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Role of the CM2 Protein in Influenza C Virus Replication: Analyses of Recombinant Viruses possessing CM2 Mutants

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Abstract

CM2 is the second membrane protein of influenza C virus. In the present study, to investigate the role(s) of CM2 in the virus replication, we generated recombinant influenza C viruses, rC65A and rN11A, which lack the palmitoylation and glycosylation sites of CM2, respectively. The rC65A virus grew as efficiently as the recombinant wild-type (rWT) virus did, whereas the rN11A grew less efficiently than the rWT virus. To study the difference more precisely, we generated influenza C virus-like particles (VLPs) lacking the CM2 glycosylation site (N11A-VLPs) and examined the VLPs and VLP-infected cells. As a result, the N11A-VLPs contain approximately 13% of the virus RNA found in wild-type VLPs (WT-VLPs), and N11A-VLP-infected HMV-II cells exhibited reduced reporter gene expression compared with that of WT-VLP-infected cells (WT : N11A = 1:0.1). Thus, supportive evidence was obtained that CM2 is involved in packaging and uncoating processes and that observed growth difference between rWT and rN11A can be attributed to the difference in the CM2 function.

Keywords: Influenza C virus; CM2 protein; Replication; Glycosylation

Introduction

The RNA segment 6 (M gene) of the influenza C/Ann Arbor/1/50 (AA/50) strain is 1,180 nucleotides in length and encodes a 242-amino acid matrix protein (M1) and a 374-amino acid P42 from the spliced and unspliced mRNA, respectively [1,2]. M1 is located beneath the virus envelope and is involved in the virion morphogenesis [3]. P42 is cleaved by signal peptidase at an internal cleavage site, generating M1' and CM2, which are composed of the N-terminal 259 amino acids and the C-terminal 115 amino acids of P42, respectively [4 - 6].

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 [7,8]. It is modified by N-glycosylation at an asparagine residue 11, and as a result, three forms of CM2 with different electrophoretic mobilities (CM2o, CM2a, and CM2b) are detected in infected cells [7,8]. A mannose-rich oligosaccharide core is added to unglycosylated CM2o to form CM2a, and the maturation of the carbohydrate chain from the high mannose type to the complex type converts CM2a into CM2b, the latter of which is modified by addition of polylactosaminoglycan.

A recent study using CM2-deficient virus-like particles (VLPs) suggested the involvement of CM2 in the genome packaging and uncoating processes [9], although the role(s) of CM2 in the context of viral replication remains to be clarified. In the present study, therefore, to clarify the role(s) of CM2 in the virus replication, the amino acids for the posttranslational modifications that are highly conserved among influenza C viruses isolated to date [10,11] were focused. We generated recombinant viruses lacking palmitoylation or N-glycosylation, and influenza C VLPs lacking the CM2 glycosylation site, and analyzed the virus particles and the infected cells.

Materials and Methods

Cells and antibodies

293T, HMV-II, and LLC-MK₂ cells were maintained as described previously [12,13]. Monoclonal antibodies (MAbs) against the HEF,

NP, and M1 proteins of AA/50, and antiserum against CM2 protein were reported previously [4,14,15].

Plasmids

The Pol I plasmids for the expression of virus RNAs (vRNAs) of AA/50 (pPolI/PB2, pPolI/PB1, pPolI/P3, pPolI/HEF, pPolI/NP, pPolI/M and pPolI/NS) were described previously [3]. The plasmids for the expression of the influenza C virus proteins (pcDNA/PB2-AA, pcDNA/PB1-AA, pcDNA/P3-AA, pME18S/HEF-AA, pCAGGS.MCS/NP-AA, pCAGGS.MCS/M1-AA, pME18S/Met-CM2-YA, pME18S/NS1-YA, and pME18S/NS2-YA) were also reported previously [12]. The Pol I plasmids, pPolI/M-CM2-C65A [16] and pPolI/M-CM2-N11A, used to generate vRNA encoding non-palmitoylated and non-glycosylated CM2, respectively, were generated based on pPolI/M. The plasmid, pME18S/Met-CM2-N11A, for the expression of the non-glycosylated CM2 protein, was constructed based on pPolI/M-CM2-N11A. Details of the primers and PCR protocols will be provided on request.

