR amplification to carry a library insert encoding for full-length FR-α. By Northern blot analysis, 7 other recovered cell clones selected for EBO-Z permissivity were positive for FR-α-specific message. Moreover, FR-α-positive F10 cells that had been selected for MBG permissivity were infectible by both MBG and EBO-Z luciferase viruses (Figure 6B). Thus, in two inde-

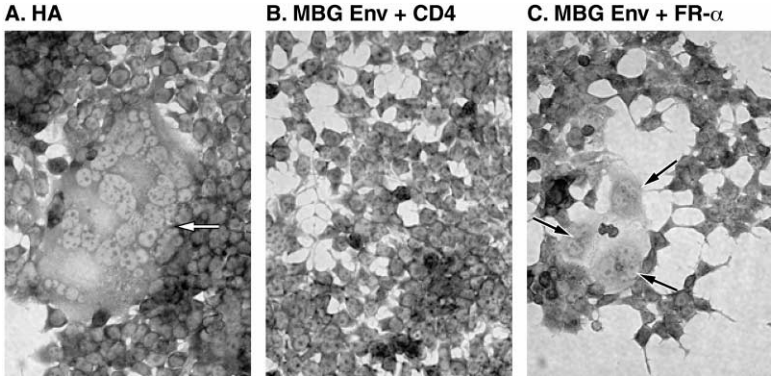


Figure 5. Syncytia Formation in 293T Cells by MBG GP Is Facilitated by FR-α

(A) Syncytium production (>100 syncytia/well, white arrow) in 293T cells expressing influenza hemaglutinin (HA) after pH 5 exposure.

(B) No detectable syncytia in cocultures expressing MBG GP with CD4 after pH 5 exposure.

(C) Syncytium production in cocultures expressing MBG GP with FR-α after pH 5 exposure (1 to 6 syncytia/well, black arrows).

pendent procedures designed to reconstitute permissivity for filovirus entry—one approach utilizing MBG pseudotypes and the other utilizing EBO-Z pseudotypes—recovered cells were found to be FR-α-positive and concurrently permissive for both MBG and EBO-Z infection. These results provide genetic evidence demonstrating that FR-α mediates infection by EBO-Z as well as by MBG virus.

To obtain direct genetic proof that FR-α reconstitutes permissivity for EBO-Z entry, Jurkat-EctR cells expressing either GFP or FR-α were challenged with blasti pseudotypes, selected in blasticidin S, and monitored for cell viability. Comparable susceptibility to VSV-blasti entry was observed in GFP-positive and FR-α-positive cells after 3 days of selection (Figure 6C). In contrast, cells expressing FR-α, but not GFP, were permissive for EBO-Z pseudotype entry and allowed for detectable cell growth after 35 days of selection. Therefore, similar to the MBG studies, these independent genetic data demonstrate that FR-α mediates cellular entry by EBO-Z virus.

Subsequently, FR-α-specific inhibition strategies were pursued. HeLa cell pretreatment with phospholipase C abolished entry by EBO-Z pseudotypes but did not affect control virus entry (data not shown). Infection of F10 cells by EBO-Z pseudotypes decreased significantly in the presence of monoclonal anti-human FR-α antibody (Figure 6D). Furthermore, when FR-α-positive, monkey Vero E6 cells, which are commonly used to propagate filoviruses in cell culture (Peters et al., 1996), were treated with a polyclonal antisera raised against bovine FBP, EBO-Z entry specifically decreased by nearly 60% (Figure 6E). Thus, EBO-Z entry was specifically inhibited in the presence of separate anti-FR-α antibodies in genetically reconstituted human cells and in untransduced monkey cells, further defining FR-α as a conserved mediator of EBO-Z virus entry in different cell types and mammalian species. Finally, while soluble FR-α (FBP) minimally affected VSV entry, it induced a substantial dose-dependent inhibition of EBO-Z infection (Figure 6F). Therefore, mirroring the MBG inhibition profiles, EBO-Z pseudotype entry was specifically abrogated by agents disrupting the interaction between virion GP and FR-α.

