JOURNAL OF VIROLOGY, Apr. 1997, p. 2786–2792 Vol. 71, No. 4
0022-538X/97/$04.00+0

Characterization of a Second Protein (CM2) Encoded by RNA Segment 6 of Influenza C Virus

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Received 12 September 1996/Accepted 9 January 1997

The biochemical properties of a second protein (CM2) encoded by RNA segment 6 of influenza C virus were investigated. Three forms of CM2 with different electrophoretic mobilities ($CM2_0$, CM2a, and CM2b) were detected in infected cells by immunoprecipitation with antiserum to the glutathione *S*-transferase (GST)–CM2 fusion protein. Treatment of infected cells with tunicamycin and digestion of immunoprecipitated proteins with endoglycosidase H or peptide-*N*-glycosidase F suggested that a mannose-rich oligosaccharide core is added to unglycosylated $CM2_0$ (M_r, ~16,000) to form CM2a (M_r, ~18,000) and that the processing of the carbohydrate chain from the high-mannose type to the complex type converts CM2a into CM2b, which is heterogeneous in electrophoretic mobility (M_r, ~22,000 to 30,000). Labeling of infected cells with [^{3}H]palmitic acid showed that CM2 is fatty acylated. The fatty acid bond was sensitive to treatment with hydroxylamine and mercaptoethanol, which indicates a labile thioester-type linkage. The CM2 protein was also found to form disulfide-linked dimers and tetramers on sodium dodecyl sulfate-polyacrylamide gels under nonreducing conditions. Trypsin treatment of infected cell surfaces as well as of microsome vesicles from infected cells followed by immunoprecipitation with antiserum to the GST fusion protein containing the 56 C-terminal amino acid residues of CM2 suggested that this C-terminal domain is intracellular and exposed to the cytoplasms of microsomes. Furthermore, evidence that a small amount of CM2 is incorporated into progeny virus particles was obtained by Western blot analysis. These results, altogether, suggest that CM2 is an integral membrane protein with biochemical properties similar to those of influenza A virus M2 and influenza B virus NB proteins.

Unspliced and spliced mRNAs are synthesized from RNA segment 7 of influenza A virus and are translated to generate the matrix protein M1 and a small integral membrane protein (97 amino acids; M_r, ~15,000) designated M2, respectively (7). M2 is oriented in membranes so that the 23 N-terminal amino acid residues are exposed extracellularly and the 54 C-terminal residues are exposed cytoplasmically (8). This protein is posttranslationally modified by the formation of intermolecular disulfide bonds (5, 19) and by palmitoylation through a thioester-type linkage (18, 24). The M2 protein is abundantly expressed at the surfaces of infected cells (8) but is underrepresented in virions, as only a few molecules are packaged into virus particles (30). M2 acts as a proton channel to allow acidification of the interior of the virion during virus uncoating and to regulate the pHs of vesicular components of the trans-Golgi network (1, 12, 19). In influenza B virus-infected cells the NB protein (100 amino acids; M_r, ~18,000) encoded by RNA segment 6 is believed to perform a function similar to that of influenza A virus M2 (19). The structural features of NB are remarkably similar to those of M2, except that NB is glycosylated whereas M2 is not (25). The carbohydrate chains on NB are unique in that they are modified to contain polylactosaminoglycan (26).

RNA segment 6 of influenza C/Yamagata/1/88 virus is 1,181 nucleotides in length and contains a single open reading frame that may code for a polypeptide of 374 amino acids with a predicted M_r of about 42,000 (6). However, the predominant mRNA synthesized from RNA segment 6 was shown to lack a region from nucleotides 755 to 982 and to encode a 242-amino-acid M1 (M_r, ~27,000) (6, 27). Unspliced mRNA from this RNA segment was recently detected, although in small quantities (~13% of spliced mRNA), in influenza C virus-infected cells (6). This mRNA species potentially encodes a polypeptide with an M_r of 42,000 that contains an additional 132 amino acids from the C terminus of the M1 protein. However, immunoprecipitation experiments with antiserum to the glutathione *S*-transferase (GST) fusion protein containing this extra C-terminal domain identified a protein with an apparent M_r of ~18,000 (designated CM2) in infected cells (6). The mechanism by which the CM2 protein is generated from the unspliced mRNA is not known. It is possible, however, that this mRNA is translated from the initiation codon at nucleotides 732 to 734 to the termination codon at nucleotides 1149 to 1151, yielding a protein composed of 139 amino acids, shown in Fig. 1 (6). We present here biochemical evidence that influenza C virus CM2 is an integral membrane protein that shares many structural features with influenza A virus M2 and influenza B virus NB.

