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Mammarenaviruses deleted from their Z gene are replicative and produce an infectious progeny in BHK-21 cells

Amélie D. Zaza[a,b], Cécile H. Herbreteau[a], Christophe N. Peyrefitte[b], Sébastien F. Emonet[b,*]

[a] Fab'entech, 24 rue Jean Baldassini, 69007 Lyon, France
[b] Unité de virologie, Département de Biologie des Agents Transmissibles, Institut de Recherche Biomédicale des Armées, 1 place général Valérie André, BP 73 91 223 Brétigny-sur-Orge cedex, France

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ABSTRACT

Mammarenaviruses bud out of infected cells via the recruitment of the endosomal sorting complex required for transport through late domain motifs localized into their Z protein. Here, we demonstrated that mammarenaviruses lacking this protein can be rescued and are replicative, despite a 3-log reduction in virion production, in BHK-21 cells, but not in five other cell lines. Mutations of putative late domain motifs identified into the viral nucleoprotein resulted in the almost complete abolition of infectious virion production by Z-deleted mammarenaviruses. This result strongly suggested that the nucleoprotein may compensate for the deletion of Z. These observations were primarily obtained using the Lymphocytic choriomeningitis virus, and further confirmed using the Old World Lassa and New World Machupo viruses, responsible of human hemorrhagic fevers. Z-deleted viruses should prove very useful tools to investigate the biology of Mammarenaviruses.

1. Introduction

Among the current 33 species of the *Mammarenavirus* genera, which belongs to the *Arenaviridae* family, seven members are responsible of human hemorrhagic fevers: the Lassa and Lujo viruses endemic in West Africa and in Zambia, and the Junín, Machupo, Guanarito, Sabiá and Chapare viruses endemic in South America (Current ICTV, 2018; Shao et al., 2015). Infections caused by those viruses represent a serious threat for local populations, due to the pathogenesis on one hand, leading to a mortality ranging from 1% to 35% in untreated patients (Günther and Lenz, 2004; Patterson et al., 2014; Enria et al., 2008), and, on the other hand, the absence of specific symptoms, preventing the early initiation of available therapies, and thereby reducing their efficiency. These therapies are limited to ribavirin in general, and to immune plasma in the case of Argentinean hemorrhagic fevers (Enria et al., 2008; Vela, 2012). The Candid #1 vaccine has been used since 1991 to protect this at-risk population (Ambrosio et al., 2011). Mammarenaviruses are rodent-borne viruses establishing into their hosts persistent infections (Salazar-Bravo et al., 2002). Humans become infected by direct contact, inhalation or ingestion of contaminated rodent excreta or secreta. Due to the severity of their induced illness, their documented human-to-human transmission, their relative easiness to be isolated and grown at high titers, their aerosol transmission and the public panic and social disruption they could provoke, the CDC classified these viruses into the category A pathogens (Category A Bioterrorism, 2018).

Mammarenaviruses are enveloped viruses with a single-stranded RNA genome composed of two segments (Meyer et al., 2002). Each segment possesses two genes encoded in an ambisens orientation, separated by an intergenic region capable to form hairpin structures. Segments are terminated by 5′ and 3′ non-coding regions. The small S segment codes for the glycoprotein complex and the nucleoprotein whereas the large L segment codes for the Z and L proteins. The main function of each viral protein is now clearly established (Buchmeier, 2002; Perez et al., 2003): the GPC protein is post-translationally cleaved to produce the GP1, GP2 and signal peptide, involved in both the cellular attachment and entry; the NP encapsidates the RNA genome; the L protein is the RNA-dependent RNA-polymerase; the Z protein plays the counterpart of the Matrix protein found in several negative-stranded viruses. Apart from those main functions, mammarenavirus proteins display other roles, such as the inhibition of the IFN response mediated by the NP and Z proteins (Martínez-Sobrido et al., 2006; Fan et al., 2010), the endonuclease activity of the L protein to snatch cellular caps to initiate the viral mRNA production (Morin et al., 2010), the interaction with cellular factors, such as the eukaryotic initiation factor 4E (Campbell Dwyer et al., 2000) and the promyelocytic leukemia (PML)

* Corresponding author.
E-mail addresses: ameliezaza@gmail.com (A.D. Zaza), cecile.herbreteau-delale@fabentech.com (C.H. Herbreteau), christophe.peyrefitte@intradef.gouv.fr (C.N. Peyrefitte), sebastien.emonet@defense.gouv.fr (S.F. Emonet).

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protein (Borden et al., 1998).

The small Z protein (11 kDa) was discovered in 1989 by Salvato and Shimomaye (Salvato and Shimomaye, 1989). This protein was first thought to be an RNA-binding protein with a regulatory role. Then, the Z protein was detected into virions and proposed as a structural component of viral particles (Salvato et al., 1992). This localization into virions as well as its requirement to produce infectious VLPs oriented the research toward a function either in the budding or the entry of viral particles (Lee et al., 2002). The central role played by Z in the egress process of mammarenaviruses was then established (Perez et al., 2003). In fact, the Z protein exhibited a self-budding activity through a canonical PPXY late domain motif found near its C-terminus. This late domain interacted with a component of the endosomal sorting complex required for transport (ESCRT) cellular machinery by the recruitment of the ubiquitin-binding ESCRT-I-component tumor susceptibility gene 101 (Tsg101).

However, a recent publication demonstrated that the late domain of the Z protein may be involved in the production of defective interfering particles rather than in the production of infectious particles (Ziegler et al., 2016). In this publication, the necessity of a functional ESCRT pathway to produce virions was also challenged.

