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Influenza C Virus: Structure and Function of M Gene and Its Products

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1. Introduction

Influenza C virus (Fig.1), which belongs to the genus *Influenza C Virus* of the family *Orthomyxoviridae*, was first isolated from a patient with respiratory illness in 1947 (Taylor, 1949). It is widely distributed throughout the world and the majority of humans acquire antibodies to the virus early in life (Homma et al., 1982; Nishimura et al., 1987). The virus usually causes a mild upper respiratory illness (Katagiri et al., 1983), but can also cause lower respiratory infections such as bronchitis and pneumonia (Moriuchi et al., 1991; Matsuzaki et al., 2006). Recently, a case of acute encephalopathy associated with influenza C virus infection has been reported for the first time (Takayanagi et al., 2009). Although influenza C virus is isolated infrequently due to lack of facilities equipped with the resources for performing efficient virus isolation, recurrent infection with this virus occurs frequently in children as well as in adults (Homma et al., 1982; Katagiri et al., 1983, 1987; Matsuzaki et al., 1990).

The genome of influenza C virus consists of seven RNA segments of negative polarity, each of which encodes three polymerase proteins (PB2, PB1, and P3), hemagglutinin-esterase-fusion (HEF) glycoprotein, nucleoprotein (NP), matrix (M1) protein and CM2, and two nonstructural proteins (NS1 and NS2/NEP) (Palese & Shaw, 2007). PB2, PB1 and P3 are subunits of the RNA polymerase of the virus (Crescenzo-Chaigne et al., 1999; Crescenzo-Chaigne & van der Werf, 2001; Nagele & Meier-Ewert 1984; Yamashita et al., 1989). HEF, which has receptor-binding, receptor-destroying and fusion activities, forms a spike on the virion (Herrler & Klenk, 1991). NP participates in forming ribonucleoproteins (RNPs) with viral RNA (vRNA), PB2, PB1 and P3 (Crescenzo-Chaigne et al., 1999; Crescenzo-Chaigne & van der Werf, 2001; Nakada et al., 1984; Sugawara et al., 1991; 2006). M1 is abundantly present beneath the envelope, which gives rigidity to the virion. CM2 is the second membrane protein of the virus. NS1 is involved in viral mRNA splicing (Muraki et al. 2010), and NS2/NEP is a nuclear export protein (Paragas et al., 2001) and is incorporated into the virions (Kohno et al. 2009).

In this chapter, the author will focus on the M gene and M gene products and describe how they contribute to the replication of influenza C virus. First, the author will show the M gene structure, including its coding strategy. Second, the author will mention the characteristics

of M1 and CM2 proteins. Finally, the author will describe recent findings on M1 and CM2 proteins with regard to their roles in virus replication.

Fig. 1. Structure of influenza C virus.

The influenza C virus contains seven single-stranded RNA segments of negative polarity. The viral ribonucleoprotein (vRNP) complex is composed of viral RNA (black line), PB2 (open circle), PB1 (open rectangle), PA (open triangle) and NP (closed circle) proteins. The structure of vRNP is depicted only for the RNA Segment 7 for simplicity. HEF is a spike protein of the virus, forming homotrimers on the envelope. M1 is located beneath the envelope. CM2 is the second membrane protein that is present as a homotetramer. A small amount of NS2/NEP is incorporated into the virions.

2. Coding strategy of influenza C virus M gene

The influenza C virus RNA segment 6 (M gene) is 1,180 nucleotides in length and encodes a 242-amino-acid matrix protein (M1) and 115-amino-acid CM2 protein. In this section, the author will describe the coding strategy of the M gene, including the brief history of M1 and CM2 discovery.

2.1 M1 protein

Yamashita et al. determined the nucleotide sequence of C/Ann Arbor/1/50 M gene and provided evidence that a 242-amino-acid M1 protein is encoded by a spliced mRNA of the gene (Yamashita et al., 1988). The spliced mRNA of the gene lacks a region from nucleotides 754 to 981 due to the introduction of the stop codon (TGA) as a result of splicing (Fig. 2). The splicing event itself introduced the stop codon TGA, with the TG coming from the 5' splicing site and the A coming from the 3' splicing site.

The mRNA for M1 represents the major M gene-specific mRNA species in virus-infected cells. It is of interest that the splicing pattern of the influenza C virus M gene is different from that of influenza A virus M gene, in that the influenza A virus M1 protein, a major product of the gene, is coded for by an unspliced mRNA, not spliced mRNA (Palese & Shaw, 2007).