MATERIALS AND METHODS

Viruses and cells. Influenza C viruses (Ann Arbor/1/50, Yamagata/64, Aomori/74, Aichi/1/81, and Yamagata/1/88) were grown in the amniotic cavities of 9-day-old, embryonated chicken eggs (28). The HMV-II line of human malignant melanoma cells was grown in RPMI 1640 medium supplemented with 10% calf serum (11).

Radioimmunoprecipitation (RIP) and SDS-PAGE. HMV-II cells infected with influenza C virus at a multiplicity of about 10 PFU/cell were labeled except where stated below with 15 to 30 μCi of [^{35}S]methionine (ARC)/ml for 1 h at 26 h postinfection (p.i.) in methionine-deficient RPMI 1640 medium. Cells were then disrupted in 0.01 M Tris-HCl (pH 7.4) containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.15 M NaCl, and a cocktail of protease inhibitors [20 μg of (*p*-amidinophenyl)methanesulfonyl fluoride/ml, 100 kallikrein units of aprotinin/ml, 0.5 μg of leupeptin/ml] and immunoprecipitated as described previously (15), with rabbit immune serum raised against either egg-grown C/Ann Arbor/1/50 virions (28) or the GST fusion protein that contains residues 253 to 374 (numbering corresponds to that shown in Fig. 1) of

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236          250          260          270          280          290          300          310          320
  MGRMA MKWLVVIIYF SITSQPASAC NLKTCLNLFN NTDAVTVHCF NENQGYMLTL ASLGLGIITM LYLLVKIIIE LVNGFVLGRW

                330          340          350          360          374
ERWCGDIKTT IMPEIDSMEK DIALFRERLD LGEDAPDETD NSPIPFSNDG IFEI
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FIG. 1. Predicted amino acid sequence of CM2. The amino acid sequence of CM2 is deduced from the nucleotide sequence of RNA segment 6 of C/Yamagata/1/88 (6). The N-terminal amino acid of CM2, although it has not yet been determined, has been suggested to correspond to residue 236 of the putative 374-amino-acid protein that may be encoded by RNA segment 6 (6). The hydrophobic domains that can interact with a lipid bilayer are underlined, and the potential N-glycosylation site is boxed.

the putative 139-amino-acid protein (GST-CM2) (6). The immunoprecipitates obtained were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 17.5% gels containing 4 M urea and processed for analysis by fluorography (28).

Endoglycosidase treatment of immunoprecipitated proteins. The protein A-Sepharose antibody-antigen complexes were boiled for 5 min in 30 μl of 0.0625 M Tris-HCl (pH 6.8) containing 1% SDS. After low-speed centrifugation, the resulting supernatant was diluted with 570 μl of either 0.1 M sodium acetate (pH 7.4) (for treatment with peptide-*N*-glycosidase F [*N*-glycanase]), 0.1 M sodium acetate (pH 5.5) containing the cocktail of protease inhibitors described above (for treatment with endoglycosidase H [endo H]), or 0.1 M sodium acetate (pH 5.5) (for treatment with endoglycosidase F [endo F] or endo-β-galactosidase [endo β]). Digestion with *N*-glycanase (300 mU), endo H (30 mU), endo F (120 mU), or endo β (30 mU) was done at 37°C for 16 h. The proteins were then acetone precipitated and analyzed by SDS-PAGE. endo H was purchased from Seikagaku Kogyo Co. Ltd., and the other endoglycosidases were purchased from Boehringer Mannheim Biochemica.

Labeling with [^{3}H]palmitic acid and hydroxylamine treatment of immunoprecipitated proteins. C/Yamagata/1/88 virus-infected cells were labeled for 1 h at 26 h p.i. with [^{3}H]palmitic acid (52 Ci/mmol; Amersham) in RPMI 1640 medium supplemented with 5 mM sodium pyruvate and then immunoprecipitated with antiserum to the GST-CM2 fusion protein. The resultant precipitates were suspended in 40 μl of 0.0625 M Tris-HCl (pH 6.8) containing 1% SDS and boiled for 5 min. The supernatant obtained after low-speed centrifugation was treated with 1 ml of 0.25 M hydroxylamine (pH 6.8) for 1 h at 37°C. The proteins were methanol precipitated and analyzed by SDS-PAGE.

