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Author

Chan, Stephen Y.

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Entry Mechanisms and Pathologic Effects Mediated by the Envelope Glycoproteins of the Human Immunodeficiency Virus Type 1, Marburg, and Ebola Viruses

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Stephen Y. Chan

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Dedication

For my parents Lois and Shung-Kai Chan,

whose wisdom and love are endless.

Preface

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All of the work described in this dissertation has been submitted and/or published in scientific journals. Chapter 2 contains an article published in the *Journal of Virology* reproduced with copyright permission from the American

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Entry Mechanisms and Pathologic Effects Mediated by the Envelope Glycoproteins of the Human Immunodeficiency Virus Type 1, Marburg, and Ebola Viruses

Stephen Y. Chan

Cellular entry by enveloped viruses is a central event in pathogenesis and depends critically on the binding of the viral envelope glycoprotein (GP) to a cell surface receptor(s) and/or coreceptor(s). The objective of this thesis project was to characterize the molecular methods by which the envelope GP of the human immunodeficiency virus type 1 (HIV-1), Marburg (MBG), and Ebola (EBO) viruses bind to host cell factors to initiate infection and to determine the pathogenic consequences of those interactions. For HIV-1, the hypothesis was investigated that HIV dementia (HIVD) results largely from alterations in the gp120 envelope protein, influencing receptor/coreceptor use. Through functional characterization of primary gp120 V3 sequences, the CCR5 chemokine receptor was identified as a primary coreceptor for brain-derived viruses regardless of HIVD diagnosis, indicating that alteration of coreceptor *specificity* is not necessary for HIVD development. In contrast, an alternate *mechanism* of coreceptor use was characterized that allows for HIV-1 entry into CD4-negative human fetal astrocytes in a coreceptor-dependent manner only when CD4 is

provided in *trans* by donor cells. Such a process may be active in facilitating neurodegeneration and inciting dementia. For the filoviruses MBG and EBO, GP-mediated entry and pathogenesis were characterized by using pseudotype viruses carrying an HIV-1 backbone and packaged by MBG or EBO GP. While these pseudotype viruses infected a comparable wide range of cell types, it was discovered that the MBG and EBO GP utilize distinct pathways to enter cells and incite cellular dysregulation. To identify cellular factors that facilitate virus uptake, a genetic complementation strategy combining selectable filovirus pseudotype viruses with a retroviral expression library was implemented to isolate cells exhibiting reconstitution of permissivity for filovirus entry. Library cDNA inserts encoding for the folate receptor-α (FR-α) protein were recovered from reconstituted cells. Utilizing further genetic and biochemical strategies, FR-α was identified as a cellular cofactor that mediates entry by both MBG and EBO viruses. Taken together, these discoveries should expand our understanding of the pathogenic life cycles of HIV-1, MBG, and EBO viruses and may facilitate the development of more effective treatments of these devastating and lethal infections.

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Chapter 1

Introduction

I. Overview

The method(s) by which a virus binds to a host cell to initiate entry is a central determinant of viral pathogenesis. Enveloped viruses, which derive their lipid membrane coat from budding off host cell membranes, typically attach to a target cell by binding to a surface receptor(s) via envelope glycoprotein (GP) spikes expressed on the virion surface. Subsequently, a fusogenic domain of the glycoprotein is activated by conformational changes associated with receptor binding and/or endocytosis and triggers fusion of the viral envelope with host membranes, allowing for entry.

Specifically, the human immunodeficiency virus type 1 (HIV-1), Marburg (MBG) virus, and Ebola (EBO) virus represent distinct enveloped viruses that carry differences in genomic structure, virion composition, cellular tropism and cause diverse disease manifestations after human infection. Despite these differences, all three human pathogens carry unique but related type I transmembrane GP that mediate virion binding to target cell receptors and subsequently allow for viral entry. In addition, GP expressed by these viruses are derived from respective precursor polyproteins that are cleaved intracellularly to two distinct proteins that aggregate as a trimeric GP complex at the virion surface. In the case of HIV-1, the *env* gene encodes for gp160 that is cleaved to gp120 and gp41 (20). While gp120 is an extracellular protein that mediates binding to target cell receptors, gp41 is a transmembrane protein that facilitates membrane fusion between the virion and target cell membranes via changes in conformation of coiled-coil motifs (3, 48). Similarly, GP1 and GP2 comprise the

mature GP complexes of MBG (45) and EBO (44) viruses. GP1 is an extracellular protein that mediates virion binding, whereas GP2 is the transmembrane protein that carries an analagous coiled-coil fusogenic domain (22, 47). Because this GP complex is the primary viral factor controlling entry of a variety of enveloped viruses, a sophisticated molecular understanding has emerged of its conserved function in initiating infection and interacting with target cells. In contrast, host determinants of entry dictated by the target cell remain largely undefined, and they vary depending on the specific virus involved. Moreover, responses by the host cell following interaction with the viral envelope GP have been poorly characterized yet still may play essential roles in allowing for further disease progression. Therefore, the primary goal of this thesis project was to characterize and compare the molecular method(s) by which the envelope GP complexes of HIV-1, MBG, and EBO viruses bind to host cell factors to initiate infection and the pathogenic consequences of that interaction. The results should expand our understanding of the life cycle and pathogenesis of these viruses and may facilitate the development of more effective treatments.

II. HIV-1 pathogenesis in the central nervous system

HIV-1, the etiologic agent of the acquired immunodeficiency syndrome (AIDS), is a retrovirus that causes a variety of chronic immunologic and systemic conditions. Particularly nebulous are the mechanisms by which HIV-1 invades the central nervous system and infects target cells to cause neurologic deficits. While a number of these sensory and motor deficits have been described during

acute infection, the more widespread and devastating neurologic complication is HIV-associated dementia (HIVD), typically diagnosed after chronic infection and AIDS progression. HIVD is defined clinically by impairments in attention and memory and in the speed of processing complex issues. Variably, it can affect fine motor skills, emotions, and interest, and in some cases, it may progress to global loss of mental function, resulting in a near vegetative state. Approximately 20% of all AIDS patients suffer from HIVD, a diagnosis that requires exclusion of concurrent opportunistic infections, tumor metastases, and substance abuse (27). HIV encephalitis refers to the pathologic changes in the brain tissue of AIDS patients regardless of clinical dementia diagnosis. They consist of brain atrophy, astrocytosis, microglial nodules, neuronal loss, increased permeability of the blood-brain barrier, and multinucleated giant cells (27). While neuronal death and dysfunction are the direct causes of deficits in motor and cognitive decline, overall neuronal loss, astrocytosis (27), and viral load (19) do not always correlate with HIVD diagnosis. Rather, HIVD patients can present with more subtle CNS disruptions. These discrepancies in disease manifestation may be the result of the complexity of unknown pathogenic events controlled both by the virus as well as the host.

Early after systemic infection, HIV-1 invades the CNS and brain parenchyma by incompletely characterized mechanisms. Robust HIV-1 replication is detected mainly in microglia and macrophages residing in brain tissue (46), $CD4^+$ cell types postulated to carry and produce much of the viral load in the CNS. However, other cell types such as astrocytes (42) and microvascular endothelial cells (24, 26) may also become productively or non-

productively infected. Given the importance of these cells in maintaining the normal integrity of the CNS, virus tropism and alternate virus-cell interactions with these $CD4^-$ cells are likely to play a prominent role in neuronal dysregulation and dementia development. Subsequently, HIV-1 infected cells primarily concentrate in cerebral white matter, basal ganglia, thalamus, and the brain stem (27) where viruses are thought to replicate as a separate reservoir distinct from the lymphoid compartment (as reviewed in (20)). Importantly, significant levels of HIV infection in neurons or oligodendrocytes have not been reported (39). While direct cytopathic effects on various non-neuronal target cells are important in neuropathology, other indirect pathways involving secreted viral and cellular factors likely contribute to neuronal death. The prevailing hypothesis attributes the development of HIVD to such a combination of direct viral infection and a subsequent deregulation of secretion of various soluble factors (including cytokines, excitatory amino acids, free oxygen radicals, arachidonic acid metabolites, and soluble viral proteins (e.g., gp120)) by various cells in the brain that derange normal cellular function in the CNS and ultimately lead to neuronal damage and death (as reviewed by ((27)). However, the primary event that triggers soluble factor release and subsequent neuronal dysregulation remains unclear.

III. Mechanism of entry by HIV-1 into neural target cells and its role in pathogenesis

Previously, studies of HIVD have centered on elucidating the viral determinants of pathogenesis as a way to discover the specific mechanism(s) leading to neurological disease. "Neurovirulence" is defined as the ability of a virus to cause disease or dysfunction in the CNS such as HIVD. It has been described for a number of retroviral (18, 23, 32, 33) as well as nonretroviral (12, 36, 38, 43) infections that rely on certain sequences of their structural genes for this property. Specifically, viral envelope gene variation has been demonstrated to control neurologic disease in a variety of viral infections through dictating either tropism requirements or other aspects of virus envelope/host cellular receptor interactions. However, a more sophisticated molecular understanding of this correlation with respect to HIV neurovirulence has been hampered previously by a lack of information about the specific host cell factors that associate with the gp120 envelope protein.

Recently, our understanding of viral entry by HIV-1 has been augmented by the discovery that HIV-1 depends critically on the binding of the viral envelope glycoprotein (gp120) to both CD4 and a member of the chemokine receptor family that serves as an essential cellular coreceptor (9). HIV-1 coreceptor utilization is the principal determinant of cellular tropism in the lymphoid system, and changes in tropism and coreceptor specificity correlate with progression of AIDS. Early after infection, primary viral isolates from the blood are homogenous in envelope sequence and are largely or exclusively

CCR5-using or macrophage-tropic (8, 50). As AIDS develops, approximately 50% of individuals experience a switch in cellular tropism to a more heterogeneous population, termed "quasispeciation," in the blood that carries CXCR4-using, or T-cell line-tropic, viruses (8, 40, 41). In addition, a number of HIV-1 strains have been described that can utilize an ever-growing list of alternate chemokine receptors in addition to CCR5 and CXCR4 under various conditions in cell culture. At the time of these studies, these receptors included CCR2b (11), CCR3 (5, 15), CCR8 (17), BOB/GPR15 (10, 14), Bonzo/STRL33 (10, 21), GPR1 (14), V28/CX_3CR1 (34), ChemR23 (37), leukotriene B4 receptor (28), APJ (4, 13) and human cytomegalovirus (HCMV)-encoded US28 (31). However, the significance, if any, of each alternate coreceptor in HIV-1 disease remains undefined.

By extrapolating this information of HIV-1 receptor biology derived from studies in the lymphoid compartment, we investigated the hypothesis that HIV-1 neurovirulence and subsequent development of HIVD result largely from distinct alterations in the gp120 envelope protein that control viral interactions with receptors and coreceptors on brain cells. Specifically, in the first section of this thesis encompassing Chapter 2 and Chapter 3, we addressed two general questions surrounding virus interactions with neural cells: 1) Do alterations in coreceptor specificity of gp120 in neurovirulent HIV strains mediate pathogenic changes in the brain? 2) Do alternate mechanisms of gp120 association with receptor/coreceptor complexes contribute to neural cell infection and development of HIVD? Chapter 2 describes a study designed to determine the receptor and coreceptor specificities of HIV-1 envelope clones derived from brain tissue of patients with and without HIVD and/or HIV encephalitis. Chapter 3

details evidence supporting the relevance of a novel *trans*-receptor mechanism for infection of $CD4^-$ human astrocytes to HIV-1 infection by elucidating the mechanistic roles of CD4 and coreceptors in this process. As a result, a more thorough understanding of the interactions of the HIV envelope with CNS cells should provide a better molecular understanding of HIV-1 entry and its role in pathogenesis as well as a more generalizable context for a comparison with entry by the MBG and EBO pathogens.

IV. Virologic and clinical characteristics of MBG and EBO viruses.

Unlike the more detailed molecular picture of HIV-1 and its host targets, our current understanding of the molecular biology of MBG and EBO viruses is quite limited. Presently, MBG and EBO viruses comprise the only defined members of the *Filoviridae* family, characterized by their filamentous morphologies. They are non-segmented single-stranded RNA viruses of negative polarity that carry a 19 kb genome with 7 sequentially arranged genes (29) and are packaged by an envelope derived from the host cell plasma membrane. No taxonomic subtypes have been described for MBG virus, but four exist for EBO virus, named for the original location where each was first isolated: Zaire, Sudan, Reston, and Ivory Coast. While nucelotide sequence analysis has revealed 53% identity among EBO subtypes, only 28% identity exists between MBG and EBO strains (30). Despite these differences, filoviruses share a number of similarities in genomic organization, virion structure, cellular tropism, and human pathogenesis.

Human infection by filoviruses have resulted in a number of lethal outbreaks of hemorrhagic fever, 4 documented for MBG (30) and 16 for EBO (6) since the initial discovery of MBG virus in 1967 (29). Approximately 1,000-2,000 individuals have been affected worldwide, and fatality rates typically range from 30%-80% of infected individuals, depending upon the specific outbreak. Relatively remote locations of central and northeastern Africa have been the sites of the majority of these epidemics, prompting speculation that unidentified African wildlife may the reservoir host(s) for propagation of filoviruses (25). Infections have also been reported in the Philippines, Europe, and the United States, inciting heightened awareness among health and government officials of the importance to prevent or effectively treat human infections before they reach major metropolitan areas. Currently, there are no specific therapies or vaccines available for clinical use, and infected individuals are typically treated by rehydration or other supportive measures.

Filovirus infection is transmitted largely through direct contact with body fluids (e.g., blood or saliva) (as reviewed in (6)), however aerosol transmission may also be possible (16). Typically, after an asymptomatic incubation period of 4-10 days, humans infected with pathogenic strains of EBO or MBG virus experience an abrupt onset of severe illness with high fevers, severe frontal headache, myalgia and systemic malaise (6, 30). After 48 hours, hemorrhage typically follows, occurring into skin, mucous membranes, numerous visceral organs, and the lumen of the gastrointestinal tract. Death can occur 1-9 weeks after emergence of initial symptoms, characterized by multi-organ failure, life-threatening hypotensive shock, and widespread infection of numerous tissues

associated with high viral loads. In survivors, convalescence can be slow, commonly accompanied by arthralgias and fatigue.

The pathophysiology of filoviral hemorrhagic fevers is unclear. During infection, explosive viral replication is found in various anatomic sites, especially the liver, spleen and lymph nodes. In addition, prominent focal necrosis of liver, spleen, lymph nodes, kidneys, gonads, and brain is observed (6, 30). However, the mechanism for hemorrhagic shock remains unknown, though release of viral and/or non-viral mediators (e.g., interleukin-2, interleukin-10, tumor necrosis factor, interferon-alpha, and interferon gamma) are potential factors (6). *In vivo*, viral infection of macrophages, fibroblasts, vascular endothelial cells, and hepatocytes predominates, but the relative contributions of each of these (or other cell types) to disease progression is also not well established (49). Study of pathogenesis has been aided by use of animal models. In addition to humans, diverse mammalian species are permissive for viral replication and development of disease, including monkeys (35), guinea pigs (7), and suckling mice (2). However, because of the currently numerous uncertainties of the filovirus life cycle, coupled with the association with high mortality and possibility of aerosol transmission, work with wildtype filoviruses has been restricted to maximum containment, biosafety level 4 (BSL4) facilities. Therefore, progress in studying the molecular aspects of filovirus infection has been limited.

V. Cellular mechanisms of binding and entry mediated by MBG and EBO envelope GP

In contrast to studies of HIV-1 virion binding and entry, little is known about the identity of cellular factors that mediate these events in filovirus infections. Previously, the liver-specific asialoglycoprotein receptor (ASGP-R) was proposed as a host factor that may play a role in infection of liver cells by MBG virus (1). However, this role for ASGP-R was never fully established, and since both MBG and EBO viruses infect a variety of ASGP-R-negative cell types, at least one additional factor must facilitate entry in other cellular contexts. Furthermore, as in the case of HIV-1 gp120 and other more fully characterized enveloped virus glycoproteins, filovirus GP interactions with cell surface factors may incite pathogenic consequences independent of entry and replication. Therefore, analagous to the initial HIV-1 studies, the second half of this thesis project characterized and compared the undefined molecular mechanisms that mediate MBG GP and EBO GP association with target cells and subsequently lead to disease. Chapter 4 specifically describes characterization of a system designed to reconstitute infection mediated by filovirus GP without the use of full-length filoviruses. In addition, initial observations about the general characteristics of entry by MBG and EBO are analyzed and compared. Chapter 5 describes a study of potential pathogenic effects of EBO envelope glycoproteins in 293T cells independent of infection or viral replication. Finally, Chapter 6 identifies and characterizes the folate receptor-α protein (FR-α) as a cellular factor that mediates entry by MBG and EBO viruses.

Taken together, the results from these studies provide a more detailed molecular picture of the interaction of virus envelope GP with target cells. As a result, they represent a fundamental advancement of our understanding of viral infection and perhaps may be used in the future as a basis for designing more effective treatments of human viral syndromes.

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Chapter 2

Central Role for CCR5 as a Coreceptor in Infection of the Central Nervous System by Human Immunodeficiency Virus Type 1

Introduction

High levels of viral replication by HIV-1 are associated with genetic evolution of *env in vivo* and correlate with progressively severe disease manifestations. A possible molecular explanation postulates that this evolution allows for production of a range of "quasispecies" of HIV-1 with distinct envelopes that have been hypothesized to use a broader range of coreceptors, to infect a larger number of host cell types, and therefore to cause more pathogenic effects (2). This model is consistent with the observation of the coreceptor usage "switch" of blood-borne HIV-1 strains from CCR5 to CXCR4 and other alternate coreceptors as AIDS progresses (1). Such a paradigm may also exist in the CNS where the envelope GP of "neurovirulent" strains may evolve to use alternate coreceptors and subsequently trigger the development of HIV-associated dementia (HIVD). To explore this hypothesis, the receptor/coreceptor specificities of brain-derived virus strains were characterized for correlation with the development of HIVD. This study demonstrated that the CCR5 chemokine receptor is a primary coreceptor for brain-derived viruses (and colon-derived viruses) regardless of dementia diagnosis and that alternate receptor/coreceptor use is not necessary for HIVD development.

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V3 Recombinants Indicate a Central Role for CCR5 as a Coreceptor in Tissue Infection by Human Immunodeficiency Virus Type 1

STEPHEN Y. CHAN,[1,2] ROBERTO F. SPECK,[1] CHRISTOPHER POWER,[3] SARAH L. GAFFEN,[1] BRUCE CHESEBRO,[4] AND MARK A. GOLDSMITH[1,2]*

Gladstone Institute of Virology and Immunology[1] and Department of Medicine, School of Medicine, University of California San Francisco,[2] San Francisco, California; Department of Clinical Neurosciences, University of Calgary, Calgary, Alberta, Canada[3]; and Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Montana[4]

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Binding of the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein gp120 to both CD4 and one of several chemokine receptors (coreceptors) permits entry of virus into target cells. Infection of tissues may establish latent viral reservoirs as well as cause direct pathologic effects that manifest as clinical disease such as HIV-associated dementia. We sought to identify the critical coreceptors recognized by HIV-1 tissue-derived strains as well as to correlate these coreceptor preferences with site of infection and dementia diagnosis. To reconstitute coreceptor use, we cloned HIV-1 envelope V3 sequences encoding the primary determinants of coreceptor specificity from 13 brain-derived and 6 colon-derived viruses into an isogenic (NL4-3) viral background. All V3 recombinants utilized the chemokine receptor CCR5 uniformly and efficiently as a coreceptor but not CXCR4, BOB/GPR15, or Bonzo/STRL33. Other receptors such as CCR3, CCR8, and US28 were inefficiently and variably used as coreceptors by various envelopes. CCR5 without CD4 present did not allow for detectable infection by any of the tested recombinants. In contrast to the pathogenic switch in coreceptor specificity frequently observed in comparisons of blood-derived viruses early after HIV-1 seroconversion and after onset of AIDS, the characteristics of these V3 recombinants suggest that CCR5 is a primary coreceptor for brain- and colon-derived viruses regardless of tissue source or diagnosis of dementia. Therefore, tissue infection may not depend significantly on viral envelope quasispeciation to broaden coreceptor range but rather selects for CCR5 use throughout disease progression.

Entry into target cells by human immunodeficiency virus type 1 (HIV-1) depends critically on binding of the viral envelope glycoprotein (gp120) to both CD4 and a cellular coreceptor (31). Recently, both definitive and putative coreceptors have been identified as members of the G-protein-coupled chemokine receptor family that confer onto cells susceptibility to infection by various isolates of HIV-1.

HIV-1 coreceptor utilization is the principal determinant of cellular tropism. While macrophage-tropic viruses characteristically employ the β-chemokine receptor CCR5 (3, 15, 21, 29, 30), T-cell line-tropic viruses use the α-chemokine receptor CXCR4 (38). Changes in tropism and coreceptor specificity correlate with progression of AIDS. Early after infection, primary viral isolates from the blood are homogeneous in envelope sequence and are largely or exclusively CCR5 using or macrophage-tropic (18, 85, 110, 111). As AIDS develops, approximately 50% of individuals experience a switch in cellular tropism to a more heterogeneous population in the blood that carries CXCR4-using or T-cell line-tropic viruses (18, 98–100). The importance of CCR5 in mediating HIV-1 infection was established by the natural occurrence of the CCR5Δ32 loss-of-function mutation. Persons homozygous for CCR5Δ32 display resistance to initial HIV-1 infection, while heterozygotes demonstrate a slower progression to AIDS after seroconversion (19, 45, 60, 77, 84). The contribution of CXCR4 to pathogenesis has also been highlighted by studies in various models of HIV-1 immunodepletion (41, 71).

High levels of viral replication are associated with genetic evolution in vivo. This allows for production of a range of quasispecies with distinct envelopes that have been hypothesized to use a broader range of coreceptors to infect a larger number of host cell types (103). Accordingly, a number of HIV-1 strains that can utilize alternate chemokine receptors in addition to CCR5 and CXCR4 under various in vitro conditions have been described. These receptors include CCR2b (29), CCR3 (15, 43), CCR8 (50), BOB/GPR15 (22, 37), Bonzo/STRL33 (22, 59), GPR1 (37), V28/CX_3CR1 (82), ChemR23 (83), leukotriene B4 receptor (69), Apj (14, 32), and human cytomegalovirus (HCMV)-encoded US28 (72). However, the significance of each alternate coreceptor in HIV-1 disease remains undefined.

Previous work that explored coreceptor use and disease progression focused mainly on primary blood isolates (18). Viral entry into tissues may also be a principal determinant of HIV-1 dissemination and pathogenesis (58), and studies have begun to examine this issue (26, 88). Tissue infection may allow for establishment of viral reservoirs that function as separate replication sites from blood. Viruses isolated from the central nervous system (CNS) (1, 6, 10, 27, 35, 46, 55, 70, 76, 87, 107), bowel (8), and other tissues (6, 27, 35, 48, 87, 112) possess genetic and phenotypic differences compared to viruses isolated from peripheral blood mononuclear cells. In addition, various cell types that reside in tissues and express alternate coreceptors may play critical roles in disease progression (5, 20, 36, 40, 53, 66, 81, 97, 102). It is unknown whether a separate

* Corresponding author. Mailing address: Gladstone Institute of Virology and Immunology, P.O. Box 419100, San Francisco, CA 94110-9100. Phone: (415) 695-3775. Fax: (415) 695-1364. E-mail: mgoldsmith@gladstone.ucsf.edu.

evolution of coreceptor use also occurs in viruses replicating in tissues. Preliminary coreceptor specificity studies have also implicated tissue-invasive strains as direct contributors to clinical disease. Such a paradigm exists for the CNS and brain, where neurotropic strains are hypothesized to infect specifically macrophages, microglia (74, 96, 102), and other components of neural tissue (5, 97). Neurovirulent strains evolving from these may subsequently trigger the development of HIV-associated dementia (HIVD) (51, 61, 62).

The V3 hypervariable region of the gp120 envelope protein carries the main determinants of both cellular tropism (11, 12, 47, 86, 89, 105, 106) and coreceptor use (15, 16, 94, 108). Transfer of a V3 loop from one virus into a distinct HIV-1 background typically retains the coreceptor profile of the V3 donor (15, 94). In addition, single amino acid changes in the V3 loop can drastically alter coreceptor specificities (94). As a result, V3 recombinants allow not only for mapping determinants of tropism but also for a methodology of screening viral coreceptor preferences. Although other regions of gp120 may influence coreceptor recognition in some settings (13, 92), relatively few unpassaged HIV-1 strains have been isolated from tissues as molecular clones that carry full-length envelope sequences (26, 88).

