CORONAVIRUS MATURATION

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INTRODUCTION

Coronaviruses cause a wide variety of diseases which includes both acute infections like infectious bronchitis of chickens and persistent infections like infectious peritonitis of cats. Current studies on the molecular biology of coronaviruses will provide essential information on the nature of the virus-cell interactions which can lead to such different outcomes of viral infection. This article will analyze the interactions of coronaviruses with host cells in order to identify host cell dependent processes which are essential for coronavirus growth. Coronavirus infection of a cell can have different results as shown in Table 1.

Since coronaviruses usually exhibit very limited cell and species tropisms, it is apparent that interactions of many cell types with coronaviruses do not result in infection. The molecular basis for this resistance to infection has not been determined. One plausible explanation would be that coronaviruses may require very specific receptors which are only found on the plasma membranes of susceptible cells.

Several types of abortive infections with coronaviruses have been identified. Abortive infection with mouse hepatitis virus (MHV) occurs in macrophages or hepatocytes of mice from strains

Table 1. Possible Results of Coronavirus Infection

No infection

Abortive infection

Productive infection

- a. Leading to cell death
  - 1. By cell fusion
  - 2. By cell lysis
- b. Leading to persistent infection
  - 1. With continued viral replication
  - 2. Without continued viral replication

genetically resistant to MHV.^{2,3} Little viral protein or RNA synthesis can be detected in these cells, but the mechanism of host cell restriction of viral replication has not yet been identified. Abortive infections may also result from MHV infection of a variety of differentiated host cell types in genetically susceptible animals. For example, in primary differentiated cultures of mouse spinal cord, virus production in glial cells was abundant whereas only limited virus replication could be detected in neurons.⁴ The strain of virus is also important in determining whether a given cell type will undergo abortive infection with a coronavirus. The neurotropic JHM strain of MHV can infect neurons in vitro much more readily than can the A59 strain of MHV.⁴ Some types of abortive infection may result in the accumulation of viral structural components within the cytoplasm. A variety of coronavirus inclusions have been described.⁵

Productive infection of a susceptible cell by a coronavirus frequently results in cell death. Unlike many other types of viruses, coronaviruses do not appear to induce rapid inhibition of host cell macromolecular synthesis. Indeed, coronavirus maturation appears to require continuation of many normal functions of the host cell. Death of infected cells is due to cell fusion or cell lysis. It is not clear what viral factor leads to cell lysis. Some coronaviruses such as bovine coronavirus (BCV)⁶ may induce lytic plaques without cell fusion in some cell types. Coronavirus induced fusion occurs in vitro and in vivo.⁷ We have recently shown that fusion of L2 cells by MHV-A59 depends on proteolytic cleavage of the E2 peplomeric glycoprotein.⁸

Because of the lack of virus-induced inhibition of cellular macromolecular synthesis and the non-lytic release of virions from infected cells, persistent coronavirus infections can be readily established in vitro and in vivo. The virus-cell interactions in persistent coronavirus infections vary considerably. Some persistently⁹ infected cultures show active virus production from all cells, while in others such as our

Figure 1. Coronavirus Replication
(Reproduced from 5 with permission)

persistent infection of 17 Cl 1 cells by MHV-A59, only 10 to 20% of the cells produce viral antigens and virions.¹⁰ In the remaining cells some viral nucleic acid must be present since infectious virus can be recovered after cloning and cocultivation with susceptible cells. MHV can also induce a persistent infection in which viral antigens are not detectable in any cells, although virus can be rescued by cocultivation.¹¹ During persistent infection, virus mutants may be selected which are less virulent (small plaque, temperature sensitive, and/or fusion defective) and cells may be selected, which are resistant to the cytopathic effects of coronaviruses.^{10,11} The specific changes in the viruses and cells during persistent infection have not yet been characterized, but may elucidate important virus-host control mechanisms.

HOST CELL PROCESSES REQUIRED FOR CORONAVIRUS MATURATION

The events that occur in a cell infected with a coronavirus will be summarized in order to identify cellular processes that are used for virus replication. These are illustrated in Figure 1.

