The Cell Biology of Coronavirus Infection

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1. INTRODUCTION

Several aspects of coronavirus interaction with host cells have been identified, the best characterized of which involve virus entry and virion assembly (for review, see Holmes and Lai, 1996). These accomplishments are critical in the understanding of virus-cell interactions; however, since such interactions occur in four dimensions (over time in the three-dimensional space of the cell) they are important landmarks on a path that for the most part remains unilluminated. To understand the dynamic relationship of the virus to the cell it is important to use approaches that can observe interactions in the context of the entire cell and throughout the course of infection.

More recently, several groups have begun to investigate the formation and fate of coronavirus replication complexes in virus-infected cells (Bi *et al.*, 1998; Denison *et al.*, 1999; Shi *et al.*, 1999; van der Meer *et al.*, 1999). Our studies on the cell biology of coronavirus infection have been based on the observation that viral RNA synthesis localizes to punctate cytoplasmic complexes that are distinct from known sites of virion assembly in the ER-Golgi-intermediate complex (ERGIC) (Bost *et al.*, 2000; Sims, Ostermann, and Denison, 2000). These results have raised questions concerning the mechanisms by which the newly synthesized viral RNA is delivered to sites of virion assembly. Answers to these questions will allow us to define the viral and cellular proteins involved in replication and transport of RNA. We have developed methods for imaging multiple MHV proteins, cellular proteins, and cytoskeletal elements MHV cells, both in fixed and live cells.

We have used confocal microscopy to generate three-dimensional reconstructions of infected cells and have investigated virus-induced changes in cells over time using video microscopy. We have demonstrated that the replication complexes vary during the course of infection, and that it is critical to observe the entire context of the cell over time to draw accurate conclusions concerning the formation, function and fate of viral replication complexes.

2. METHODS AND MATERIALS

2.1 Virus Infection and Immunofluorescence

DBT cells were grown on glass coverslips as previously described (Bost *et al.*, 2000). In general, cells were seeded at lower densities and grown for 36-48 h prior to infection. Cells were infected at moi's of 10-20 pfu/cell. Cells were fixed and permeabilized by aspiration of media and direct addition of 100% methanol (-20°C) to the well. Cells were fixed for at least 1 hr prior to processing for immunofluorescence. Cells were processed for immunofluorescence as previously described (Denison *et al.*, 1999).

2.2 Confocal Microscopy

All fixed cell images were acquired on Zeiss LSM 410 laser scanning confocal microscope (Denison *et al.*, 1999). Unless otherwise stated, images were acquired using lasers at 488nm(green) and 568nm (red). Images were obtained with brightness and contrast settings to optimize the resolution and maximize the signal-to-noise ratio. Images were acquired using a 40x, NA 1.3 oil objective at a magnification of 3.1 to obtain resolution in x,y and z dimensions of ~0.2 μm /pixel. The confocal pinhole was set to optimize the confocal "slice" size to create a non-overlapping volume set.

2.3 Live Cell Imaging

DBT cells were grown on glass 40mm glass coverslips and infected as above. Prior to imaging the coverslip was transferred to a Biopetechs FCS live-cell imaging chamber. Cells were simultaneously imaged using Nomarski DIC and fluorescence. Cells were transfected with constructs expressing viral N fused to enhanced green fluorescent protein (GFP). For experiments in this report, images were obtained at 30 second intervals for up to 8 h, beginning 3-4 h p.i. For maximum brightness over time, fluorescent signal was acquired using "nonconfocal" settings.

2.4 Post-Acquisition Image Processing

Images were acquired in the LSM software as 512 x 512 pixel TIFF images. Image modifications were made using Adobe Photoshop 5.0, NIH image 1.62, Adobe Premiere 4.0, and Apple Quicktime 4.0 Pro. Original data was archived as LSM TIFFs prior to any image modification. To reconstruct 3D images of fixed cells, “stacks” of individual confocal images were constructed, and rotations of the x,y,z volume were made.

3. RESULTS AND DISCUSSION

3.1 3D Reconstructions of MHV-Infected Cells Show the Relationships of Gene 1 and Structural Proteins

When MHV infected cells were dual-probed for the gene 1 protein p65 and the structural M protein at 6.5 h p.i. the proteins appeared to be partially co-localized when viewed by nonconfocal indirect immunofluorescence.