Thus, the most feasible approach to screening in vivo coreceptor specificity is to use the V3 and flanking sequences previously identified by postmortem PCR analyses of brain and colon tissue from AIDS patients (27, 76). Isolates from brain tissue were chosen to identify selection pressures present in the CNS viral reservoir (10, 54) that may affect development of HIVD. Isolates from colon tissue were chosen for comparison to explore the possible existence of an alternate viral reservoir that imposes distinct selection pressures. Using an isogenic proviral backbone, we constructed V3 recombinants that carried the specific coreceptor usages of the original clinical isolates. Subsequent functional analyses allowed us to identify the biologically relevant coreceptors that mediate tissue infection of the brain and colon and then correlate coreceptor specificity with site of infection (brain versus colon) and the diagnosis of HIVD.

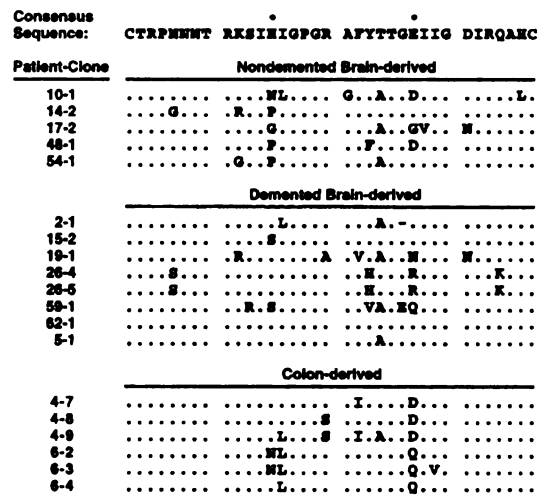


FIG. 1. Three groups of V3 loop amino acid sequences, isolated by postmortem PCR from brain tissue and colon tissue of AIDS patients, show homology to a macrophage-tropic consensus sequence (11) deduced from blood-borne strains. A period denotes identity with the consensus, while a dash denotes deletion. Bullets denote V3 positions 13 and 25 in the consensus sequence. Sequences of colon-derived viruses and demented brain-derived virus (5-1) were isolated from three AIDS patients (27). Remaining brain-derived virus sequences were isolated from 11 AIDS patients diagnosed with HIVD (76). Recombinant virus 59-1 was previously termed 59-3. Recombinant viruses 26-4 and 26-5 (previously termed 26-1 and 26-2, respectively) have identical V3 sequences as noted above but vary at gp120 position 335 in the C3 region, as described elsewhere (76).

MATERIALS AND METHODS

Cell lines. 293T (American Type Culture Collection, Rockville, Md.) and human osteosarcoma (GHOST) indicator (provided by D. Littman) cell lines were cultured in Dulbecco modified Eagle medium (Mediatech, Herndon, Va.) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products, Calabasas, Calif.) and 100 μg of penicillin-streptomycin (Mediatech) per ml. The parental GHOST clone 34 stably maintaining human CD4 expression was under constant selection with G418 (500 μg/ml; Life Technologies, Grand Island, N.Y.) and hygromycin B (100 μg/ml; Boehringer Mannheim, Mannheim, Germany). GHOST transfectants stably expressing a specific coreceptor (CCR5, CXCR4, BOB/GPR15, or Bonzo/STRL33) were under additional selection with puromycin (1 μg/ml; Sigma Chemical Co., St. Louis, Mo.). COS-7 cells (American Type Culture Collection) were routinely cultured in Iscove's medium (Mediatech) supplemented with 10% FBS, 100 μg of penicillin-streptomycin per ml, and 2 mM L-glutamine (Mediatech). COS-7 transfections were carried out with LipofectAMINE (Life Technologies) according to the manufacturer's instructions. All infections were performed in Dulbecco modified Eagle medium–10% FBS–100 μg of penicillin-streptomycin per ml.

Plasmids and construction of recombinant proviruses. Molecular clones pYU-2, pNL4-3, and pNL-Luc-E$^-$R$^-$ (17) (the NL4-3 provirus backbone with a luciferase reporter gene insertion) were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases. Expression plasmids encoding CCR3 (pcDNA3-CCR3p), HCMV US28 (pRC/CMV-US28), ADA gp160 envelope (ADApEnv), CD4 (pCD4neo), and CCR5 (pCMVFCCR5) were previously described (4, 42, 72, 82, 105). The expression plasmid encoding CCR8 (pAW-CCR8F) was provided by I. Charo.

Plasmids (p2-1, p10-1, p14-2, p15-2, p17-2, p19-1, p26-4, p26-5, p48-1, p54-1, p59-1, and p62-1) encoding chimeric NL4-3 provirus with a 428-bp insertion (*StuI-NheI*) that includes the V3 and portions of the C2 and C3 regions of brain-derived gp120 sequences were constructed as previously described (76). Plasmid p4-14 (11) encoding NL4-3 provirus (with a 16-bp polylinker *MluI-NheI* inserted in place of the 126-bp region coding portions of gp120 V3-C3 region) was used as the isogenic viral backbone for constructing additional V3 replacements based on published amino acid sequences (27, 74, 76). V3 nucleotide sequences were deduced by inserting degenerate codons most prevalent in the mammalian genome. An oligonucleotide pair representing the deduced V3 sequence for brain-derived 5-1 (Fig. 1) along with deduced 5′ C3 sequence for NL4-3 was annealed as described by the manufacturer (Oligos, Etc., Wilsonville, Oreg.) and contained restriction site overhangs 5′ *MluI*-3′ *NheI* with unique sites *ApaI*, *SmaI*, and *XbaI* inserted internally. The annealed oligonucleotides were cloned initially into a holding vector. After digestion of both p4-14 and the holding vector containing 5-1 with *MluI* and *NheI* and ligation of 5-1 insert with p4-14 backbone, the full-length chimeric provirus p5-1 was used as the parental vector for construction of other proviruses (p4-7, p4-8, p4-9, p6-2, p6-3, and p6-4) since it carried convenient restriction sites. Oligonucleotide pairs 4-7 and 4-8 were annealed (5′ *SmaI*-3′ *XbaI*), inserted into a holding vector, digested (*SmaI/XbaI*), and ligated with the linearized p5-1 backbone (*SmaI/XbaI*) to make p4-7 and p4-8 proviruses. Oligonucleotide pairs 4-9, 6-2, 6-3, and 6-4 were annealed (5′ *MluI*-3′ *XbaI*), inserted into a holding vector, digested (*MluI/XbaI*), and ligated with the linearized p5-1 (*MluI/XbaI*) to make p4-9, p6-2, p6-3, and p6-4 proviruses. Inserted sequences were verified by ABI Prism Dye terminator cycle sequencing (Perkin-Elmer, Foster City, Calif.).

Preparation of virus stocks. To prepare experimental V3 recombinant viruses and full-length control viruses (YU-2 and NL4-3) for spreading infections, proviral plasmids were transfected into 293T cells by the $CaPO_4$ method (Promega, Madison, Wis.) as previously described (42). Recombinant viral stocks were sterile filtered (0.45-μm-pore-size-filters) and harvested after both 36- and 60-h incubations. The $p24^{Gag}$ concentration was assessed by enzyme-linked immunosorbent assay (ELISA; New England Nuclear Life Sciences, Boston, Mass.), and stocks greater than 1,500 ng/ml were used for infections. Simian immunodeficiency virus (SIV) strain Mac239 (550 ng of $p27^{Gag}$ per ml) (52) was provided by R. Grant.

To prepare pseudotype virus carrying the luciferase gene (Luc^+) with the ADA envelope for single-round infections, pNL-Luc-E$^-$R$^-$ (2 μg/well) was cotransfected with ADApEnv (2 μg/well) in 293T cells growing in six-well plates as previously described (22). Pseudotype Luc^+ viruses carrying the tissue-derived V3 envelopes were similarly prepared by cotransfecting pNL-Luc-E$^-$R$^-$ (3 μg/well) along with full-length proviral plasmids described above (1 μg/well).

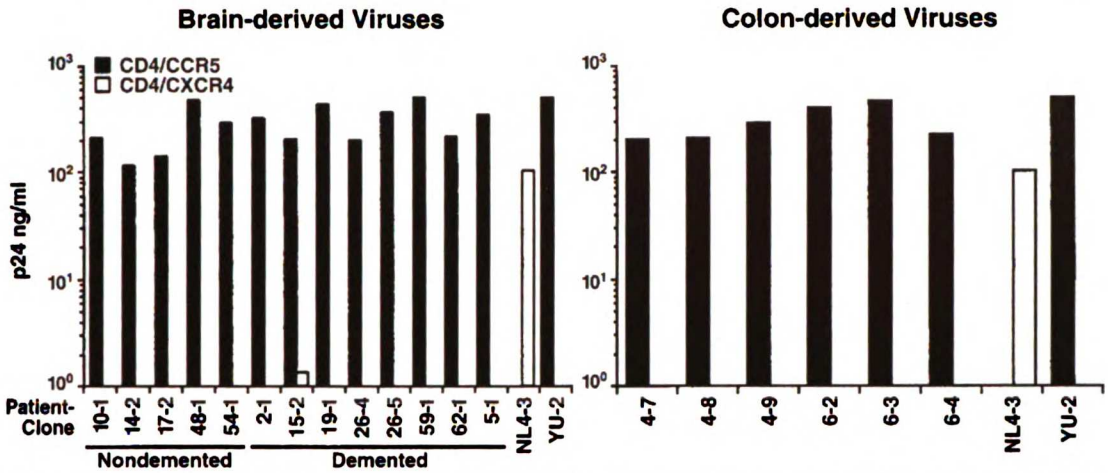


FIG. 2. Efficient and uniform use of CCR5 but not CXCR4 as a coreceptor by all V3 recombinant viruses. HIV-1 YU-2 (CCR5 dependent) and NL4-3 (CXCR4 dependent) were used as controls. Displayed values are representative of day 8 supernatant p24 concentrations seen in two separate infections performed with these recombinants.

Pseudotype recombinant virus stocks greater than 110 ng/ml were used for infections.

Coreceptor utilization assay for spreading infection. GHOST cell lines expressing CD4 alone or in combination with CCR5 or CXCR4 were plated in 96-well plates (7,000 cells/well) and infected as previously described (93). Cells were washed once with phosphate-buffered saline, and medium was replaced 12 h after infection. Day 0 and day 8 supernatant aliquots (10 μl) were removed for secreted $p24^{Gag}$ measurements. Cell lines were infected with equivalent inocula of recombinant virus normalized by utilization of CCR5 as a coreceptor (Fig. 2). The amount of recombinant virus added to different GHOST cell lines never varied for a given infection. Infection of GHOST cell lines stably expressing CD4 with BOB or Bonzo was assessed as described above except that cells were plated (20,000 cells/well) in 12-well plates. The amount of added recombinant virus never varied between these cell lines for a given infection. To detect infection by SIV Mac239, secreted $p27^{Gag}$ concentration was measured by ELISA (Coulter, Miami, Fla.). For all infections, microscopic observations of syncytium formation were also recorded up to 14 days after infection.

Coreceptor utilization assay for single-round infection. COS-7 cells were plated in six-well plates and transfected with pCMVFCCR5 alone, pCD4neo alone, or pCD4neo in combination with pCMVFCCR5, pcDNA3-CCR3p, pAW-CCR8F, or pRC/CMV-US28 as previously described (4). After 24 h, cells were infected with pseudotype Luc^+ recombinant viruses. V3 recombinant viruses were normalized by utilization of CCR5 as a coreceptor (Fig. 3A), and amounts of added virus never varied between cell lines in a specific transfection/infection experiment. After 48 h, luciferase expression was assessed by enzymatic activity (light units/milligram of protein) as previously described (65), as instructed by the manufacturer (Analytical Luminescence Laboratories, Sparks, Md.).

RESULTS

Envelope sequences from brain and colon tissue isolates of HIV-1 inserted into the NL4-3 provirus. Previous studies demonstrated that V3 loop insertion into an alternate viral backbone such as NL4-3 can reconstitute the tropism (47, 106) and coreceptor phenotype (15, 16, 94) of the V3 donor. For the present study, we surveyed the literature for V3 loop amino acid sequences derived from colon and brain samples of AIDS patients for whom HIVD diagnosis was documented (27, 76). To correlate coreceptor preferences with presence of HIVD, we chose five sequences from five nondemented patients and eight sequences from seven demented patients for analysis. To correlate coreceptor preferences with site of infection, we selected six colon sequences from two patients for comparison to the brain-derived recombinants (Fig. 1).

All brain- and colon-derived V3 sequences showed homology to a macrophage-tropic consensus sequence (76). Furthermore, all samples carried a moderately positive net V3 loop charge (ranging from +2 to +6), in contrast to the highly positive net charge commonly observed in CXCR4-using V3 loops (49). Of note, V3 loop position 13 appeared to favor proline in nondemented subjects compared to a consensus histidine in demented patients when a larger collection than this listed set was considered (76). In addition, all colon-derived viral sequences carried either an aspartate or a glutamine residue at V3 loop position 25 rather than the consensus glutamate. In the present study, DNA sequences representing the selected V3 loops with adjacent conserved sequences (for details, see Materials and Methods) were inserted into the NL4-3 backbone. The resulting chimeric viruses were used to test definitively the coreceptor utilization of tissue-specific HIV-1 isolates.

Efficient use of CCR5, but not of CXCR4, as coreceptors for cellular entry. Human osteosarcoma (GHOST) cell lines stably expressing human CD4/CCR5 or human CD4/CXCR4 were infected with each recombinant virus in vitro. Detection of $p24^{Gag}$ by ELISA was performed on the culture supernatants at days 0 and 8 after infection as a marker of spreading infection. Since it demands multiple rounds of infection, such an assay is most biologically pertinent and interpretable. All V3 recombinants representing brain-derived (nondemented and demented) and colon-derived viruses displayed uniformly high replication in GHOST CCR5/CD4 cells (Fig. 2). By day 8, microscopic examination revealed syncytia in all cultures. Conversely, no brain- or colon-derived recombinant propagated significant spreading infection in GHOST CXCR4/CD4 cells, and no syncytia were observed at any time in the cultures (Fig. 2). The parental NL4-3 virus, known to employ CXCR4 as a coreceptor, infected these cells robustly. Therefore, all of the experimental recombinant viruses displayed efficient CCR5 coreceptor use, while none demonstrated detectable use of CXCR4.

Nearly uniform failure to use either BOB/GPR15 or Bonzo/STRL33 as an alternate coreceptor for cellular entry. We sought to determine if BOB/GPR15 or Bonzo/STRL33, two

orphan chemokine receptor-like proteins identified as putative HIV-1 coreceptors (22, 59), could be used for cellular entry. We employed SIV Mac239 (52) as a positive control since it uses both coreceptors more efficiently than some HIV-1 strains (data not shown) and is able to establish more robust spreading infections in culture (as detected in a p27 ELISA). The supernatant p24 measurements of infection of GHOST cells stably expressing CD4/BOB or CD4/Bonzo were compared to Mac239 p27 levels after days 0 and 10. One brain-derived recombinant (2-1) inefficiently employed BOB/GPR15 to sustain spreading infection (4 ng of p24 per ml measured at day 10), but no syncytia were observed by day 14. In contrast, no other V3 recombinant representing a brain-derived virus (nondemented or demented) or colon-derived virus detectably used BOB/GPR15 or Bonzo/STRL33 for spreading infection (data not shown).

Inefficient and variable use of CCR3, CCR8, and US28 as alternate coreceptors for cellular entry. In contrast to CCR5, CXCR4, BOB/GPR15, and Bonzo/STRL33, the chemokine receptors CCR3, CCR8, and HCMV US28 presented several technical challenges to studying their role as coreceptors. Importantly, our initial experiments revealed that HIV-1 strains previously reported to recognize these alternate coreceptors (15) may not utilize them efficiently for cellular entry. Thus, we used an enzymatic assay in which COS-7 cells transiently expressing CD4 and a putative coreceptor were infected with pseudotype viruses carrying the envelopes of interest and the luciferase reporter gene (22). Viral infection was quantitated by measuring entry of the pseudotype Luc^+ recombinant strains through readout of luciferase enzymatic activity. This allowed for a sensitive assessment of coreceptor specificity that was not achieved in a p24 spreading infection assay.

In corroboration of the results from the spreading infection assay, all of the V3 recombinants representing the brain- and colon-derived strains efficiently used CCR5 for cellular entry (Fig. 3A), generating signals in the range of 10^6 to 10^7 light units/mg of protein. Since several recent studies have identified CD4-independent infection by a neurovirulent strain of SIV (33) and select strains of HIV-2 (34, 79), viral infection in the presence of CCR5 without CD4 was also assessed. None of the brain- or colon-derived recombinants infected cells detectably in the absence of CD4 expression (Fig. 3A).

Substantially lower and more variable luciferase signals were observed in single-round infections of COS-7 cells expressing CD4/CCR3 compared with CD4/CCR5 (Fig. 3B). Whereas all recombinants used CCR5 as a coreceptor for entry, one (10-1) did not detectably use CCR3, two (17-2, 2-1) slightly recognized CCR3, while others (for example, 14-2, 54-1; 26-4, 62-1, and 6-4) measurably used CCR3, albeit less efficiently than CCR5. Of those that used CCR3, nearly all recombinants generated luciferase counts less than 10^6 light units/mg of protein, which were typically 90 to 99% lower than comparable readings seen in the CCR5-dependent infections. Importantly, comparison of V3 recombinants representing brain-derived viruses from nondemented and demented patients revealed no distinct pattern of CCR3 use, with both groups displaying a spectrum of viral phenotypes with regard to CCR3. Similarly variable phenotypes of CCR3 use were observed in the colon-derived collection compared with the brain-specific set.

Inefficient and variable infections were also observed with the putative coreceptor CCR8 after single-round infection (Fig. 3C). While some brain-derived recombinants from the nondemented (14-2 and 48-1) and demented (2-1, 26-4, 26-5, 62-1, and 5-1) groups exhibited modest infection, other recombinants from both brain collections (10-1, 17-2, 54-1, 15-2, 19-1, and 59-1) showed little infection using CCR8 as a coreceptor. Similarly, only one colon-derived recombinant (4-9) failed to use CCR8, while the others recognized CCR8 to a slight degree. Signals from the CCR8 infections were less than 10^5 light units/mg of protein and at least 90% lower than those for CCR5 infections.

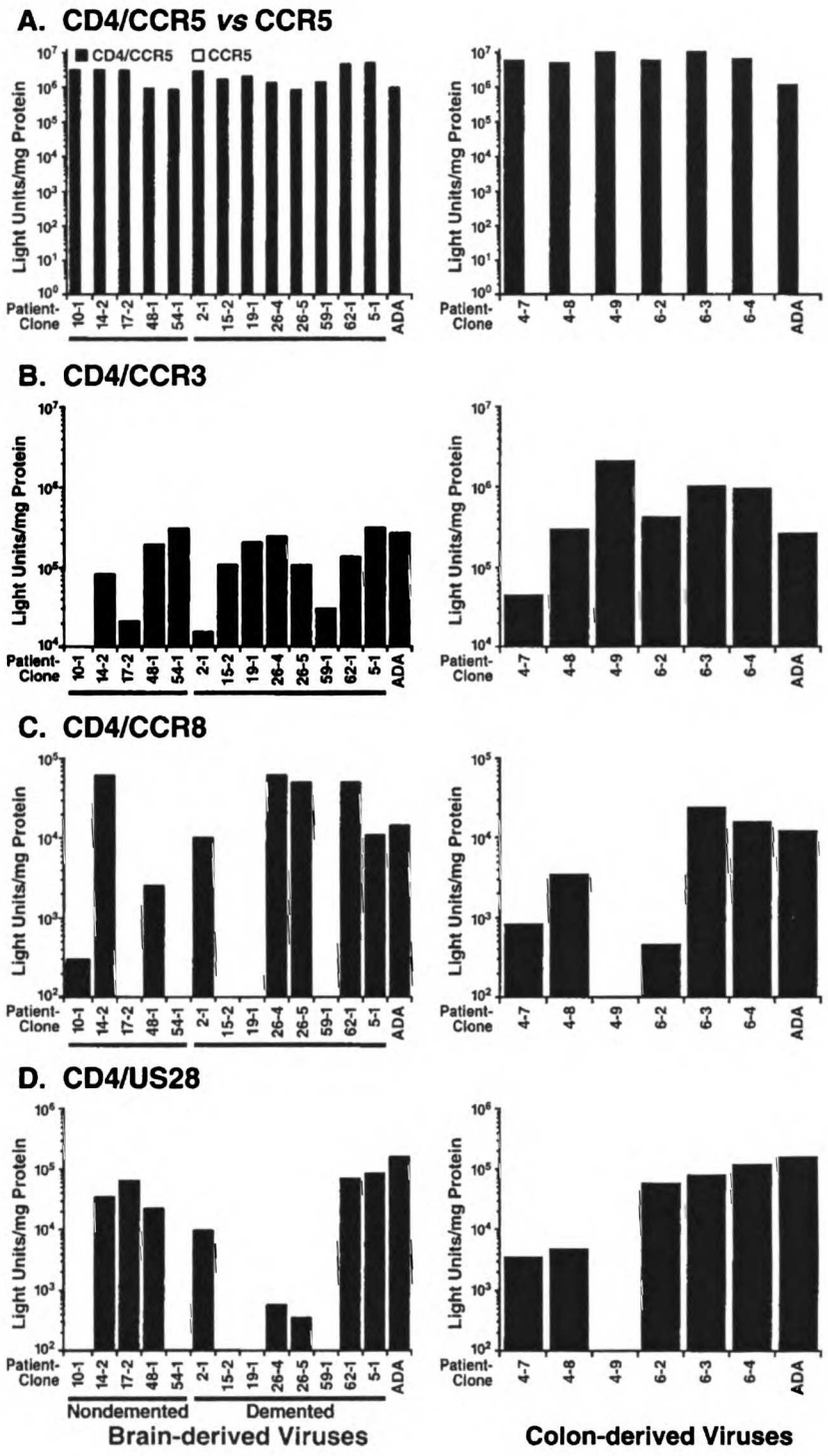
Similar results of low and variable infectivity were obtained with HCMV US28 (Fig. 3D). Because it is encoded by HCMV rather than the human host genome, US28 has been proposed to enhance HIV-1 disease progression during HCMV coinfection commonly seen in AIDS patients (7). Inefficient infection through US28 was observed with recombinants from certain nondemented (14-2, 17-2, and 48-1) and demented (2-1, 26-4, 26-5, 62-1, and 5-1) patients, while other strains (10-1, 54-1, 15-2, 19-1, and 59-1) from both groups displayed no cellular entry. Similar to the CCR8 profile, one colon-derived recombinant (4-9) did not use US28, while the rest of the set recognized it to a small degree. Nearly all recombinant viral infections mediated by US28 exhibited luciferase readings less than 10^5 light units/mg of protein and at least 90% lower than those from CCR5.

Further comparison of the virus infection profiles revealed no uniform correlation among particular coreceptor specificities in any recombinant collection. One recombinant (10-1) exhibited virtually no use of any of the alternate coreceptors tested, while others (14-2, 48-1, 2-1, 62-1, 5-1, 4-7, and 6-2) used all three alternate coreceptors to some degree. Moreover, recombinants such as 26-5 recognized CCR3 and CCR8 but only slightly US28, while 17-2 recognized CCR3 and US28 but not CCR8, and 4-9 recognized CCR3 but not CCR8 or US28. Thus, these V3 recombinant viruses did not display a simple and predictable pattern in their coreceptor specificity profiles. Rather, they all demonstrated variable alternate coreceptor patterns compared to their highly efficient and uniform utilization of CCR5.

DISCUSSION

In this study, we sought to characterize coreceptor preferences among V3 recombinants representing brain- and colon-derived HIV-1 isolates as well as to correlate HIVD diagnosis and site of infection with these coreceptor recognition profiles. We determined that all demented and nondemented brain-specific recombinants and colon-specific recombinants utilized CCR5 efficiently but did not use CXCR4, BOB/GPR15, or Bonzo/STRL33 detectably by spreading infection. In addition, these recombinants recognized CCR3, CCR8, and US28 relatively inefficiently and variably as coreceptors as assayed by sensitive single-round infection methods.