Binding of coronaviruses to cells takes place by means of the peplomeric glycoprotein E2 which binds to specific receptors on the plasma membrane. Some cells such as spleen cells have large numbers of receptors, whereas others have few or no receptors. It is not yet clear whether coronaviruses enter cells

by fusion of the viral envelope with the plasma membrane or by an endocytic pathway.

Virion RNA can apparently act as mRNA to direct the synthesis of a viral specific RNA dependent RNA polymerase. This enzyme has been identified as early as 1 hour after infection.¹² The enzyme directs the synthesis of a full length negative strand of RNA and then synthesis of genomic RNA and a nested set of 6 subgenomic mRNAs.¹³ No host dependent factors have yet been identified in this aspect of coronavirus replication.

The synthesis of viral polypeptides does depend on many cellular mechanisms. These include synthesis on free ribosomes of the nucleocapsid protein, N, and synthesis on membrane bound ribosomes of E1 and E2¹⁴ and possibly some N.

Phosphorylation of the nucleocapsid protein, N,¹⁵ may use a cellular protein kinase. Acylation of the E2 glycoprotein but not the E1 glycoprotein has been demonstrated.¹⁶ This is probably also a cell dependent modification.¹⁷

Glycosylation of the two viral glycoproteins of MHV-A59 appears to depend upon two different host cell pathways. Oligosaccharides of the E2 glycoprotein are attached to asparagine residues by N-glycosidic bonds. The oligosaccharides of the E1 glycoprotein are markedly different, and are attached post-translationally by a tunicamycin resistant mechanism^{16,18,19} to serine and threonine residues via O-glycosidic bonds. Thus MHV-A59 may be a useful model system for study of the formation of O-glycosidic bonds.

Proteolytic cleavage of viral proteins may depend either on host cell or virus specific proteases. For coronaviruses, there is no evidence to suggest viral specific protease activity. As recently demonstrated in our laboratories,⁸ the E2 glycoprotein of the peplomers of MHV undergoes a proteolytic cleavage to yield two cleavage products of identical molecular weight which we have called 90A and 90B. This cleavage is critical for the activation of MHV induced cell fusion.

Coronaviruses also make use of two different pathways for the intracellular transport of glycoproteins. We have used monospecific antisera to the E1 and E2 glycoproteins²⁰ to study the intracellular transport of these glycoproteins.²¹ We found that E2 is rapidly dispersed throughout the cytoplasmic membrane system and appears on the plasma membrane much like N-linked glycoproteins of other enveloped viruses. In contrast,²¹ the E1 glycoprotein did not migrate past the Golgi apparatus. These two different patterns of intracellular transport are also observed with monoclonal antibodies to E1 and E2 as shown in

Figure 2. Different patterns of intracellular transport of the two glycoproteins of MHV-A59. Fluorescent antibody labeling with monoclonal antisera to the peplomeric glycoprotein E2 (A) and to the membrane glycoprotein E1 (B) shows that E1 is limited to the Golgi apparatus while E2 migrates throughout the cytoplasmic membranes.

Figure 2. We have suggested that the localization of E1 to the rough endoplasmic reticulum (RER) and Golgi apparatus determines the intracellular location of virus budding.²¹

A variety of experiments has suggested the functions of the E1 and E2 glycoproteins that are listed in Table 2.

To determine the order in which MHV-A59 glycoproteins are synthesized, processed and incorporated into virions, cell fractionation, and pulse labeling studies have been performed.^{16,21} On the RER, E1 is not glycosylated and sugars are added post-translationally in the Golgi apparatus.¹⁶ Although core sugars of E2 are apparently added to the 120K apoprotein during translation on the RER, the trimming and elaboration of the oligosaccharides probably occurs in the Golgi apparatus. The proteolytic cleavage of E2 appears to be a host²¹ dependent process which occurs at or near the plasma membrane. These processes are illustrated in Figure 3.

