Nucleocapsid-like Structures of Ebola Virus Reconstructed Using Electron Tomography

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ABSTRACT. Electron tomography (ET) is a new technique for high resolution, three-dimensional (3D) reconstruction of pleiomorphic macromolecular complexes, such as virus components. By employing this technique, we resolved the 3D structure of Ebola virus nucleocapsid-like (NC-like) structures in the cytoplasm of cells expressing NP, VP24, and VP35: the minimum components required to form these NC-like structures. Reconstruction of these tubular NC-like structures of Ebola virus showed them to be composed of left-handed helices spaced at short intervals, which is structurally consistent with other non-segmented negative-strand RNA viruses.

KEY WORDS: Ebola virus, electron tomography, nucleocapsid.

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Transmission electron microscopy (TEM) has been a useful technique for examining fine structures of a variety of objects, its primary limitation being that the information derived is only in 2 dimensions. Electron tomography (ET), however, allows high resolution, three-dimensional (3D) reconstruction of macromolecular complexes, such as cells and organelles. While the principle of ET was outlined more than three decades ago [4], only recently has the value of this technique been demonstrated and its use gained popularity. ET involves collecting a tilt series of images of an object, which are then computationally combined to obtain a 3D density map [1, 7]. In addition, ET permits the visualization of pleiomorphic macromolecules, thereby offering a distinct advantage over single particle analysis, which is restricted to the reconstruction of symmetrical objects. Thus, ET is a powerful tool for acquiring 3D images of virus components and their spatial arrangement within infected cells.

Ebola virus is an enveloped, non-segmented, negative-strand RNA virus. It is a member of the family *Filoviridae*, in the order of *Mononegavirales* [13] and causes a syndrome known as hemorrhagic fever. Its enveloped virions are filamentous, measuring approximately 80 nm in diameter. Along the central axis of each virion resides a tubular nucleocapsid. As infection progresses, a large number of these nucleocapsids are synthesized in the cytoplasm of the virus-infected cells [8]. The nucleocapsid is thought to be a helical structure (50 nm in diameter), with the helices spaced at 5 nm intervals. It contains an axial channel of 10–15 nm in diameter surrounded by a high electron-dense layer of 20 nm in diameter [6]. Recently, conventional TEM demon-

strated that three proteins, nucleoprotein (NP), putative minor matrix protein (VP24), and polymerase cofactor (VP35) were necessary and sufficient for the formation of nucleocapsid-like (NC-like) structures in a mammalian expression system [10].

To obtain 3D structural information on these NC-like structures, we used ET to study NC-like structures present in the cytoplasm of cells expressing NP, VP24, and VP35.

First, to confirm the formation of the NC-like structures, we transfected 10⁶ human embryonic kidney (293T) cells with 2 µg of pCEZ-NP, 2 µg of pCEZ-VP24, and 2 µg of pCEZ-VP35, which express NP, VP24, and VP35, respectively [14]. At 72 hr post-transfection, 293T cells expressing these viral proteins were prefixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), and washed with the same buffer. After being post-fixed with 2% Osmium tetroxide and washed with distilled water, the specimen was dehydrated using an ethanol gradient and sequential propylene oxide exposure. The specimen was then embedded in Epon 812 Resin mixture, and polymerized at 70°C for 2 days. Thin sections were stained with uranyl acetate and lead citrate and subsequently examined with a JEM-1200EX electron microscope at 80 kV. These sections revealed NC-like structures in the cytoplasm, as previously reported by Huang *et al.*, 2002. The NC-like structures (50 nm in diameter) possessed a central axial space (10–15 nm in diameter) (Fig. 1A). In addition, they were filamentous and showed helical structures spaced at short intervals (Fig. 1B).

We then carried out a 3D structural analysis of the NC-like structures in plasmid-transfected cells. Sections (approximately 200 nm thick) were affixed on both sides with 10 nm-colloidal gold particles, which served as markers for subsequent image alignment. The grid was then placed on a CompStage specimen holder (FEI Company

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Fig. 1. NC-like structures produced by the expression of NP, VP24, and VP35. Transverse section shows NC-like structures of 50 nm in diameter with an axial space of 10–15 nm in the center (A). Longitudinal section of NC-like structures (B). Bars; 100 nm.

Japan Ltd.) and observed in a TECNAI G2 SHERA (FEI Company Japan Ltd.) at 200 kV. Electron micrographs were taken at 50,000 magnification every 2° (range from -70° to +70°) and recorded with a CCD camera (1024 Å–1024 pixels with 0.49 nm/pixel). The alignment of the projections was calculated by R-weighted back projection with IMOD software [11], and these calculations were then used to construct 3D images of the NC-like structures. Reconstructed NC-like structures were visualized with Amira software (Template Graphics Software Inc.). Figure 2 shows galleries of 3D images of NC-like structures at lower and higher magnifications. At the lower magnification, filamentous NC-like structures with an axial channel in the center can be clearly observed (Fig. 2A). Further, ET revealed that these NC-like structures are aligned but are not perfectly parallel, information that has not been obtained by conventional TEM. At the higher magnification (Fig. 2B), the helical grooves spaced at short intervals on the surface of the NC-like structures are more visible than those observed in longitudinal sections by conventional TEM (Fig. 1B). In addition, we were able to determine that the helices were left-handed (Fig. 2B), a finding made only with ET. Thus, ET is a superior tool for acquiring detailed 3D structural information.

The left-handed helix of nucleocapsids has not been reported previously for Ebola virus, but has been documented for other non-segmented negative-strand RNA viruses, such as Sendai virus [5], measles virus [2], simian virus 5, and mumps virus [3]. The nucleocapsids of these viruses also comprise filamentous structures with an axial space in the center, identical to those of Ebola virus. Although the biological significance of this left-handed helix is currently unclear, the structural similarity among these negative-strand RNA viruses suggests common mechanisms

in the morphogenesis of nucleocapsids, incorporation of viral RNAs into nucleocapsids, and transcription and replication of viral RNAs. For this reason, information obtained by further studies on Ebola virus nucleocapsids would likely be applicable to these other viruses.

In conclusion, we have used ET to obtain more detailed structural information on Ebola virus NC-like structures than has been obtained to date using conventional TEM analysis. The ET technique can be applied to complexes as large as an entire virus particle [9], and when combined with cryo-electron microscopy, can provide 3D structures of individual pleiomorphic macromolecules at a near-molecular resolution [12]. Thus, ET is a powerful method to generate fundamental structural information and its application in diverse biological fields should provide a wealth of information regarding the three-dimensional arrangement of cellular components.

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Fig. 2. Reconstructed NC-like structures by ET. Reconstructed NC-like structures were obtained from a 141 tilt series of 200 nm-thick sections, reconstructed by R-weighted back projection with IMOD, and visualized with Amira software. The galleries of 3D NC-like structures are shown at a low (A) and a high (B) magnification.

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