Incomplete eradication of HIV-1 after triple-drug therapy has been attributed in part to the hypothesized separation and relative protection of tissue reservoirs of latent virus from viral populations residing in blood (54, 101, 107). Previous comparisons of V3 envelope sequences (9, 24, 27, 35, 48, 55, 56, 76, 78, 101) and, in some cases, cellular target specificities (26, 74, 88) of unpassaged, tissue-derived viruses sought to define a pattern(s) necessary for viral propagation in tissues different from that observed in blood. In the present study, we examined V3 recombinant viruses carrying some sequence variability within the tissue-specific V3 loops, particularly at positions 13 and 25 (see Results). Brain-derived HIV-1 strains such as those represented here are thought to replicate in vivo as an isolated population, while colon-derived strains may not evolve separately from blood-borne viruses, based on a recent phylogenetic analysis (101). Despite these differences, we discovered a pattern of uniform CCR5 use that indicated a higher degree of functional similarity among tissue-specific viruses than initially


A. CD4/CCR5 vs CCR5
CD4/CCR5
CCR5
Light Units/mg Protein
Patient-Clone
10-1 14-2 17-2 48-1 54-1 2-1 15-2 19-1 26-4 26-5 59-1 62-1 5-1 ADA
4-7 4-8 4-9 6-2 6-3 6-4 ADA
B. CD4/CCR3
C. CD4/CCR8
D. CD4/US28
Nondemented
Demented
Brain-derived Viruses
Colon-derived Viruses

suspected. Indeed, additional sequence comparisons of these recombinants with the V3 loops of known CCR5-using strains (64) revealed extensive similarities at other potentially critical amino acid positions. Although we were unable to detect the presence of a distinct viral reservoir present in colon tissue based on a lack of differences in coreceptor specificity, the uniformity of CCR5 utilization did indicate the probable existence of a common selective pressure in multiple tissues. These results strengthen the claim that antigen-presenting cells, such as microglia, macrophages, and follicular dendritic cells, which commonly reside in tissues and express CD4 and CCR5, are the principal cells that establish and maintain virus populations in all tissues (23, 53, 74, 91, 95, 102).

The nearly absent usage of CXCR4, BOB/GPR15, and Bonzo/STRL33 suggests the inability of these coreceptors to mediate tissue infection despite their expression on certain tissue-resident cell types (22, 57, 109). While no evidence has directly demonstrated the in vivo importance of BOB/GPR15 and Bonzo/STRL33, the role of CXCR4 in pathogenesis has been described for certain viruses residing in the blood (41, 71). From our results, it is reasonable to postulate that unidentified pressures in the brain and colon select against viral use of CXCR4, perhaps in part by the exclusion of infected CXCR4-expressing T cells from trafficking into these tissues.

While most of the brain- and colon-derived recombinants were found to recognize CCR3 as a coreceptor to a variable degree, all could use CCR5 better than CCR3, with most generating at least a 10-fold difference in infection levels. Previously, it was demonstrated that the level of HIV-1 infection mediated by CCR3 varies with the level of coreceptor expression, and inefficient use of CCR3 (15, 82) was attributed to the difficulty of maintaining high levels of expression in cell culture (73). For our analyses, we used the best-reported expression construct (pcDNA3-CCR3p) (82) and a sensitive enzymatic readout to increase our ability to detect CCR3-dependent entry. Conceivably, the presence of at least some degree of CCR3 recognition in nearly all tested recombinants may point to a positive selective pressure in the brain and colon in light of recent evidence of high expression levels in vivo of CCR3 in the CNS and lamina propria of the colon (109). Nonetheless, the global efficiency of CCR5 utilization strongly highlights CCR5 as a predominant coreceptor in mediating tissue-specific infections.

Recently, it was reported that certain HIV-1 strains isolated from brain-derived cells can use CCR8 as a coreceptor (50). In addition, US28 has been implicated in HIV-1 pathogenesis through HCMV opportunistic coinfections in various tissues (39, 63, 67, 90). Our results indicated that neither CCR8 nor HCMV US28 supports efficient infection by any of the recombinants tested. Furthermore, the variability in infection efficiency among different recombinants suggested that no consistent selection pattern is imposed on viruses based on CCR8 or HCMV US28 use. Of note, all colon-derived recombinants from patient 6 did display relatively uniform use of US28. It is tempting to speculate that this coreceptor specificity may be related to the presence of HCMV in the colon, perhaps causing the mucosal atrophy and inflammation observed in this patient (28). However, in light of the rest of our results and the fact that US28 is expressed only during the HCMV lytic growth phase in vitro (104), it is unlikely that US28 has major in vivo significance by itself as a coreceptor for infection of the brain or colon.

We also sought to correlate coreceptor preferences with the presence of HIVD (74, 76, 96). Evidence that both supports and argues against the hypothesis that CCR3 is preferentially recognized by neurotropic and/or neurovirulent viruses (43, 88) that may dictate dementia development has been presented. We observed uniform coreceptor recognition of CCR5 along with inefficient and variable use of alternate putative coreceptors including CCR3 by all demented and nondemented brain-derived V3 recombinants. Importantly, comparison of coreceptor use by brain-derived recombinants with that by presumably nonneurotropic colon-derived recombinants yielded no significant difference in relative specificity profiles. These results imply that a change or selection for CCR3 or other coreceptor specificity is not sufficient to trigger HIVD development. In addition, our results suggest that CD4-independent infection may not contribute to progression of dementia. Alternatively, it has been postulated that HIV-1 may use CCR3 in combination with CCR5 or other coreceptors to enhance infection of the brain (43). However, our initial experiments showed no significant difference in levels of infection among cells transiently expressing CD4 with both CCR5 and CCR3 and cells expressing CD4 with CCR5 alone (data not shown). Collectively, these observations argue against alternate coreceptors or viral CD4 independence as key factors in the pathogenesis of HIVD.

Our strategy employing an isogenic viral backbone allowed us to normalize postentry events, such as viral replication and transcription rates that may vary among different primary isolates. However, it also limited our ability to test all possible determinants of coreceptor specificity and cellular tropism of these clinical strains. Although the V3 loop primarily controls coreceptor specificity, other envelope regions such as V1/2 can be important with certain viruses for specific coreceptor utilization (44, 68, 80). To address this issue, we obtained recombinant NL4-3 proviruses carrying regions V1 to V3 of five of the original brain-derived viruses, including two from the demented group and three from the nondemented group (75). Comparable coreceptor utilization profiles were achieved for both the V1-V3 strains and the matched V3 strains upon testing CCR5, CXCR4, BOB/GRRI5, Bonzo/STRL33, CCR3, CCR8, and US28. Therefore, these results further substantiate the claim that usage patterns derived from the V3 recombinants constructed for this study approximate the coreceptor specificities of the original primary tissue-derived isolates.

It is also conceivable that coreceptor recognition and infection of primary cells in vivo are modulated by parameters different from those that allow for in vitro infections (25). However, of the 19 recombinant strains, 13 had previously

FIG. 3. Variable use of CCR3, CCR8, and US28 as coreceptors by all V3 recombinant viruses compared to use of CCR5. (A) Comparison of infections by pseudotype V3 recombinant viruses of COS-7 cells transiently expressing CCR5 with and without CD4. No tested recombinant displayed infection mediated by CCR5 in the absence of CD4. (B) Infection of COS-7 cells transiently expressing CD4 and CCR3. Recombinant virus 10-1 displayed no detectable infection mediated by CCR3. (C) Infection of COS-7 cells transiently expressing CD4 and CCR8. Recombinant viruses 17-2, 54-1, 15-2, 19-1, 59-1, and 4-9 displayed no detectable infection mediated by CCR8. (D) Infection of COS-7 cells transiently expressing CD4 and US28. Recombinant viruses 10-1, 54-1, 15-2, 19-1, 59-1, and 4-9 displayed no detectable infection mediated by US28. In all studies, Luc$^+$ pseudotype HIV-1 carrying the ADA envelope was used as a positive control. Background readings were obtained from infection of COS-7 cells transiently expressing CD4 without coreceptor and ranged from 0 to 5,000 light units/mg of protein. Displayed values were normalized by subtraction of background signals, and they are representative of the relative ratios of coreceptor specificity seen in two to three separate infections performed with Luc$^+$ pseudotype recombinant viruses.

been shown to infect macrophages in culture (74), demonstrating that the efficiency of CCR5 use by these viruses correlates well with macrophage tropism. Furthermore, previous reports have emphasized that different coreceptor assays display a range of intrinsic sensitivities that can lead to conflicting results in specificity (2, 13, 88). Therefore, differences in the various assays must be noted in order to compare accurately coreceptor utilization data among different studies.

Despite these caveats, the consistent patterns and large differences in coreceptor specificity among these representative recombinants strengthen our conclusion of the predominant role of CCR5 in tissue invasion. Therefore, the coreceptor switch from CCR5 to CXCR4 characteristically seen in blood-borne viruses does not seem typical of tissue-specific isolates. Absence of a predictable coreceptor evolution pattern also suggests that other pathogenic events in the brain are responsible for causing HIVD. It remains an intriguing possibility that other recently described putative coreceptors, such as V28/CX_3CR1 (79, 82) and Apj (14, 32), that are highly expressed in the brain play a role in development of HIVD. Alternatively, differences observed in the V3 loop sequence between isolates from demented and nondemented patients may have an impact on HIVD progression by differentially altering production of soluble neurotoxic factors by infected macrophages (75), perhaps through alterations in signal transduction that are independent of tropism determination. Nonetheless, the results of the present study should provide optimism for the potential effectiveness of drug therapies aimed at preventing CCR5-mediated cellular entry.

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Chapter 3

A Trans-Receptor Mechanism Mediates Entry by Human Immunodeficiency Virus Type 1 into CD4-Negative Primary Human Fetal Astrocytes

Introduction

Since changes in coreceptor specificity of brain-derived HIV-1 envelopes are not necessary for dementia progression (see Chap. 2), we investigated the hypothesis that the mechanism of CD4/coreceptor utilization may not be uniform for infection of all brain cells. It is well established that HIV-1 can infect brain cells such as microglia and macrophages by binding both CD4 and a coreceptor on the target cell surface to induce viral fusion. However, previous studies have consistently demonstrated both cell culture (10) and *in vivo* (9) infections by HIV-1 of $CD4^-$ astrocytes, presumably through an unknown alternate mechanism (as reviewed in (1)). Astrocytes maintain a homeostatic ionic, electrical, and pH environment for proper neuronal function, they engulf cellular debris during CNS cell turnover, and they secrete soluble cytokines important for regulation and communication with other CNS cell types (5). Therefore, infection by HIV-1 may result in subsequent cellular dysregulation and lead to progressive neural decline and clinical dementia.

Proposed hypotheses for an alternate mode of virus entry into $CD4^-$ brain cells include CD4-independent association of the HIV gp120 with a coreceptor as observed with some neurovirulent SIV strains (6) and HIV-2 strains (2, 7) or with an alternate cell surface receptor such as galactosylceramide (4). In addition, virus spread may occur through cell-cell fusion or engulfment of HIV-infected $CD4^+$ macrophages, microglia, or T-lymphocytes trafficking through the CNS

with these $CD4^-$ cells. However, direct evidence that these mechanisms actively mediate the infection of astrocytes is lacking.

In this study, using the $CD4^-$ 293 cell line, we discovered a novel mechanism of infection by which CD4 displayed on neighboring cells (8) or soluble CD4 molecules alone (3) may be used by HIV-1 gp120 to facilitate infection of $CD4^-$ cells while engaging coreceptors expressed on target cells. Consistent with this mechanism, infection of primary human fetal astrocytes has been observed in the presence of co-cultured HIV-1 infected $CD4^+$ lymphocytes (10). The brain parenchyma provides a possible environment where $CD4^+$ macrophages, microglia, and trafficking T-lymphocytes reside in close proximity to $CD4^-$ astrocytes to facilitate such infection. Therefore, experiments were performed with primary human fetal astrocytes to determine the mechanism of infection of these cells. Subsequently, this study demonstrated that a trans-CD4 receptor/gp120/coreceptor interaction facilitates infection of fetal astrocytes by HIV-1.

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A *trans*-receptor mechanism for infection of CD4-negative cells by human immunodeficiency virus type 1

Roberto F. Speck*†, Ursula Esser*†, Michael L. Penn*, Daniel A. Eckstein*, Lynn Pulliam‡§¶, Stephen Y. Chan* and Mark A. Goldsmith*‡

Chemokine receptors, particularly CCR5 and CXCR4, act as essential coreceptors in concert with CD4 for cellular entry by human immunodeficiency virus type 1 (HIV-1; reviewed in [1]). But infection of CD4⁻ cells has also been encountered in various tissues *in vivo*, including astrocytes, neurons and microvascular endothelial cells of the brain [2–6], epithelial cells [5,7], CD4⁻ lymphocytes and thymocytes [8,9], and cardiomyocytes [10]. Here, we present evidence for the infection of CD4⁻ cell lines bearing coreceptors by well-known HIV-1 strains when co-cultured with CD4⁺ cells. This process requires contact between the coreceptor-bearing and CD4⁺ cells and supports the full viral replication cycle within the coreceptor-bearing target cell. Furthermore, CD4 provided *in trans* facilitates infection of primary human cells, such as brain-derived astrocytes. Although the pathobiological significance of infection of CD4⁻ cells *in vivo* remains to be elucidated, this *trans*-receptor mechanism may facilitate generation of hidden reservoirs of latent virus that confound antiviral therapies and that contribute to specific AIDS-associated clinical syndromes.

Addresses: *Gladstone Institute of Virology and Immunology, PO Box 419100, San Francisco, California 94141, USA. Departments of †Medicine and of §Laboratory Medicine, School of Medicine, University of California San Francisco and ¶Veterans Affairs Medical Center, 4150 Clement Street, 113A, San Francisco, California 94121, USA.

†R.F.S. and U.E. contributed equally to this work.

Correspondence: Mark A. Goldsmith
E-mail: mgoldsmith@gladstone.ucsf.edu

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Results and discussion

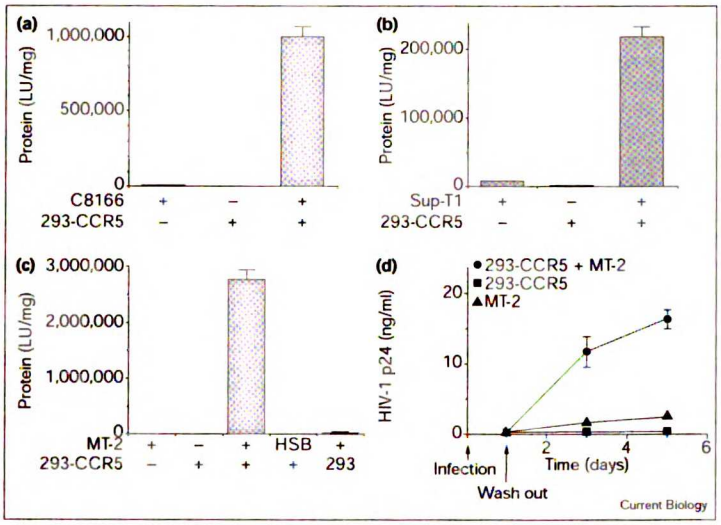
In view of the present coreceptor paradigm for CD4-dependent infection by HIV-1, we considered the possibility that CD4 molecules on neighboring lymphocytes may be provided to CD4⁻ target cells *in trans*, which might permit triggering of virus–cell fusion. To test this hypothesis, we first established a co-culture system in which human CD4⁺ T cells lacking CCR5 were mixed with HEK-293 cells expressing CCR5 (293-CCR5 cells) and challenged with pseudotype HIV-1 bearing CCR5-dependent envelopes. Viral stocks were generated with the envelope-negative NL4-3 proviral plasmid encoding luciferase (pNL-Luc-E⁻R⁻ [11]) and plasmids encoding various HIV-1 envelopes. A very low luciferase signal was evident in 293-CCR5 cells and in the T-cell lines C8166, Sup-T1 and MT-2 upon exposure to the CCR5-dependent viral strains ADA or JRFL (Figure 1a–c). A large signal was generated, however, when the ADA or JRFL pseudotype viruses were used to challenge co-cultures of 293-CCR5 cells and C8166, Sup-T1 or MT-2 cells, which were used as the source of CD4. This signal, which was directly proportional to the number of cells infected, was approximately 4% of that seen when 293 cells co-expressing CCR5 and CD4 were inoculated in parallel (data not shown). Co-cultures exposed to other CCR5-dependent pseudotype viruses also yielded substantial infection signals (R.F.S. and M.A.G., unpublished observations). Thus, CD4 and CCR5 need not be expressed together on the target cell, but can cooperate when expressed on neighboring cells *in trans*.

To determine whether this process applies to other HIV coreceptors, we tested a CD4⁻ human B-cell line (Raji) that naturally expresses the coreceptor CXCR4. Raji cells alone exhibited no permissivity for pseudotype luciferase HIV-1 carrying the envelope glycoproteins of HXB2, a CXCR4-dependent strain (51 ± 31 light units (LU)/mg). But co-culture with a CXCR4⁻ rodent cell line engineered to express human CD4 (Rat2-CD4) permitted significant infection by the reporter virus (5,427 ± 683 LU/mg), indicating that CXCR4 and CD4 can also cooperate *in trans*.

We further assessed the importance of cell-surface CD4 and the coreceptor in this co-culture system by replacing MT-2 cells with HSB cells, a CD4⁻ T lymphoblastoid cell line, or by replacing 293-CCR5 cells with parental 293 cells, which lack CCR5 (Figure 1c). Infection was completely prevented in both experimental conditions. In addition, the neutralizing anti-CD4 monoclonal antibody 13B.8.2 inhibited infection in the co-culture assay by more than 95% at 5 μg/ml, whereas no neutralization was seen with an isotype-matched control monoclonal antibody (data not shown).

We next determined whether such a *trans*-receptor mechanism could support the production of mature virions and viral spread in the culture. Co-cultures of 293-CCR5 cells and MT-2 cells were inoculated with a CCR5-dependent

Figure 1



Co-cultures of CD4⁺ CCR5⁻ T-cell lines and CD4⁻ 293-CCR5 cells exposed to CCR5-dependent viruses. **(a–c)** Various CD4⁺ T-cell lines (C8166, Sup-T1 or MT-2) mixed with 293-CCR5 cells were exposed to the pseudotype viruses (a,b) ADA or (c) JRFL. Pseudotype viruses comprised the envelope-deficient backbone NL4-3 (pNL-Luc-E⁻R⁻) encoding the firefly enzyme luciferase and the specified HIV-1 envelopes. Two days after infection, the co-cultures were analyzed for their luciferase activity, as previously described [22]. LU, light units. (c) The contribution of cell-surface markers was tested by replacing MT-2 cells, a CD4⁺ cell line, with HSB, a CD4⁻ T-cell line and by replacing 293-CCR5 cells with parental 293 cells in the co-culture assay system. **(d)** Infection and spread of replication-competent HIV-1. MT-2 cells co-cultured with 293-CCR5 cells were infected with YU-2, a CCR5-dependent viral strain. The amount of p24 released into the supernatant was measured, at the indicated time after infection, by enzyme-linked immunosorbent assay (ELISA) (Dupont). Data represent the mean ± standard error of the mean (SEM) of experiments performed in triplicate.

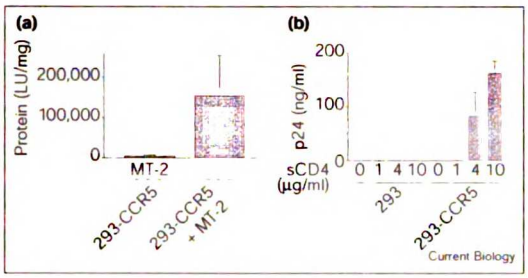
strain of HIV-1 (YU-2), washed extensively, and monitored for production of the HIV-1 secreted protein Gag p24 antigen (p24) over time. Abundant and progressive production of p24 was detected in supernatants of these co-cultures, which indicates that the system supported the complete viral replication cycle (Figure 1d).

To identify the specific cell type infected in the present assay system, we used fluorescence-activated cell sorting (FACS) to separate 293-CCR5 cells and MT-2 cells that had been co-cultured and exposed to the pseudotype virus JRFL for 2 days. The co-cultures were immunostained with an anti-CD25 monoclonal antibody to define MT-2 cells and an anti-CCR5 monoclonal antibody to define 293-CCR5 cells. The cells were separated by FACS into CD25⁺ and CCR5⁺ subsets, and analyzed for luciferase content. Of the total luciferase signal, 90% was detected in the 293-CCR5 cell fraction and 10% in the MT-2 cell fraction. Therefore, the CCR5-bearing cells appear to be the predominant target of HIV-1 in these co-cultures. Within the sensitivity limits of this technology, no CD4 was detected in the CCR5⁺ cell fraction.

It was important to distinguish whether the infectivity by a *trans*-receptor mechanism requires direct cell–cell contact rather than release of cellular factors (for example, soluble forms of CD4) from the CD4⁺ lymphocytes. We therefore separated MT-2 cells from the 293-CCR5 cells by a semi-permeable membrane (pore size, 3 μm) that permits the diffusion of molecules and virions but not cells. Infection was detected when both cell types were plated together in the bottom chamber, but was completely prevented when the two cell types were separated by the membrane (Figure 2a). Thus, direct cell–cell contact was mandatory to generate an effective viral receptor complex, and soluble factors released by either cell type are not sufficient to promote infection. Furthermore, serial interaction with CD4 in one chamber followed by CCR5 in the second chamber was insufficient to mediate this process.

We next investigated whether a soluble form of CD4 (sCD4) representing the D1–D4 extracellular domains of the molecule [12] could mimic CD4⁺ cells in these cultures. We exposed 293-CCR5 cells or 293 parental cells to the CCR5-dependent virus BaL in the presence of sCD4. The sCD4 conferred susceptibility to HIV-1 infection leading to productive replication of virus onto 293-CCR5 cells, but had no effect on cells lacking CCR5 (Figure 2b); this process exhibited dose-dependence. We have also found that the amino-terminal D1 domain is both necessary and sufficient to induce infection by this pathway (U.E. and M.A.G., unpublished observations). Therefore, although optimal effects may be triggered when CD4 is membrane-bound, these results are reminiscent of earlier observations with HIV-2 [13] and strongly suggest a molecular basis by which CD4 and CCR5 cooperate *in trans* to permit cellular entry by HIV-1.

Figure 2



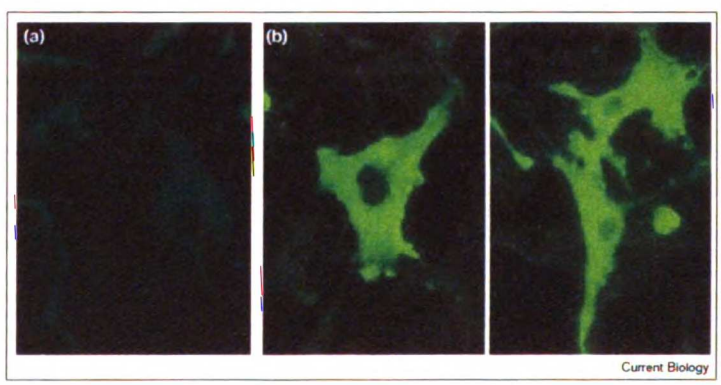
CD4 provided in membrane-anchored or soluble forms. **(a)** MT-2 cells and 293-CCR5 cells were cultured in chambers separated by a semi-permeable membrane (indicated by the dashed line). Effective diffusion of viruses across the membrane was assessed by adding the pseudotype virus JRFL to the top chamber and both cell types to the bottom chamber (right). The requirement for physical contact was tested by culturing MT-2 cells in the top chamber and 293-CCR5 cells in the bottom chamber, with JRFL added to both (left). **(b)** Soluble CD4 (sCD4), consisting of domains D1–D4 (amino acids 1–369), was added to 293-CCR5 cells or 293 parental cells at various concentrations at the time of infection with the CCR5-dependent virus BaL. The cultures were washed 24 h after infection and sCD4 was replenished at the same concentrations. Virion production was assessed by measuring the amount of p24 in the supernatant 9 days after infection. The values from triplicate wells are presented as mean ± SEM.

Finally, we sought to establish whether such a process may promote infection of primary CD4⁻ cell types, such as astrocytes, that have been reported to be infected *in vivo* and thus implicated in brain pathology. Primary human fetal astrocytes were therefore isolated and immunostained for glial fibrillary acidic protein (GFAP), CD4 and HIV-1 coreceptors. Fluorescence microscopy of these cells revealed uniform (>95% of cells) expression of GFAP and CXCR4 but not of CCR5, and an absence of CD4 (S.Y.C. and M.A.G., unpublished observations). Astrocytes cultured alone and exposed to the replication-competent CXCR4-dependent virus NL4-3 exhibited no detectable intracellular p24, as detected by immunostaining (Figure 3a). In contrast, in infected co-cultures containing primary astrocytes and CD4⁺ Sup-T1 cells, approximately 2–5% of the astrocytes were found to express intracellular p24, despite the persistent absence of CD4 on the cells (Figure 3b). Moreover, infection was fully abrogated by treatment with the specific CXCR4-antagonist peptide T22 [14] (S.Y.C. and M.A.G., unpublished observations). These findings extend earlier co-cultivation studies [15] and indicate that primary astrocytes acquire susceptibility to coreceptor-dependent infection by HIV-1 upon mixing with CD4⁺ lymphocytes. Although it remains to be established whether this process occurs *in vivo*, it may represent an important pathway for entry into astrocytes and other CD4⁻ cells during HIV disease.

