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Cryo-electron microscopy and single molecule fluorescent microscopy detect CD4 receptor induced HIV size expansion prior to cell entry

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ABSTRACT

Viruses are often thought to have static structure, and they only remodel after the viruses have entered target cells. Here, we detected a size expansion of virus particles prior to viral entry using cryo-electron microscopy (cryo-EM) and single molecule fluorescence imaging. HIV expanded both under cell-free conditions with soluble receptor CD4 (sCD4) targeting the CD4 binding site on the HIV-1 envelope protein (Env) and when HIV binds to receptor on cellular membrane. We have shown that the HIV Env is needed to facilitate receptor induced virus size expansions, showing that the 'lynchpin' for size expansion is highly specific. We demonstrate that the size expansion required maturation of HIV and an internal capsid core with wild type stability, suggesting that different HIV compartments are linked and are involved in remodelling. Our work reveals a previously unknown event in HIV entry, and we propose that this pre-entry priming process enables HIV particles to facilitate the subsequent steps in infection.

Introduction

Cell-free infectious viruses are ultramicroscopic infectious agents that are often viewed as being metabolically inert and static in structure, as viruses can only replicate within cells of living hosts. Human immunodeficiency virus (HIV) particles have electron dense cone-shaped cores, and are pleomorphic in size, with diameters that range between 100 nm and 200 nm (Briggs et al., 2003). The interactions amongst HIV envelope (Env) proteins, cellular receptor CD4 and co-receptors (CXCR4 or CCR5) during entry are well documented (Wilen et al., 2012), and a series of structural reorganisations of the viral envelope proteins and the cellular receptors must follow to facilitate membrane fusion and viral entry (Wilen et al., 2012). It is currently unknown whether structural reorganisation would occur within the internal compartments of HIV prior to entry to facilitate infection. Observations with the herpes virus system suggest that receptor engagement of pre-entry viruses may alter the internal capsid core by releasing the viral tegument protein from the capsid shell (Meckes and Wills, 2008).

The combination of the miniature- and the pleomorphic-nature of HIV (Briggs et al., 2003; Benjamin et al., 2005), and the resolution limits of conventional fluorescence microscopy techniques (Muller and Heilemann, 2013; Chojnacki and Muller, 2013) have made it difficult to discern whether any nano-scale internal structural reorganisation may exist at the pre-entry step of HIV infection. Recent applications of state-of-the-art imaging and fluorescent probing techniques have revealed previously unattainable details of HIV replication, such as the detection of surface HIV Env trimer redistribution during virus maturation (Chojnacki et al., 2012), the mechanism of tetherin-mediated HIV restriction (Lehmann et al., 2011), the assembly of HIV Gag and Pol proteins (Van Engelenburg et al., 2014; Gunzenhauser et al., 2012; Bharat et al., 2014; Schur et al., 2015; Lelek et al., 2012, 2014), the recruitment of HIV Env and ESCRT during viral assembly (Van Engelenburg et al., 2014; Muranyi et al., 2013; Prescher et al., 2015), and the structural details of HIV

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Env at the early stages of infection (Bartesaghi et al., 2013; Lyumkis et al., 2013; Munro et al., 2014).

In the current work, we have used: (1) three dimensional (3D) structural reconstruction from cryo-electron microscopy (EM) tomography; (2) two dimensional (2D) projection images from cryo-EM; and (3) cluster distributions of protein localisations recorded with direct stochastic optical reconstruction microscopy (*d*STORM) super-resolution fluorescent imaging; to show that cell-free HIV underwent size expansion upon receptor engagement with soluble CD4 and when HIV binds to cellular membrane CD4 receptor during infection. Our data showed that both the outer parameters of viral particles and the internal viral core expanded as detected by cryo-EM and *d*STORM analyses. Further, expansion was highly specific and required the engagement of the CD4 binding sites on the HIV Env. The cryo-EM virus size estimations of untreated and soluble CD4 treated HIV were recorded (blinded, and with independent operators) using either pairwise analysis and/or with multiple batches of viruses to ensure that the pleomorphic size of HIV was not a confounding factor. We have also demonstrated that blocking virus maturation or cross-linking of capsid proteins within the viral core prevents virus particle size expansions upon receptor engagement. Our *d*STORM super-resolution fluorescent imaging data on the early steps of HIV infection in T cells provided evidence that these receptor engagement mediated virus size expansions occur at the pre-entry stage of HIV infection.

Results

Cryo-EM tomograms demonstrate that soluble CD4 caused cell-free HIV to expand

Using *d*STORM super-resolution fluorescent imaging analysis, we previously reported that the clusters of both matrix (MA) and capsid (CA) proteins associated with HIV particles undergo significant redistribution after infection (Pereira et al., 2012). To independently assess whether the engagement of the primary receptor CD4 with HIV is sufficient to induce size expansion, we compared the sizes of both the viral exterior (matrix protein lattice) and the internal capsid core in cell-free viruses before and after treatment with soluble CD4 (sCD4) receptor. Both high (500 μg/ml) and low (50 μg/ml) concentrations of sCD4 were used to mimic the sCD4 suppressive concentration (Groenink et al., 1995). Blinded- pairwise-analyses were carried out with multiple operators to ensure impartiality.