Trypsin treatment of microsomes and infected cell surfaces. Intracellular microsomal vesicles were prepared from C/Yamagata/1/88 virus-infected cells labeled with [^{35}S]methionine for 20 min at 26 h p.i. and treated with tosylamide-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (50 μg/ml) for 30 min at 37°C as previously described (10). After addition of soybean trypsin inhibitor (50 μg/ml), microsomes were pelleted by centrifugation at 100,000 × *g* for 30 min and then subjected to RIP with rabbit immune serum raised against the GST fusion protein that contains the C-terminal region of CM2 (residues 319 to 374 of Fig. 1) (GST–CM2-CTR). Construction of plasmid pGEX/CM2-CTR used for expressing the GST–CM2-CTR fusion protein as well as preparation of GST–CM2-CTR antiserum was carried out according to the procedures described previously (6). For trypsin treatment of cell surfaces, infected cells labeled with [^{35}S]methionine for 30 min at 26 h p.i. and chased for 2 h were scraped into phosphate-buffered saline (pH 7.2) lacking Ca^{2+} and Mg^{2+} and then treated with TPCK-trypsin (100 μg/ml) for 30 min at 37°C. After addition of soybean trypsin inhibitor (100 μg/ml), cells were pelleted by low-speed centrifugation and then immunoprecipitated with antiserum to the GST–CM2-CTR fusion protein. The resulting precipitates, either before or after treatment with *N*-glycanase, were analyzed by SDS-PAGE.

Western blotting. After SDS-PAGE of C/Yamagata/1/88 virions grown in eggs and purified as described previously (14), viral proteins were electrotransferred (15 V, 60 min) to a Durapore membrane (Millipore) in a buffer consisting of 48 mM Tris-base, 39 mM glycine, and 20% methanol. To prevent nonspecific adsorption of antibody, the membrane was treated at 4°C overnight with 4% skim milk (Difco) in 20 mM Tris-HCl (pH 8.0) containing 100 mM NaCl and then cut into strips. Each strip was incubated for 1 h at room temperature with anti-GST–CM2 serum diluted 1:100 in TNS buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1% skim milk). The strip was then washed with TNS buffer and further incubated at room temperature for 1 h with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Seikagaku Kogyo) diluted appropriately in the same buffer. After being washed extensively, the strip was developed by incubation with the substrate solution composed of 0.025% 3,3′-diaminobenzidine tetrahydrochloride (Sigma), 0.01% H_2O_2, 0.1 M NaCl, and 20 mM Tris-HCl (pH 8.0).

RESULTS

Synthesis of CM2 in HMV-II cells infected with various influenza C virus strains. We previously identified CM2 (M_r, ~18,000) in HMV-II cells infected with C/Yamagata/1/88 (6). To confirm that this polypeptide is synthesized in cells infected with other virus strains as well, HMV-II cells were infected with each of five strains (C/Ann Arbor/1/50, C/Yamagata/64, C/Aomori/74, C/Aichi/1/81, and C/Yamagata/1/88), labeled with [^{35}S]methionine, and then analyzed by RIP with anti-GST–CM2 serum. A polypeptide with an M_r of ~18,000 (designated here CM2a) could be readily detected in cells infected with any of the strains examined (Fig. 2). However, strain-dependent differences in its mobility were clearly seen; the CM2a of C/Yamagata/1/88 migrated faster than those of the other strains, which supports the notion that it is virus coded. In addition to CM2a, a material heterogeneous in electrophoretic mobility (M_r, ~22,000 to 30,000) was also detectable in infected cells irrespective of the virus strain used and is designated here CM2b.

To see if there is a precursor-product relationship between CM2a and CM2b, a pulse-chase experiment was done with cells infected with C/Yamagata/1/88. The cells were pulse-labeled with [^{35}S]methionine for 30 min at 40 h p.i. and then chased for 1 h in RPMI 1640 medium containing 1 mM cold methionine. Immediately after a pulse or after a subsequent chase, cells were disrupted and subjected to RIP. Figure 3 shows that the amount of CM2a was greatly reduced during the chase, with a concomitant increase in the amount of CM2b, suggesting that CM2a is modified, presumably by the processing of oligosaccharide chains, to a heterogeneous form with a higher M_r, CM2b.

N-linked glycosylation of CM2. Labeling experiments with [^{3}H]glucosamine showed that CM2a and CM2b are both glycosylated (data not shown). To determine whether the carbo-