We thus describe a process by which CD4 expressed on neighboring cells appears to prime the HIV-1 envelope protein to fuse with a target cell expressing appropriate coreceptors. Although additional studies are needed to clarify and extend these principles, a cooperative *trans*-receptor mechanism may permit HIV-1 to infect a broader range of cell types expressing chemokine receptors with low or absent CD4 in the central nervous system, lymphoid organs, inflammatory lesions, or other sites in which CD4⁺ cells are juxtaposed with other potential targets. While the mechanism appears to be less efficient than the typical CD4-dependent pathway, it corresponds to the relative infrequency of HIV-infected CD4⁻ cells *in vivo*. The expanded range of targets may nonetheless contribute to disease pathogenesis and may also represent substantial

Figure 3

Productive infection of primary human astrocytes upon co-culture with CD4⁺ cells. Human fetal brain astrocytes were isolated as described [18] and cultured to 80% confluence in chamber slides. **(a)** Astrocytes alone or **(b)** astrocytes co-cultured with CD4⁺ Sup-T1 cells were infected with the replication-competent HIV-1 strain NL4-3. After 72 h, cells were fixed in 2% paraformaldehyde and permeabilized with 0.1% Triton X-100. Intracellular p24 expression was assessed by immunofluorescence (NEN Life Science Renaissance TSA-Direct kit) using an anti-p24 IgG1 primary monoclonal antibody. Cells with characteristic astrocyte morphology were observed throughout, and examples of typical p24-positive astrocytes are shown in (b); no such cells were seen in the absence of CD4⁺ cells (a).



reservoirs for latent or replicating viruses. Furthermore, binding of ligands via *trans*-receptor mechanisms may be a general theme that affects other biological processes.

Materials and methods

Cells and co-culture assays

The T-cell lines C8166, MT-2 and HSB (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH), and Sup-T1 (ATCC CRL-1942; American Type Culture Collection), were cultured in RPMI medium (Mediatech), supplemented with 10% fetal calf serum (Gemini Bio Products), 1% glutamine (Mediatech) and 1% penicillin/streptomycin (Mediatech). 293-CCR5 cells [16] were kindly provided by I. Charo. T cells (2×10^6) were added to 293-CCR5 cells that had reached a confluence of 60–80% in a well of a 6-well plate, whereupon the co-cultures were immediately challenged with pseudotype viruses. Cells were harvested 2 days after infection and were assessed for luciferase activity as previously described [17].

Virus stocks

Pseudotype viral stocks were generated by calcium-phosphate transfection of 293T cells with the proviral plasmid pNL-Luc-E$^-$R$^-$ containing a luciferase reporter gene and expression plasmids for ADA and JRFL gp160 envelope (plasmids kindly provided by N. Landau through the NIH AIDS Research and Reference Reagent Program). Viral stocks of YU-2 were obtained by transfecting 293T cells with the molecular clones pYU-2 (provided by B. Hahn through the NIH AIDS Research and Reference Reagent Program). The BaL virus stock was prepared on human macrophages. Stocks with p24 amounts of higher than 150 ng/ml were used for all experiments.

Infection in the presence of sCD4

293-CCR5 cells and parental 293 cells were plated the day prior to infection in a 24-well plate at 70,000 cells/well. The following day, sCD4 was added to the cultures at the indicated concentrations immediately prior to adding 50 µl BaL stock. The following day, cell cultures were washed twice with medium, and fresh media containing sCD4 was added. Cells were cultured for 8 more days and secreted p24 was assessed. The sCD4 (D1–D4) was kindly provided by R. Sweet (SmithKline Beecham) through the NIH AIDS Research and Reference Reagent Program.

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Chapter 4

Distinct Mechanisms of Entry by Envelope Glycoproteins of Marburg and Ebola (Zaire) Viruses

Introduction

Unlike the case for HIV-1, the mechanisms of cellular entry used by Marburg (MBG) and Ebola (EBO) viruses are largely uncharacterized. Since MBG and EBO viruses themselves carry genomic sequence homology, comparable virion structures, and cause similar diseases, it is reasonable to postulate that they associate with target cells via envelope GP by similar mechanisms.

To test this hypothesis, a system was established to compare these mechanisms of infection in the absence of a maximum level biosafety level 4 (BSL4) containment facility. Previously, it was demonstrated that a number of heterologous envelope GP, including the vesicular stomatitis virus-G protein (VSV-G) as well as the amphotropic (Ampho) and ecotropic (Eco) murine leukemia virus (MLV) GP (as reviewed by (1)), could be packaged into pseudotype virions carrying HIV genomes. Given the similarities in structure of filovirus GP with that of HIV-1 gp120/gp41 (2), we hypothesized that expression of either MBG and EBO GP in the lipid virion envelope could stably form infectious pseudovirions containing an HIV genome as well. Because of access to a number of genetically manipulated HIV-1 *env*-negative proviral constructs, pseudotype virions were produced that carried entry specificity dictated by either MBG or EBO GP yet could only sustain a single round of infection. Through chemical modification of target cells followed challenge with these pseudtype viruses, experiments were conducted in a BSL3 facility by to

determine the general pathways of entry utilized by filoviruses as a prelude to identifying a specific cellular host factor(s) that mediates virus entry. As a result, this study provided the first proof that, while they carry certain functional similarities, the MBG and EBO GP utilize distinct pathways to enter cells.

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Distinct Mechanisms of Entry by Envelope Glycoproteins of Marburg and Ebola (Zaire) Viruses

STEPHEN Y. CHAN,[1,2] ROBERTO F. SPECK,[1]† MELISSA C. MA,[1] AND MARK A. GOLDSMITH[1,2]*

Gladstone Institute of Virology and Immunology[1] and University of California San Francisco,[2] San Francisco, California 94141-9100

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Since the Marburg (MBG) and Ebola (EBO) viruses have sequence homology and cause similar diseases, we hypothesized that they associate with target cells by similar mechanisms. Pseudotype viruses prepared with a luciferase-containing human immunodeficiency virus type 1 backbone and packaged by the MBG virus or the Zaire subtype EBO virus glycoproteins (GP) mediated infection of a comparable wide range of mammalian cell types, and both were inhibited by ammonium chloride. In contrast, they exhibited differential sensitivities to treatment of target cells with tunicamycin, endoglycosidase H, or protease (pronase). Therefore, while they exhibit certain functional similarities, the MBG and EBO virus GP interact with target cells by distinct processes.

The Marburg virus (MBG) and the Ebola virus (EBO) are filoviruses that have caused lethal outbreaks of hemorrhagic fever (8). Both are RNA viruses that carry host-derived envelopes and unique but related transmembrane glycoproteins (GP) that likely mediate cellular binding and fusion. The highly glycosylated GP exhibit conservation in the N- and C-terminal regions but more variability in the middle region (9, 17). Similarities in virus organization, GP structure (15), and pathogenesis suggest that MBG and EBO use similar mechanisms to enter target cells. However, since these viruses share only 31% identity in GP amino acid sequence (11) and exhibit differences in GP transcriptional processing (10), it is also conceivable that different filoviruses use alternate mechanisms to infect cells and incite disease.

To construct a system for comparing these mechanisms of infection, genes encoding the MBG GP and the Zaire subtype EBO (EBO-Z) GP (provided by A. Sanchez, Centers for Disease Control and Prevention), cloned into the mammalian expression vector pCMV4neo (3), were incorporated into replication-incompetent pseudotype viruses carrying a human immunodeficiency virus type 1 (HIV-1) provirus NL4-3 lacking *env* but carrying a luciferase reporter gene (2) as previously described (1). Both MBG and EBO-Z pseudotypes infected HeLa cells to a significant degree, whereas no infection was observed by pseudovirions expressing the CCR5- and CD4-dependent envelope of HIV-1 JR-FL (provided by N. Landau, Salk Institute) (Fig. 1A). Expression of CD4 and CCR5 restored JR-FL infection with no change in the infection patterns of MBG or EBO-Z (Fig. 1B). Furthermore, no infection of the T-cell line SupT1 was observed for the MBG and EBO-Z pseudotypes, while robust infection was mediated by the envelope of CXCR4-dependent HIV-1 NL4-3 (Fig. 1C). Thus, MBG and EBO-Z GP can package HIV-1 virions and dictate distinct specificities of cellular infection.

To define the cellular range of infection controlled by MBG and EBO-Z GP, a panel of mammalian cells was tested as targets for entry. The MBG pseudotype yielded variable but significant signals (up to 10^5 relative light units) in diverse target cells, including human osteosarcoma (HOS), 293T, HeLa, HepG2, and primary HUVEC cells (provided by A. van Zante and S. Rosen, University of California, San Francisco), as well as all adherent monkey, hamster, and dog cell lines (Fig. 2A). In contrast, human U87, murine NIH 3T3, and all suspension T-cell lines (C8166, SupT1, MT-2, and Jurkat) were nonpermissive for MBG. The hamster cell lines CHO and BHK were exceptional in supporting low levels of infection by both MBG and amphotropic Moloney leukemia virus (Ampho) (5) pseudotypes. Because the activity of the luciferase gene promoter, the HIV-1 long terminal repeat, in these pseudotype viruses is compromised in human astrocytes and murine cells, the lack of detectable infection of U87 and NIH-3T3 cells may have resulted from weak promoter function rather than failure of entry. Therefore, infections of U87, NIH-3T3, and Jurkat control cells were repeated by using MBG pseudotypes carrying an HIV-1 vector (HIV-puro) containing a puromycin resistance gene driven by the simian virus 40 promoter (provided by R. Sutton, Baylor University). After 10 days of selection of infected U87 and NIH-3T3 cells with puromycin (1 μg/ml), a significant number of antibiotic-resistant colonies survived in the MBG-infected samples, while none survived in the mock-infected cultures (data not shown). In contrast, while a significant number of Jurkat cells were viable after infection with pseudotypes carrying the vesicular stomatitis virus G protein (VSV-G) (provided by J. Burns, University of California, San Diego) and subsequent puromycin selection, no MBG-infected Jurkat cells survived. Therefore, both U87 and NIH 3T3 cells are, in fact, susceptible to entry mediated by MBG GP (Fig. 2A), and human T cells were the only cells identified as nonpermissive for MBG.

Similarly, EBO-Z pseudotypes selectively infected various human cell lines, including HOS, 293T, HeLa, HepG2, and HUVEC, and all tested monkey, hamster, and dog cells (Fig. 2B). Like the MBG pattern, U87 and NIH 3T3 cells and all T-cell lines were nonpermissive for EBO-Z entry, as assessed by luciferase expression. However, puromycin selection studies with HIV-puro virions carrying EBO-Z GP as described above indicated that both U87 and NIH 3T3 cells were susceptible to

* Corresponding author. Mailing address: Gladstone Institute of Virology and Immunology, P.O. Box 419100, San Francisco, CA 94141-9100. Phone: (415) 695-3775. Fax: (415) 695-1364. Email: mgoldsmith@gladstone.ucsf.edu.

† Present address: University Hospital Zürich, Zürich, Switzerland CH-8091.

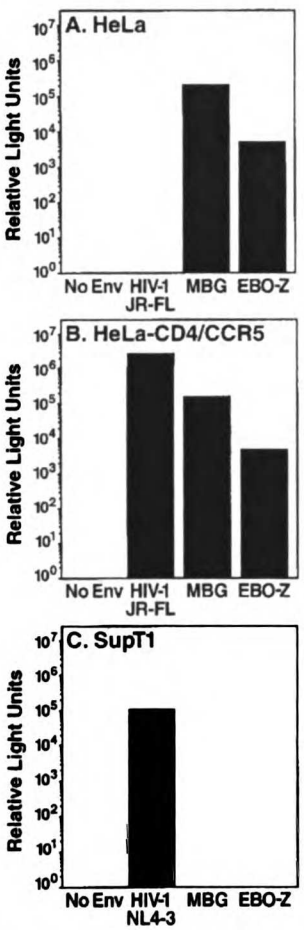

FIG. 1. HIV-1 pseudotypes packaged by HIV-1 (JR-FL or NL4-3), MBG, or EBO-Z GP display distinct specificities for virus entry. To determine range of infection by pseudotype viruses, HeLa cells (A), HeLa-CD4/CCR5 cells (B), or SupT1 T cells (C) were challenged with constant inocula of HIV-1 Luc$^+$ pseudotypes. After 48 h, luciferase expression was assessed as previously described (1). Displayed values are typical of three separate infections.

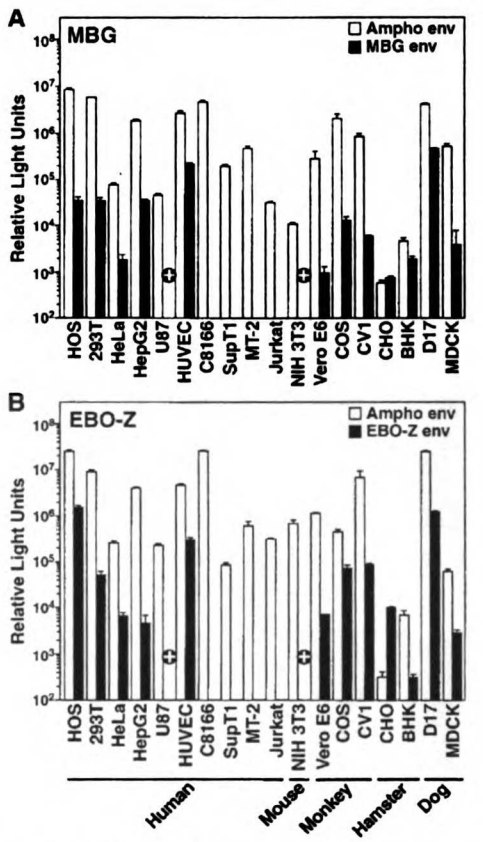

FIG. 2. MBG and EBO-Z GP dictate similar ranges of infection in a panel of mammalian cell types. Infections with constant inocula of MBG (A) and EBO-Z (B) luciferase pseudotypes were performed for 48 h in parallel with infections by Ampho pseudotype virus to estimate cell type variability in luciferase reporter expression after virus entry. Data are the means derived from three separate infections (± standard error of the mean). +, cell line permissive to infection by pseudotype virions carrying the puromycin resistance gene driven by a simian virus 40 promoter but nonpermissive as assessed through infection by pseudovirions carrying the luciferase reporter gene driven by the HIV-1 long terminal repeat promoter.

EBO-Z pseudotypes while Jurkat controls were not (Fig. 2B). Therefore, both MBG and EBO-Z pseudotype virions display similarly broad, yet selective, ranges of infectivity.

The extensive range of infection by these viruses is consistent with other studies that have reported the in vitro tropism dictated by EBO-Z GP (18, 19), but there have been no previous reports of a similar comprehensive review for MBG GP. The broad target range correlates well with the widespread tissue necrosis after MBG and EBO infections (8). These similarities suggest that the cellular receptor(s) that mediates infection by these viruses not only is expressed in a variety of different tissues but also is highly conserved among mammalian species. Interestingly, all four suspension cell lines tested were not infectable by either virus, in agreement with previous reports regarding both EBO-Z and the Reston (EBO-R) subtypes of EBO (18, 19). Therefore, we postulated that the cellular receptor(s) mediating filovirus infection may play a role in cellular attachment and perhaps is a member of the highly conserved integrin family. However, antibody neutralization across a range of integrin complexes did not reproducibly inhibit entry by either pseudotype virus (data not shown). Nonetheless, the comparable infection profiles support the hypothesis that both filoviruses cause disease in part by infection and cytopathicity in a broad range of body tissues. Furthermore, the identification of both infectable and noninfectable cells should prove useful in combination with these pseudotype viruses to identify the cellular receptor(s) for these filoviruses.

To determine the efficiency of single-round infection by MBG and EBO-Z pseudotypes, we utilized HOS cells that express a green fluorescent protein (GFP) reporter only in the presence of HIV-1 Tat protein (14) (GHOST cells), and thus after successful infection by HIV-1 pseudotype virions. While negligible basal GFP expression was observed in these cells as

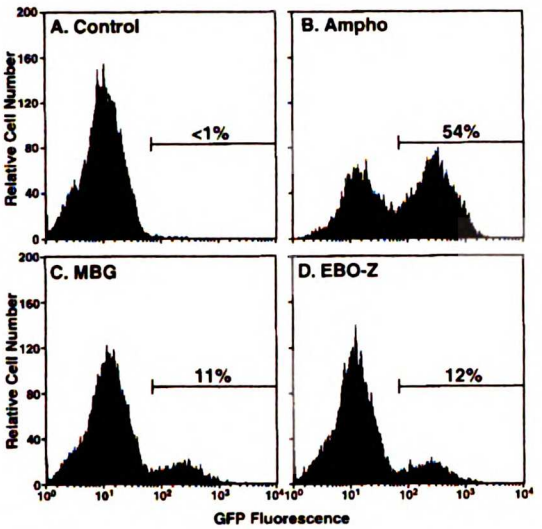

FIG. 3. Efficient single-round infection of GHOST cells by pseudotypes of HIV-1 carrying Ampho, MBG, or EBO-Z GP. To determine the percentage of infected cells, GHOST cells were infected for 48 h with no virus (A) or Ampho (B), MBG (C), or EBO-Z (D) pseudotypes and analyzed for GFP expression by flow cytometry.

assessed by flow cytometry (Fig. 3A), Ampho pseudotype virus infected 54% of cells (Fig. 3B) and MBG and EBO-Z pseudotypes infected 11 and 12% of target cells, respectively (Fig. 3C and D). Since different viral stocks were used in the studies to determine range (Fig. 2) and efficiency (Fig. 3) of infection, percentages of infected cells cannot be correlated directly with luciferase activities in these two experiments. Nonetheless, both MBG and EBO-Z GP package HIV-1 genomes relatively efficiently, yielding significant and comparable titers of infectious viruses.

Previous studies have reported analogous strategies to pseudotype a murine leukemia virus vector with EBO-Z GP (18, 19) or a VSV vector with EBO-R GP (13), but no such result has been described with MBG GP. We also attempted to use murine leukemia virus for MBG or EBO-Z pseudotyping but were unable to obtain titers that supported >1% infection. Nonetheless, modest efficiency may not have precluded their use for entry studies due to the high sensitivity of the luciferase assay system. In the present study, MBG and EBO-Z pseudotypes based on the HIV-1 backbone infected >10% of target cells when the highest achievable titers were utilized. The discovery of an efficient viral packaging system for filoviruses will likely prove useful in future studies of virus infection that can be performed without a Biosafety Level 4 facility.

To identify processes critical to viral entry by MBG and EBO-Z, HeLa-CD4/CCR5 cells were subjected to chemical treatment to alter functions (e.g., receptor presentation) that may influence infection. First, cells were preincubated (2 h) and incubated during infection with ammonium chloride (3 h), a lysosomotropic reagent that prevents acidification of endosomes and vesicles. After 48 h, infection indicated by luciferase expression would be expected to be inhibited for viruses internalized by endosomes (e.g., VSV) but not for viruses gaining access through plasma membrane fusion at the cell surface (e.g., HIV-1). Infection by the HIV-1 JR-FL pseudotype did

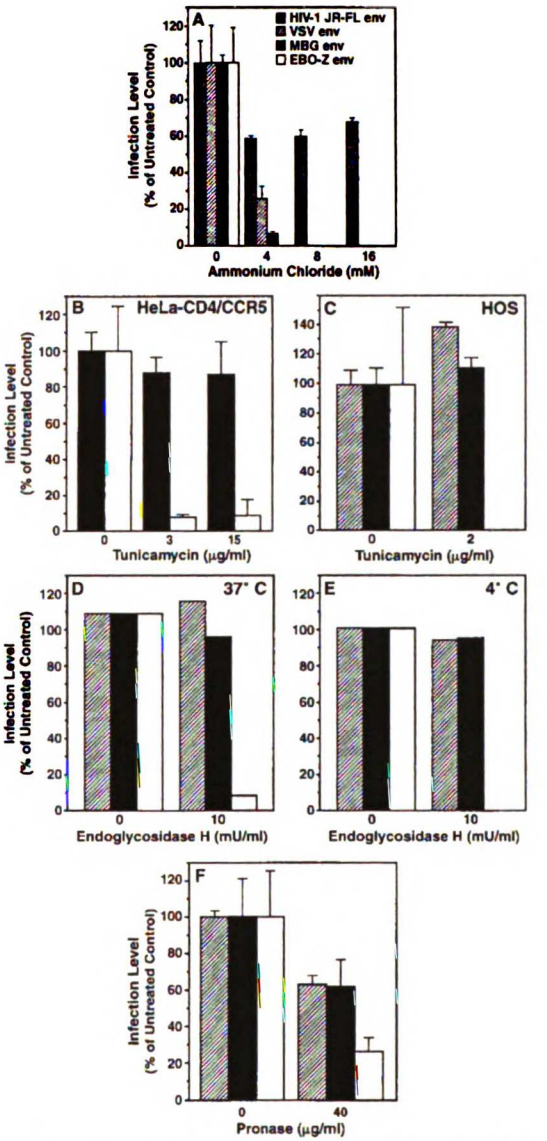

FIG. 4. Comparison of alterations in infectability of MBG and EBO-Z pseudotypes after chemical modification of target cells. Shown are treatments of HeLa-CD4/CCR5 cells with ammonium chloride (A), with tunicamycin (B), with endoglycosidase H (D), with endoglycosidase H at 4°C and in the presence of protease inhibitors (E), and with pronase protease (F) and a treatment of HOS cells with tunicamycin (C). Displayed values are means (± standard error of the mean) of luciferase activity from three separate infections (A to C, F) or are typical of two separate infections (D and E).

not exhibit a dose-dependent decrease compared to the untreated condition (Fig. 4A). We did note that treatment by ammonium chloride decreased infection by 30 to 40% of the untreated signal, but this dose-independent phenomenon is most likely attributable to nonspecific effects. In contrast, the

VSV pseudotype demonstrated a dose-dependent and complete abrogation of infection. Likewise, infections by both the MBG and EBO-Z pseudotypes exhibited marked, dose-dependent decreases following treatment. Therefore, both MBG and EBO-Z GP mediate viral entry into target cells by a pH-dependent process. Similar findings were reported for pseudotype viruses carrying EBO-R (13) and EBO-Z (18) GP, and acidification has been implicated in an undefined aspect of the MBG replication cycle (7). Furthermore, the recently solved crystal structure of the transmembrane portion of EBO GP2 reveals structural similarities to the low-pH-induced HA2 protein that regulates influenza virus fusion (6, 16). Together, these data suggest that virus fusion by both MBG and EBO-Z likely depends on postendocytic acid-dependent conformational changes in the virus GP. Thus, our studies extend the set of diverse virus GP that appears to rely on a common final pathway for mediating entry.

Second, to investigate the role of N-glycosylation of surface proteins on target cells, HeLa cells were preincubated with tunicamycin (24 h) or endoglycosidase H (2 h) in serum-free media before inoculation with the pseudotype viruses. Tunicamycin inhibits intracellular N-glycosylation of proteins, while endoglycosidase H cleaves high-mannose type N-glycosylated carbohydrate moieties at the cell surface. After inoculation (48 h), infection by MBG was not altered in cells treated with a range of tunicamycin concentrations (Fig. 4B). However, infection by EBO-Z decreased by >90% at a concentration of either 3 or 15 μg/ml. To rule out the possibility that variability in the virus titers allowed for this distinction, cells were challenged with equivalent inocula of MBG and EBO-Z pseudotypes and normalized by luciferase expression, and essentially the same pattern of inhibition was observed (data not shown). Identical profiles of inhibition were also observed in HOS cells (Fig. 4C). Similarly, infection by neither the MBG nor the VSV pseudotype was altered after pretreatment of cells (37°C for 2 h) with endoglycosidase H. In contrast, infection by the EBO-Z pseudotype decreased by >90% (Fig. 4D). Separate experiments with endoglycosidase H were performed at 4°C to prevent internalization of the enzyme and thus to ensure carbohydrate cleavage only at the cell surface. In addition, a protease inhibitor cocktail (leupeptin [10 μg/ml], pepstatin A [1 μg/ml], aprotinin [10 μg/ml], and phenylmethylsulfonyl fluoride [1 mM]) was used to ensure neutralization of undetected proteases; specific inhibition of EBO-Z was observed (Fig. 4E). These complementary experiments utilizing tunicamycin and endoglycosidase H revealed that alterations in N-glycosylation in target cells selectively impact virus entry mediated by EBO-Z GP, but not by MBG GP, and therefore suggest that MBG and EBO-Z are dependent on different cell surface moieties for cellular entry.