FR-α Binds to Cells Expressing MBG GP or EBO-Z GP

To determine if FR-α binds MBG or EBO-Z GP, immunofluorescence was used to detect FBP attached to the surface of cells expressing either MBG GP or EBO-Z GP (Figure 7). FR-α-negative CHO-K1 cells (Weitman et al., 1992a), expressing either Ampho, MBG, or EBO-Z GP, were incubated with bovine FBP and a polyclonal anti-bovine FBP antibody that was nonneutralizing for MBG or EBO-Z entry (data not shown). Samples were then fixed and stained with a fluorescein-conjugated secondary antibody in order to highlight selectively those cells with FBP bound to their surface. Transfected cells exposed to anti-FBP and secondary antibody in the absence of FBP yielded negligible staining. Additionally, cells transfected with Ampho GP exhibited only a low level of background staining in the presence of FBP. In contrast, cells expressing MBG GP or EBO-Z GP that were incubated with FBP displayed ring-like cell surface staining. This significant and specific staining pattern indicates that MBG GP or EBO-Z GP can bind FR-α. Taken together with the recovery of FR-α in separate library transductions challenged with either MBG- or EBO-Z-blasti viruses, various FR-α-specific inhibition assays, the MBG GP-driven induction of cell-cell fusion by FR-α, and the functional reconstitution of permissivity with FR-α expression constructs, we conclude that FR-α is a cell surface factor that facilitates cellular entry of MBG and EBO-Z viruses.

Discussion

In this study, a genetic strategy combining selectable pseudotype viruses packaged by filovirus GP with a retroviral expression library was implemented to select for reconstituted cells permissive for filovirus entry. Pseudotype viruses have been used extensively for studying entry by native filoviruses (Takada et al., 1997; Wool-Lewis and Bates, 1998; Yang et al., 1998; Chan et al., 2000b). Furthermore, a comparison of entry requirements of pseudotype (Figure 2A) and wild-type (Figures 3B and 3C) filoviruses confirmed their dependence on the same cellular pathway and validated this experimental approach. Based on this strategy, genetically reconstituted Jurkat cells were successfully selected for permissivity for either MBG or EBO-Z entry. In both populations, surviving cells were found to be permissive for entry by both MBG and EBO-Z viruses. Correspondingly, FR-α cDNA inserts were recovered from both populations, demonstrating genetically that FR-α reconstitutes deficiencies for entry by both types of filoviruses.