An open reading frame (ORF) in the influenza C virus M gene could potentially code for a protein of 374 amino acids, and small quantities of an mRNA collinear with RNA segment 6 was actually detected since a faint signal corresponding to the mRNA species was observed by S1 analysis (Yamashita et al., 1988). At that moment, however, the significance of the collinear mRNA species remained unknown.

2.2 CM2 protein

Analysis of the nucleotide sequences of the M gene from five influenza C virus isolates, including C/Ann Arbor/1/50, revealed that the gene contains a single ORF capable of coding for a 374-amino-acid protein (Hongo et al., 1994; Yamashita et al., 1988). Therefore, attempts were made to identify the unspliced mRNA of the M gene, and the mRNA was found to be synthesized in 1/10 amount of the spliced mRNA in the virus-infected cells (Hongo et al., 1994). To identify the protein encoded by the unspliced mRNA, whose approximate molecular weight is 42,000, the antiserum against the glutathione S-transferase fusion protein containing the extra C-terminal domain of the protein (Fig. 2) was prepared. Unexpectedly, immunoprecipitation experiments with the antiserum identified a protein of Mr ~18,000 in virus-infected cells (Hongo et al., 1994). The authors designated the protein as CM2, a second protein encoded by the influenza C virus M gene, and identified three forms of CM2 (CM2o, CM2a, and CM2b) depending on the moieties of glycosylation (Hongo et al., 1994).

A previously unrecognized 374-amino-acid protein was identified in virus-infected cells using the antiserum against CM2, and the protein was named as P42 according to the molecular weight. P42 is demonstrated to be modified by the addition of a high-mannose oligosaccharide chain to generate P44. The tryptic peptide map of either P42 or P44 was indistinguishable from the map of the mixture of M1 and CM2. Thus, the authors concluded that P42 and P44 correspond to the 374 amino acid protein encoded by unspliced RNA segment 6 mRNA and its N-glycosylated form, respectively (Hongo et al., 1998).

At that moment, the translational mechanism of CM2 was still unclear. It was reported previously that the P42 is an integral membrane protein having two internal hydrophobic domains, one of which (residues 241 to 252) is followed by two sequences (252 Ile-Thr-Ser and 257 Ala-Ser-Ala) favourable for cleavage by signal peptidase. To examine the possibility that P42 is cleaved by signal peptidase to yield CM2, a series of mutant M gene cDNAs were constructed and transfected into cultured cells (Hongo et al., 1999; Pekosz & Lamb, 1998). As a result, biosynthesis of CM2 has been demonstrated to proceed in the following manner; (i) Unspliced mRNA from RNA segment 6 is first translated into a 374-amino-acid protein, P42. (ii) Cotranslationally or immediately after the completion of translation, P42 begins to be inserted into the endoplasmic reticulum with the aid of the first hydrophobic domain, composed of amino acid residues 241 to 252. (iii) Translocation of P42 through the membrane is halted by the presence of the second hydrophobic domain, consisting of residues 287 to 318. (iv) P42 is then N-glycosylated at Asn residue 270, generating P44. (v)

Either before or after the addition of an oligosaccharide chain, P42/P44 is cleaved by signal peptidase at the C-terminal side of Ala residue 259, producing the M1' and CM2 proteins, composed of the N-terminal 259 amino acids and the C-terminal 115 amino acids, respectively.

Fig. 2. Coding strategy of influenza C virus M gene.

RNA segments 6 (M gene) of C/Ann Arbor/1/50 is shown in positive-sense orientation.

Numbers indicate the nucleotide positions along the genes. The lines at the 5' and 3' termini represent the non-coding regions. The boxes represent the coding region of viral proteins encoded by the gene. The intron is shown by V-shaped lines. M1 is encoded by a spliced mRNA (upper panel), into which the stop codon TGA is introduced at 752, 753 and 982, as a result of splicing. The P42 protein (M1'+CM2), encoded by a collinear transcript of the gene (lower panel), is cleaved by signal peptidase at an internal cleavage site (closed triangle) to generate M1' and CM2.