Third, target cells were preincubated with pronase protease to cleave surface proteins nonspecifically prior to infection (13). Although VSV infection may not utilize a protein receptor (12), infection by the VSV pseudotype decreased by 40% compared to that of the untreated culture (Fig. 4F), a previously reported nonspecific effect of pronase treatment on cells (13). Similarly, infection by MBG decreased by 40%. In contrast, entry mediated by EBO-Z GP was more significantly inhibited, decreasing by 73%. These effects of pronase were quantitatively variable across multiple experiments, but the pattern of specific inhibition of EBO-Z GP above the background effects was reproducible. Therefore, since alteration of proper protein presentation on target cells via treatment with both pronase and inhibitors of N-glycosylation suppressed EBO-Z but not MBG entry, their infection processes must not be fully identical.

Treatment of target cells with tunicamycin, endoglycosidase H, and pronase delineated potentially important distinctions between MBG and EBO-Z GP. While inhibition of infection by EBO-Z after loss of either surface proteins or N-glycosylated moieties on target cells was consistent with earlier findings regarding the EBO-R subtype (13), these treatments had little effect upon infection by MBG. It is also possible that variability in GP incorporation into MBG and EBO-Z pseudotype virions may contribute to these distinctions. However, the same pattern of infection was observed when using equivalent MBG and EBO-Z inocula normalized by luciferase expression in target cells. Furthermore, since MBG GP and EBO-Z GP were expressed at significant levels in 293T cells for virus preparations (data not shown), and both pseudotype viruses infected with similar efficiencies when used at high titers (Fig. 3), it is likely that they carry similar amounts of GP in their envelopes and that variability in pseudotype virus titers was not the cause of this distinction.

These results indicate that EBO-Z GP either interacts with a cell surface protein receptor to initiate viral entry or relies on the function of a surface protein to increase infection efficiency, as exemplified by disruption of HIV-1 infection by inhibition of LFA-1 (4). On the other hand, like MBG, infection by HIV-1 JR-FL was not inhibited by pronase (data not shown) despite the fact that entry by this virus is dependent on the binding of two protein receptors (CD4 and CCR5). Therefore, the unaffected MBG infection profiles do not exclude the possibility that MBG GP utilizes a surface protein as a receptor. Rather, these data highlight the fact that MBG and EBO-Z infections depend differentially on the presentation of target cell surface proteins, which may uniquely influence the viral life cycle and/or pathogenesis. Because only partial identity in amino acid sequence exists between MBG GP and EBO-Z GP, it is not unreasonable to expect functional differences in entry requirements to have evolved. Future investigations aimed at identifying the cellular receptor(s) for these filoviruses are necessary to characterize these mechanisms definitively.

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Chapter 5

Differential Induction of Cellular Detachment by Envelope Glycoproteins of Marburg and Ebola (Zaire) Viruses

Introduction

The major role of envelope GP is to mediate entry into target cells, but the complete functions of all filovirus GP products remain unclear. Because they typically bind host factors on the target cell surface to gain entry, viral envelope GP can also serve as signaling ligands to initiate intracellular responses and pathogenic changes independent of viral replication. Such is the case for HIV-1 gp120, expressed in the brain in the absence of infection and independent of intact HIV-1 virions (1-6). In these experiments, the possibility that filovirus GP expression may directly disrupt target cell function and therefore contribute to pathogenesis was investigated. As a result of fortuitous observations made during production of pseudotype viruses, expression of EBO-Z GP, but not MBG GP, in 293T cells was found to induce significant levels of cellular detachment in the absence of cell death and independent of virus replication. Therefore, this study demonstrated that the MBG and EBO GP interact with target cells by distinct processes to incite cellular dysregulation and perhaps to contribute to disease manifestations.

Note: Similar findings were reported at the same time by Yang et al. (7). While many of our observations were confirmed, Yang et al. localized the ability to induce cellular dysregulation to a domain of GP1 rather than GP2 as found in this study. This discrepancy may be explained by the fact that both GP1 and GP2

carry sequences necessary for induction of this phenomenon. Deletion of either one may significantly or completely abrogate the ability to induce cellular dysfunction, as both may be necessary together for a robust phenotype.

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Differential induction of cellular detachment by envelope glycoproteins of Marburg and Ebola (Zaire) viruses

Stephen Y. Chan,[1,2] Melissa C. Ma[1] and Mark A. Goldsmith[1,2]

[1] Gladstone Institute of Virology and Immunology, PO Box 419100, San Francisco, CA 94141-9100, USA
[2] Department of Medicine, School of Medicine, University of California San Francisco, San Francisco, CA 94141-9100, USA

Human infection by Marburg (MBG) or Ebola (EBO) virus is associated with fatal haemorrhagic fevers. While these filoviruses may both incite disease as a result of explosive virus replication, we hypothesized that expression of individual viral gene products, such as the envelope glycoprotein (GP), may directly alter target cells and contribute to pathogenesis. We found that expression of EBO GP in 293T cells caused significant levels of cellular detachment in the absence of cell death or virus replication. This detachment was induced most potently by membrane-bound EBO GP, rather than the shed glycoprotein products (sGP or GP1), and was largely attributable to a domain within the extracellular region of GP2. Furthermore, detachment was blocked by the Ser/Thr kinase inhibitor 2-aminopurine, suggesting the importance of a phosphorylation-dependent signalling cascade in inducing detachment. Since MBG GP did not induce similar cellular detachment, MBG and EBO GP interact with target cells by distinct processes to elicit cellular dysregulation.

Marburg (MBG) and Ebola (EBO) viruses are human pathogens that have caused fatal epidemics of haemorrhagic fever characterized by massive virus replication, widespread infection of numerous tissues and fatal tissue necrosis (Peters *et al.*, 1996). The molecular mechanism(s) underlying these disease manifestations are poorly understood. MBG and EBO each express a unique but related type I transmembrane glycoprotein (GP) (Sanchez *et al.*, 1993; Will *et al.*, 1993) that probably mediates binding and fusion with target cells. However, the complete functions of all GP products remain unknown. Here, we investigated the possibility that filovirus GP expression may directly disrupt target cell function and therefore contribute to pathogenesis independent of virus replication.

Author for correspondence: Mark Goldsmith (at Gladstone Institute of Virology and Immunology). Fax +1 415 695 1364.
e-mail mgoldsmith@gladstone.ucsf.edu

EBO GP produced in an infected cell is cleaved intracellularly to produce membrane-bound GP2, a transmembrane protein that mediates membrane fusion, and associated GP1, an extracellular protein that presumably mediates virus attachment to a receptor at the cell surface (Volchkov *et al.*, 1998*a*). Free GP1 is also shed in a nonvirion-bound form and has been hypothesized to be pathogenic (Volchkov *et al.*, 1998*b*). MBG GP is processed similarly (Volchkov *et al.*, 2000). In addition, pre-translational processing of EBO GP results in the production of two EBO glycoprotein products, a shorter secreted product (sGP) derived from unedited mRNA transcripts and a polyprotein precursor to the longer membrane-bound form (GP1/GP2) encoded by edited transcripts (Sanchez *et al.*, 1996; Volchkov *et al.*, 1995). While GP1/GP2 mediates infection in a variety of cells, EBO sGP has been reported to bind to uninfected cells such as neutrophils (Yang *et al.*, 1998); the interpretation of these findings has been challenged (Maruyama *et al.*, 1998), and the precise functional relevance of EBO sGP in pathogenesis is unknown. In contrast, MBG GP transcripts apparently do not undergo such editing (Bukreyev *et al.*, 1995; Will *et al.*, 1993), and express exclusively the GP1/GP2 polyprotein which is cleaved and assembled into the full-length membrane-bound GP1/GP2 complex. These distinctions underscore the possibility that GP products from different filoviruses may induce different dysregulatory phenotypes in host cells.

In initial studies, genes encoding MBG GP and the Zaire (Z) subtype of EBO GP (provided by A. Sanchez, Centers for Disease Control and Prevention, Atlanta, GA, USA) were cloned into the mammalian expression vector pCMV4neo (Goldsmith *et al.*, 1994) and separately co-transfected with pNL-Luc-E$^-$R$^-$ (Connor *et al.*, 1995), the HIV-1 NL4-3 provirus carrying a luciferase reporter gene driven by the 5′ LTR (provided by N. Landau, Salk Institute, La Jolla, CA, USA, via the AIDS Research and Reference Reagent Program) into 293T cells in order to produce pseudotype virus stocks as previously described (Chan *et al.*, 2000). To assess and compare production

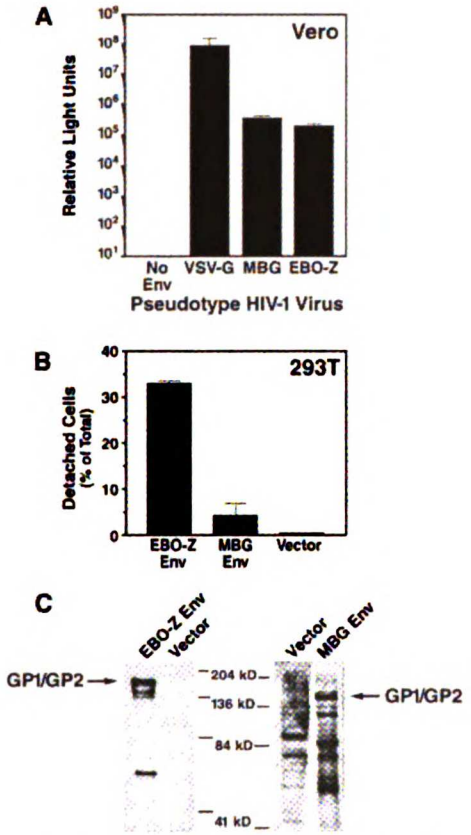

Fig. 1. Induction of cellular detachment by EBO-Z GP, but not MBG GP. (A) Production of similar levels of functional MBG and EBO-Z GP by 293T cells as assessed by infection of Vero cells by HIV-1 pseudotype viruses packaged by VSV-G, MBG or EBO-Z GP and produced from transfected 293T cells. Displayed values are typical of three separate infections. (B) Assessment of detachment after transient transfection of EBO-Z or MBG GP into 293T cells. Detachment was quantified as described in the text, and was assessed in parallel with determination of expression levels in (C). Values are typical of three separate transfections. (C) Consistent expression of full-length EBO-Z GP (left) and MBG GP (right) in 293T cells after transient transfection as assessed by SDS–PAGE of cellular lysates under reducing conditions and Western blot using chemiluminescent detection with the ECL system (Amersham) using separate polyclonal guinea pig antisera recognizing EBO-Z GP and MBG GP products, respectively. Although these polyclonal antisera recognized a number of variable background bands, the labelled GP products were reproducibly detectable. Consistent expression of sGP was not observed as expected since the EBO-Z GP cDNA used for these studies encoded only edited, full-length GP1/GP2 products and not pre-edited sGP products.

and function of MBG and EBO-Z GP in this system, Vero cells were challenged with Luc$^+$ pseudotype viruses packaged by no GP, vesicular stomatitis virus (VSV) G protein (provided by J. Burns, University of California, San Diego, CA, USA), MBG GP or EBO-Z GP (Fig. 1A), and luciferase expression was used to quantify virus entry as previously described (Chan *et al.*, 2000). Both MBG and EBO-Z pseudotypes infected Vero cells to comparable and significant levels, demonstrating that GP1/GP2 complexes encoded by both MBG and EBO-Z constructs were functionally competent for packaging virus and initiating target cell infections. Furthermore, MBG and EBO-Z pseudotype viruses generated from 293T transfections infected the same proportion of target cells at highest achievable titres (Chan *et al.*, 2000), indicating similar expression of functional GP in both preparations.

The present studies were based on the fortuitous observation that EBO-Z pseudotype preparations were associated with substantial 293T cell detachment after transfection, a phenomenon that was also observed when EBO-Z GP alone was expressed in 293T cells without other viral genes. To quantify this effect, 293T cells were transfected with expression vectors carrying the EBO-Z or MBG GP as well as the parental pCMV4neo control vector as previously described (Chan *et al.*, 1999). After a 48 h incubation, detached cells were collected, attached cells were recovered by trypsinization, and both populations were counted on a haemocytometer. Transient expression of the EBO-Z GP construct caused release of 33% of total cells (Fig. 1B). Despite using equivalent amounts of expression vectors, transfection of the MBG GP resulted in $< 5\%$ detachment. Of the detached cells in EBO-Z transfections, $> 99\%$ were viable as assessed by Trypan blue exclusion; transmission electron microscopy of detached cells also revealed morphology consistent with cell viability (data not shown). Therefore, expression of the EBO-Z envelope glycoprotein(s) was not cytotoxic to 293T cells, but rather it interfered specifically with the mechanism of cellular attachment.

To assess directly MBG and EBO-Z GP expression levels in 293T cells, SDS–PAGE under reducing conditions and Western blotting were performed as previously described (Liu *et al.*, 1997) on lysates [1% NP-40 lysis buffer containing 1 × stock protease inhibitor cocktail set I (CalBiochem)] obtained from transfected samples. For detection, we used polyclonal guinea pig antisera (1:1000 dilution) raised against MBG (Musoke strain) or EBO-Z (Mayinga strain) virus, respectively (provided by A. Schmaljohn, United States Army Medical Research Institute for Infectious Diseases, Fort Detrick, MD, USA), and an HRP-conjugated goat anti-guinea pig IgG (H + L) secondary antibody (1:5000 dilution; Accurate Chemical and Scientific Corp.) (Fig. 1C). Despite the presence of variable background bands, expression of both MBG and EBO-Z GP1/GP2 polyproteins was consistently and readily detected. As expected, sGP was undetectable since the cDNA used to express EBO-Z GP encoded only the edited full-length, membrane-bound GP and not the pre-edited cDNA encoding sGP (Xu *et al.*, 1998). In view of the comparable infectious titres of both pseudotype viruses generated from 293T transfections (Fig.

1A) and the detectable expression of envelope glycoproteins of both viruses, the stark difference in detachment between EBO-Z and MBG GP transfected samples indicates that EBO-Z GP induces a distinct cellular dysregulatory effect compared with that of MBG.

To determine whether detachment of cells was caused specifically by cell-associated or released GP products, 293T cells were transfected with pEBO-Z-IRES2-GFP, an expression vector encoding EBO-Z GP upstream of an internal ribosome entry site (IRES) site separately driving translation of an enhanced green fluorescent protein (GFP) reporter gene. In this configuration, every transfected cell that produced EBO-Z GP products was marked by GFP expression. After 12 h, transfected cells were re-plated together with untransfected 293T cells at a 1:1 ratio, and detachment was measured at 24 h. If secreted GP products induce detachment in *trans*, a similar ratio of GFP-positive (transfected) and GFP-negative (untransfected) cells would be expected to be evident in the detached cell population as in the attached cell population. However, if cell-associated GP causes detachment in *cis*, only GFP-positive transfected cells should be present in the detached population. In fact, flow cytometry revealed that 25% of cells remaining attached were GFP-positive in this experiment (Fig. 2 A), while nearly all (95%) of released cells were GFP-positive. In other experiments, the proportion of attached cells that was GFP-positive varied with the specific mixture of input cells, but the GFP-positive proportion in the detached fraction was always nearly 100%. As a specificity control, 293T cells were similarly transfected with the parental vector pIRES2-GFP and re-plated with untransfected cells. No detachment was detected in these cultures, and 28% of adherent cells expressed GFP. These results clearly demonstrate that detachment of 293T cells is caused by cell-associated EBO-Z GP rather than shed GP products.

To map the general region of EBO-Z GP necessary to induce cellular detachment, a panel of EBO-Z GP expression vectors was constructed that carry FLAG epitopes at the C-terminal end encoding: (1) full-length GP1/GP2; (2) a variant of GP1/GP2 truncated artificially at the transmembrane segment of GP2 (GP1/GP2 Trunc.) and carrying the extracellular portion of GP2; and (3) GP1 alone (expected to express a FLAG-tagged GP1 but not sGP, unless unexpected and previously unreported 'reverse editing' occurs). Using the monoclonal M2 antibody (International Biotechnologies Inc.) recognizing the FLAG epitope, a Western blot was performed on cellular lysates of 293T cells 48 h after transfection of these constructs. Titration of the amount of expression vector used in each transfection allowed for equivalent levels of expression from each GP construct (Fig. 2B). In parallel, attached and released cells were counted as before. Despite comparable levels of expression by all constructs, only full-length GP1/GP2 (> 7%) and GP1/GP2 Trunc. (> 2%) forms reproducibly caused significant detachment while GP1 alone did not (Fig. 2C). Therefore, a portion of EBO-Z GP necessary for eliciting

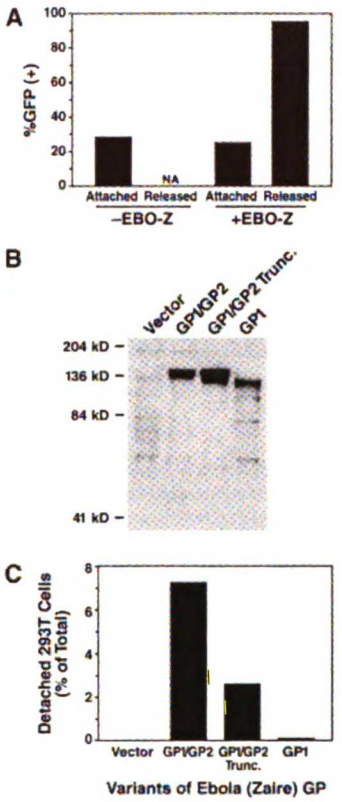

Fig. 2. The extracellular region of membrane-bound EBO-Z GP2 is necessary to cause detachment of 293T cells. (A) Detachment induced by cell-associated EBO-Z GP in *cis*. Cells expressing both EBO-Z GP and GFP were mixed and plated with untransfected cells as described in the text, and detachment was quantified. Displayed values were derived from analysis by flow cytometry of GFP expression in detached and adherent cell fractions (+EBO-Z). In parallel, cells expressing GFP alone (−EBO-Z) were handled similarly, and no released cells were observed after mixing with untransfected cells and re-plating (NA, not applicable). Displayed values are typical of two separate transfection studies. (B) Comparable expression of FLAG-tagged full-length EBO-Z GP1/GP2 (GP1/GP2), EBO-Z GP1/GP2 truncated at the transmembrane region of GP2 (GP1/GP2 Trunc.) and EBO-Z GP1 (GP1), as assessed by Western blot of cellular lysates using monoclonal anti-FLAG antibody. sGP was not expressed since the EBO-Z GP cDNA used to construct these truncated GP products was edited to encode only for GP1/GP2 products. (C) Detachment in 293T cells after transient transfection of FLAG-tagged EBO-Z GP constructs. Quantification was done in parallel with determination of expression levels in (B). Values are typical of three separate transfections.

detachment in 293T cells must be located in the extracellular domain of GP2, a region absent from either soluble GP1 or sGP products. It is important to emphasize that GP2 is normally anchored in the plasma membrane when wild-type GP1/GP2 is expressed. Therefore, in the context of our observation in Fig. 2(A) that detachment is observed only in

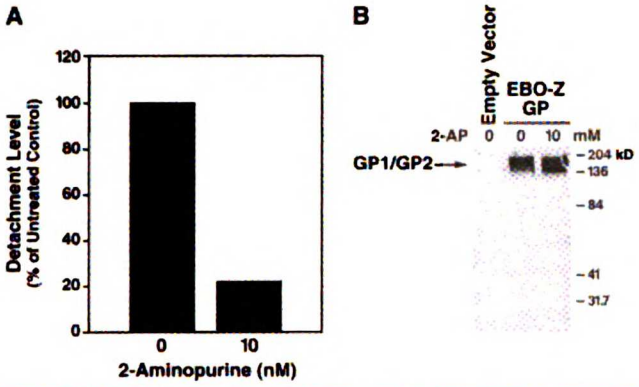

Fig. 3. The Ser/Thr kinase inhibitor 2-AP prevents EBO-Z GP-induced detachment. (A) Inhibition of detachment by treatment of EBO-Z GP-transfected 293T cells with 2-AP (10 mM) 6 h post-transfection compared with untreated transfected sample. Displayed values are representative of three separate trials. (B) Comparable expression of EBO-Z GP in transfected 293T cells, either treated with 2-AP (10 mM) or not at 6 h post-transfection, as assessed by Western blot of cellular lysates using guinea pig polyclonal antisera raised against EBO-Z (Mayinga) virus.

cells directly expressing GP products and not in the neighbouring cells exposed to other secreted GP products, we conclude that the detachment effect is caused by the extracellular domain of GP2 anchored in the host cell membrane. Furthermore, since alterations in cellular adhesion potentially play an important role in pathogenesis, this novel process of cellular dysregulation driven only by EBO-Z GP highlights the fact that MBG and EBO-Z interact with host cells differently and may elicit disease by distinct mechanisms.

Finally, we hypothesized that expression of EBO-Z GP results in cellular detachment as a consequence of modulating a specific intracellular signalling pathway(s). To identify such a signalling cascade in target cells, we screened a panel of phosphorylation inhibitors for the ability to block 293T cell detachment and found that the Ser/Thr kinase inhibitor 2-aminopurine (2-AP) potently inhibited detachment. When 2-AP (10 mM) was added to cultures 6 h after EBO-Z GP transfection, cellular detachment at 48 h was reduced by 77% as compared to untreated transfected controls (Fig. 3A). A Western blot using the guinea pig antisera raised against EBO-Z virus on separate lysates from control transfections and transfections treated with 2-AP confirmed equivalent expression of EBO-Z GP1/GP2 in both cultures (Fig. 3B). Therefore, an as yet undefined Ser/Thr kinase activity induced by EBO-Z GP expression must be instrumental in mediating cellular detachment.

We discovered that expression of EBO-Z GP causes marked detachment of 293T adherent cells by a process that is independent of cell death and is mediated by a signalling pathway involving Ser/Thr phosphorylation. A similar effect was not evident for MBG GP, despite detection of GP expression and similar levels of envelope-mediated infectivity in pseudotype virus production as compared to EBO-Z GP. Therefore, these data reveal a functional difference between MBG GP and EBO-Z GP, perhaps not an unexpected result since they share only a 31% identity in amino acid sequence (Sanchez *et al.*, 1998). The weak adherence of 293T cells may have made these cells especially susceptible to the detachment effect of EBO-Z GP, since more strongly adherent cells such as COS and CHO cells did not display such a response (data not shown). Nonetheless, this finding provides the first indication in a functional assay that EBO GP gene products themselves may cause cellular dysregulation, as is the case with HIV-1 gp120 and its pathogenic potential in the central nervous system (Toggas *et al.*, 1994). It has been speculated EBO GP products shed from virions or infected cells may be important for inducing disease manifestations (Volchkov *et al.*, 1998*b*; Yang *et al.*, 1998). However, our results identify the extracellular region of the membrane-bound GP2, rather than secreted products such as sGP or shed GP1, as necessary for induction of the cellular detachment manifest in our assay system. Furthermore, the responsible GP domain maps to a region that is absent from the sGP coding sequence. Since sGP selectively binds neutrophils rather than endothelial cells (Yang *et al.*, 1998), it thus would not be expected to have direct effects on endothelial cells. In contrast, the present findings are potentially important since dysfunction of blood vessel endothelial cells previously has been implicated in the widespread haemorrhage in visceral organs after EBO infections (Georges-Courbot *et al.*, 1997), and these cells could be key targets of GP1/GP2-induced cellular detachment. Future experiments aimed at identifying the *in vivo* pathogenic potential of MBG and EBO-Z GP should clarify their impact on disease progression as well as hasten the design of more effective treatments for lethal filovirus outbreaks in the future.

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Chapter 6

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

Introduction

As described in Chapter 4, the production of pseudotype viruses that carry the target cell specificities dictated by either MBG or EBO-Z GP was optimized to recapitulate the entry processes of filoviruses. In doing so, a number of mammalian cell lines were identified that were permissive or non-permissive for filovirus entry. With this information, this final project employed a genetic complementation strategy designed to identify host cell proteins that mediate entry by the MBG or EBO-Z viruses. By using a number of independent genetic and biochemical strategies, the folate receptor-α (FR-α) protein was identified as a cellular cofactor that mediates entry of both MBG and EBO-Z viruses. Such a discovery appears to represent a scientific milestone in advancing our understanding of the molecular life cycle of filoviruses. In addition, by providing an attractive drug target for blocking filovirus entry, it offers hope for successful treatment of these typically fatal infections.

