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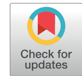
Insights into the homo-oligomerization properties of N-terminal coiled-coil domain of Ebola virus VP35 protein



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ABSTRACT

The multifunctional Ebola virus (EBOV) VP35 protein is a key determinant of virulence. VP35 is essential for EBOV replication, is a component of the viral RNA polymerase and participates in nucleocapsid formation. Furthermore, VP35 contributes to EBOV evasion of the host innate immune response by suppressing RNA silencing and blocking RIG-I like receptors' pathways that lead to type I interferon (IFN) production. VP35 homo-oligomerization has been reported to be critical for its replicative function and to increase its IFN-antagonism properties. Moreover, homo-oligomerization is mediated by a predicted coiled-coil (CC) domain located within its N-terminal region. Here we report the homo-oligomerization profile of full-length recombinant EBOV VP35 (rVP35) assessed by size-exclusion chromatography and native polyacrylamide gel electrophoresis. Based on our biochemical results and in agreement with previous experimental observations, we have built an *in silico* 3D model of the so-far structurally unsolved EBOV VP35 CC domain and performed self-assembly homo-oligomerization simulations by means of molecular dynamics. Our model advances the understanding of how VP35 may associate in different homo-oligomeric species, a crucial process for EBOV replication and pathogenicity.

1. Introduction

Ebola virus (EBOV) is the prototype of a group of five species of enveloped, non-segmented and negative-stranded RNA viruses with filamentous morphology that belong to the genus *Ebolavirus* of the family *Filoviridae*, order *Mononegavirales* (Kuhn et al., 2010). EBOV causes haemorrhagic fever in both humans and non-human primates, namely EBOV disease (EVD), for which neither antivirals (Wu and Liu, 2017) nor vaccines (Reynolds and Marzi, 2017) are currently approved. Since its first appearance in 1976, EBOV has been responsible of a series of human outbreaks with mortality rates as high as 90% which, although sporadic and limited to equatorial regions of sub-Saharan Africa, posed a serious threat to global health due to the possibility of spread from travel-imported cases (Feldmann and Geisbert, 2010). This concern became reality in 2013–16, when an EBOV outbreak larger than all previous combined hit West Africa reporting 28646 cases, several infections across multiple international boundaries and 11323 deaths (Coltart et al., 2017). The fatal outcome of EVD can be attributed, at least in part, to the virus ability in suppressing innate immune response while undergoing a rapid viral replication in infected cells. Impairment of the host cell defenses, in turn, triggers multiple pathogenic mechanisms that dysregulate the vascular and coagulation systems, and subvert the adaptive immune responses (Messaoudi et al., 2015; Zinzula and Tramontano, 2013). Among the nine viral products encoded by EBOV genome, the multifunctional VP35 protein plays critical roles in both virus replication and innate immune suppression. In association with the L protein, VP35 constitutes the EBOV RNA-dependent RNA polymerase holoenzyme, bridging it to the nucleoprotein (NP)-complexed viral RNA (Boehmann et al., 2005; hlberger et al., 1998, 1999). VP35 also serves as a factor for packaging of viral genome (Johnson et al., 2006) and for nucleocapsid assembly (Beniac et al., 2012; Bharat et al., 2012; Huang et al., 2002; Noda et al., 2011), and it is an antagonist of the host cell antiviral response. In this regard, VP35 has been reported to abolish IFN-α/β production in a dose-dependent mode (Cannas et al., 2015) by *i*) inhibiting IRF-3 phosphorylation-dimerization and translocation to the nucleus (Basler et al., 2000, 2003; Hartman et al., 2006, 2008) *ii*) enhancing PIAS1-induced SUMOylation of IRF-7 (Chang et al., 2009); *iii*) impairing the activity of IKK-epsilon and TBK-1 kinases (Prins et al., 2009) *iv*) binding viral dsRNA for its sequestration to RIG-I recognition (Cárdenas et al., 2006); *v*) binding