3. Characteristics of the M1 and CM2 proteins

3.1 M1 protein

The influenza C virion contains three major structural polypeptides: a large glycoprotein gp88 (HEF), a nucleoprotein (NP) and matrix protein (M1) (Palese & Shaw, 2007). Furthermore, Yokota et al. identified the M1 protein synthesized in C/Ann Arbor/1/50-infected MDCK cells (Yokota et al., 1981). To characterize the protein, the monoclonal antibodies against M1 have been produced (Sugawara et al., 1991). Using a panel of the nine MAbs against M1, the M1 protein was shown to contain two non-overlapping antigenic regions that are highly resistant to conformational changes, and to exhibit no antigenic variations among 23 influenza C virus strains isolated over 41 year period. The C/Ann Arbor/1/50-infected cells were examined for the localization of the M1 protein using one of these MAbs (L2), and as a result, M1 is shown to be localized in the nucleus at 24 h postinfection. The latter finding provided evidence for the requirement of nuclear function for influenza C virus replication. In addition, the association of M1 with the nucleoli was observed in the C/Yamagata/1/88-infected cells, a phenomenon that remains to be elucidated with respect to viral replication (Sugawara et al., 1991).

3.2 CM2 protein

The biochemical characteristics of CM2 in virus-infected cells were precisely determined, and as a result, CM2 is demonstrated to be the counterpart of the influenza A virus M2 protein (Hongo et al., 1997; Pekosz & Lamb, 1997; Tada et al., 1998). CM2 is a type III integral membrane protein that is oriented in membranes with a 23-amino-acid N-terminal extracellular domain, a 23-amino-acid transmembrane domain and a 69-amino-acid C-terminal cytoplasmic domain. It forms homodimer and homotetramer, and is phosphorylated, palmitoylated and N-glycosylated, and is incorporated into progeny virions. Site-specific mutational analyses of the M gene identified the amino acid sites for the posttranslational modifications on CM2 molecules as follows; i) an asparagine residue 11 is an N-glycosylation site, ii) cysteines at residue 1, 6 and 20 are all involved in disulfide bond formation, iii) a cysteine residue at 65 is palmitoylated through labile thioester linkage, and iv) serine residues at 78, 103, 108 and a proline at 104 are phosphorylation sites (Li et al., 2001; Pekosz & Lamb, 1997).

CM2 has also been investigated from an electrophysiological point of view. A number of studies have shown that the CM2 protein appears to have ion channel activities. CM2 forms a voltage-activated ion channel permeable to Cl^- (Hongo et al., 2004). When co-expressed with a pH-sensitive hemagglutinin (HA) from influenza A virus, the CM2 protein has a capacity to reduce the acidity of the exocytic pathway and reduce conversion of the pH-sensitive HA to its low pH conformation during transport to the cell surface, suggesting that CM2 is permeable to H^+ (Betáková & Hay, 2007). Preliminary electrophysiological studies of CM2-expressing mouse erythroleukemia cells have identified Na^+ -activated proton permeability in addition to the low pH-activated Cl^- permeability (Muraki & Hay, 2009; Muraki et al., personal communications). However, the relationship between the channel function and the role(s) of CM2 in the virus replication (see below) remains to be clarified.

4. Role of the M gene-products in virus replication

4.1 M1 protein

The M1 protein of influenza C virus is involved in virion morphogenesis. Nishimura et al. reported that cord-like structures (CLSs) which had lengths up to $\sim 500 \mu\text{m}$ or greater were protruding from the surface of C/Yamagata/1/88-infected HMV-II cells (Nishimura et al., 1990). Electron microscopic analysis showed that CLS consists of numerous filamentous particles in the process of budding, each of which is covered with a layer of surface projections and aggregated with their long axes. Further analyses using a series of reassortant viruses between C/Yamagata/1/88 and C/Taylor/1233/47, the latter of which is a unique strain incapable of forming CLS, showed that reassortants with the M gene from C/Taylor/1233/47 could not form CLS on infected cells. Comparison of the M gene sequences of influenza C virus strains, including C/Yamagata/1/88 and C/Taylor/1233/47, suggested that either or both of amino acid changes at positions 24 and 133 of the M1 protein were responsible for differences in cord-forming ability between the two strains (Nishimura et al., 1994).

The amino acid on the M1 protein that is responsible for CLS formation and virion morphology was determined. Upon establishment of influenza C virus-like particle (VLP) generation system, Muraki et al. observed that CLS was protruding from the 293T cells

transfected with a set of plasmid DNAs for VLP generation (Muraki et al., 2004). CLSs were observed on the 293T cells transfected with the plasmid for M1 of C/Ann Arbor/1/50, whereas CLSs were not observed when the plasmid for M1 of C/Ann Arbor/1/50 was replaced with that of C/Taylor/1233/47. Expression of the M1 protein possessing alanine or threonine at residue 24 together with the other virus components resulted in the generation of filamentous or spherical VLPs, respectively. These findings indicate that an amino acid at residue 24 of the M1 protein is a determinant for virion morphology.