We have observed that the proteolytic cleavage of the E2 glycoprotein is cell dependent. There is considerable variation in the ratio of the 180K to the 90K forms of E2 in virions released from different cell types as shown in Figure 4. Virions released from 17 Cl 1 cells contain approximately equal amounts of 180 and 90K E2. In contrast, virions released from Sac- cells contain E2 almost totally in the 90K forms. This host cell

Table 2. Functions of Coronavirus Glycoproteins

E2, the Peplomeric Glycoprotein

1. Binding to receptors on the cell membrane
(adsorption and/or hemagglutination)
2. Inducing neutralizing antibody
3. Eliciting cell-mediated cytotoxicity
4. Causing pH-dependent thermolability of
coronaviruses
5. Inducing cell fusion;
Activated by proteolytic cleavage
6. Fusing viral envelope with cell membrane;
May be activated by proteolytic cleavage

E1, the Membrane Glycoprotein

1. Determining location of viral budding
2. Forming viral envelope
3. Interacting with viral nucleocapsid

dependent cleavage of E2 correlates with the different extent of cell fusion induced in these cell types by infection with MHV-A59 as shown in Figure 5. In 17Cl 1 cells, MHV-A59 establishes a moderate infection in which there is little fusion for 20 hours. The same virus in Sac- cells produces rapid cell fusion and death within 10 hours. We suggest that differences in the ability of different cell types to incorporate viral glycoproteins efficiently into virions or to transport them to the plasma membrane as well as differences in the rate of cleavage of the E2 glycoprotein may determine whether the outcome of coronavirus infection is moderate or cytotoxic.

ASSEMBLY AND RELEASE OF VIRIONS

Budding of coronaviruses occurs in the RER and Golgi apparatus but not at the plasma membrane. It appears likely that this intracellular budding site for coronaviruses is determined by the restricted intracellular transport of the E1 glycoprotein which forms the viral membrane. In the budding virions, the helical nucleocapsids appear as tubular structures in a regular array under the viral envelope^{4,10,21,23} and the virion has an electron-lucent center. Virions isolated from within the cell appear to have a normal complement of peplomers, suggesting that the virus may be fully formed in the RER and Golgi and that the glycoproteins may be processed on the virion as they migrate through these cellular compartments. In 17 Cl 1 cells, the virions change markedly in shape during this migration as shown

Figure 3. Processing of MHV-A59 proteins in different cell membrane fractions. MHV infected cells were fractionated on sucrose density gradients²³ at 4, 6, 8, 12 and 26 hours after inoculation. Proteins were analyzed by PAGE and Western blotting using anti-virion antibody and ¹²⁵I labeled protein A. In the pellet N accumulates and is processed into a faster migrating species. The glycoproteins E1 and E2 are synthesized in the RER fractions 6 and 7. E1 appears to be glycosylated as it migrates into smooth membrane fractions 3, 4 and 5. Proteolytic cleavage of E2 from 180K to 90K appears to be a late step, as the 90K is most apparent in fraction 4 which contains mature virions as well as plasma membrane and Golgi membranes. Markers on virion proteins indicate E2 (180 and 90K), N (50K), and E1 (23K).

Figure 4. Host dependent differences in proteolytic cleavage of the E2 glycoprotein of MHV-A59 virions. 17 Cl 1 cells (A) and Sac⁻ cells (B) were labeled with ³⁵S-methionine for 15 min. and virus released from the cells 3 hr. later was pelleted and analyzed by PAGE.

in Figure 6. In the smooth walled, post-Golgi vesicles, the virions become flattened and disk shaped, and the nucleocapsid appears as a rather homogeneous electron-dense mass within the virion. This morphological change may be due to different ionic conditions within the cellular compartments or to processing of viral components in these compartments. These changes may be similar to those seen in aggregates of cellular secretory proteins which are condensed and concentrated in post-Golgi vesicles of secretory cells. Thus, coronaviruses may make use of the cellular secretory process for release from infected cells. Virions from 17 Cl 1 cells are also in the flattened mature shape when they are released or reabsorbed onto the plasma membrane as shown in Figure 7.

Figure 5. Coronavirus induced fusion of different cell types. Infection with MHV-A59 causes much less fusion in 17 CL 1 cells than in Sac- cells. Cell fusion correlates with the extent of cleavage of the cell dependent E2 glycoprotein.