To establish directly this role in filovirus entry, several

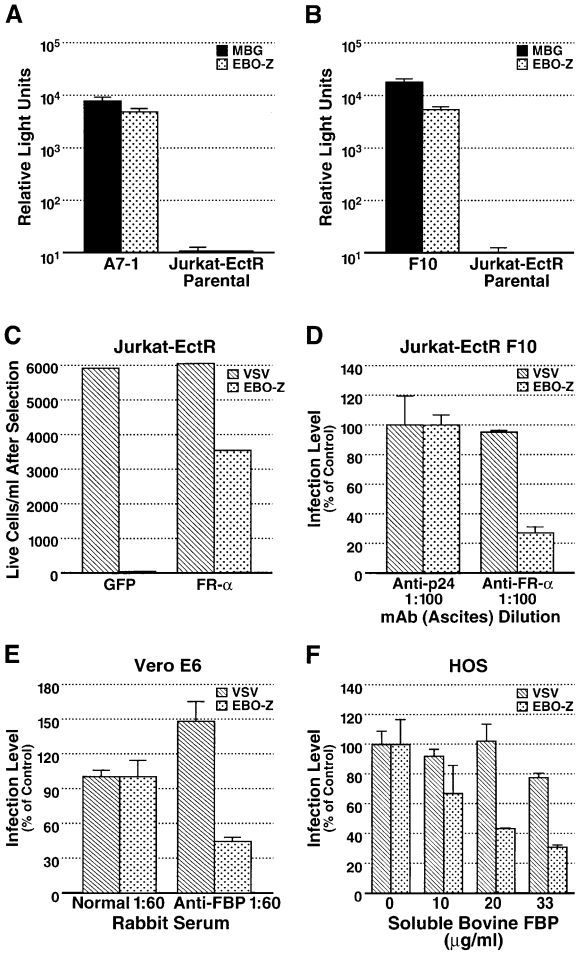


Figure 6. FR-α Mediates EBO-Z Virus Entry

(A) FR-α-positive A7-1 cells selected for EBO-Z permissivity and (B) FR-α-positive F10 cells selected for MBG permissivity are infectible by both MBG and EBO-Z luciferase viruses. (C) Jurkat-EctR cells expressing FR-α are permissive for entry by EBO-Z-blasti virus. Cells expressing GFP or FR-α were inoculated with blasti viruses, selected in blasticidin S, and monitored for cell viability. Displayed values are representative of two separate reconstitution procedures. (D) EBO-Z pseudotype entry into F10 cells is abrogated in the presence of anti-human FR-α IgG1. (E) EBO-Z pseudotype entry into Vero E6 cells is inhibited by polyclonal anti-bovine FBP. (F) Dose-dependent decrease of EBO-Z infection of HOS cells in the presence of soluble bovine FBP. Displayed values in (A), (B), and (D)–(F) are the means derived from three separate infections (± SEM).

experimental avenues were implemented. These included reconstitution of permissivity for filovirus entry through FR-α expression, inhibition of infection by blocking association of filovirus GP with FR-α, generation of MBG GP-triggered membrane fusion by FR-α, and demonstration of purified soluble FR-α specifically bound to cells expressing either MBG or EBO-Z GP. These independent genetic and biochemical studies converge upon the same conclusion that FR-α acts as a significant cofactor that mediates infection by MBG or EBO-Z virus.

FR-α is a widely expressed 38–39 kDa GPI-linked cell surface protein that binds extracellular folic acid for cellular uptake (Antony, 1996). It is one of four receptor isoforms (α, β, and γ, and γ′) that carry 68%–79% identity in amino acid sequence (Maziarz et al., 1999) and differ in their size, tissue distribution (Ross et al., 1994), or ligand binding sites (Maziarz et al., 1999). Only the FR-α isoform has been detected in normal, nonhematopoietic tissue other than placenta (Ross et al., 1999). Upon binding folic acid, FR-α is endocytosed in vesicles that have been characterized as caveolae via a pathway implicated in the uptake of other GPI-linked proteins (as reviewed by Anderson et al., 1992). However, the exact mechanism of endocytosis (Mayor et al., 1994) and type of vesicle (Rijnboutt et al., 1996) used for this uptake is controversial. After endocytosis, folic acid is released via a decrease in intravesicular pH, followed by recycling of FR-α to the cell surface (Kamen et al., 1988; Lee et al., 1996).

The identification of FR-α as a cofactor for MBG and EBO-Z entry is plausible in the biologic context of filoviruses. The pH-dependence both of filovirus entry into target cells (Takada et al., 1997; Wool-Lewis and Bates, 1998; Chan et al., 2000b) and of membrane fusion mediated by MBG GP (Figure 5C) correlates with the pathway of endocytosis and acidification exploited by FR-α for folic acid uptake. In addition, pretreatment of target cells with caveolae inhibitors specifically abrogated entry by MBG and EBO-Z pseudotype viruses, arguing that the caveolar pathway thought to be associated with endocytosis of FR-α also mediates filovirus infection (C.J.E. and M.A.G., unpublished observations). Future characterization of the endocytic pathway downstream of the initial interaction with the virion GP will be important for understanding the filovirus life cycle and defining the exact composition of the vesicles used by FR-α and other GPI-linked receptors for ligand uptake.

Much, but perhaps not all, of the pathology of filovirus infection also correlates with the range of FR-α expression. The fact that FR-α is highly conserved in a number of mammalian species (Antony, 1996) corresponds with the wide tropism of filoviruses in cell culture (Chan et al., 2000b) and animal models (Geisbert and Jaax, 1998; Bray et al., 1999; Connolly et al., 1999). Furthermore, FR-α is significantly expressed in a variety of epithelial and parenchymal cells in the lung, gonads, gastrointestinal (GI) tract, genitourinary (GU) tract (Antony, 1996), pancreas, thyroid (Weitman et al., 1992b), and arteries (Weitman et al., 1992a), but not in lymphocytes (Franklin et al., 1994). Concordantly, in humans and in animal models, filoviruses have been documented to replicate in parenchymal cells of the lung (Johnson et al., 1995), gonads, GI tract, thyroid (Connolly et al., 1999), and pancreas (Geisbert and Jaax, 1998), as well as in epithelial cells of the GU tract (Baskerville et al., 1985; Connolly et al., 1999), but not in lymphocytes (Geisbert et al., 2000). In addition, major targets of direct infection by filoviruses include macrophages/monocytes and fibroblasts, which are present in numerous susceptible tissues, as well as hepatocytes and endothelial cells (Zaki and Goldsmith, 1999). Importantly, significant expression of FR-α has been documented in both macrophages (Franklin et al., 1994) and fibroblasts (Antony, 1996). Furthermore, we found that naturally permissive HOS cells, derived from fibroblast-like sarcoma cells, and Vero E6 cells, derived from fibroblast-like kidney cells, both express folate receptor that is active in mediating filovirus entry (Figures 4D and 6E). Although high