The above finding obtained from VLP generation system has been confirmed using a recombinant virus generated by reverse genetics. The parental recombinant influenza C virus, which has the consensus sequences of C/Ann Arbor/1/50 (the residue 24 of M1 is alanine), exhibited a filamentous morphology. In contrast, a recombinant mutant virus, rMG96A, which has an Ala→Thr mutation at residue 24 of M1, exhibited spherical morphology. The mutant M1 protein had lower membrane affinity than did the wild type M1, suggesting that the difference in the affinity affects the virion morphology (Muraki et al., 2007). Furthermore, the flotation analysis showed that budding of influenza C virus does not occur from the lipid raft domain of the plasma membrane. These studies indicate that M1 plays an important role in virus budding, and imply that a region around the residue 24 of M1 may represent a late domain of the protein and that budding mechanism of influenza C virus differs from that of influenza A virus.

4.2 CM2 protein

The fact that the biochemical characteristics of CM2 are closely similar to those of influenza A virus M2 and that CM2 seems to have permeability to proton suggests that CM2 is also crucial in influenza C virus replication. To clarify the role(s) of CM2 in the influenza C virus replication cycle, attempts were made to generate a recombinant CM2-deficient virus using reverse genetics. However, infectious recombinants lacking CM2 have not been rescued to date (Furukawa et al., 2011), which highly suggests that CM2 is essential to influenza C virus replication.

Using VLP generation system, evidence was obtained that CM2 is involved in the packaging and uncoating processes. The CM2-deficient influenza C VLPs were successfully generated and the amount of GFP-vRNA in the VLPs was quantified. The data showed that the CM2-deficient VLPs contain approximately 37% of the vRNA found in wild-type VLPs, although no significant differences were detected in the expression levels of viral components in VLP-producing cells as well as in the number and morphology of the generated VLPs (Furukawa et al., 2011). This finding suggests that CM2 is involved in the mini-genome packaging into VLPs. In addition, HMV-II cells infected with CM2-deficient VLPs exhibited significantly reduced GFP expression. Although CM2-deficient VLPs could be internalized into HMV-II cells as efficiently as wild-type VLPs, a smaller amount of GFP-vRNA was detected in the nuclear fraction of CM2-deficient VLP-infected cells than in that of wild-type VLP-infected cells, suggesting that the uncoating process of the CM2-deficient VLPs in the infected cells did not proceed in an appropriate manner (Furukawa et al., 2011). Thus, CM2 appears to play a role in the packaging and uncoating processes of the virus replication cycle. However, whether and how the CM2 channel activities relate to the roles remains unclear.

A recombinant influenza C virus lacking CM2 palmitoylation, rCM2-C65A, was successfully rescued (Muraki et al., 2011). The rCM2-C65A, in which the cysteine at residue 65 of CM2 was substituted to alanine, grew as efficiently as did the parental recombinant virus in cultured cells and embryonated chicken eggs. The transport, maturation and localization of HEF, NP, M1 and CM2 in the cells infected with rCM2-C65A were virtually identical to those of the parental virus-infected cells. These findings suggest that CM2 palmitoylation is not required for virus replication. The fact that a recombinant lacking CM2 palmitoylation could be rescued suggests that CM2 mutant(s) at posttranslational site(s) is available for the analysis of CM2 role(s) in the virus replication.

The ion channel proteins of influenza A and B viruses play critical roles in the viral replication. The influenza A virus M2 protein functions as a proton channel during the entry of the virus into cells by allowing the acidification of the virion interior (Palese & Shaw, 2007). Recently, the M2 proton-selective ion channel function mediates virus budding (Rossman et al., 2010b; 2011). The M2 cytoplasmic tail also plays a role in progeny virus production and virion morphology (Chen et al., 2008; Iwatsuki-Horimoto et al. 2006; McCown & Pekosz, 2005; Rossman et al., 2010a; Zhang et al., 2000). The influenza B virus BM2 protein has a proton channel activity (Mould et al., 2003), and is involved in the incorporation of the virus genome into virions (Imai et al., 2004; 2008). Thus CM2 needs to be further investigated from these points of view.

4.3 M1' and P42

As mentioned above, P42, encoded by a collinear transcript of the RNA segment 6 (M gene), is cleaved by signal peptidase to generate CM2, composed of the C-terminal 115 amino acids, in addition to the M1' protein, composed of the N-terminal 259 amino acids (Hongo S et al., 1999; Pekosz & Lamb 1998). It is of interest to clarify the role(s) of P42 and M1' in virus replication. The characteristics of the P42 and M1' (or p31) proteins were precisely determined as follow.

