Virology 419 (2011) 1–9

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Differential requirements for clathrin endocytic pathway components in cellular entry by Ebola and Marburg glycoprotein pseudovirions

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ARTICLE INFO

Article history:
Received 13 April 2011
Returned to author for revision 24 May 2011
Accepted 29 July 2011
Available online 19 August 2011

Keywords:
Filoviral GP
Pseudovirions
Entry
Clathrin-mediated endocytosis
Eps15
AP-2
DAB2
ARRB1
AP-1

ABSTRACT

Clathrin-mediated endocytosis was previously implicated as one of the cellular pathways involved in filoviral glycoprotein mediated viral entry into target cells. Here we have further dissected the requirements for different components of this pathway in Ebola versus Marburg virus glycoprotein (GP) mediated viral infection. Although a number of these components were involved in both cases; Ebola GP-dependent viral entry specifically required the cargo recognition proteins Eps15 and DAB2 as well as the clathrin adaptor protein AP-2. In contrast, Marburg GP-mediated infection was independent of these three proteins and instead required beta-arrestin 1 (ARRB1). These findings have revealed an unexpected difference between the clathrin pathway requirements for Ebola GP versus Marburg GP pseudovirion infection. Anthrax toxin also uses a clathrin-, and ARRB1-dependent pathway for cellular entry, indicating that the mechanism used by Marburg GP pseudovirions may be more generally important for pathogen entry.

Introduction

The *Filoviridae* family comprises of *Marburg virus* (MARV) and *Ebola virus* (EBOV), the causative agents of viral hemorrhagic fever (Schnittler and Feldmann, 2003). There have been several sporadic outbreaks of these virus infections since the late 1960's, the most recent occurring in 2008 in Uganda (MARV), and in 2009 in the Democratic Republic of the Congo (EBOV). The high fatality rates associated with these viruses represents a potential global health challenge and also makes them ideal candidates for use as biological weapons. Consequently, these viruses have been classified as Category A Bioterrorism Agents by the US Centers for Disease Control and Prevention (CDC). There are currently no effective drugs or licensed vaccines to protect humans against filovirus infection (Sullivan et al., 2009). Therefore, there is an urgent need to better understand the mechanisms that control filovirus replication for designing effective therapeutic measures.

The target cells for filoviral infection are monocytes, macrophages, dendritic cells and endothelial cells (Connolly et al., 1999;Geisbert et al., 2003). Several cell surface proteins have been implicated in filovirus entry including folate receptor alpha (Chan et al., 2001), lectins (Alvarez et al., 2002; Ji et al., 2005; Marzi et al., 2006; Simmons et al., 2003a;Takada et al., 2004), beta 1 integrins (Takada et al., 2000) and TAM receptors (Shimojima et al., 2006). T-cell immunoglobulin and mucin domain 1 (TIM-1) was recently reported to be a receptor for Ebola as well as Marburg virus (Kondratowicz et al., 2011), suggesting that these viruses bind to a common receptor.

Filovirus entry is mediated by the virus-encoded glycoprotein (GP), located on the viral surface lipid bilayer. The filoviral GP is a homotrimeric, class I viral fusion protein, expressed as a precursor that is post-translationally cleaved in the Trans Golgi Network (TGN) by a cellular proprotein convertase furin into the disulfide-linked GP1 (140 kD) and GP2 (26 kD) subunits (Jeffers et al., 2002;Volchkov et al., 1998). GP1 is primarily involved in receptor binding whereas GP2 facilitates virus–cell membrane fusion (White et al., 2008).