Generation of recombinant viruses by reverse genetics

A recombinant wild-type (rWT) virus, rC65A, a mutant recombinant virus in which the cysteine at residue 65 of CM2 was substituted to alanine, and rN11A in which the asparagine at residue 11 of CM2 was substituted to alanine, were generated by transfecting the above-mentioned plasmids into 293T cells as described previously [3, 16].

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Received March 26, 2012; Accepted April 11, 2012; Published April 13, 2012

Citation: Muraki Y, Okuwa T, Himeda T, Ohara Y (2012) Role of the CM2 Protein in Influenza C Virus Replication: Analyses of Recombinant Viruses possessing CM2 Mutants. Metabolomics S1:003. doi:10.4172/2153-0769.S1-003

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Plaque titration

The infectious titers of the recombinants and the supernatants of recombinant-infected cells were determined according to the procedure reported previously [16,17].

Radioimmunoprecipitation

HMV-II cells infected with recombinant viruses were pulse-labeled with [³⁵S]methionine at 26 hr postinfection (p.i.) and chased for 2 hrs. Cells were then disrupted and immunoprecipitated with MABs against HEF, NP and M1, or anti-CM2 serum, followed by SDS-PAGE under reducing or non-reducing conditions [16,18]. The immunoprecipitated CM2 proteins were digested with N-glycanase under conditions as described previously [7], followed by SDS-PAGE.

Immunoblotting

Immunoblotting for the purified VLPs, plasmid-transfected 293T cells, and recombinant virus- or VLP-infected HMV-II cells were carried out as described previously [12]. Band intensities were measured by ImageJ software, version 1.42 (W. Rasband, National Institutes of Health [<http://rsb.info.nih.gov/ij/>]).

Indirect immunofluorescence

HMV-II cells grown on glass coverslips were infected with recombinant viruses, and incubated for 48 h. The cells were fixed in 10% formalin, permeabilized with 0.2% Triton X-100, and analyzed by indirect immunofluorescence using anti-CM2 serum. The samples were examined by an LSM710 confocal laser scanning microscope.

Flow cytometry of HMV-II cells infected with recombinants and VLPs

Flow cytometric analyses of VLP-infected HMV-II cells were performed as described previously using the anti-HEF MAB [9].

Generation, purification and infection of VLPs

WT-VLPs and N11A-VLPs were generated and purified as described previously [9], though the amounts of each of the 10 plasmids used for transfection were re-evaluated based on the gene transfer efficiency by the generated VLPs (data not shown). The purified VLPs were treated with N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (20 µg/ml) at 37°C for 10 mins, followed by the addition of soybean trypsin inhibitor. The monolayered HMV-II cells were infected with the VLPs at 33°C for 60 mins and subsequently infected with the helper virus (AA/50) at a multiplicity of infection (MOI) of 5, and then incubated for 48 hrs.

Statistical analysis

Data between groups were analyzed using a Student's *t* test. A *p* value of less than 0.05 was determined as statistically significant.

Results

Generation and growth kinetics of rC65A

To investigate the effect of CM2 palmitoylation on the influenza C virus replication, we generated rC65A, a recombinant influenza C virus lacking the CM2 palmitoylation site. As reported previously [16], there were no significant differences in the growth kinetics between rWT and rC65A. Therefore we decided to generate a recombinant virus possessing another mutation.

Generation and growth kinetics of rN11A

To investigate the effect of CM2 glycosylation on the influenza C virus replication, we generated rN11A, a recombinant influenza C virus lacking the CM2 glycosylation site. The radioimmunoprecipitation experiment using the MABs and anti-CM2 serum revealed that i) the synthesis and maturation of the HEF, NP and M1 proteins exhibited no significant differences between the rWT- and rN11A-infected cells (data not shown) and ii) no glycosylated forms of CM2 (CM2a and CM2b) were detected in the rN11A-infected cells (Figure 1).