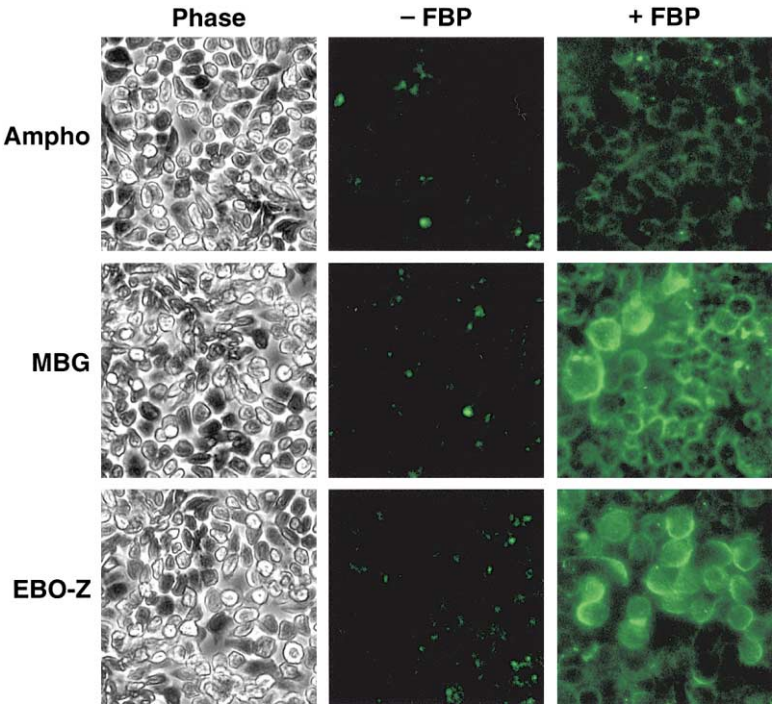


Figure 7. Soluble FR-α (FBP) Binds Specifically to Cells Expressing MBG GP or EBO-Z GP

CHO-K1 cells expressing Ampho, MBG, or EBO-Z GP were incubated with bovine FBP and polyclonal anti-FBP (+ FBP). Control wells were incubated in the presence of only polyclonal anti-FBP (Phase and – FBP). Cells were then stained with a fluorescein-conjugated secondary antibody and analyzed by immunofluorescence (+ FBP and – FBP). Corresponding phase microscopy of the control wells is displayed for reference (Phase).

levels of FR-α have not been reported in hepatocytes and endothelial cells, FR protein was detected in pig hepatocytes (Villanueva et al., 1998) as well as in rat liver tissue (da Costa et al., 2000), while FR-α mRNA was detected by RT-PCR in human liver (Ross et al., 1994). It is possible that these low levels of FR-α are sufficient to facilitate filovirus entry into hepatocytes or that further expression is induced in vivo (Antony, 1996). It currently remains unclear if endothelial cells also express low yet functional receptor levels. Nonetheless, the otherwise vast range of permissive cells with significant FR-α expression largely matches known features of the filovirus life cycle and strengthens our conclusion that FR-α plays a role in propagating the spread of filovirus infection in relevant cell types.

The fact that FR-α mediates entry by either MBG or EBO-Z virus also reinforces the hypothesis that these viruses exploit fundamentally similar cellular pathways to infect cells. However, we previously described biochemical differences in target cell entry mediated by EBO-Z GP compared with that by MBG GP (Chan et al., 2000b). Therefore, although both MBG and EBO-Z viruses similarly utilize FR-α for initiation of entry, it will be important to determine how their entry processes diverge. It is possible that their envelope GP may interact differentially with other unknown cellular factors. In support of this hypothesis, we discovered that not all cell types that are naturally permissive for MBG or EBO-Z entry express FR-α (data not shown). Therefore, although FR-α mediates MBG and EBO-Z virus entry in certain cell types, at least one alternate factor must share this function in other cellular contexts. Similar to other viruses that can interact with one of various receptors to enter cells such as HIV (as reviewed by Berger et al., 1999), we hypothesize that filoviruses can utilize a family of receptors to facilitate their life cycle in a broad range of cell types.

