Comparison of Replicase Localization in Different Types of Mouse Hepatitis Virus (MHV)-infected Cells

RAINER GOSERT[1], AMORNRAT KANJANAHALUETHAI[2], DENISE EGGER[1], KURT BIENZ,[1] AND SUSAN C. BAKER[2]
[1]*Institute for Medical Microbiology, Basel, Switzerland.* [2]*Department of Microbiology and Immunology, Loyola University of Chicago, Stritch School of Medicine, Maywood, IL, USA*

1. INTRODUCTION

The replication complex (RC) of virtually all positive strand RNA viruses has been shown to be intimately associated with cellular membranes. However, different RNA viruses seem to target or recruit distinct membranes for the assembly of their RCs. For example, poliovirus replicates its RNA on the surface of membranous vesicles which seem to evolve from the endoplasmic reticulum (ER). The vesicles form a rosette-like structure (Bienz *et al* 1992; Egger *et al* 1996). For tobacco etch potyvirus viral replication takes place at ER-derived vesicles and results in a collapse of the ER (Schaad *et al* 1997). In contrast, alphaviruses appear to use the cytosolic surface of endocytic organelles for the formation of their RCs (Froshauer *et al* 1988). For Nidoviruses such as arteriviruses and coronaviruses, the story is even more complex. Recently, it was shown that equine arteritis virus (EAV) generates ER-derived double-membrane vesicles (DMVs) (Pedersen *et al* 1999). In contrast, studies of the coronavirus mouse hepatitis virus (MHV) implicated a role for late endosomes in the formation of the RC (van der Meer *et al* 1999). Recently, we have shown that translation products of the MHV replicase gene localized to different membrane structures in different cell lines. For a human cell line, viral replicase products colocalized with golgi markers, but in a murine cell line, the viral products and ER-derived membranes colocalized (Shi *et al* 1999). To extend our studies, we wanted to determine the viral and cellular factor(s) that drive the generation of the MHV RC at distinct membranes in the different cell lines.

The Nidoviruses (Coronaviruses and Arteriviruses).
Edited by Ehud Lavi *et al.*, Kluwer Academic/Plenum Publishers, 2001.

To investigate the localization of the MHV RC, we initiated ultrastructural studies. Here, we present our preliminary analysis in the electron microscope (EM) and report that our results supported our earlier localization studies. We detected a difference in the distribution of MHV-induced DMVs in the human and murine cell lines. We also initiated biochemical studies to identify MHV proteins that allow the assembly of a membrane-associated RC. We found that the MHV replicase product p44 acts as an integral membrane protein, implicating it as a likely membrane-anchor protein for the MHV RC.

2. MATERIALS AND METHODS

2.1 Cell lines

17Cl-1 cells were maintained and passaged as described by Sturman and Takemoto (1972). HeLa-MHVR cells, expressing high levels of the MHV receptor, were maintained and passaged as described by Gallagher (1996).

2.2 Triton X-114 extraction

HeLa-MHVR cells were infected with MHV-A59 at moi 1. Newly synthesized proteins were radiolabeled with 100 μCi/ml of (^{35}S)-translabel from 3.5-5.5 h post-infection (pi) and extractions were carried out essentially as described by Border (1981). Pellet and soluble fractions were subjected to immunoprecipitation as previously described (Schiller *et al* 1998).

2.3 Electron microscopy

Confluent monolayers of HeLa-MHVR or 17Cl-1 cells were infected at moi 1 with MHV-A59. At 5 h pi, cells were fixed with 2.5% glutaraldehyde and 2% OsO_4 and embedded in Poly/Bed 812 (Polysciences) according to standard protocols. The ultrathin sections were examined with a Philips CM 100 EM.

3. RESULTS AND DISCUSSION

In this study, we describe our preliminary biochemical and ultrastructural analysis of the MHV RC. We wanted to determine which replicase subunit(s) may act as putative integral membrane proteins to anchor the RC to intracellular membranes and to determine if MHV-infection induces the

formation of intracellular vesicles which are likely the sites for MHV replication.