Recently we have observed flattened disk-shaped virions on the surface of 17 CL 1 cells infected with MHV-A59 and prepared for high voltage electron microscopy by the critical point drying technique. Late in the infectious cycle, many of the cells had become detached from the substrate. Adhering to the tiny bits of cytoplasm remaining on the substrate were large numbers of disk shaped virions as shown in Figure 8. These probably represented virions which had been released from the under surface of the 17 CL 1 fibroblasts and remained adsorbed to the remnants of the membrane left behind when the cells rounded up and peeled off.

Late in the infectious cycle of coronaviruses, abnormal formation of virions and accumulations of structural elements of virions may be observed in infected cells. Inclusions consisting of viral nucleocapsids have been observed in MHV infected cultures of mouse spinal cord.⁴ Reticular inclusions formed of highly convoluted membranes of the ER have been observed in several cell types.⁵ A final type of intracellular structure associated with coronavirus infection is the formation of long tubules within the lumen of the RER as shown in Figure 9.^{4,5} These tubules have also been observed in infected cells treated with tunicamycin.¹⁸ We believe that these may represent accumulations of the matrix glycoprotein, E1. Tubules somewhat similar to these have been formed in vitro from the M protein of parainfluenza virus which performs a similar function in the formation of the viral membrane.²⁴ We have recently found that

Figure 6. Structure of mature and immature coronaviruses. In 17 Cl 1 cells infected with MHV-A59 the virions in the RER are spherical with electron-lucent centers (arrow). Virions in smooth walled vesicles (arrowheads) or adsorbed to the plasma membrane are flattened and disk shaped with electron-dense nucleocapsids. Bar=200nm.

these tubular forms can be isolated from fractionated cells by sucrose density gradient ultracentrifugation as shown in Figure 10. Experiments are now in progress to identify the components of these tubular inclusions.

In this manuscript, we have reviewed the many processes of host cells which are required for the elaboration and release of coronaviruses. These processes are summarized in Table 3. It is apparent that the rate and extent of each of these processes may vary from one cell to another. This may affect the outcome of coronavirus infection. Thus 17 Cl 1 cells represent a cell type which shows a good balance of synthesis and transport of viral structural components, together with limited ability to cleave the E2 glycoprotein which is required for cell fusion. Thus

Figure 7. Gradient purified MHV-A59 virions. Gradient purified virions released from 17 Cl 1 cells were pelleted and examined in thin sections. Many virions were flattened and disk-shaped (arrowheads). Bar=100nm.

MHV-A59 may cause moderate infection in these cells. Cells such as Sac- or L2 cell lines, which cause rapid and extensive cleavage of the E2 glycoprotein, undergo rapid cell fusion and cell death. Because of the extensive dependence of coronavirus maturation on the host cell processes, it appears likely that host controlled modification of virus replication may determine the outcome of coronavirus infection.

Figure 8. High voltage microscopy of mature virions. Cells grown on carbon films on grids were fixed, dehydrated by the critical point technique and examined with 1M KV. The virions released from the under side of the cells were flattened, disk shaped mature particles (arrow). Bar=200nm.

Table 3. Cellular Functions Required for Coronavirus Replication

Uptake of virions (?)

Synthesis of proteins

Processing of proteins:

N = phosphorylation (?)

E1 = glycosylation with O-linked sugars

E2 = glycosylation with N-linked sugars
acylation

proteolytic cleavage

Intracellular transport of proteins

E1 = to Golgi

E2 = through Golgi to plasma membrane

Figure 9. Tubular inclusions in a coronavirus infected cell. In a 17 Cl 1 cell infected with MHV-A59, long, rigid tubular inclusions are found in the lumen of the RER. Arrow shows tubule in longitudinal section; arrowhead, tubule in cross section. Bar=100nm.

Figure 10. Tubular inclusion isolated from coronavirus infected cells. The inclusion was released from cells by homogenization, isolated by sucrose gradient ultracentrifugation and examined in a negatively stained preparation. Arrow indicates tubule. Bar=100nm.

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