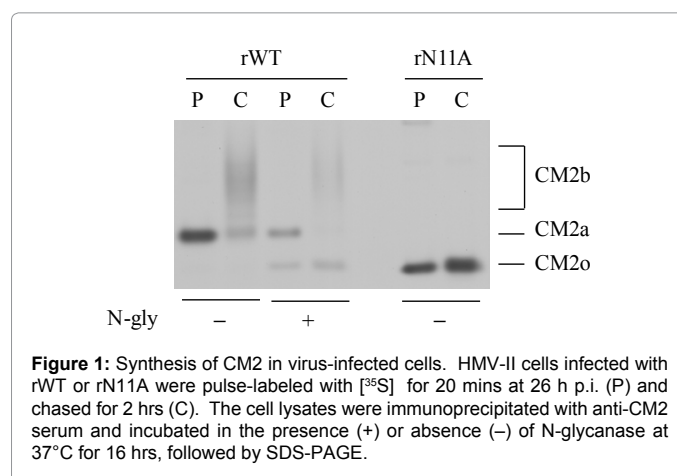
The rWT or rN11A viruses were infected to HMV-II cells at an MOI of 5 and incubated at 33°C for up to 120 hrs. The rWT virus grew more efficiently than the rN11A virus did, though the difference (2 to 3 times) did not reach statistical significance (data not shown). The recombinants were infected to HMV-II cells at an MOI of 0.001 and incubated at 33°C for 120 hrs in the presence of trypsin (20 µg/ml). Three independent experiments revealed that the rN11A virus grew less efficiently than rWT did (Figure 2). Therefore, the glycosylation of CM2 appears to have some effect on the generation of infectious viruses in cultured cells, and we decided to analyze the rN11A virus and its infected cells to clarify the role(s) of CM2 in the virus replication.

Maturation and transport of CM2 in infected cells

The immunoprecipitated CM2 protein labeled with [³⁵S]methionine were analyzed by SDS-PAGE under non-reducing conditions. The bands corresponding to monomer, dimer and tetramer forms of CM2 were detected in both the rWT- and rN11A-infected cells (data not shown). Indirect immunofluorescent analysis using anti-CM2 serum showed that the CM2 protein was expressed on the cell surface in both the rWT- and rN11A-infected cells (Figure 3). Thus no significant differences were observed between rWT- and rN11A-infected cells in terms of synthesis, maturation and transport of CM2.

Analysis of influenza C VLPs possessing non-glycosylated CM2

Although the rN11A virus grew less efficiently than the rWT virus (Figure 2), we could not find any differences in the above experiments except for the glycosylation of CM2. The influenza C VLP generation system has recently been shown to be a useful tool to study the effect of mutations on the influenza C virus replication [9]. In the present study, we first re-evaluated the amounts of each plasmid transfected, based on the gene transfer efficiency by the generated VLPs (see Materials and Methods). We then generated N11A-VLPs, VLPs possessing non-



glycosylated CM2 proteins, and analyzed the VLPs, VLP-producing cells and VLP-infected cells to elucidate the role(s) of CM2 in the virus replication.

We examined viral proteins and GFP-vRNA in the generated VLPs. An equal amount of the purified VLPs was subjected to immunoblotting. No significant difference was observed in the amount of HEF, M1 and CM2 between the WT-VLPs and N11A-VLPs, whereas the amount of NP in the N11A-VLPs was smaller than that in the WT-VLPs (data not shown). Furthermore, three independent experiments revealed that the amount of GFP-vRNA in the N11A-VLPs and CM2-deficient VLPs and was approximately 13% and 1.0% of that in the WT-VLPs, respectively ($p < 0.01$). Taken together, these findings indicate that the amount of virus ribonucleoprotein (vRNP) in N11A-VLPs is smaller than that in WT-VLPs. Thus, we conclude that the CM2 glycosylation is involved in the genome packaging process, since the amounts of the virus components expressed in the N11A-VLP-producing 293T cells was virtually identical to those of WT-VLP-producing cells (data not shown).