Our first approach was to characterize MHV replicase products biochemically using Triton X-114 extraction (Bordier 1981). This procedure separates proteins principally by their hydrophobic properties, with hydrophobic, integral membrane proteins partitioning into the detergent phase, while hydrophilic proteins remain in the aqueous phase. As shown in Figure 1, the amino-terminal replicase product, p28, remains in the soluble fraction whereas the putative membrane-spanning protein 1, MP1 (p44), is detected in the membranous pellet fraction. As expected, MHV virion integral membrane matrix protein M, partitions to the membranous pellet fraction. Overall, these results show that the MHV replicase product MP1 (p44) does indeed act as an integral membrane protein and may serve as an important anchor protein for the RC.

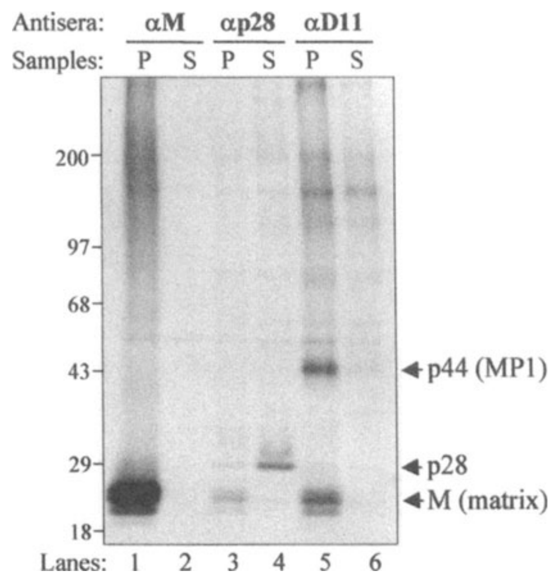

Figure 1. Triton X-114 extraction analysis of MHV replicase products. A schematic diagram depicting the region to which the specific antisera was generated and a map of the known MHV replicase products of ORF1a is shown at the top. MHV products detected after extraction with Triton X-114 and immunoprecipitation with specific antisera are shown below. The pelleted (P) fraction contains integral membrane proteins such as the matrix (M) protein of the MHV virion. The supernatant (S) fraction contains cytosolic proteins and non-integral membrane proteins.

Our second approach was to examine MHV-infected cells by EM to determine intracellular membrane alterations. We tested two distinct cell lines, human cells encoding the MHV receptor, HeLa-MHVR cells, and a mouse fibroblast cell line, 17Cl-1 cells. Our previous immunofluorescene results indicated that the MHV replicase products localized to different sites in these two cell lines (Shi *et al* 1999), therefore we wanted to investigate these distinct localization patterns at the EM level.