Following cell surface receptor binding, filoviruses are taken up by endocytosis. Using multiple approaches and cell lines we have previously shown that Ebola GP pseudovirus uses clathrin-mediated endocytosis as an entry pathway (Bhattacharyya et al., 2010). We also found that treatment with chlorpromazine, which was previously

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doi:10.1016/j.virol.2011.07.018

reported to block clathrin-dependent entry (Wang et al., 1993), inhibited wild type (WT) Ebola. However, it is now known that chlorpromazine also inhibits macropinocytosis and hence is not a specific inhibitor of the clathrin pathway (Ivanov, 2008). Clathrin and macropinocytic pathways have also been implicated in filovirus infection by other groups (Quinn et al., 2009;Sanchez, 2007); while the role of caveolae pathway has been both implicated and refuted (Empig and Goldsmith, 2002;Simmons et al., 2003b). It was recently suggested that while filoviral glycoprotein pseudotyped viruses enter via the clathrin pathway (Hunt et al., 2010); wild type filoviruses predominantly use macropinocytosis for entry (Nanbo et al., 2010; Saeed et al., 2010). These differences in entry pathway requirements could be due to the differences in size and shape of pseudovirions versus WT viruses (Cureton et al., 2010).

Upon entry, filoviruses are trafficked by cellular endocytic machinery to an acidic endosomal compartment, which is the site of virus–cell membrane fusion. The Ebola virus GP is activated to trigger fusion through proteolytic cleavage mediated by cellular lysosomal cysteine proteases, cathepsins B and L (Chandran et al., 2005; Kaletsky et al., 2007; Sanchez, 2007;Schornberg et al., 2006).

In this report we have investigated the specific requirements for different components of the clathrin endocytic machinery in Ebola GP versus Marburg GP pseudovirion entry. These studies have demonstrated that these two highly related glycoproteins exhibit differential requirements for several players of this pathway, uncovering critical differences in their entry mechanisms. Moreover, the factors required for Marburg GP mediated entry are very similar to those previously described for anthrax toxin entry (Abrami et al., 2010), suggesting that these components of the clathrin pathway may be broadly required by various pathogens to enter target cells.

Results

To examine virus entry mediated by MARVGP and EbGP, we exploited a previously used envelope protein-deficient lentiviral (HIV-1) vector system (Bhattacharyya et al., 2010) pseudotyped with the Ebola or Marburg GP. As expected, infection of HOS cells by both types of filovirus GP pseudovirions was dependent upon low endosomal pH as well as cathepsin (Cat) L cleavage since it was blocked by treatment with the vacuolar ATPase inhibitor Bafilomycin A1 and the Cat L inhibitor FYdmk, respectively (Supplemental Fig. 1). For control purposes, we showed that these treatments did not impair infection with an isogenic HIV vector carrying the wild-type envelope protein, which directs pH-independent cellular entry (Miyauchi et al., 2009;Stein et al., 1987) or HIV pseudotyped with VSVg, which mediates low-pH dependent but Cat L independent entry (Chandran et al., 2005) (Supplemental Fig. 1).

Previously, we showed that EbGP-dependent virus infection was blocked by treatment with two chemical inhibitors of the clathrin endocytic pathway; chlorpromazine and sucrose, and by RNAi-mediated knockdown of the clathrin heavy chain (CHC) (Bhattacharyya et al., 2010). To assess the role of clathrin-mediated endocytosis in MARVGP pseudovirion entry, we evaluated the effects of these treatments on infection. Similar to EbGP-mediated virus infection, MARVGP-mediated infection of HOS cells was blocked by treatment with either 10 μg/ml chlorpromazine (Fig. 1A) or 0.45 M sucrose (Fig. 1B), or by treatment with both inhibitors (Fig. 1C). These inhibitors also blocked MARVGP-dependent infection in physiologically relevant human microvascular endothelial cells (HMEC) (Fig. 1D). MARVGP-mediated infection was also blocked specifically by two independent siRNAs, which significantly knocked down the levels of CHC mRNA without causing any overt cytotoxicity (Figs. 1E–G). As expected, none of these treatments inhibited infection by the control HIV-1 virus. Taken together, these results demonstrate that MARVGP pseudovirions utilize a clathrin-dependent pathway for cellular entry.