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Folate Receptor-α is a Cofactor for Cellular Entry

by Marburg and Ebola Viruses

Stephen Y. Chan[1,2], Cyril J. Empig[1], Frank J. Welte[1], Roberto F. Speck[1‡],

Alan Schmaljohn[3], Jason F. Kreisberg[1], and Mark A. Goldsmith[1,2*]

[1]Gladstone Institute of Virology and Immunology, San Francisco, CA; [2]School of Medicine, University of California San Francisco, San Francisco, CA 94141-9100; [3]Virology Division, United States Army Medical Research Institute for Infectious Diseases, Fort Detrick, Frederick, MD 21702

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*Corresponding author:
Gladstone Institute of Virology and Immunology
P.O. Box 419100
San Francisco, CA 94141-9100
Telephone (415) 695-3775; Fax (415) 695-1364
Email mgoldsmith@gladstone.ucsf.edu

‡Current address:
University Hospital Zürich
Rämistrasse 100, U Pol 33
Zürich, Switzerland CH-8091

Summary

Human infections by Marburg (MBG) and Ebola (EBO) viruses result in lethal hemorrhagic fever. To select for cellular genes employed by MBG virus for entry, non-infectible Jurkat T-cells were transduced with a retroviral expression library derived from permissive HeLa cells and challenged with a selectable pseudotype virus packaged by MBG glycoproteins (GP). Cells were isolated that exhibited reconstitution of viral entry. A cDNA encoding the folate receptor-α (FR-α) protein was recovered from permissive clones. In a similar strategy employing a pseudotype virus packaged by EBO GP, a library insert encoding FR-α was also recovered from reconstituted cells. Expression of FR-α in Jurkat cells allowed for entry by either MBG or EBO pseudotypes. Infection by MBG or EBO was inhibited by FR-specific blocking reagents. FR-α also mediated cell-cell fusion of 293T cells triggered by MBG GP. Finally, FR-α bound cells expressing MBG or EBO GP. Thus, FR-α is a significant cofactor for cellular entry by both MBG and EBO. The identification of such a host factor presents hope that blocking its function can provide therapeutic benefit for fatal infections by these viruses.

Introduction

Marburg (MBG) and Ebola (EBO) viruses, comprising the *Filoviridae* family, cause fatal hemorrhagic fevers characterized by high viral load and widespread tissue infection and destruction (27). These viruses all carry single-stranded RNA genomes that are packaged by lipid membrane envelopes into filamentous virions. Despite primary sequence variation, all filoviruses have substantial similarity in genomic and virion structure (27). Furthermore, both MBG and EBO viruses infect an extensive and comparable range of other mammalian cell types and species (9, 43, 44).

Filovirus entry into target cells is mediated by the binding of transmembrane virus envelope glycoproteins (GP) to an unknown cell surface factor(s) (27). Both MBG GP (40) and EBO GP (39) 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 pre-translational processing of EBO GP but not MBG GP, an additional EBO GP product, sGP, is produced along with the longer membrane-bound form (31, 38). 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. (8, 45). Third, prevention of N-glycosylation of target cell proteins specifically inhibits entry of virions mediated by EBO GP but not MBG GP (9, 34).

It remains unknown whether MBG and EBO viruses use fundamentally similar or distinct processes to gain entry into target cells. In particular, little is known about the identity of cellular factors that mediate entry of filoviruses. Previously, it was proposed that the liver-specific asialoglycoprotein receptor (ASGP-R) may play a role in infection of liver cells by MBG virus (5). However, since both MBG and EBO viruses infect a comparable and extensive range of mammalian cell types in a number of different species (9, 43, 44) that do not express ASGP-R, other relevant cellular factors must exist that facilitate entry by these viruses. It is likely that these factors are widely expressed and highly conserved.

In previous studies using pseudotype viruses packaged by MBG or EBO GP, we and others demonstrated that T-cells are non-permissive for entry by filoviruses (9, 43, 44). We utilized this information to design a genetic complementation protocol to identify cellular factors responsible for mediating entry by MBG or EBO viruses. From functionally reconstituted cells permissive for MBG and the Zaire subtype of EBO (EBO-Z) infection, we identified the folate receptor-α (FR-α) as a common factor utilized by both MBG and EBO-Z viruses to gain entry into cells. Identification of a cell surface mediator of filovirus entry

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.

Experimental Procedures

Cell lines. HeLa, 293T, Jurkat, and Vero E6 cells were cultured as recommended by the American Type Culture Collection (ATCC). 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 (18). The PT67 packaging cell line (26) was cultured as described (Clontech). Human osteosarcoma (HOS) indicator cells carrying a human green fluorescent protein (GFP) reporter gene driven by an HIV-2 Tat-dependent LTR were provided by Dr. D. Littman, Skirball Institute (35).

Plasmids and cDNA library amplification. The HIV-1 provirus carrying a luciferase reporter gene driven by the 5' LTR (along with mutations in *env*, *nef*, and *vpr*), pNL-Luc-E$^-$R$^-$ (11), was a gift of Dr. N. Landau (Salk Institute) via the AIDS Research and Reference Reagent Program. The HIV-1 provirus carrying the blasticidin S deaminase gene driven by the 5' LTR along with null mutations in *env*, *nef*, *vpu*, *vif*, and *vpr*, pHIV-blasti, was provided by Dr. R. Sutton (Baylor University). Mammalian 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* (22) 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 (9). The full length cDNA and truncated cDNA encoding FR-α were recovered by PCR from the HeLa retroviral library as described below and subcloned into pCMV4neo. For reconstitution studies, pBabe-FR-α was constructed by subcloning the full-length FR-α gene into the MLV retroviral vector pBabeMN (21), 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 cDNA library derived from HeLa cells (pLIB-HeLa, $2x10^6$ independent clones) was amplified as described by the manufacturer (Clontech). The plasmid pLIB-GFP encoding GFP in the MLV backbone was used as a marker for quantitating efficiency of transduction.

Antibodies. For detection of wild-type MBG virus infection, guinea pig anti-MBG antisera were used (17). 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) (14), 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 co-transfected with a GP expression vector in 293T cells as previously described (9). 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 ($8.0x10^7$) were then divided into 10 separate batches and transduced with library-containing viral supernatants with polybrene (5 μg/ml) via spin infection ($2.5x10^3$ RPM, 32 °C, 2 h). 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 h 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 viruses and wildtype filoviruses. To determine permissivity for entry by pseudotype luciferase viruses, target cells were incubated with constant virus inocula for 48-72 h, and luciferase expression was quantitated as previously described (9).

To determine permissivity for entry by wildtype MBG virus, Vero E6, parental Jurkat-EctR and reconstituted Jurkat-EctR (F10 clone) cells were inoculated with wildtype MBG virus at increasing MOI of 0.1, 1, and 10. On days 1, 3 and 6, post-infection cells were washed, dried on spot-slides, fixed with acetone, irradiated, and then immunostained with guinea pig antisera raised against MBG virus as previously described (32). Positive cells were counted by fluorescence microscopy.

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. MLV retroviral expression vectors encoding either FR-α (pBabe-FR-α) or GFP (pMSCV2.2-IRES-GFP) were packaged into pseudovirions by PT67 cell transfection. Supernatant was then harvested to transduce parental Jurkat-EctR cells following the protocol for HeLa library delivery. Transduction efficiency of pMSCV2.2-IRES-GFP was quantitated by flow cytometry. To determine cell surface expression of FR-α, pBabe-FR-α-transduced cells were stained with a

monoclonal primary anti-FR-α antibody for flow cytometry analysis. An equivalent number of transduced cells (6 X 10^6 cells) stably expressing either FR-α on the cell surface or intracellular GFP were inoculated (48 h, 37 °C) with blasti pseudotype viruses (1 ml virus supernatant/1 X 10^6 cells) 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.

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 h at 37 °C, washed, and challenged with luciferase viruses for 4 h at 37 °C. Luciferase expression was assessed after 72 h. F10 cells were pre-incubated with anti-human FR-α (IgG1) or anti-HIV p24 Gag (IgG1) for 15 minutes at 4 °C. Cells were challenged with luciferase virus in the presence of antisera, followed by assessment of luciferase expression. Vero E6 cells were pre-incubated with medium containing polyclonal anti-FBP (with 0.1% sodium azide) or normal rabbit sera (with 0.1% sodium azide) for 15 minutes at 4 °C, followed by virus

challenge as above. HOS cells were pre-incubated at 4 °C for 30 minutes 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 h, culture medium was replaced, and luciferase expression was assessed after 48 h. Finally, pseudotype virus produced in the absence of serum or folic acid was pre-incubated with soluble FR-α (FBP, (33)) derived from bovine milk (Sigma) for 15 minutes at 4 °C. HOS cells were challenged with virus supernatants alone or with virus mixtures containing FBP for 4 h at 37 °C. Cells were then washed, medium was replaced, and luciferase expression was assessed after 72 h.

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 (9). After 24 h, cells were detached using Cell Dissociation Buffer (enzyme-free, PBS-based, Life Technologies). The appropriately transfected cells were mixed (1:1 ratio), cultured on poly-L-lysine-coated (0.01% solution, Sigma) 24-well plates as previously described (1), and incubated at 37 °C for 48 h. To trigger syncytia (≥ 3 nuclei), cells were incubated in either 75 mM MES (2-[N-morpholino]ethanesulfonic acid)/10 mM HEPES buffer (pH 5) or 10 mM HEPES buffer (pH 7) for 10 minutes at 37 °C as

previously described (36). The buffer was then replaced with medium. Syncytia were scored after 24 h.

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 h, bovine FBP (33 μg/ml) was incubated in the presence of non-neutralizing goat anti-bovine FBP (250 μg/ml) for 15 minutes 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 minutes at 4 °C. Cells were washed (3 X ice cold PBS), fixed in 2% paraformaldehyde, washed, and incubated with fluorescein-conjugated secondary antibody (4 μg/ml) for 45 minutes at 25 °C in the dark. Slides were washed, mounted, and analyzed by fluorescence microscopy.

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 (9). 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 (Fig. 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 (Fig. 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, Jurkat-EctR, which stably express the ecotropic murine leukemia virus (MLV) receptor yet are non-permissive 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 re-challenged 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 (Fig. 2A). Importantly, these recovered cells remained non-permissive 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 non-specifically 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 (Fig. 2B), on the F10 subclone derived from reconstituted Jurkat-EctR culture 2-23 (Fig. 2C), and on other permissive cell types such as Vero E6 (Fig. 2D), typically used to passage filoviruses in cell culture (27). 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 (Fig. 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 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 (Fig. 3B-C). 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 (3). 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 pre-treated 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 (Fig. 4A). A specific, dose-dependent effect on MBG entry was also seen upon treatment of F10 cells (Fig. 4B) at non-cytotoxic 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 (14). MBG infection was specifically and potently reduced by anti-FR-α (Fig. 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 (9) 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 (Fig. 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 pre-incubated 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 (Fig. 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 over-expressing MBG GP were co-cultivated with cells over-expressing either CD4 or FR-α. Because fusion mediated by MBG GP may be pH-dependent (9), syncytia formation (≥ 3 nuclei) was quantitated after pulsing cells at pH 5 or pH 7 and allowing for recovery. As described previously (36), expression of the influenza hemaglutinin protein (HA) allowed for robust formation of syncytia (> 100 per well) when exposed to pH 5 but not pH 7 (Fig. 5A). No significant fusion was detected at either pH in cultures expressing MBG GP with CD4 (Fig. 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 co-cultures expressing MBG GP with FR-α (Fig. 5C). While less robust than that seen with HA, this significant 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 (9, 44). 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 (Fig. 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 (Fig. 6B). Thus, in two independent 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.

Subsequently, FR-α-specific inhibition strategies were pursued, as in the MBG studies. HeLa cell pre-treatment 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 (Fig. 6C). Furthermore, when FR-α-positive, monkey Vero E6 cells, which are commonly used to propagate filoviruses in cell culture (27), were treated with a polyclonal antisera raised against bovine FBP, EBO-Z entry specifically decreased by nearly 60% (Fig. 6D). 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 (Fig. 6E). Therefore, mirroring the MBG inhibition

profiles, EBO-Z pseudotype entry was specifically abrogated by agents disrupting the interaction between virion GP and FR-α.

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 (Fig. 6F). 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 complement the biochemical analyses and demonstrate that FR-α mediates cellular entry by EBO-Z virus.

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 (Fig. 7). FR-α-negative CHO-K1 cells (41), expressing either Ampho, MBG, or EBO-Z GP, were incubated with bovine FBP and an anti-bovine FBP antibody that was non-neutralizing 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 (9, 34, 43, 44). Furthermore, a comparison of entry requirements of pseudotype (Fig. 2A) and wild-type (Fig. 3B-C) 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 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 kD GPI-linked cell surface protein that binds extracellular folic acid for cellular uptake (3). It is one of four receptor isoforms (α, β, and γ, and γ') that carry 68–79% identity in amino acid sequence (25) and differ in their size, tissue distribution (29), or ligand binding sites (25). Only the FR-α isoform has been detected in normal, non-hematopoietic tissue other than placenta (30). 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 (2)). However, the exact mechanism of endocytosis (24) and type of vesicle (28) 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 (20, 23).

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 (9, 34, 43) and of membrane fusion mediated by MBG GP (Fig. 5C) correlates with the pathway of endocytosis and acidification exploited by FR-α for folic acid uptake. In addition, pre-treatment 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). To our knowledge, filoviruses constitute the only identified family of enveloped viruses that rely upon a GPI-linked receptor for cellular entry. 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 (3) corresponds with the wide tropism of filoviruses in cell culture (9) and animal models (7, 10, 16). Furthermore, FR-α is significantly expressed in a variety of epithelial and parenchymal cells in the lung, gonads, gastrointestinal (GI) tract, genitourinary (GU) tract (3), pancreas, thyroid (42), and arteries (41), but not in lymphocytes (14). Concordantly, in humans and in animal models, filoviruses have been documented to replicate in parenchymal cells of the lung (19), gonads, GI tract, thyroid (10), and pancreas (16), as well as in epithelial cells of the GU tract (4, 10), but not in lymphocytes (15). 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 (46). Importantly, significant expression of FR-α has been documented in both

macrophages (14) and fibroblasts (3). 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 (Figs. 4D, 6D). Although high levels of FR-α have not been reported in hepatocytes and endothelial cells, FR protein was detected in pig hepatocytes (37) as well as in rat liver tissue (12), while FR-α mRNA was detected by RT-PCR in human liver (29). 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* (3). 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 (9). 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 (6)), 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 (Fig. 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 (9, 43, 44) and in animal models (15). 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 (13) 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 non-manipulated, 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.

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Figure Legends

Fig. 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 (± S.E.M.). (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).

Fig. 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 (± S.E.M.). (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).

Fig. 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 2 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.

Fig. 4. Inhibition of MBG pseudotype entry with FR-specific blocking reagents. Pre-treatment 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 (± S.E.M.).

Fig. 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 co-cultures expressing MBG GP with CD4 after pH 5 exposure. (C) Syncytium production in co-cultures expressing MBG GP with FR-α after pH 5 exposure (1 to 6 syncytia/well, black arrows).

Fig. 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) EBO-Z pseudotype entry into F10 cells is abrogated in the presence of anti-human FR-α IgG1. (D) EBO-Z pseudotype entry into Vero E6 cells is inhibited by polyclonal anti-bovine FBP. (E) Dose-dependent decrease of EBO-Z infection of HOS cells in the presence of soluble bovine FBP. (F) 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 2 separate reconstitution

procedures. Displayed values in (A)-(E) are the means derived from three separate infections (± S.E.M.).

Fig. 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).

Figure 1

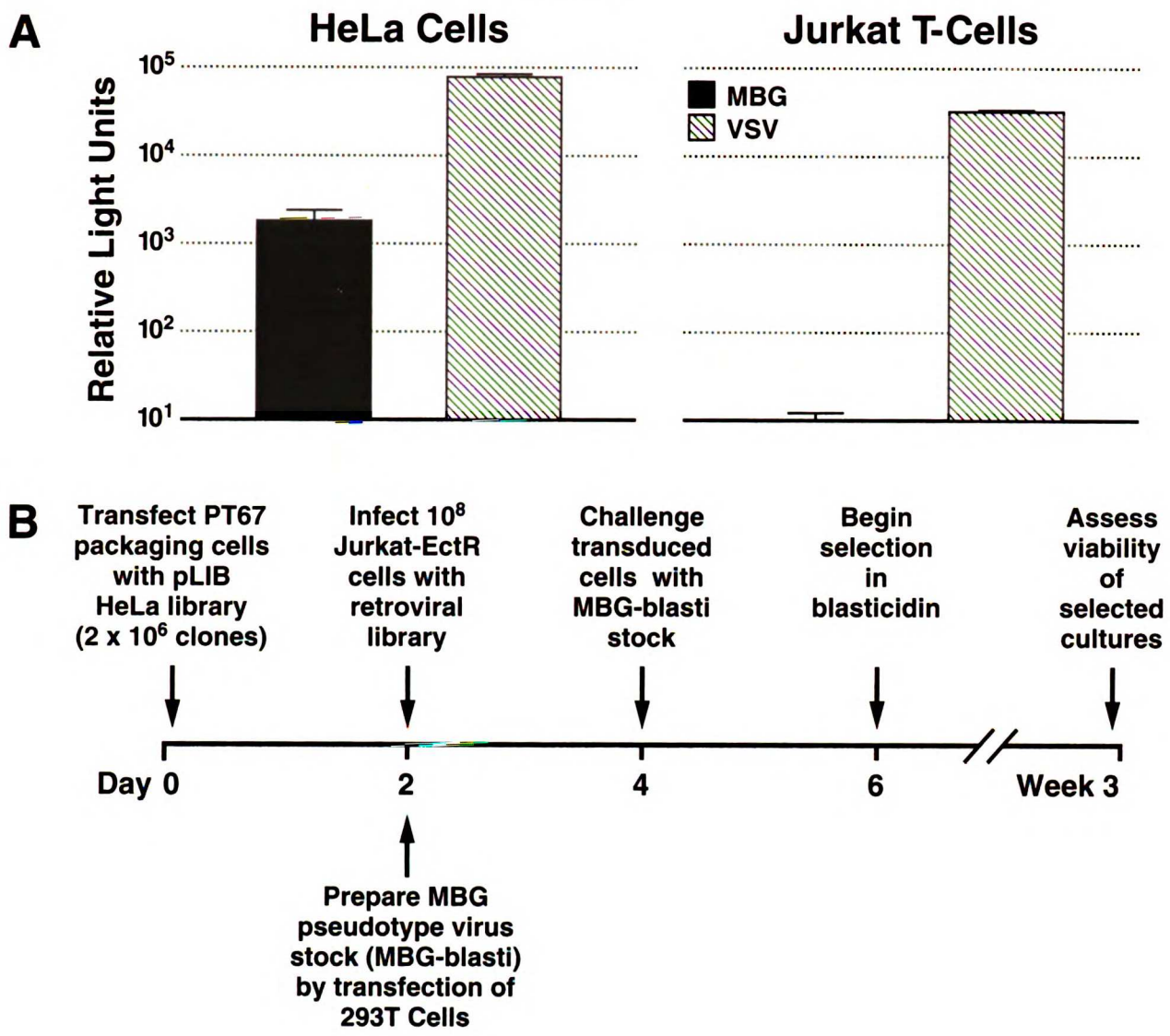

A
HeLa Cells
Jurkat T-Cells
Relative Light Units
10^5
10^4
10^3
10^2
10^1
MBG
VSV
B
Transfect PT67 packaging cells with pLIB HeLa library (2 x 10^6 clones)
Infect 10^8 Jurkat-EctR cells with retroviral library
Challenge transduced cells with MBG-blasti stock
Begin selection in blasticidin
Assess viability of selected cultures
Day 0
2
4
6
Week 3
Prepare MBG pseudotype virus stock (MBG-blasti) by transfection of 293T Cells

Figure 2

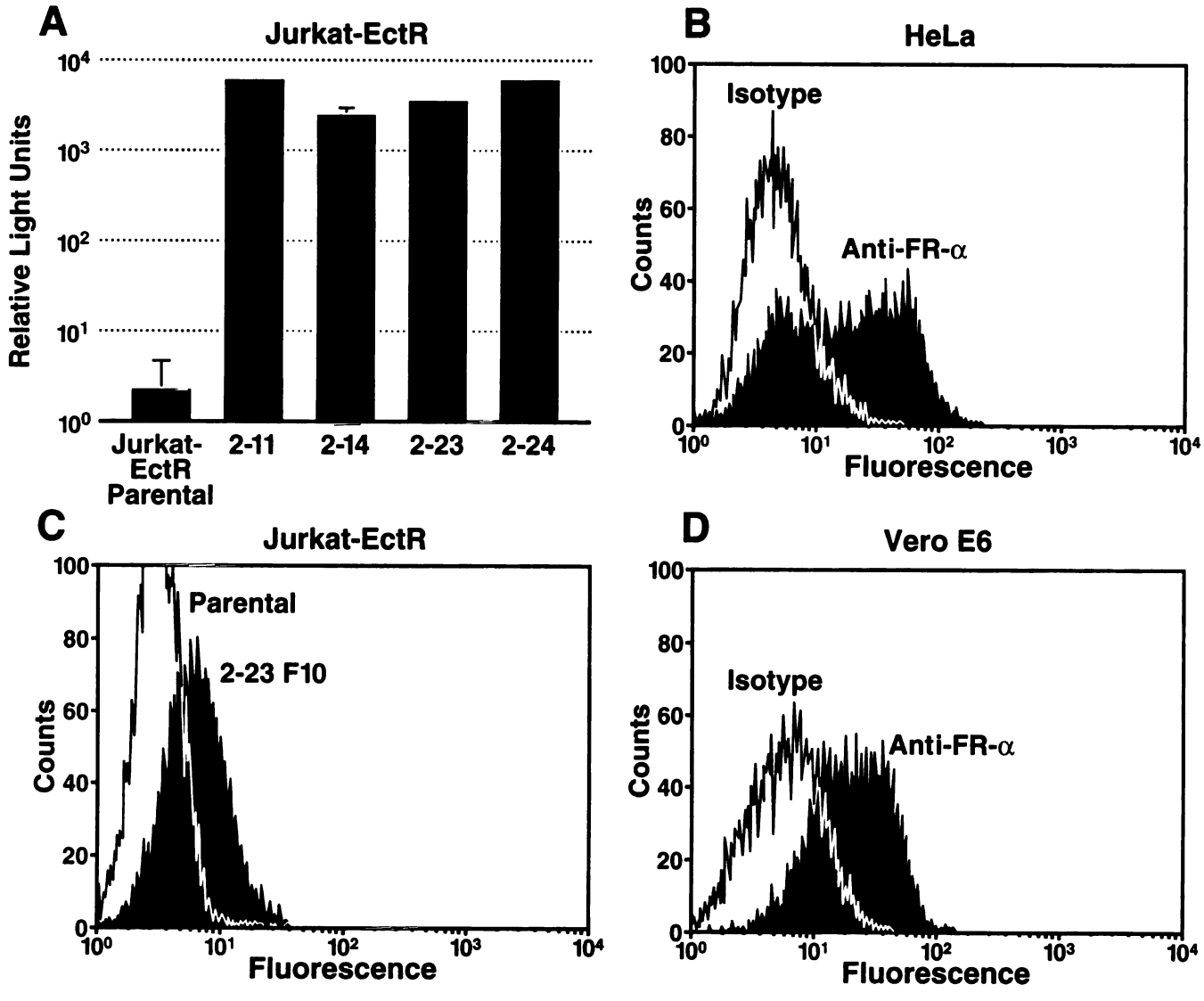
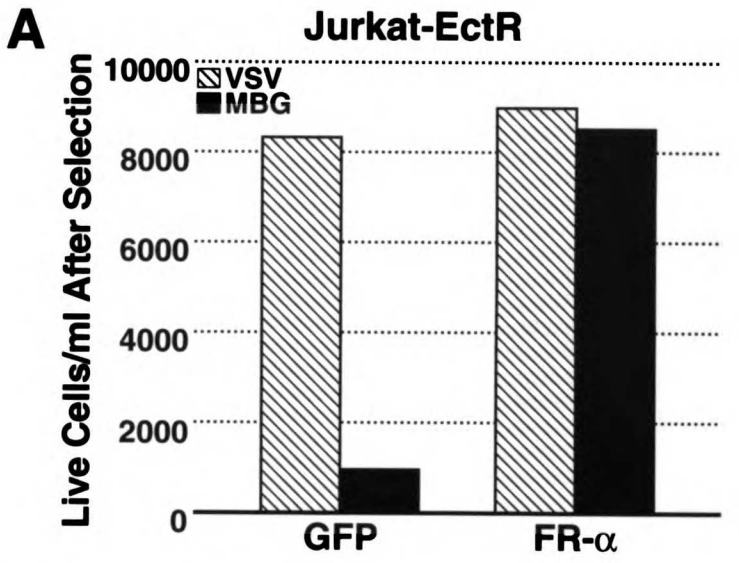
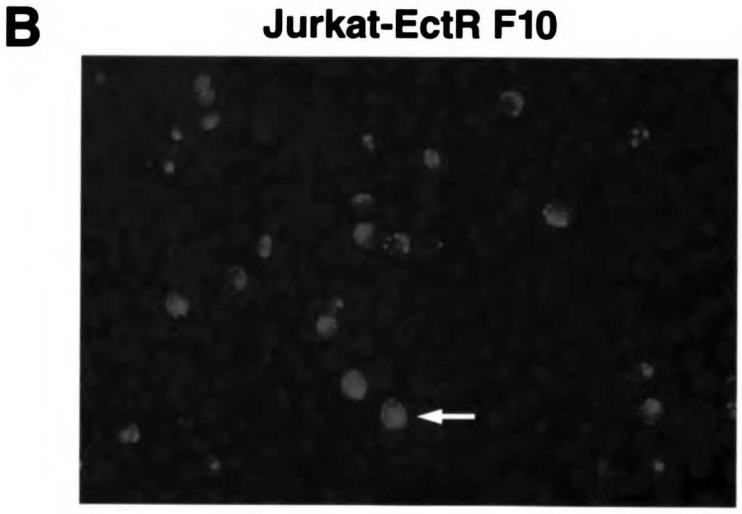