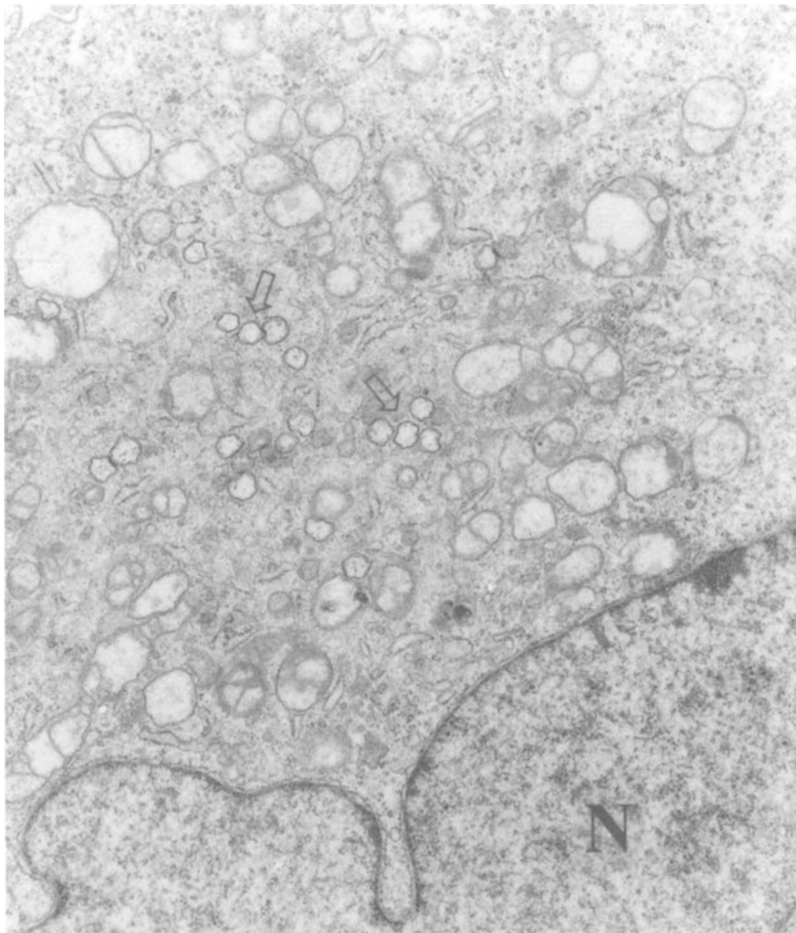

Figure 2. EM analysis of intracellular membrane structures in MHV-A59 infected HeLa-MHVR cells at 5 h pi. Magnification: 15,000x. N: Nucleus. MHV-induced vesicles are indicated by the open arrows.

MHV-infected HeLa-MHVR cells contained virus-induced vesicles in the cytoplasm that were found alone or in small clusters (Figure 2). The vesicle membranes appear as electron dense structures, with a thickness

more consistent with double membranes. High resolution images indicate that the vesicle membranes consist of 2 lipid bilayers (data not shown). EM analysis of MHV-infected 17Cl-1 cells revealed similar, virus-induced vesicles (Figure 3). However, fewer vesicles were detected and the vesicles were found primarily as vesicle clusters. We also noted a clear dilatation of the ER in MHV-infected 17Cl-1 cells, while the golgi complex remained intact at 5 h pi (data not shown).