Figure 1. Three dimensional reconstruction of MHV infected DBT cell. The left image shows the x/y image with a flattened “z projection” of 70 “slices” (14µm in z depth). Labeling for p65 as medium grey and M is white. Horizontal lines (a-h) represent location of reslicing of the x/y/z volume set perpendicular or “into” the plane of the image. The respective x/z slices are seen to the right as viewed from the direction of the white arrow in the image to the left. The location of foci of M on both sets of images are shown by the white arrowheads. The “bottom” of each image to the left represents the plane of the coverslip.

However, when the same cell was imaged using confocal settings, the apparent coincidence of signals was eliminated and p65 and M had entirely distinct patterns of distribution with little or no overlap. To determine the three-dimensional relationship of M and p65, 70 images were obtained at 0.2 μ m intervals in Z, the images were “stacked” and the stack or volume data was used to analyze the relationship of p65 and M (Fig. 1). The volume was analyzed in rotations and stereo images, or post image “reslicing” of the cell in different planes. When the dual-labeled, infected cell was analyzed in this way, it was immediately apparent that the proteins were localized to extensive structures that were organized in distinct parts of the cell. P65 localized to punctate structures that were generally in the peripheral cytoplasm and not tightly associated with the nucleus. In contrast, M was localized to structures closely juxtaposed to the nucleus and predominantly eccentrically localized to one side of the nucleus, consistent with the distribution of the (Fig. 1). In addition, the reconstructions demonstrated that the perinuclear distribution of M was “above” the cytoplasmic foci of p65 when the coverslip was viewed as the “bottom” of the cell. These reconstructions also revealed the relative height in z of the nucleus compared to the more distributed cytoplasm.

3.2 Live Cell Imaging of MHV-Infected Cells

The distribution of gene 1 and structural proteins showed consistent patterns across entire monolayers, but it was also obvious that there was evolution of the pattern depending on the time p.i. and on whether the cells were involved in syncytia formation. We used a live cell imaging closed chamber system to analyze changes occurring in infected cells over time (Fig. 2). To assess the directionality of fusion and distribution of intracellular contents, we overexpressed N as a fusion with enhanced GFP in the context of infection. What we observed was active movement and division of cells in the monolayer for the first four hours followed by loss of sharpness of cell membrane margins of closely approximated cells. This was succeeded by rapid fusion of cell membranes and coalescence of nuclei and all easily visible intracellular membrane-bound organelles to the center of syncytia. In addition, we observed that “recruitment” of cells into syncytia was often initiated by contact of extended processes, followed by fusion at the point of contact and rapid movement of the uninvolved cell toward the multinucleated cell. Our results suggested that the process of syncytia formation involved the linking of cytoskeletal elements and was active rather than passive. Although syncytia have not been thought to be critical in the normal lifecycle and pathogenesis of MHV infection, our images raise the possibility that cell contact through extended filopodia might play a role in cell-to-cell transmission of virus. GFP:N was not

Figure 2. Confocal Video Microscopy of MHV-infected DBT cells expressing an N-GFP fusion protein. Cells were transfected with constructs expressing N protein fused to enhanced green fluorescent protein (GFP:N) for 48 h, infected, and imaged from 4-12 h p.i. Simultaneous DIC and fluorescent images obtained every 30 seconds. Equally spaced images are shown. The black signal in cells and syncytia indicates expression of GFP-N. The black arrowhead shows the sites of filopodia contact, directional recruitment, and incorporation of cell contents.

detected in the nuclei of transfected cells, but was detected in the cytoplasm in both a diffuse cytoplasmic distribution and in punctate foci of various size that were never seen with GFP expression alone. The punctate character of GFP:N foci was retained even during the alterations of cells during syncytia formation. Finally, rapid directional redistribution of the contents of one cell into a syncytium was frequently observed, supporting the concept of active involvement of cytoskeletal elements such as actin and/or microtubules in MHV syncytia formation.

4. SUMMARY

The ability to obtain entire volume data on infected cells will allow us to define much more accurately the interactions of viral proteins with host cell structures such as ER, Golgi, and cytoskeletal elements. In addition, the demonstrated ability to express viral proteins fused to fluorescent markers in live cells will allow us to follow specific proteins or complexes during the course of infection and to determine if exogenously expressed proteins are able to target to sites of active viral replication. This in turn will allow new approaches to the study of viral and cellular protein-protein interactions, as methods to study the biology and pathogenesis of MHV infection at a cellular level. Finally, the approaches described here will allow us to define

protein complementation of defective viruses at a cellular level, rather than being dependent on population measurements of RNA, protein, or progeny virus. By combining these approaches with available biochemical and molecular biological approaches and the emerging reverse genetic and recombinant genetic approaches, rapid progress in understanding the details of coronavirus-cell interactions should be possible.

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