A
Jurkat-EctR
Relative Light Units
10^4
10^3
10^2
10^1
10^0
Jurkat-EctR Parental
2-11
2-14
2-23
2-24
B
HeLa
Isotype
Anti-FR-α
Counts
Fluorescence
C
Jurkat-EctR
Parental
2-23 F10
Counts
Fluorescence
D
Vero E6
Isotype
Anti-FR-α
Counts
Fluorescence

Figure 3





C

Cell Line	Positive Cells by IFA (Approximate % of Total Cells) 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 4

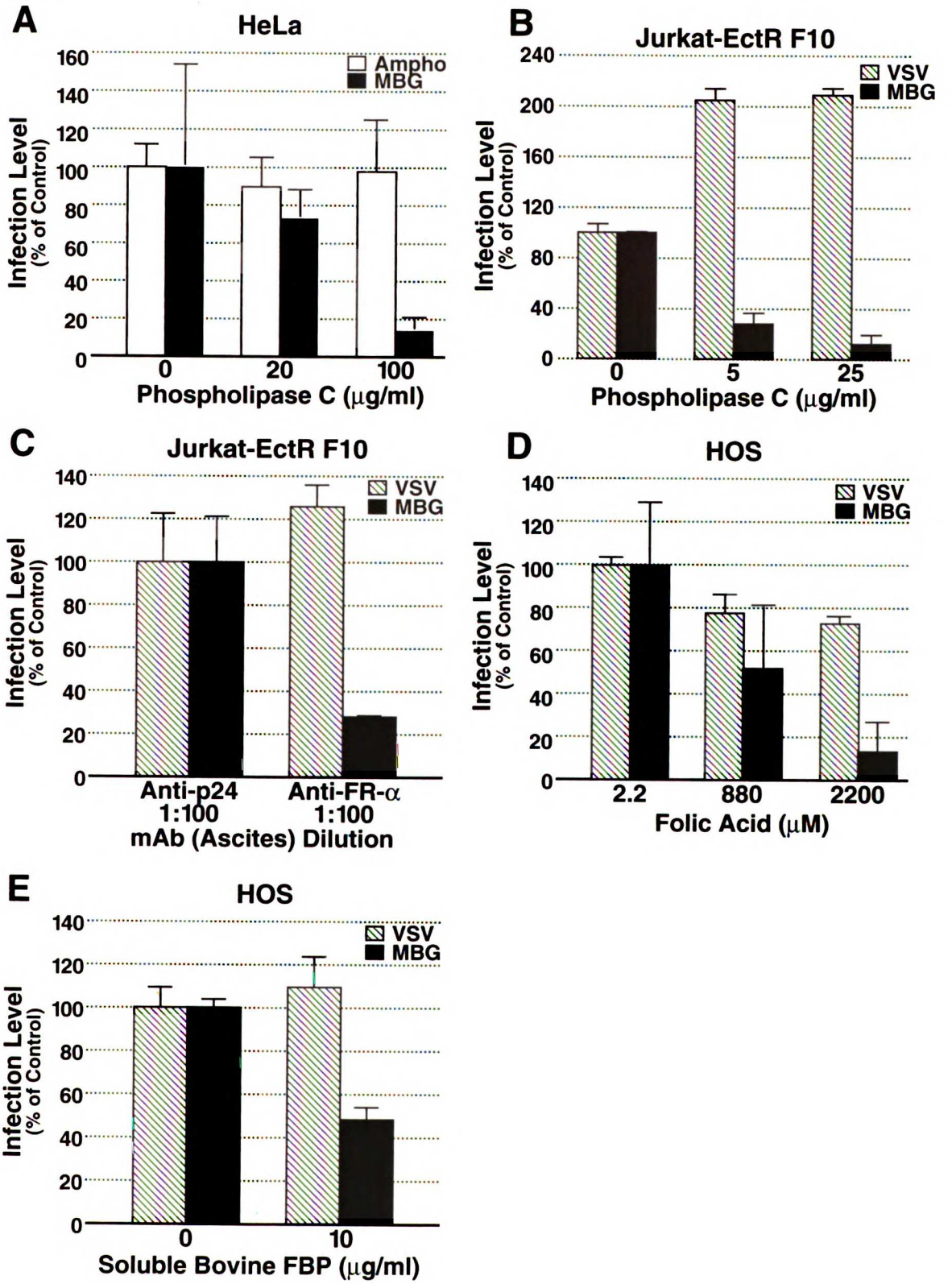

A
HeLa
Ampho
MBG
Infection Level (% of Control)
160
140
120
100
80
60
40
20
0
0
20
100
Phospholipase C (μg/ml)
B
Jurkat-EctR F10
VSV
MBG
Infection Level (% of Control)
240
200
160
120
80
40
0
0
5
25
Phospholipase C (μg/ml)
C
Jurkat-EctR F10
VSV
MBG
Infection Level (% of Control)
140
120
100
80
60
40
20
0
Anti-p24 1:100
Anti-FR-α 1:100
mAb (Ascites) Dilution
D
HOS
VSV
MBG
Infection Level (% of Control)
140
120
100
80
60
40
20
0
2.2
880
2200
Folic Acid (μM)
E
HOS
VSV
MBG
Infection Level (% of Control)
140
120
100
80
60
40
20
0
0
10
Soluble Bovine FBP (μg/ml)

Figure 5

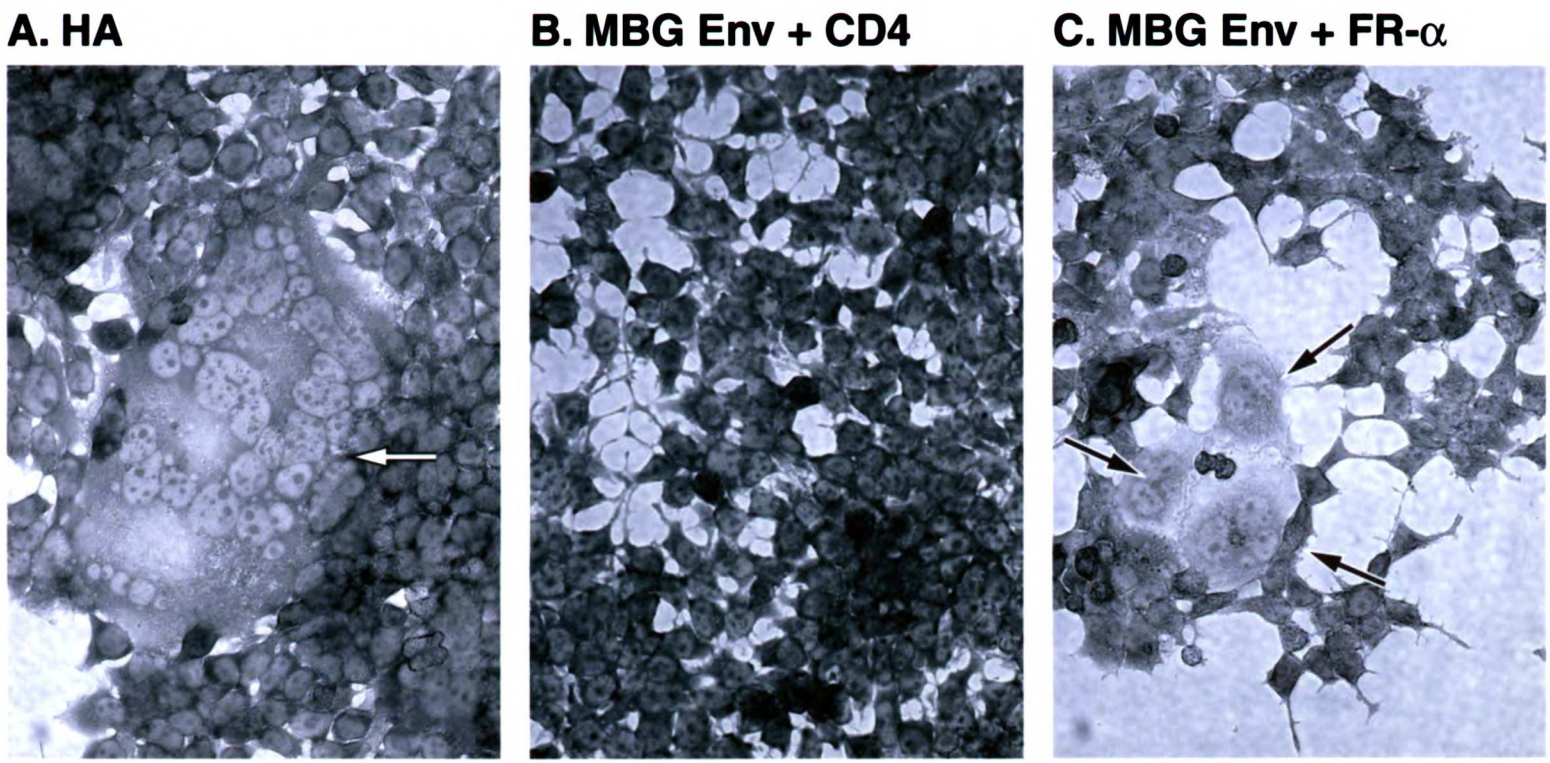


Figure 6

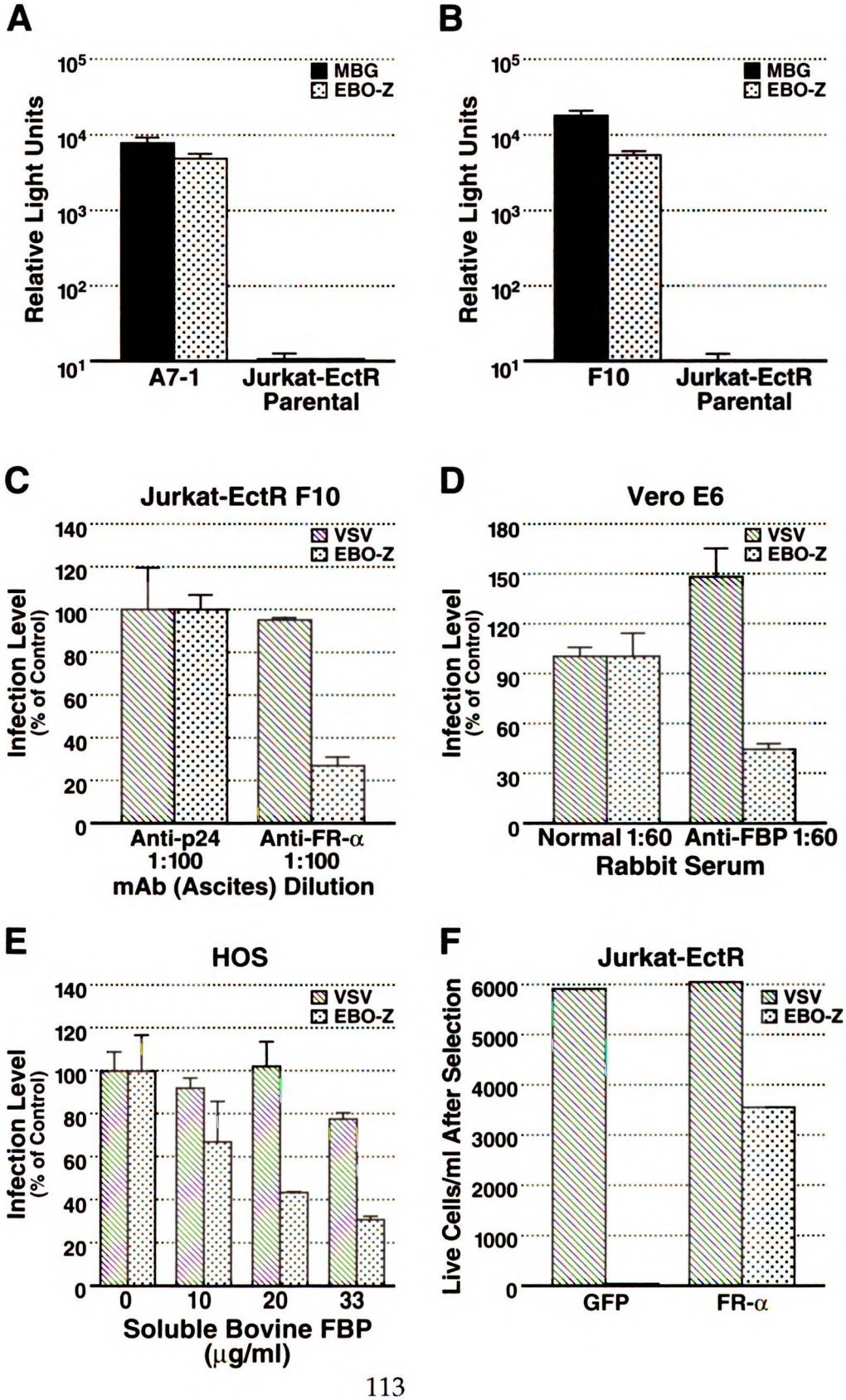
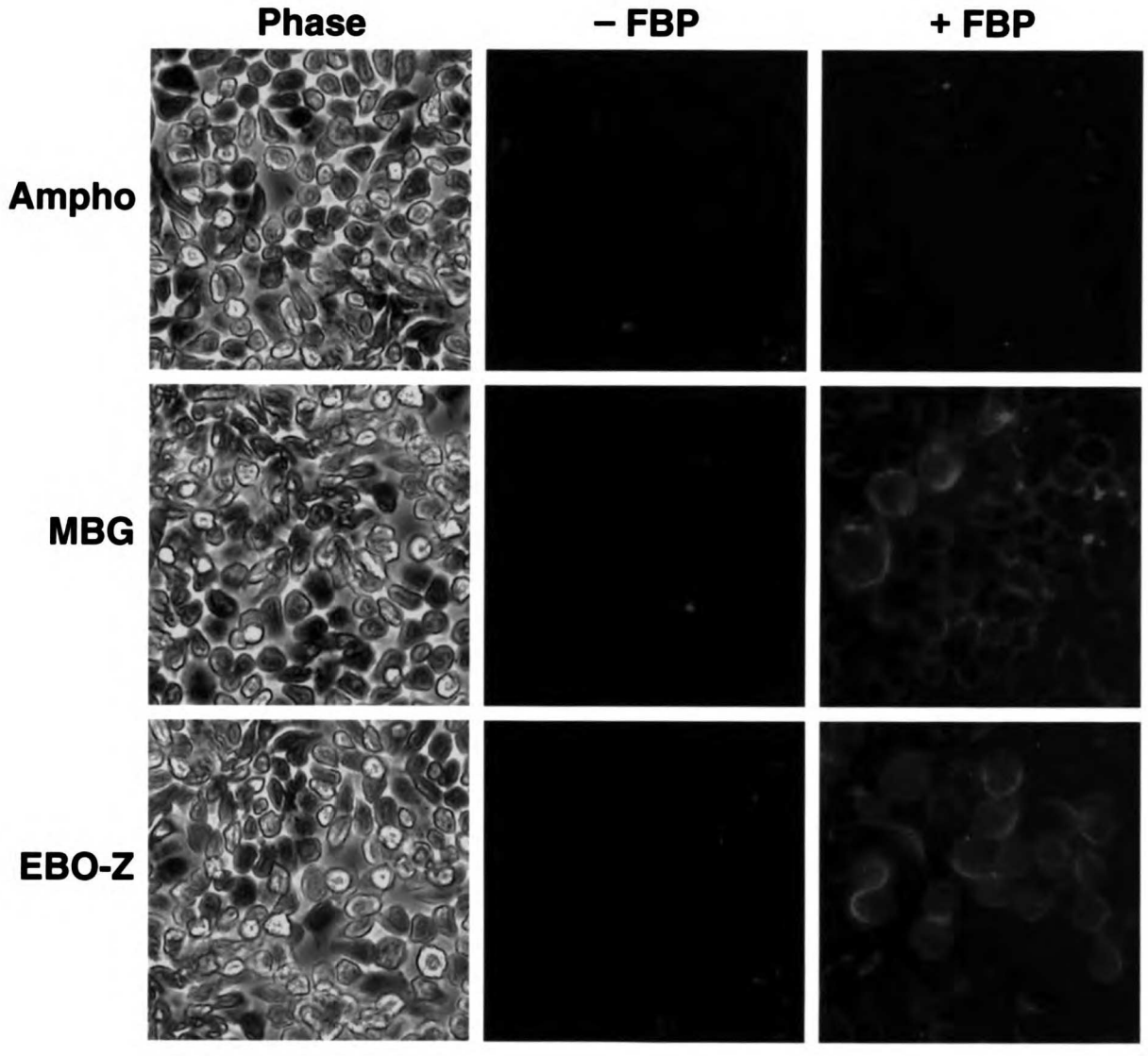

A
MBG
EBO-Z
Relative Light Units
10^5
10^4
10^3
10^2
10^1
A7-1
Jurkat-EctR Parental
B
MBG
EBO-Z
Relative Light Units
F10
Jurkat-EctR Parental
C
Jurkat-EctR F10
VSV
EBO-Z
Infection Level (% of Control)
Anti-p24 1:100
Anti-FR-α 1:100
mAb (Ascites) Dilution
D
Vero E6
VSV
EBO-Z
Infection Level (% of Control)
Normal 1:60
Anti-FBP 1:60
Rabbit Serum
E
HOS
VSV
EBO-Z
Infection Level (% of Control)
0
10
20
33
Soluble Bovine FBP (μg/ml)
F
Jurkat-EctR
VSV
EBO-Z
Live Cells/ml After Selection
GFP
FR-α

Figure 7



Chapter 7

Conclusion

The molecular mechanisms by which enveloped viruses bind to and enter target cells are essential both for initiation of the viral replication cycle and for the development of a variety of pathologic conditions. In this thesis, I identified and characterized host cell factors as well as associated cellular pathways important for entry and pathogenesis of the viruses HIV-1, MBG, and EBO. As a result, a clearer understanding of these processes critical for pathogenesis should translate into novel therapeutic avenues designed to inhibit specific molecular interactions and hopefully ameliorate disease.

I. HIV-1 coreceptor utilization in the infection of brain tissue: Questions of pathogenic potential

In the case of HIV-1, a detailed description of the CD4/coreceptor-dependent entry process and its correlation to cellular depletion was previously described for lymphoid cells (as reviewed in (4)). However, the entry process and its importance for dementia development had been poorly characterized for the brain and the central nervous system (CNS), long presumed to be an isolated viral reservoir for HIV-1 replication and pathogenesis (as reviewed in (23)). Therefore, we explored the hypothesis that a similar pattern of evolution of CD4/coreceptor dependence with subsequent expansion of the range of susceptible target cell types is an important contributor to pathogenic changes in

the brain (Chapter 2). While mutations in HIV-1 gp120 cause changes in coreceptor use by blood-borne strains and correlate with progressively severe immune deficiencies, the same pathogenic mechanism was not implicated in neuronal dysfunction observed in HIV dementia (HIVD). In contrast to serial blood-borne isolates that alter their preference for CCR5 to alternate coreceptors over time, all tested brain tissue-derived strains uniformly used CCR5 as a coreceptor along with CD4 to gain entry into brain cells regardless of clinical diagnosis.

As a result, a model of HIV infection in the brain can be generated in which CCR5-dependent strains invade the central nervous system from the bloodstream initially after an adult individual is infected. Subsequently, they infect mostly, if not only, tissue macrophages or microglia, since these cells are the only cells in the brain that have been found to express both CD4 and CCR5 in cell culture and *in vivo* (as reviewed in (17)). Years later, these CCR5-dependent strains in the blood evolve into multiple quasispecies that can utilize alternate coreceptors and allow for the progressive decline of $CD4^+$ T-lymphocytes. However, our results indicate that uncharacterized selective pressures apparently do not allow these quasispecies to survive in the central nervous system and infect brain cells. The origin of this absence of coreceptor evolution in the CNS may simply be that no cell type in the brain expresses sufficient levels of both CD4 and a coreceptor besides CCR5 to sustain the viral life cycle of such

quasispecies that develop after initial entry into the CNS. Or, perhaps in combination with this scenario, blood-borne quasispecies of alternate coreceptor specificity may be deficient in the ability to cross the blood-brain barrier and further invade the CNS. Regardless of the molecular explanation, these results strengthen the hypothesis that the central nervous system is a viral reservoir separate from the circulating blood. Therefore, it seems to be subject to different selective pressures and pathogenic mechanisms other than simple viral coreceptor "switching" that is seen in the progressive infection of lymphoid cells.

In the absence of changes in coreceptor *specificity* in brain-derived viruses, we next explored the hypothesis that rather an alternate *mechanism* of coreceptor use may be relevant in the CNS and HIVD progression (Chapter 3). Specifically, while the process by which CD4/CCR5-positive macrophages and microglia are infected had been identified, the method of viral entry into CD4-negative astrocytes was unknown. Given the importance of astrocytes in the production of secreted neurotoxic factors in a number of neurodegenerative diseases (24) as well as in HIV-1 infection (30), it is plausible that infection of this cell type plays a significant role in the progression of HIVD. We characterized a novel mechanism by which CD4-negative, CXCR4-positive primary human fetal astrocytes may be infected in cell culture by a CXCR4-dependent strain NL4-3 only in the presence of CD4-positive cells. This infection was abrogated either by treatment with AZT or with the CXCR4-specific peptide inhibitor T22. Therefore,

this entry by HIV-1 into primary fetal astrocytes occurs through a coreceptor-dependent pathway when CD4 is provided in *trans* by donor cells. Such a finding provides a plausible molecular mechanism to allow for HIV-1 entry into CD4-negative astrocytes in the typical course of infection *in vivo.* However, it seems incongruous with the previously described presence of only CCR5-dependent viruses in brain tissue throughout the disease process. This discrepancy may lie in the fact that primary human *adult* astrocytes express CCR5 as shown previously by *in situ* immunostaining of brain tissue (35), but primary human *fetal* astrocytes express only CXCR4 (39) even in the presence of inducing cytokine stimulation (S.Y.C., data not shown). Therefore, future verification of CCR5-dependent viral entry into CCR5-positive astrocytes will be important to strengthen the significance of this mechanism for HIV-1 infection of CD4-negative astrocytes derived from adult patients.

On the other hand, the process of CXCR4-dependent entry into fetal astrocytes may be directly relevant for infections of infants via vertically transmitted HIV-1. HIVD can develop in both adults and infants. However, in infants, neurodegeneration typically occurs much more rapidly and with more severe deterioration due to unknown pathogenic mediators (18). Because a higher level of infection of astrocytes has routinely been observed in children with HIV encephalopathy, it has been proposed as an important contributor to accelerated disease progression (as reviewed in (5)). Correspondingly, in contrast

to my study of the adult CNS, evidence exists of a switch in coreceptor specificity from CCR5 to CXCR4 of viral gp120 in the pediatric CNS. In contrast to relatively homogenous gp120 V3 sequences derived from adult brain tissue predicted as CCR5-dependent from sequence analysis (9) and later confirmed by functional criteria (8), reported gp120 V3 sequences derived from infant brain tissue (12) are more varied and carry significant positive charge indicative of CXCR4 dependence (22). Successful confirmation of this hypothesis through functional assays would suggest that the specific selective pressures in the CNS of the adult are different or entirely absent in the infant. Moreover, the presence of CXCR4-dependent strains in the CNS not only would correlate with the observation that CXCR4-positive cells such as astrocytes are infected at significant levels in infant neural tissue but also would strengthen the likelihood that a *trans*-receptor mechanism actively mediates viral entry into these CD4-negative cell types. Therefore, while the adult CNS may not allow for alternate coreceptor use to develop, viral usage of CXCR4 may play more of a pathogenic role in pediatric HIVD by exploiting CD4 expressed on neighboring cells in *trans*. This concept should be further explored in future studies.