In the present work, we demonstrated that mammarenaviruses lacking the Z gene can be rescued and propagated onto BHK-21 cells. Several evidences suggest that the viral NP may compensate this deletion through the late domain motifs identified into its sequence. The mutation of the NP late motifs did not affect the main function of the NP but drastically reduced the already low infectious recombinant virus production. The ability to produce VLPs in absence of the Z protein was not only observed using the prototypic Lymphocyctic choriomeningitis virus (LCMV) but also using Lassa and Machupo viruses, two mammarenaviruses responsible of human hemorrhagic fevers. The understanding of the role played by Z in the mammarenavirus life cycle will likely benefit from the possibility to generate and study mammarenaviruses deleted from this protein. Similar achievements were published for different viruses (Cathomen et al., 1998; Dietzel et al., 2015; Inoue et al., 2003; Mebatsion et al., 1999). Such viruses could be used to study the impact of the deletion of Z onto their packaging, budding or any other function presumably assigned to Z."

2. Results

2.1. VLPs are produced in absence of the specific LCMV Z protein

To demonstrate the functionality of our newly generated LCMV reverse genetics system, we tried to reproduce previously published results using a minigenome (MG) assay. The transfection of the vector expressing the MG alone resulted in the production of a background level of GLuc (Fig. 1A), while no eGFP expression was observed (Fig. 1B). The co-transfection of one of the vectors expressing the viral proteins did not improve the reporter gene expression. A 3500 fold increase of GLuc expression as well as the observation of eGFP production into transfected cells was observed when cells were transfected with a combination of vectors expressing the MG as well as the NP and L genes. The addition of the plasmid expressing the GPC gene (pC-GPC) to the previous combination did not modify the production of GLuc, whereas the addition of a plasmid expressing the Z protein (pC-Z) was associated with a decrease of the GLuc expression (by factors of 41 and 10 when pC-Z was co-transfected with a combination of pC-NP and pC-L, or with a combination of pC-NP, pC-L and pC-GPC, respectively). In the presence of all viral proteins eGFP-expressing cells, patches of eGFP-expressing cells were observed, resembling viral foci (Fig. 1B). Surprisingly, we also detected such patches in cells transfected with all plasmids but the one expressing Z (Fig. 1B). This observation suggested a MG transmission to adjacent cells when Z was absent similar to the one observed in cells transfected with the combination of plasmids expressing all the viral proteins. Despite the increase of the number of MG-expressing cells, the GLuc expression was not higher in cells transfected with the MG + NP + L + GPC plasmid combination than in cells with transfected with the MG + NP + L combination. This observation is likely related to the slight inhibition induced by GPC of the MG RNA replication (Fig. 2).

To verify that the observed foci were the result of the MG propagation to adjacent cells through VLPs, a VLP titration was performed. The tissue culture supernatants (TCS) were collected from transfected cells. The TCS were then used to replace the TCS from cells transfected only with pC-NP and pC-L. When the TCS originated from cells transfected with a combination of vectors expressing all four viral proteins plus the MG, 895 green fluorescent cells were observed in average with a standard deviation [SD] of 125 (Fig. 1C). On the contrary, when the TCS originated from cells transfected only with the minimal NP + L + MG combination, no eGFP expression was observed. These results demonstrated that the eGFP expression originated from the expression of the MG brought by infectious VLPs into cells producing the minimal viral *trans*-acting factors but did not resulted from a contamination by eGFP-expressing cells coming from the first transfection. When TCS originated from cells transfected with plasmids expressing the MG plus all viral proteins but Z, 104 green fluorescent cells in average (SD of 40) were observed.

In conclusion, the cells expressing the MG and the NP, L and GPC viral proteins were able to produce infectious VLPs. This experiment demonstrated that Z was not strictly necessary to produce infectious VLPs.

2.2. LCMV NP protein is associated with an increased amount of MG RNA released in the TCS

The above experiments did not permit to discriminate between the viral proteins necessary to produce VLPs from those implicated into their infectivity. To answer this question, a western blot experiment was designed using tagged proteins. However, the addition of the widely used and small HA tag to the LCMV NP protein resulted in the blockade of the production of VLPs in the absence of the Z protein (S1 Fig).

A second approach, based on the detection of the MG RNA into the TCS from cells transfected with different combinations of vectors, was performed. The amount of transfected plasmids per well was standardized using a mCherry-expressing plasmid. The eGFP expression and foci production mirrored the previous results (compare Fig. 1A and S2), ruling out any bias coming from the transfection of a different quantity of plasmids per well. Low levels of MG RNA were detected when pol-MG was transfected alone (9×10^1 RNA copies/µl) (Fig. 2), likely due to the presence in the TCS of the MG RNA either into detached cells or released from dead cells. When cells were co-transfected with pC-NP and pol-MG, we noted a 9 fold increase in the quantity of MG RNA in the TCS, whereas the co-transfection of pol-MG with either pC-L or pC-GPC had no effect. Since the MG RNA increased by only a 2-fold factor in the intracellular compartment, this increase was not solely due a higher half-life of the encapsidated MG RNA. The addition of pC-GPC or pC-Z to the MG + NP combination did not modify the MG RNA quantities. When cells were co-transfected with the MG + NP + L combination, the quantity of MG RNA observed in the TCS was increased 25 times (27 times for the intracellular MG RNA) when compared with the MG + NP combination, likely due to the intracellular MG replication, and therefore increased the number of MG RNA available for encapsidation and particle formation. The increase factor went up to 27 when the pC-GPC was added to the MG + NP + L combination (increase factor of 16 for the intracellular MG RNA), probably because produced VLPs started to be infectious and therefore, to propagate the MG RNA, resulting in a higher amount of VLP-producing cells. These results suggested that NP does not only enhanced the viral RNA half-life but also can trigger the formation of viral particles.