While FR-α was successfully recovered as a cofactor for filovirus entry in this approach, use of Jurkat cells provided an imperfect cellular context to reconstitute high levels of MBG or EBO-Z entry with either pseudotype or wild-type (Figure 3C) viruses. T cells were selected for this study since lymphocytes represent the only mammalian cell type tested that consistently resists robust infection by filoviruses in cell culture (Yang et al., 1998; Wool-Lewis and Bates, 1998; Chan et al., 2000b) and in animal models (Geisbert et al., 2000). However, not only do Jurkat cells express little to no FR-α, but also they are typically deficient in certain endocytic processes such as caveolae formation (Fra et al., 1994) proposed to be instrumental for FR-α-mediated folic acid uptake. Therefore, it is probable that Jurkat T cells do not support robust infection because they carry multiple impediments to filovirus entry. Consequently, additional cellular factors beyond FR-α may be necessary to reconstitute permissivity to high levels observed in nonmanipulated, infectible cells. Nonetheless, in combination with the high sensitivity of the genetic complementation protocol, the use of Jurkat T cells was successful in recovering FR-α for characterization as a mediator of filovirus entry by other independent strategies. Delivery of cDNA libraries derived from FR-α-negative permissive cells into Jurkat cells may allow for recovery of other host factors that play a role independently or in combination with FR-α in the filovirus entry process.

Finally, the identification of FR-α as a cofactor that mediates filovirus entry may offer a novel therapeutic avenue for effectively treating the hemorrhagic fevers caused by infection. The observation that soluble FBP, anti-FR-α antisera, and folic acid can inhibit entry by filoviruses may provide a basis for establishing treatment regimens designed to block FR-α from associating with extracellular filoviruses. However, as indicated earlier, FR-α may not facilitate virus entry into all cell types.

Nonetheless, the characterization of FR-α as a cofactor that mediates filovirus entry is an important first step both in gaining a molecular understanding of how filoviruses associate with cells and in designing therapeutic measures to intervene in these key events.

Experimental Procedures

Cell Lines

Jurkat T cells stably expressing the ecotropic murine leukemia virus (MLV) receptor (Jurkat-EctR, also known as Jurkat.ecoR) were provided by Dr. G. Nolan (Stanford University). PT67 packaging cells were cultured as described (Clontech). Human osteosarcoma (HOS) cells carrying a human green fluorescent protein reporter gene driven by an HIV-2 Tat-dependent LTR were provided by Dr. D. Littman (Skirball Institute). Other cell types were cultured as recommended by the American Type Culture Collection (ATCC).

Plasmids and cDNA Library Amplification

The *env*-negative, HIV-1 provirus carrying a luciferase reporter gene, pNL-Luc-E$^-$R$^-$ (Connor et al., 1995), was provided by Dr. N. Landau (Salk Institute). The *env*-negative, HIV-1 provirus carrying the blasticidin S deaminase gene, pHIV-blasti, was provided by Dr. R. Sutton (Baylor University). Expression plasmid pVSV-G encoding the vesicular stomatitis virus-G (VSV) protein was provided by Dr. J. Burns (University of California, San Diego), and pMULV-A encoding the amphotropic (Ampho) MLV *env* was provided by Dr. K. Page (University of California, San Francisco). The cDNA clones encoding MBG GP and EBO-Z GP were provided by Dr. A. Sanchez (Centers for Disease Control and Prevention) and cloned into the pCMV4neo expression vector (Chan et al., 2000b). The full-length library cDNA and truncated cDNA encoding FR-α were recovered by PCR as described below. For reconstitution studies, pBabe-FR-α was constructed by subcloning the full-length FR-α gene into the MLV retroviral vector pBabeMN, provided by Dr. G. Nolan (Stanford University), and the MLV retroviral vector encoding GFP, pMSCV2.2-IRES-GFP, was provided by Dr. W. Sha (University of California, Berkeley). The expression vector encoding the influenza hemaglutinin protein (HA) was provided by Dr. W.F. Anderson (University of Southern California).