Many molecular aspects underlying the development of HIVD still remain mysterious. With our results, we can now conclude that alterations in coreceptor specificity and changes in target cell range are likely not predominant factors driving HIV pathogenesis in the adult CNS viral reservoir. However, evolution

of coreceptor specificity may still accelerate neurodegeneration in specialized contexts such as neonatal or pediatric infections. On the other hand, an alternate mechanism of *trans*-CD4/coreceptor usage may be active in allowing for entry into CD4-negative astrocytes. The significance of this process in HIVD progression should be explored further. Nonetheless, it is likely that modulation of CD4/coreceptor usage by HIV-1 is merely one of several host and viral determinants leading to neurodegeneration. While some viral disease conditions can be directly traced to changes in tropism, it is becoming clear that HIV-1 neurovirulence will never be mapped entirely to gp120 and changes in its receptor and coreceptor preferences and interactions. Therefore, to complete our understanding of the molecular mechanism of HIVD development, future studies should also focus on defining pathogenic processes that operate independently of coreceptor and entry requirements.

II. Identification of a molecular pathway for entry and pathogenesis by filoviruses.

Unlike the situation for HIV-1, in the cases of MBG and EBO viruses, no cellular pathway or host factor had previously been identified definitively that associates with the envelope GP to facilitate viral entry. In addition, while filoviruses share similarities in genomic structure, virion composition, and

clinical disease, it was unclear if MBG and EBO viruses utilize identical or distinct cellular pathways for this process. Therefore, in the second part of this thesis work, the processes of filovirus entry into target cells were characterized and compared at a molecular level for ascertainment of their role in pathogenesis and applicability for therapeutic intervention.

In the initial studies, we constructed and characterized a pseudotype system for studying filovirus entry by packaging an HIV-1 genome with envelopes carrying either the MBG or EBO-Z GP complexes (Chapter 4). These pseudotype viruses mediated infection of a comparable wide range of mammalian cell types, and both were inhibited by treatment of target cells with ammonium chloride, an agent that prevents vesicular acidification, thus suggesting the importance of endocytosis for the entry of both viruses. Therefore, the envelope GP of both MBG and EBO viruses share certain functional similarities in exploiting the same general pathway for entry. However, only EBO-Z pseudotype virus infection, but not MBG, exhibited sensitivity to treatment of target cells with either N-glycosylation inhibitors tunicamycin and endoglycosidase H or protease (Pronase). While these results indicate that the MBG and EBO GP interact with target cells by distinct processes, it is unclear which cellular components play a role in these differences. As a result, the divergence of their entry processes may involve cofactors that are active at any point between the initial cell surface binding event of the virion to the final

fusion step after endocytosis. Future studies that elaborate on the entire molecular pathway for filovirus entry will be important to clarify these functional distinctions.

In addition to exploring differences in their entry pathways, I also discovered that EBO-Z GP, but not MBG GP, induces cellular detachment of the normally adherent 293T cell line in the absence of cell death or viral infection (Chapter 5). This phenotype was mapped to the extracellular membrane-anchored domain of EBO-Z GP2. Furthermore, while the host cell factors that mediate this cellular dysregulation are unknown, we discovered that a Ser/Thr phosphorylation signaling pathway is essential for its execution. This fortuitous finding demonstrates further functional distinctions between the envelope GP of MBG and EBO viruses beyond only facilitating virus entry. Furthermore, this mechanism may play a role in the pathogenic dysfunction of blood vessel endothelial cells which are key targets of infection and have been previously implicated in the widespread hemorrhage in visceral organs after Ebola infections (19).

However, the pathogenic implications of this phenotype must be interpreted cautiously. At the same time as the report of these findings, another study was published describing a similar phenomenon (44). In addition to confirming the bulk of our observations, GP-induced cellular detachment and dysfunction were demonstrated in endothelial cells grown in cell culture as well

as those lining human saphenous vein explants. This group therefore identified EBO GP as the "main viral determinant of vascular cell cytotoxicity and injury" in EBO virus infections. The breadth of their conclusions has been questioned. First, both our results and their findings have demonstrated this phenotype only after overexpression of EBO GP, which may not accurately reflect *in vivo* expression levels after infection. Second, infection of endothelial cells, and subsequently expression of EBO GP, is only detectable days after the first clinical signs of hemorrhage appear (V. Volchkov, personal communication), suggesting GP-induced change is likely not the only factor in endothelial cell death after EBO infection. Third, our findings suggest that hemorrhage during MBG virus infections must result from other pathogenic processes altogether, such as aberrant cytokine release (13), since overexpression of MBG GP does not induce any detectable cellular dysfunction. Therefore, the contribution of GP-induced cellular dysregulation to filovirus disease progression is presently unclear. Future experiments designed both to identify the disrupted cellular pathway affected by GP expression and to study this novel activity in the context of mammalian infections are necessary to accurately ascribe a primary pathogenic role to EBO GP cytotoxicity.

Finally, to gain insight into these similarities and distinctions of filovirus GP function at a molecular level, I embarked on my major project to identify the cellular factor(s) that mediate entry by MBG and EBO viruses (Chapter 6). To

recover genes that complement cellular deficiencies in entry by filovirus, a retroviral expression library derived from infectable HeLa cells was delivered to normally non-infectable Jurkat T-cells. Transduced cells were then challenged with a selectable MBG pseudotype virus to recover cells that are permissive for MBG virus entry and separately challenged with an EBO-Z pseudotype to recover cells that are permissive for EBO-Z entry. In both populations, surviving cells were permissive for entry by both MBG and EBO-Z. Correspondingly, cDNA inserts were recovered from both populations that encoded for the folate receptor-α protein (FR-α). To establish directly a role for FR-α in MBG and EBO-Z virus entry, several experimental avenues were implemented that have classically been used to characterize host factors important for entry of other enveloped viruses (3, 11, 14, 28). They included reconstitution of permissivity for filovirus entry through expression of FR-α, inhibition of infection by blocking association of filovirus GP with FR-α at the cell surface, generation of MBG GP-triggered membrane fusion by FR-α, and demonstration of direct binding of FR-α with cells expressing MBG or EBO-Z GP. Taken together, the results of these independent genetic and biochemical studies converge upon the same conclusion that FR-α is a cofactor that mediates infection of either MBG or EBO-Z virus.

In combination with the characterization of the general pathway of infection derived from our initial studies, the identification of the importance of FR-α in filovirus entry permits us to construct a molecular model of infection

based on the previously studied pathway of folic acid internalization by FR-α. The simplest and most likely model predicts that the filovirus GP present on the extracellular virion envelope can attach to FR-α at the target cell surface for initiation of virus entry. An alternate explanation proposes that FR-α instead binds to and regulates an unidentified cell surface factor that then can directly interact with filovirus GP complexes. Therefore, while FR-α would act as a mediator of filovirus entry, it would not function as a filovirus receptor. However, since a soluble form of the FR-α (FBP) can both act as a competitive inhibitor of MBG and EBO infection as well as directly bind cells expressing MBG or EBO-Z GP, it is highly likely that FR-α can interact with either MBG or EBO-Z GP to exert its function as a cellular receptor allowing for filovirus entry. While the sites on FR-α that are recognized by MBG or EBO GP may differ, the function appears to be the same: to initiate contact with extracellular filoviruses and mediate internalization.

Following this event, the entry processes utilized by MBG and EBO viruses could diverge at a number of different points. As a result of GP binding, FR-α may multimerize as it does upon binding folic acid (26), triggering an uncharacterized endocytic process for uptake of attached virions. Virus endocytosis has been described previously where virion structure such as that of influenza (150-300 nm) or VSV (70-85 × 130-380 nm) can easily pack into intracellular vesicles (as reviewed in (15)). However, extracellular filoviruses are

characterized by long, coiled, filamentous morphologies (80 × 790-14,000 nm) that may not easily assume compact structures necessary for endocytosis (15). Therefore, an uncharacterized process modulating the flexibility of the virion conformation may be necessary to allow for such endocytosis to occur efficiently. In addition, the mechanism of endocytosis and the type of vesicle that internalizes filoviruses are unknown. Nonetheless, at a further downstream point in this pathway, vesicular acidification occurs which is necessary for infection (7). Presumably, the decrease in pH allows for GP detachment from FR-α and a conformational shift in GP2 to a fusogenic-competent state (25, 43). Cellular entry is then completed via fusion between the viral envelope and the endocytic membrane, releasing filovirus RNA as well as perhaps additional factors into the cytoplasm.

Identification of entry cofactors is a vital first step for comprehending the biology of virus infection and disease. As a result, these findings further advance our understanding of the life cycle of filoviruses in general. Importantly, it provides a working model for future studies that should be applicable to the medical as well as the purely scientific domain. First, it will be important to characterize the unknown downstream events in this cellular process. Second, it is imperative to determine the *in vivo* significance of FR-α in susceptible cell types critical for disease progression during typical filovirus infections. Upon doing so, we will be better equipped to develop successful therapeutic

treatments that inhibit filovirus entry not only at the cell surface but also at multiple points in the endocytic pathway.

III. Future Directions

This body of work has more clearly defined the molecular mechanisms and pathogenic implications of initial entry into target cells by various enveloped viruses. As a result, a number of future lines of investigation should be pursued to continue building more refined models for virus entry by HIV-1 and by filoviruses. Consequently, these may lead to the design of specific therapeutic regimens that inhibit these pathogenic processes and ultimately alleviate human diseases.

A. The Pathogenic Impact of Coreceptor Specificity by HIV-1 gp120

We concluded that infection of brain tissue by only CCR5-dependent viruses is sufficient for development of HIVD in adults and therefore a "switch" in coreceptor usage is not necessary for pathologic changes unlike in the lymphoid compartment. However, it is still unclear if CXCR4-dependent strains can accelerate or augment the severity of brain pathogenesis if allowed to propagate in this isolated reservoir in specific situations. While it sampled enough strains from demented and non-demented individuals to detect a significant CCR5-dependent pattern, our study was unable to definitively

conclude that CXCR4-using strains are entirely absent from the CNS, specifically in the presence of a documented coreceptor "switch" in the lymphoid compartment of the same individual. In addition, analysis of brain specimens from infant infections by HIV-1 suggests that CXCR4 strains may be present in infant tissue, and therefore, this presence correlates with more severe neurologic dysfunction.

To test the extent of the selection for only CCR5-dependent strains in the brain, a series of samples of both blood and cerebrospinal fluid could be obtained from a cohort of HIV-infected individuals over time. Subsequently, viral isolates could be isolated, and their envelope genes sequenced. While more laborious, sequencing the entire contiguous gp120 V1-V5 regions would allow for a more definitive characterization of coreceptor usage, as it is known that some strains of HIV-1 in certain contexts depend upon more than just the V3 sequences for coreceptor specificity (21, 34). These full-length sequences could then be inserted into an isogenic provirus, and their coreceptor preferences determined as before. As a number of individuals in the cohort become diagnosed with HIVD over time, the "evolution" of coreceptor usage can be correlated with disease conditions and be directly compared in the lymphoid and CNS compartments. However, certain drawbacks exist for this strategy. First, sampling viral isolates from spinal fluid rather than directly from brain tissue can result in the isolation of blood-borne strains transiently traveling in and out of the CNS in lymphoid

cells but not invading the brain directly. Second, with the advent of effective treatment regimens such as highly active anti-retroviral therapy (HAART), the incidence of HIVD is low (29), making sufficient patient sampling difficult.

Therefore, a complementary approach entails identifying deceased demented and non-demented patients as in our initial study and isolating viruses derived from both brain and spleen tissue from the same individuals for coreceptor usage analysis. In addition, similar matched sets of samples can be obtained from deceased HIV-infected infants. Since it has been estimated that 50%-100% of all HIV-infected individuals carry CXCR4-dependent strains in the blood at some point (36, 37), it is likely that a number of demented and non-demented individuals in these cohorts would carry CXCR4-specific blood-borne strains. In these individuals, the absence of CXCR4-using strains in the brain would considerably strengthen the theory of selection for only CCR5-dependent strains in the CNS and more convincingly negate alternate coreceptor usage as an important pathogenic mechanism in adult or infant HIVD. On the other hand, the presence of CXCR4-specific viruses in the brain should provide an impetus for further exploration of CXCR4 usage as a pathogenic process. Furthermore, given the increased neurologic dysfunction typically observed in HIV-positive infants compared to adults, it would be interesting to correlate the prevalence of CXCR4-using strains in the CNS with age of the patient and severity of disease manifestations. As a result, this next round of studies should more completely

define the relevance of alternate coreceptor usage in a number of specific pathologic contexts that typically result in accelerated development of HIVD.

The possibility of CXCR4-dependent strains in the brain is also especially attractive in light of our discovery of a CXCR4-dependent, *trans*-CD4 receptor mechanism for entry of CD4-negative astrocytes. While a *trans* CD4/coreceptor/gp120 ternary complex was previously characterized in detail for the infection of 293T cells, its direct formation in the infection of astrocytes was suggested but not rigorously established.

First, an alternate hypothesis of astrocyte infection proposes that a secreted factor such as a cytokine produced by the $CD4^+$ donor cells rather than the surface CD4 itself is important for virus entry. Assessment of HIV-1 infection could be performed in the presence of $CD4^+$ donor cells separated from astrocytes by a semipermeable membrane. The membrane allows for the crossing of secreted factors but not whole cells. Therefore, this experiment would determine if HIV-1 infection depends on physical contact of target cells with the $CD4^+$ donor cell. Furthermore, inhibition of viral entry with neutralizing antibodies raised against CD4 would also demonstrate the importance of *trans* CD4 in astrocyte infection rather than another unidentified factor.

Second, since the donor cells used in the initial study expressed CXCR4 and were also infectable by HIV-1, a number of mechanisms could have mediated the infection of astrocytes that did not involve the ternary complex

model but still required a coreceptor on the astrocytes, CD4 on the donor cell, and contact between the cell types. These possibilities include HIV-1 entry into astrocytes by either fusion with or engulfment of infected $CD4^+$ donor cells. To distinguish among these hypotheses, CXCR4-dependent HIV-1 infection of astrocytes can be assessed upon co-culturing with a $CD4^+$ and $CD4^-$ donor cell line that does not express CXCR4 and is itself not infectable (e.g., CH4.G10 murine B cell line). Without a confounding $CD4^+$ donor cell infection, detection of HIV-1 infection of astrocytes would prove more rigorously that the *trans* CD4 ternary complex is important in this system.

Third, it will be necessary to determine if the predominant CCR5-dependent strains can infect astrocytes using the same process, given CCR5 expression on adult astrocytes as previously described (35). To verify this possibility, it will be important to reconfirm CCR5 expression by immunostaining adult astrocytes grown in cell culture, to demonstrate viral entry by a CCR5-dependent strain, and to successfully block entry by using a neutralizing antibody specific for CCR5 (e.g., 2D7). Consequently, this novel entry mechanism would carry more biologic significance in the context of the overwhelming population of CCR5-dependent strains observed in adult brain tissue.

Taken together, the results from these experiments should more completely characterize the impact of receptor and coreceptor interactions on

HIV infection of the brain. However, results derived from cell culture analyses need to be interpreted cautiously. Since cell culture observations do not always correlate with *in vivo* events, it is still unclear how modulation of these molecular interactions affects natural infections and resulting disease. Demonstration of the pathogenic impact of such processes will rely upon an animal model that recapitulates human infection and disease. The development of such a model system lies beyond the scope of this proposal. However, a number of studies may be envisioned to address these same questions by genetic manipulation of coreceptor and CD4 presentation in the brain in conjunction with variation of gp120 coreceptor specificity. With such *in vivo* data to complement these cell culture studies, a much clearer understanding should then emerge of the relationship between viral coreceptor dependence for entry and subsequent pathogenesis in the brain.

B. A Pathway of Entry for Filoviruses: Impact on Pathogenesis

We established a pseudotype system for use in BSL3 (biosafety level 3) facilities to study the molecular events that control specifically the interaction of filovirus envelope GP with target cells. Using this system, we fortuitously discovered functional differences of filovirus GP in the ability to incite detachment of 293T cells by expression of EBO GP, but not MBG GP. Although it has been proposed that this cellular dysfunction in endothelial cells directly results in hemorrhage (44), further investigation is necessary for an accurate

conclusion. Use of specific inhibitors of known Ser/Thr kinase cascades to block GP-induced detachment should be useful to more thoroughly map the signaling pathway responsible for this phenotype. While interesting from a scientific standpoint, this study should be combined with other investigations of its pathogenic and medical relevance. To demonstrate endothelial cell dysfunction *in vivo*, a transgenic mouse can be constructed that expresses EBO-Z GP under an endothelial cell-specific promoter and subsequently monitored for visceral organ hemorrhage. Although it would establish *in vivo* activity, this study may not accurately reflect the timing and level of GP expression observed during natural EBO virus infections. Therefore, a complementary experiment entails replacing the sequence encoding for the EBO-Z GP gene, which can induce detachment, with that of either MBG GP (6) or the GP of Reston strain of EBO (EBO-R) (44), neither of which induces detachment, into cloned EBO-Z virus sequences that can produce full-length, infectious virions as recently described (42). Infection of an animal with this chimeric virus followed by absent or delayed hemorrhagic symptoms compared with wildtype virus infection would clearly demonstrate the pathogenic relevance of cellular dysregulation induced specifically by EBO-Z GP in the context of a natural infection.

For my main project, using a genetic reconstitution strategy, a cell surface cofactor, FR-α, was identified as an important mediator of entry by both MBG and EBO viruses. Two technologies were exploited in this strategy: efficient

retroviral delivery of an expression library derived from permissive cells, and production of selectable, single-cycle pseudotype viruses that utilize the same entry mechanism as wildtype filoviruses. Because heterologous envelope GP can pseudotype the HIV genome efficiently, it is reasonable that this complementation protocol could be used to identify unknown cellular factors important for entry of a variety of different viruses. The applicability of this approach is currently being assessed for studying target cell entry by the West Nile flavivirus.

The specific discovery of FR-α and its cellular trafficking patterns as important for filovirus entry serves as an important beginning to more completely dissect this process and to understand its implications for the development of hemorrhagic fever. First, to characterize more completely the interaction of FR-α with MBG and EBO GP at a molecular level, specific FR-blocking reagents could be used to inhibit the association of FR-α with GP-expressing cells as a method to demonstrate direct binding, as suggested by our initial findings. Consequently, mutation, truncation, or substitution of various domains of FR-α could be used to map the regions essential for this interaction and/or for facilitation of filovirus entry. Finally, a long-term collaborative project may entail crystallization of FR-α bound to envelope GP to demonstrate definitively the relevant protein structure and amino acid components necessary for binding and subsequent activity. As a result, improved antibodies or

antagonists for treatment of human infection could be prepared that more efficiently target these relevent domains or amino acids.

Second, to gain a clearer understanding of the entire entry process, it will be important to identify other necessary factors that facilitate virus entry. In our initial work, Jurkat T-cells stably expressing FR-α exhibited only modest permissivity for filovirus entry in contrast to the robust permissivity displayed in naturally susceptible cell types such as HeLa cells. As a result, we hypothesize that Jurkat T-cells do not support robust infection because they carry multiple impediments to filovirus entry. Consequently, other cellular factors beyond FR-α may be necessary to reconstitute permissivity to the high levels observed in non-manipulated infectable cell types. Moreover, we previously identified FR-α-negative cell types that are nonetheless susceptible to both MBG and EBO virus entry. Therefore, we hypothesized that a family of cellular factors exists, of which FR-α is the first identified member, which facilitate viral entry into a variety of cellular contexts. Since FR-α is one of four receptor isoforms (α, β, and γ, and γ') that carry 68–79% identity in amino acid sequence (27), it is plausible that the membrane-bound FR-β can also facilitate filovirus entry and should be explored further. Moreover, delivery of cDNA libraries derived from FR-α-negative permissive cells into Jurkat T-cells may allow for recovery of other non-homologous host factors predicted to play a role independently or in combination with FR-α in the filovirus entry process. Identification of these key

players is vital for a complete molecular understanding of filovirus entry and subsequently, for the identification of the exact points in the processes where MBG and EBO GP functions diverge.

Third, an alternate method to dissect this entry process entails defining the exact endocytic pathway used for internalization of FR-α. While numerous reports have suggested that a caveolar vesicular system transports ligand-bound FR-α into the cell interior (1, 32, 45), others suggest that clathrin-coated pits (26, 31, 40) or other undefined vesicular complexes (38) play a role in this and other GPI-linked receptor uptake. Use of inhibitors that target specific endocytic pathways or utilization of confocal microscopy to determine co-localization of FR-α or GP with specialized vesicular components should point out the relevance of each pathway in viral uptake. Since filoviruses constitute the only identified family of enveloped viruses that rely upon a GPI-linked receptor for cellular entry, they may use a specialized uptake system that could be specifically abrogated for therapeutic benefit.

Fourth, since three distinct EBO subtypes exist besides EBO-Z and cause fatal hemorrhagic fevers in various mammalian species, the role of FR-α for these other viruses should be further explored. In particular, it would be interesting if EBO-R virus, which is pathogenic for monkeys yet non-pathogenic for humans, relies upon an alternate factor besides FR-α for facilitating target cell entry.

In addition to further exploring the mechanism of filovirus entry, a parallel approach should be pursued to correlate these molecular events with their *in vivo* relevance. Towards this end, it will be important to determine the range of susceptible cell types that employ FR-α to facilitate MBG and EBO virus entry. Of particular interest are those cells defined as major targets of direct infection by filoviruses including macrophages/monocytes and fibroblasts, which are present in numerous susceptible tissues, as well as hepatocytes and endothelial cells (46). Importantly, we found that naturally permissive Vero E6 cells, derived from fibroblast-like kidney cells, and HOS cells, derived from fibroblast-like osteosarcoma cells, both express FR-α that is utilized by MBG and EBO-Z to gain cellular entry. Furthermore, expression of folate receptor has been documented in macrophages (16), fibroblasts (2), and hepatocytes (10, 33, 41). Expression of FR-α has not been reported in endothelial cells, but it is possible that low levels are nonetheless present or may be induced *in vivo*, as documented in a variety of other cell types due to the dynamic regulation of this receptor (2). Further characterization of FR-α expression and function in all of these relevant cell types should be pursued to strengthen the claim that FR-α can facilitate virus entry during the natural course of infection of mammalian hosts.

Subsequently, this information would offer hope that the design of antagonists to block the interaction of filoviruses with FR-α in those cell types could successfully treat typically fatal human infections. Our previous

observation in cell culture that antibodies directed against FR-α, folic acid, and soluble FBP can inhibit entry by MBG or EBO-Z virus provides proof of concept that already existing specific antagonists can be modified for therapeutic application. Initially, the efficacy should be tested of these neutralizing antibodies against wildtype filoviruses in cell culture. Subsequently, inhibition of infection and resulting disease should be assessed in mammalian animal models (e.g., guinea pigs, suckling mice). In addition, a soluble form of FR-α with an increased *in vivo* half-life may be useful as a competitive inhibitor of active infection. Preliminary studies have been initiated assessing the inhibitory activity of such a soluble FR-α fusion protein linked for stability *in vivo* to the IgG Fc region. In the same manner, two of the four homologous FR isoforms, FR-γ and FR-γ', are also expressed as soluble proteins and carry binding affinities for folic acid. Therefore, these receptor forms possibly could be manipulated to act as endogenous inhibitors of virus entry if they also bind filovirus GP and their expression levels can be augmented *in vivo*. Finally, since folic acid and its derivatives are readily deliverable and are associated with little patient toxicity (20), it is important to obtain further evidence of the efficacy of such small molecule ligands as entry inhibitors. As a result, a number of therapeutic options can be envisioned to specifically block the association of FR-α with filovirus GP and hopefully result in reduced pathogenic consequences during human epidemics.

Therefore, while broad in scope, this body of work essentially focused on two objectives: to advance our understanding of the molecular mechanisms of entry of enveloped viruses and to define their pathogenic consequences more clearly. As a result, a number of future investigations can now be pursued that ultimately may result in successful treatment of these lethal human diseases.

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