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PACT to impede its interaction with RIG-I, thereby inhibiting PACT-induced RIG-I ATPase activity (Luthra et al., 2013) and *vi*) blocking dsRNA-mediated activation of PKR (Feng et al., 2007; Schümann et al., 2009). Furthermore, EBOV VP35 was found to suppress mammalian RNA silencing (Fabozzi et al., 2011; Haasnoot et al., 2007; Zhu et al., 2012) blocking stress granule (SG) assembly through interaction with multiple SG components, including G3BP1, eIF3 and eEF2 (Le Sage et al., 2017) and inhibit T cell activation through the impairment of dendritic cells maturation (Jin et al., 2010). Additionally, as a hallmark of the multifunctional nature of this protein, interactions have been identified between VP35 and cellular factors involved in trafficking or in potential host cell defense, such as DLC8 (Kubota et al., 2009) and DSRB76 (Shabman et al., 2011) proteins, suggesting that VP35 contribution to EBOV virulence and pathogenesis is far from being exhaustively characterized.

The entire tertiary structure of EBOV VP35 is unknown and the available crystallographic data only encompass a short N-terminal peptide and the C-terminal 120 amino acids. The former is a 28 residues-long domain important for VP35 replicative function, which displaces NP protomers on the nucleocapsid to release the viral RNA and also chaperones free NP on the newly-synthesized viral genome (Kirchdoerfer et al., 2015; Leung et al., 2015; Zhu et al., 2017). The latter, termed VP35 interferon inhibitory domain (IID), encodes a dsRNA binding domain essential for suppression of innate immunity (Cárdenas et al., 2006; Hartman et al., 2004) that has been solved either alone (Leung et al., 2009, 2010b), in complex with short dsRNA molecules (Kimberlin et al., 2010; Leung et al., 2010a) and with small molecule inhibitors (Brown et al., 2014).

Located between the VP35 NP-chaperoning peptide and the IID, there is a coiled-coil (CC) domain that mediates VP35 homo-oligomerization, a process indispensable for binding to the viral polymerase L (Möller et al., 2005) and for maximal inhibition of IRF-3 pathways and suppression of IFN production (Möller et al., 2005; Reid et al., 2005). However, the presence of a complete amino acid sequence retaining homo-oligomeric properties might be also crucial for the full exertion of other VP35 functions, such as: *i*) blocking DCs maturation, since VP35 mutants lacking amino acids 1–190 were unable to abolish expression of CD80 and CD86 surface markers (Jin et al., 2010); *ii*) binding to DLC8, which was found to take place through VP35 residues 40–79 (Kubota et al., 2009); *iii*) interaction with PIAS1 and IRF-7, since VP35 N-terminal half was essential for co-precipitation (Chang et al., 2009); *iv*) association with DRBP76 protein, since full length VP35 was required for co-precipitation (Shabman et al., 2011); *v*) inhibition of PKR, since VP35 lacking residues 1–200 (dN200) failed to impair its functionality (Feng et al., 2007) and *vi*) binding to dsRNA, since the dN200 mutant failed to bind poly(IC) (Feng et al., 2007), whereas dsRNA binding affinity of full length recombinant VP35 (rVP35) was significantly higher than reported for the sole IID (Zinzula et al., 2009).

CC are α-helical motifs widely spread in proteins of eukaryotes, prokaryotes and Archaea (Liu and Rost, 2001), where they are associated with different functions, particularly oligomerization (Rackham et al., 2010). Typically, a CC structure consists of two or more amphipathic α-helices twisting around each other in the so-called “knobs-into-holes” packing. In this arrangement, apolar side chains of one helix fall into the hydrophobic core of the facing one (Grigoryan and Keating, 2008). This results in a heptad-repeating amino acid pattern, namely *abcdefg*, where residues at positions *a* and *d* are predominantly hydrophobic and influence helix orientation, structural specificity and oligomeric state (Ciani et al., 2010; Woolfson, 2005). Instead, those at positions *e* and *g* are often charged and, by forming salt bridges and electrostatic interactions, stabilize the CC structure (Woolfson, 2005).