Eps15 and AP-2 are specifically required for EbGP mediated entry

The cellular protein Eps15 links ubiquitinated cargo to clathrin through its binding to the alpha-subunit of AP-2 (Traub, 2003). Previously, we showed that expression of a dominant-negative version of Eps15 (DIII) blocks infection by EbGP-pseudotyped virus (Bhattacharyya et al., 2010). We tested the effect of this molecular inhibitor on MARVGP pseudotyped virus and surprisingly, it had no impact on infection (Fig. 2). Since Eps15 is known to constitutively associate with AP-2 (Benmerah et al., 1995), we then examined the effect of RNAi-mediated knockdown of AP-2 on both EbGP and MARVGP-dependent entry. As anticipated, EbGP-dependent infection was blocked by two siRNAs that knocked down the mRNA expression levels of AP-2 without altering cell viability, whereas MARVGP-mediated infection was not blocked by these siRNAs (Figs. 3A–C). We conclude that the mechanism of EbGP-mediated infection is dependent on both Eps15 and AP-2 whereas MARVGP-mediated infection is independent of these two cellular factors.

Differential requirements for ARRB1 and DAB2 in Marburg GP versus Ebola GP mediated entry

To further investigate the clathrin pathway requirements for EbGP and MARVGP-dependent entry, we tested the roles played by several members of this pathway including epsin 1, intersectin 1 (ITSN1), dynamin 2 (DYN2), adaptor-related protein complex 1, mu 1 subunit (AP1M1), disabled homolog 2 (DAB2), low density lipoprotein receptor adaptor protein 1 (LDLRAP1), inositol polyphosphate phosphatase-like 1 (INPPL1), phosphatidylinositol binding clathrin assembly protein (PICALM), beta-arrestin 1 (ARRB1), huntingtin interacting protein 1 (HIP1), *Drosophila* numb homolog (NUMB), RALBP1 associated Eps domain containing 1 (REPS1) and RALBP1 associated Eps domain containing 2 (REPS2). The roles of these factors in the clathrin pathway are described in the Discussion section.

Two independent siRNAs were employed that significantly knocked down mRNA expression levels of each of these factors without adversely impacting cell viability (Supplemental Fig. 3). Some of these siRNAs were extensively validated in a previous study (Huang et al., 2004), while the remaining siRNAs were chosen from a well-characterized Dharmacon library. For practical purposes, the genes were tested in 3 groups: group 1 genes are shown in black bars, group 2 in dark gray bars and group 3 in light gray bars (Fig. 4).

These studies revealed that epsin 1, ITSN1, LDLRAP1, INPPL1, PICALM, NUMB, REPS1 and REPS2 were required by both MARVGP and EbGP suggesting a conserved requirement for these factors in filoviral GP mediated entry. The siRNAs targeting these factors had no impact on infection by the isogenic vector containing the HIV-1 envelope protein, indicating that they do not influence a post-entry step of infection (Fig. 4). By contrast, DYN2 and AP-1 also inhibited infection by HIV-1, suggesting that these proteins may be required for the endocytosis-dependent infection of the viruses. DYN2 could possibly mediate HIV-1 entry via macropinocytosis (Liu et al., 2008; Marechal et al., 2001) and endosomal fusion of HIV-1 particles (Miyauchi et al., 2009). AP-2 and beta-arrestins are known to mediate endocytosis of G protein-coupled receptors (GPCRs) (Shimizu et al., 2009) and HIV-1 co-receptors CXCR4 and CCR5 are GPCRs (Unutmaz et al., 1998), which could explain the inhibitory effects of the AP-2 and ARRB1 siRNAs on HIV-1 infection. siRNA-mediated knockdown of HIP-1 also reduced infection by all three of these viruses, although statistically significant levels of inhibition with both siRNAs were obtained only with the EbGP pseudotyped virus (Fig. 4). Most importantly, these studies demonstrated that EbGP and MARVGP specifically employ DAB2 and ARRB1, respectively for entry (Fig. 4).