In general, cryo-EM tomography is the predominant technique to determine the exact size of HIV-1 particles over conventional negative contrast EM imaging (Briggs et al., 2003; Benjamin et al., 2005; Subramaniam et al., 2007). Virus particles were identified in EM based on the presence of internal electron dense core like structure. Images of cryo-electron tomography (cryo-ET) tilt series were collected with 2° increment, and 3D re-constructions were performed to obtain structural models of viral particles (for the principles of cryo-ET, see Baumeister and Steven (2000)). By scanning through structure of various reconstructed models of partial HIV particles (that has a thickness ranging from 15 nm to 50 nm, with a slice thickness increment of 1.37 nm), the 1.37 nm slice with the largest area was assigned as the 'centre slice' of the virus. Most HIV particles are ellipsoid and deviate from a perfect sphere; however, diameter is often used as a reference to estimate the size of HIV particles. Here, diameters of virus from 3D cryo-ET data were estimated by taking the average of the distance between two perpendicular axes from the 'centre slice'.

Multiple batches of HIV were treated with low (50 μg/ml) or high (500 μg/ml) concentrations of sCD4 for independent-, blinded-, pairwise-diameter and -volumetric analyses (Figs. 1, 2, S1). Mean matrix (MA) diameter difference was detected when high (Fig. 1C and D) concentration of sCD4 was used to treat HIV. 3D cryo-ET reconstruction showed that cell-free HIV had a mean diameter of 124 nm (SEM ± 3.2 nm), which significantly increased ($P < 0.01$) to 134 nm (SEM ± 2.5 nm) after incubation with high concentrations of soluble CD4 (Fig. 1C and D).

As cryo-ET 3D reconstruction was not feasible for large amounts of 'full volume' virus particles, we compared the volumes within the MA lattice and the capsid (CA) core before and after sCD4 treatment using the 15 nm thick central sections (partial virus volume) of the cryo-ET reconstructions (Figs. 1E–H, 2). A significant size difference of central MA volume of 33% ($P < 0.01$) was detected when the viruses were treated with high concentrations (500 μg/ml) of sCD4 (Fig. 2A and B). Likewise, a significant volume increase of 26% was detected with the 15 nm thick central capsid slice (Fig. 2C and D). Treatment of HIV with low concentrations of sCD4 (50 μg/ml) did not yield significant changes in diameter (Fig. 1A and B) or 15 nm central volume of the matrix (Fig. 2E and F), but a significant increase within the 15 nm central volume of capsid was detectable (Fig. 2G and H).

CD4 receptor mediated pre-entry virus size expansion is supported by 2D cryo-EM projection and dSTORM super-resolution fluorescence image analyses

As HIV is pleomorphic, the low throughput nature of cryo-EM tomography limits the number of conditions that can be sampled to dissect the biological details of this pre-entry HIV Env and CD4 receptor interaction. We therefore examined whether alternative and more rapid procedures could be used as independent tool to validate that soluble receptor engagement induced virus size expansion. Six batches of viruses and nearly 1500 HIV particles of cryo-EM 2D projections (Fig. 3A and B) were used for independent pairwise, blinded virus diameter estimations (Fig. 3A and E). Diameters were calculated as the average distance between two perpendicular axes of the 2D cryo-EM projection images. All six batches of viruses consistently showed an increase of virus diameter upon sCD4 treatment, which increased from 122.6 nm (SEM ± 2.4 nm) to 133.8 nm (SEM ± 2.3 nm) (Fig. 3C and D).

*d*STORM super-resolution imaging was also used to independently assess whether virus-associated MA and CA molecules redistribute upon receptor engagement. The locations of viral proteins (MA and CA) were estimated using appropriate primary anti-viral protein antibodies and corresponding species-specific secondary antibodies that are tagged with *d*STORM compatible fluorophores. A localisation precision of 20 nm resolutions was achieved with *d*STORM (Fig. 4). Virus particle-associated GFP-Vpr was used as a locator to identify true virus-associated MA or CA proteins from background (Fig. 4A and B). Gaussian fitting model, cluster detection and alignment were used to determine the morphological features and the projected dimensions of the virus-associated MA and CA clusters (Fig. 4C and G). Repeated analyses showed that both the cluster areas of virus-associated MA and CA molecules have increased significantly upon sCD4 treatments (Fig. 5A and F), supporting the notion that cell-free HIV underwent size expansion upon sCD4 engagement.

*d*STORM comparison between (1) the newly formed HIV from infected Jurkat cells; and (2) HIV particles that have been arrested at the early stages of infection using CXCR4 co-receptor blocker AMD3100 (Donzella et al., 1998) also revealed an increase of the clustering areas of MA molecules just prior to virus entry (Fig. 5G–I), showing that the size expansion of HIV also occurs in natural infection process prior to virus entry.

Although both cryo-EM 2D and *d*STORM analyses are less well suited to estimate the true dimensions of the virus particles