The EBOV VP35 CC was predicted to be located between residues 82 and 118 (Reid et al., 2005), whereas a similar domain locates within residues 70–120 of the protein in the closely related Marburg virus (MARV) (Möller et al., 2005). Recently, the crystal structure of MARV VP35 oligomerization domain was presented as a trimer (Bruhn et al., 2017). Nevertheless, a CC is ubiquitously present in the oligomerization domain of a VP35-homolog, namely the phosphoprotein (P), among members of the family *Paramyxoviridae*, the closest to filoviruses in the order *Mononegavirales* (Longhi et al., 2017). Noteworthy, experimentally solved structures of this domain of P were shown to be trimeric in the case of Hendra (HeV) (Beltrandi et al., 2015) and Nipah virus (NeV) (Blocquel et al., 2013) as well as tetrameric for Measles (MeV) (Blocquel et al., 2014; Communie et al., 2013), Mumps (MuV) (Cox et al., 2013), NeV (Bruhn et al., 2014) and Sendai (SeV) (Tarbouriech et al., 2000a,b) viruses. By contrast, which quaternary structure originates from the CC assembly in EBOV VP35 is yet to be elucidated. As initially suggested by size exclusion chromatography (SEC) and chemical cross-linking, VP35 expressed in eukaryotic cells existed in an oligomeric state consistent with either trimeric or tetrameric homo-complexes, with the homo-trimeric form favored by the observation that when a truncated C-terminal VP35, which alone displayed reduced IFN-antagonism, was fused to a heterologous trimerization domain, its anti-IFN function was restored (Reid et al., 2005). Moreover, a trimer was observed by small angle x-ray scattering analysis on a VP35 deleted of its first 20 residues (Kimberlin et al., 2010), whereas SEC coupled with multi-angle light scattering (MALS) performed on truncated VP35 constructs that retained the CC domain showed tetramers in solution (Luthra et al., 2015; Bruhn et al., 2017). On the other hand, during initial characterization of the full length rVP35, we observed the formation of dimers and higher complexes that were consistent with both homo-trimers and homo-tetramers (Zinzula et al., 2009).

Here we have implemented the biochemical characterization of the oligomerization profile of EBOV rVP35 by SEC and native-polyacrylamide gel electrophoresis (PAGE). Furthermore, we have built an *in silico* 3D atomic model of the EBOV VP35 CC domain and used it to perform self-assembly homo-oligomerization simulations by means of molecular dynamics (MD). Combining biochemical observation with computational results, we propose a mechanistic and dynamic explanation for EBOV VP35 propensity to assemble into multiple homo-oligomeric species.

2. Materials and methods

2.1. SEC analysis

Full length EBOV rVP35 was expressed in *E. coli* cells and purified by IMAC as previously described (Zinzula et al., 2012). Eluted fractions were pooled and concentrated using a 10,000 MWCO Vivaspin 20 concentrator (Sartorius). Protein sample (≈1 mg/mL) was briefly centrifuged prior to injection and loaded on a Superdex 200 HR 10/30 gel filtration column (GE healthcare) of 25 mL bead volume, connected to an FPLC system (ÄKTA Purifier, GE Healthcare) and previously equilibrated in buffer A (50 mM sodium phosphate pH 7.5, 300 mM NaCl, 3% glycerol, 1 mM TCEP). Gel filtration run was carried out at 4 °C and at a flow rate of 0.4 mL/min, collecting fractions of 0.25 mL and monitoring elution at 280 nm. A calibration curve of the Superdex 200 HR 10/30 column was obtained running a molecular weight standard (BioRad) containing bovine thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa) and Vitamin B_{12} (1.35 kDa). A second molecular weight standard (Sigma-Aldrich) containing bovine thyroglobulin (670 kDa), horse apoferritin (443 kDa), sweet potato β-amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa) and bovine carbonic anhydrase (29 kDa) was also run in buffer A. The calibration curve equation was calculated to be $y = -0.23\,x + 4.74$ ($r = 0.991$) and interpolation of the peak elution volumes allowed to determine apparent molecular weights.

2.2. Native-PAGE analysis

Purified rVP35 of the same concentrated pool used for SEC was also

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