The M1' protein was found to undergo rapid degradation after cleavage from P42, since addition of the 26S proteasome inhibitor lactacystin to the cells infected with influenza C virus or transfected with M gene cDNA drastically reduced M1' degradation (Pekosz & Lamb 2000). In addition, the hydrophobic nature, not the specific amino acid sequence, of the 17-amino-acid C terminus of M1' was demonstrated to act as the signal for targeting the protein to membranes and for degradation (Pekosz & Lamb 2000). Thus, M1' seems to be dispensable to virus replication.

A series of mutant M gene cDNAs were transfected into COS-1 cells to examine the characteristics of P42 (Li et al., 2004). P44, the N-glycosylated form of P42, forms disulfide-linked dimers and tetramers. P44 is transported to the Golgi apparatus, but not to the trans-Golgi, since P44 is sensitive to endoglycosidase H. P44 and P42 are unstable irrespective of N-glycosylation or oligomerization. The addition of lactacystin prevented the degradation of P42 as well as M1', but not that of P44 efficiently, suggesting that P44 is degraded by another protease besides the 26S proteasome (Li et al., 2004). The role(s) of P42/P44 in virus replication remains to be clarified.

5. Regulation of M1 and CM2 synthesis

In influenza A virus-infected cells, the regulatory mechanism for viral mRNA splicing has been extensively investigated. It has been reported that the steady-state level of spliced viral

transcripts is only 10% that of unspliced viral transcripts (Palese & Shaw, 2007). The inefficient splicing can be understood partly by the fact that influenza A virus NS1 protein is associated with spliceosomes and inhibits pre-mRNA splicing (Fortes et al., 1994; Garaigorta & Ortin, 2007; Lu et al., 1994). The cis-acting sequences in the NS1 transcript also negatively regulates splicing (Nemeroff et al., 1992). The splicing of influenza A virus M1 mRNA is controlled by the rate of nuclear export (Valcarcel et al., 1993), and the splicing of influenza A virus M1 mRNA is regulated by the binding of the viral polymerase complex and cellular splicing factor SF2/ASF (Shih et al., 1995; Shih & Krug, 1996).

The influenza C virus NS1 protein (C/NS1), a product of the RNA segment 7 (NS gene), regulates the splicing efficiency of the M gene (Muraki et al., 2010). In influenza C virus-infected cells, the predominant transcript from the M gene is a spliced mRNA (Hongo et al., 1994). Ribonuclease protection assays showed that the splicing efficiency of the M gene mRNA in infected cells was much higher than that in M gene cDNA-transfected cells, suggesting that viral factor(s) facilitates the M gene mRNA splicing. To investigate the factor(s) involved in the splicing, Muraki et al. analyzed cells co-transfected with M and NS gene-cDNAs, and showed that the splicing of the M gene mRNA was enhanced by expressing C/NS1 (Muraki et al., 2010). The fact was obtained that splicing ratio of influenza A virus M gene mRNA was also increased by C/NS1, suggesting that C/NS1 has an intrinsic ability of up-regulating viral mRNA splicing. Therefore, we speculate that efficient splicing of M gene mRNA leads to a high and reduced level of M1 and CM2 expression, respectively, creating conditions that are optimal for virus replication.

6. Conclusion

The RNA segment 6 (M gene) of influenza C virus encodes the M1 and CM2 proteins. It has been demonstrated that the M1 protein is a determinant for virion morphology and CM2 is involved in packaging and uncoating processes. A further study will be needed to clarify the structure-function relationship more precisely.

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This book covers various aspects of Molecular Virology. The first chapter discusses HIV-1 reservoirs and latency and how these twin phenomena have remained a challenge to eradication. Aspects regarding the molecular evolution of hepatitis viruses including their genetic diversities with implications for vaccine development are treated in the second chapter. Metabolic disorders that are a consequence of hepatitis C virus infection are discussed in the succeeding chapter. The following two chapters discuss influenza C virus and the applications of viral vectors in therapeutic research. Avian influenza is handled in the sixth chapter and the therapeutic potential of belladonna-200 against japanese encephalitis virus infection is discussed in the succeeding chapter. The last two chapters discuss baculoviruses and their interaction with polydnviruses. Researchers, lecturers and students will find this book an indispensable companion.

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