DNA from the MLV retroviral HeLa cDNA library (pLIB-HeLa, 2×10^6 independent clones) was amplified as described by Clontech. The plasmid pLIB-GFP encoding GFP in the MLV backbone was used for quantitating transduction efficiency.

Antibodies

For detection of wild-type MBG virus infection, guinea pig anti-MBG antisera were used (Hevey et al., 1997). For inhibition of pseudotype virus entry, a monoclonal mouse anti-human FR-α (IgG1) ascites (No. 458), provided by Dr. W. Franklin (University of Colorado Health Sciences Center) (Franklin et al., 1994), and monoclonal mouse anti-HIV-1 p24 Gag (IgG1) ascites were compared. Similarly, polyclonal antisera raised against bovine folate binding protein (FBP, Biogenesis) were compared with normal rabbit sera. A mouse monoclonal anti-soluble bovine FR-α (IgG1 clone 42/033, Biogenesis) or IgG1 isotype control was used for flow cytometry of human cells. To detect simian FR-α on Vero E6 cells more efficiently, the mouse anti-human FR-α (IgG1) was used. To detect cell surface binding of FBP, goat anti-bovine FBP (Rockland) and fluorescein-conjugated rabbit anti-goat IgG F(c) (Rockland) were used.

Pseudotype Virus Preparation and Genetic Reconstitution of Permissivity for Filovirus Entry

To prepare HIV-1 pseudotype virus packaged by viral GP, an HIV-1 proviral construct was cotransfected with a GP expression vector in 293T cells as previously described (Chan et al., 2000b). To reconstitute permissivity for MBG entry, library DNA (3 μg/well of 6-well plate) or pLIB-GFP (3 μg/well) was packaged into pseudovirions by transfecting PT67 cells and harvesting culture supernatants. Jurkat-EctR cells (8.0×10^7) were then divided into 10 separate batches and transduced with library-containing viral supernatants with polybrene (5 μg/ml) via spin infection (2.5×10^3 RPM, 32°C, 2 hr). In a parallel batch, Jurkat-EctR cells were transduced with pseudovirions carrying pLIB-GFP. By quantitating GFP-positive Jurkat-EctR cells two days after transduction, library infection was optimized to achieve a reproducible 30%–40% transduction efficiency. Selectable MBG-blasti or VSV-blasti virus was harvested and used to challenge parental cells or cells transduced with the library 48 hr earlier, followed by transfer into medium containing blasticidin S (ICN Pharmaceuticals, Inc., 40 μg/ml). During selection for 2–3 weeks, cells were monitored for viability by Trypan Blue exclusion. Selected cells were expanded and subjected to limiting dilution to obtain monoclonal cell populations. To select for cells permissive for EBO-Z virus entry, similar procedures were performed using EBO-Z-blasti virus.

Challenge of Cells with Pseudotype and Wild-Type Filoviruses

To determine permissivity for entry by pseudotype luciferase viruses, target cells were incubated with virus for 48–72 hr, and luciferase expression was quantitated as previously described (Chan et al., 2000b). Alternatively, Vero E6, parental, and reconstituted Jurkat-EctR cells were inoculated with wild-type MBG virus. Immunostaining was performed as previously described (Schmaljohn et al., 1995).

PCR Recovery of cDNA Library Inserts from Transduced Jurkat-EctR Cells

Genomic DNA was extracted from Jurkat-EctR cells selected for permissivity for MBG entry using the "Easy DNA" kit (Invitrogen). Extracted DNA was used as a template for PCR-based amplification using the Expand PCR kit (Roche Molecular Biochemicals) and oligonucleotide primers derived from sequences flanking the library inserts. Specific DNA bands amplified in experimental samples, but not control samples, were used for TA cloning (Invitrogen). Insert sequences were compared to known genomic and cDNA sequences using Entrez BLAST software.

To recover cDNA inserts from cells selected for permissivity for EBO-Z entry, RT-PCR was used for greater efficiency of library insert retrieval. Total RNA was extracted from cells by the RNA STAT 60 method (Tel-Test, Inc.). Using total RNA as a template, RT-PCR was performed using the First Strand cDNA Synthesis Kit for RT-PCR (Roche Molecular Biochemicals), followed by PCR of resulting products, TA cloning, and sequencing.