Reporter gene transfer to HMV-II cells by VLPs

We next examined VLP-infected cells to examine whether the non-glycosylated CM2 in the VLPs has some effect(s). Flow cytometric analysis showed that the attachment and entry of WT-VLP was virtually identical to that of N11A-VLPs (data not shown). HMV-II cells were infected with VLPs so that the copy number of GFP-vRNA included in WT-VLPs was equal to that in N11A-VLPs, followed by superinfection with the helper virus (AA/50). The expression level of GFP in the N11A-VLP-infected cells was approximately 10% of that of WT-VLP-infected cells as shown by immunoblotting (Figure 4). This

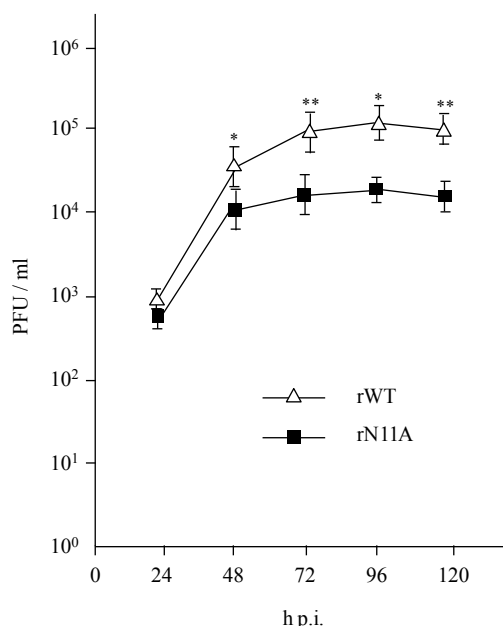


Figure 2: Growth kinetics of the rN11A virus. HMV-II cells were infected with rWT or rN11A at an MOI of 0.001 and then incubated at 33°C for 120 hrs. The culture media were used for the determination of infectious titers using LLC-MK₂ cells. A representative result from three independent experiments is shown. Data are expressed as the mean \pm standard deviation. All comparisons between groups were statistically evaluated by using unpaired Student's *t* test (*, $p < 0.05$; **, $p < 0.01$).

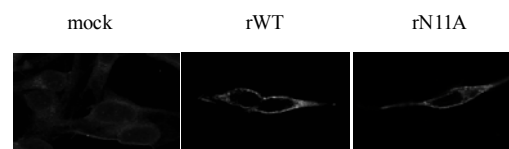


Figure 3: Immunofluorescence for CM2. HMV-II cells were infected with rWT or rN11A, and incubated for 48 hrs. The cells were stained with anti-CM2 serum and a fluorescein isothiocyanated anti-rabbit IgG, as the primary and secondary antibodies, respectively. The cells were examined by confocal microscopy.

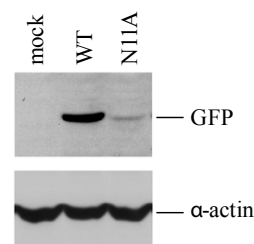


Figure 4: GFP expression in VLP-infected HMV-II cells. HMV-II cells were infected with WT- or N11A-VLPs, followed by superinfection with AA/50, and then incubated for 48 hrs. The cells were analyzed by immunoblotting using anti-GFP MAb and anti α -actin Ab.

finding suggests that glycosylation of CM2 is involved in the uncoating process of the VLPs.

Discussion

The results obtained in the present study using N11A-VLPs have raised the possibility that glycosylation of CM2 affects the uncoating and packaging processes of virus replication cycle, and the growth difference between rWT and rN11A (Figure 2) can be attributed to the difference in the CM2 function. This observation should be taken into account in relation to the ion channel function of CM2. Hongo et al. [4] hypothesized that the Cl⁻ channel activity of CM2 facilitates the interaction of M1 with vRNP by reducing the ionic strength beneath the viral budding site of the plasma membrane. If this is the case, non-glycosylated CM2 may possess the reduced permeability for Cl⁻. On the other hand, CM2 has the ability to reduce the pH in the trans-Golgi network (Betakova & Hay, 2007), suggesting that CM2 forms a channel permeable to proton. Like the influenza A virus M2 protein, proton permeability of CM2 in the virus envelope may be required for uncoating. If so, non-glycosylated CM2 may possess the reduced permeability for proton, leading to the impaired uncoating efficiency. Taken together, electrophysiological analysis of glycosylation-deficient CM2 will be of help in the elucidation of the structure-function relationship of CM2.

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