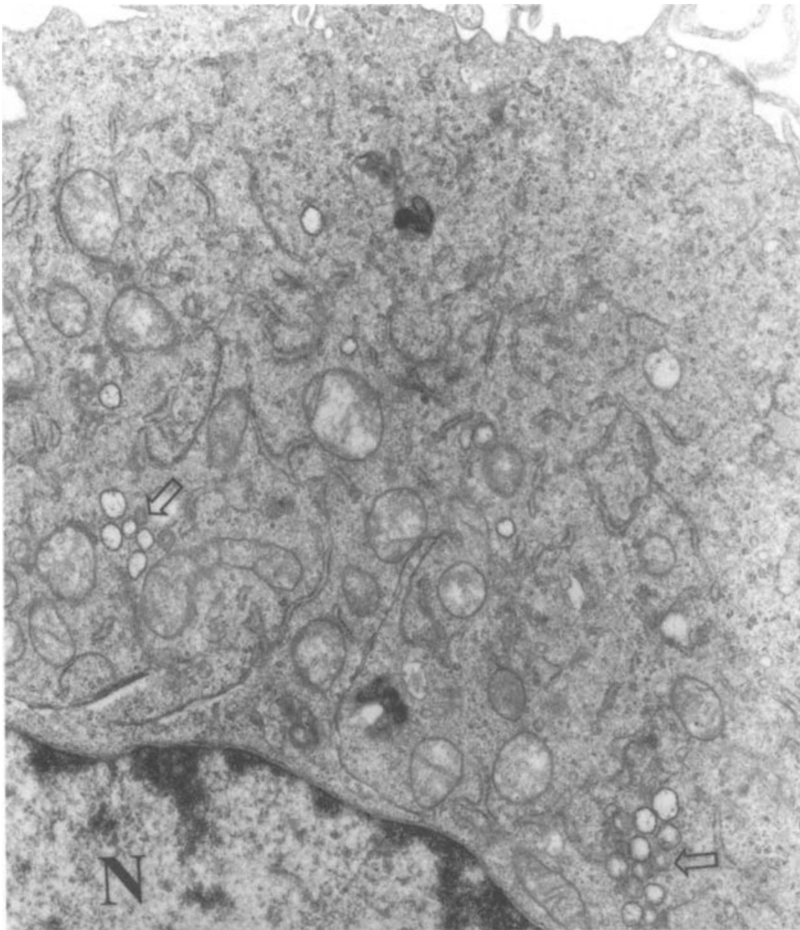

Figure 3. EM analysis of intracellular membrane structures in MHV-A59 infected 17Cl-1 cells at 5 h pi. Magnification: 15,000x. N: Nucleus. MHV-induced vesicle clusters are indicated by the open arrows. Swollen ER is seen in the central area of the picture.

In MHV-infected HeLa-MHVR cells, the large number of vesicles in a clustered area seen by EM compared well to the intense gene 1 translation

product staining that colocalized with markers of the golgi complex shown by IF studies in the confocal microscope (Shi *et al* 1999). For MHV-infected 17Cl-1 cells, the dispersed vesicle clusters detected by EM correlate with a perinuclear punctuate signal of viral proteins. Further analysis including immuno-EM and in situ hybridization with MHV-specific riboprobes will be required to characterize the protein and RNA content of the DMVs. Detection of newly synthesized RNA by BrUTP incorporation at the ultrastructural level will identify the location of MHV replication. In addition, important questions remain concerning which viral and cellular factor(s) drive the formation of the vesicles and how these factors influence vesicle distribution in different cell lines. These studies should be helpful in defining the viral and cellular processes that drive MHV RNA synthesis.

ACKNOWLEDGEMENTS

This work was supported by Public Health Service Research Grant AI 32065 (to S.C.B.) and grant 31-055397.98/1 from the Swiss National Foundation (to K.B.).

REFERENCES

Bienz, K., Egger, D., Pfister, T., and Troxler, M. 1992. Structural and functional characterization of the poliovirus replication complex. *J. Virol.* **66**:2740-2747.

Bordier C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* **256**:1604-1607.

Egger, D., Pasamontes,L., Bolten,R., Boyko,V., and Bienz, K. 1996. Reversible dissociation of the poliovirus replication complex: Functions and interactions of its components in viral RNA synthesis. *J. Virol.* **70**:8675-8683.

Froshauer, S., Kartenbeck, J. and Helenius, A. 1988. Alphavirus RNA replicase is located on the cytoplasmic surface of endosomes and lysosomes. *J. Cell Biol.* **107**:2075-2086.

Gallagher, T.M. 1996. Murine coronavirus membrane fusion is blocked by modification of thiols buried within the spike protein. *J. Virol.* **70**:4683-4690.

Pedersen, K.W., van der Meer, Y., Roos, N., and Snijder, E.J. 1999. Open reading frame 1a-encoded subunits of the arterivirus replicase induce endoplasmic reticulum-derived double-membrane vesicles which carry the viral replication complex. *J. Virol.* **73**:2016-2026.

Schaad, M.C., Jensen, P.E., and Carrington, J.C. 1997. Formation of plant RNA virus replication complexes on membranes: Role of an endoplasmic reticulum-targeted viral protein. *EMBO J.* **16**:4049-4059.

Shi, S.T., Schiller, J.J., Kanjanahaluethai, A., Baker, S.C., Oh, J.-W., and Lai, M.M.C. 1999. Colocalization and membrane association of mouse hepatitis virus gene 1 products and de novo-synthesized viral RNA in infected cells. *J. Virol.* **73**:5957-5969.

Sturman, L.S., and Takemoto, K.K. 1972. Enhanced growth of a murine coronavirus in transformed mouse cells. *Infect. Immun.* **6**:501-507.

van der Meer, Y., Snijder, E.J., Dobbe, J.C., Schleich, S., Denison, M.R., Spaan, W.J.M., and Locker, J.K. 1999. Localization of mouse hepatitis virus nonstructural proteins and RNA synthesis indicates a role for late endosomes in viral replication. *J. Virol.* **73**:7641-7657.