Reconstitution of Permissivity for MBG and EBO-Z Entry by Expression of FR-α

Retroviral expression vectors encoding either FR-α or GFP were transduced into Jurkat-EctR cells as above for HeLa library delivery. Equal numbers of transduced cells were inoculated (48 hr, 37°C) with blasti viruses with polybrene (5 μg/ml) followed by transfer into medium containing blasticidin S (ICN, 40 μg/ml). During selection for 2–5 weeks, cells were monitored for viability by Trypan Blue exclusion and counted on a hemacytometer. Growth of cultures challenged with VSV-blasti virus was quantitated after 3 days of selection. Growth of cells challenged with MBG-blasti virus or EBO-Z-blasti virus was quantitated 3 days following the initial visualization of viable cells in the transduced cultures. Alternatively, Jurkat-EctR cells were transfected with the relevant expression vectors and challenged with luciferase viruses as described above.

Inhibition of Pseudotype Virus Entry by FR-α-Specific Blockers

HeLa cells or reconstituted Jurkat-EctR F10 cells were treated with phospholipase C (ICN) for 2 hr at 37°C, washed, and challenged with luciferase viruses for 4 hr at 37°C. Luciferase expression was assessed after 72 hr. F10 cells were preincubated with anti-human FR-α (IgG1) or anti-HIV p24 Gag (IgG1) for 15 min at 4°C. Cells were challenged with luciferase virus in the presence of antisera, followed by assessment of luciferase expression. Vero E6 cells were preincubated with medium containing polyclonal anti-FBP (with 0.1% sodium azide) or normal rabbit sera (with 0.1% sodium azide) for 15 min at 4°C, followed by virus challenge as above. HOS cells were preincubated at 4°C for 30 min with media (pH 8.3) containing folic acid (ICN) added from a 220 mM stock (1 M NaOH). Cells were challenged with luciferase viruses in the presence of folic acid. After 12 hr, culture medium was replaced, and luciferase expression was assessed after 48 hr. Finally, pseudotype virus produced in the

absence of serum or folic acid was preincubated with soluble FR-α (FBP; Svendsen et al., 1982) derived from bovine milk (Sigma) for 15 min at 4°C. HOS cells were challenged with virus supernatants alone or with virus mixtures containing FBP for 4 hr at 37°C. Cells were then washed, medium was replaced, and luciferase expression was assessed after 72 hr.

Quantitation of Cell-Cell Fusion Generated by MBG GP

293T cells were transfected with envelope GP (MBG GP or HA) or receptor expression vector (CD4 or FR-α) as previously described (Chan et al., 2000b). After 24 hr, cells were detached using enzyme-free Cell Dissociation Buffer (Life Technologies). The appropriately transfected cells were mixed (1:1 ratio), cultured on poly-L-lysine-coated (Sigma) 24-well plates as previously described (Allan, 2000), and incubated at 37°C for 48 hr. To trigger syncytia (≥3 nuclei), cells were incubated in either 75 mM MES/10 mM HEPES buffer (pH 5) or 10 mM HEPES buffer (pH 7) for 10 min at 37°C as previously described (Vey et al., 1992). The buffer was then replaced with medium. Syncytia were scored after 24 hr.

Binding of Cells Expressing MBG GP or EBO-Z GP with FBP

CHO-K1 cells were plated in chamber slides coated with poly-L-lysine and transfected with MBG GP, EBO-Z GP, or Ampho GP expression vectors using LipofectAMINE (Life Technologies, Inc.). After 48 hr, bovine FBP (33 μg/ml) was incubated in the presence of nonneutralizing polyclonal goat anti-bovine FBP (250 μg/ml) for 15 min at 4°C in RPMI 1640 devoid of folic acid. Cells were then incubated in the presence of the FBP/anti-FBP mixture or in the presence of anti-FBP alone for 30 min at 4°C. Cells were washed (3 × ice cold PBS), fixed in 2% paraformaldehyde, washed, and incubated with fluorescein-conjugated secondary antibody (4 μg/ml) for 45 min at 25°C in the dark. Slides were washed, mounted, and analyzed by fluorescence microscopy.

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