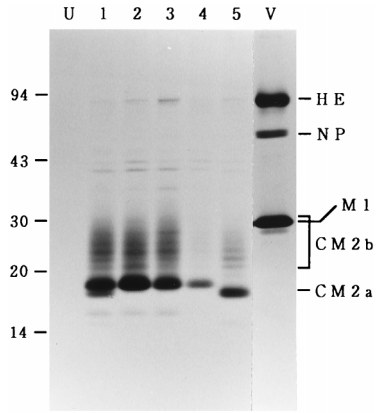

FIG. 2. Synthesis of CM2 in HMV-II cells infected with various strains of influenza C virus. HMV-II cells mock infected (lane U) or infected with C/Ann Arbor/1/50 (lane 1), C/Yamagata/64 (lane 2), C/Aomori/74 (lane 3), C/Aichi/1/81 (lane 4), or C/Yamagata/1/88 (lane 5) were labeled with [^{35}S]methionine for 1 h at 26 h p.i. Cells were then immunoprecipitated with antiserum to the GST-CM2 fusion protein, and the immunoprecipitates obtained were analyzed by SDS-PAGE. In lane V, C/Yamagata/1/88 virus-infected cells were immunoprecipitated with antiserum to C/Ann Arbor/1/50 virions. Molecular weight markers, whose approximate weights (in thousands) are noted at the left, were phosphorylase *b* (M_r, 94,000), ovalbumin (M_r, 43,000), carbonic anhydrase (M_r, 30,000), trypsin inhibitor (M_r, 20,100), and α-lactalbumin (M_r, 14,400).

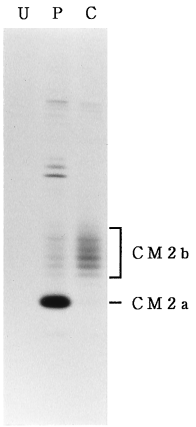

FIG. 3. Existence of a precusor-product relationship between CM2a and CM2b. HMV-II cells infected with C/Yamagata/1/88 were pulse-labeled with [^{35}S]methionine for 30 min at 40 h p.i. and chased for 1 h. Either immediately after a pulse (lane P) or after a subsequent chase (lane C), cells were immunoprecipitated with anti-GST–CM2 serum and the resulting precipitates were analyzed by SDS-PAGE. In lane U, mock-infected cells were pulse-labeled and then immunoprecipitated with anti-GST–CM2 serum.

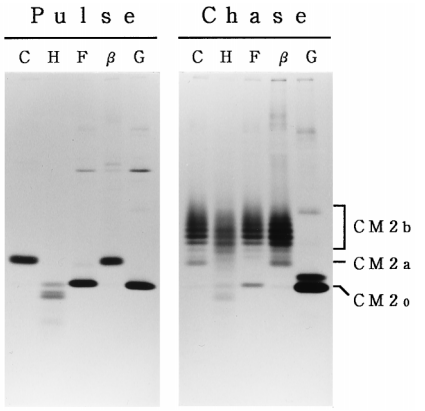

FIG. 5. Susceptibilities of CM2a and CM2b to various endoglycosidases. HMV-II cells infected with C/Yamagata/1/88 were pulse-labeled with [^{35}S]methionine for 20 min at 26 h p.i. and chased for 1 h. Either after a pulse or after a subsequent chase, cells were subjected to RIP with anti-GST–CM2 serum. The immunoprecipitated proteins were incubated at 37°C for 16 h in the absence of endoglycosidases (lanes C) or in the presence of endo H (lanes H), endo F (lanes F), endo β (lanes β), or *N*-glycanase (lanes G) and then analyzed by SDS-PAGE.

hydrates on these proteins are N linked, C/Yamagata/1/88 virus-infected cells were labeled with [^{35}S]methionine in the presence or absence of tunicamycin (5 μg/ml), a specific inhibitor of N-linked glycosylation (20). Cells were then lysed and immunoprecipitated with either antiviral serum or anti-GST–CM2 serum, and the resulting precipitates were analyzed by SDS-PAGE (Fig. 4). In tunicamycin-treated cells, CM2a and CM2b were no longer observed and only a polypeptide with an apparent M_r of ~16,000, designated $CM2_0$, could be detected (lane 4). $CM2_0$ is likely to correspond to the nonglycosylated form of CM2. It is interesting that $CM2_0$ was present in control, untreated cells, although its quantity was very low compared with those of CM2a and CM2b (lane 3), raising the possibility that the CM2 molecules, if not all of the molecules, may become core glycosylated posttranslationally.

To obtain more-detailed information concerning the structure of oligosaccharide chains associated with CM2a and CM2b, infected cells were pulse-labeled with [^{35}S]methionine for 20 min at 26 h p.i. and then chased for 1 h. Cell lysates were prepared either immediately after a pulse (for analyzing the carbohydrate on CM2a) or after a 1-h chase (for analyzing the carbohydrate on CM2b) and immunoprecipitated with GST-CM2 antiserum. The resultant precipitates were digested with various endoglycosidases and then analyzed by SDS-PAGE. As shown in Fig. 5, digestion with *N*-glycanase removed carbohydrates from both CM2a and CM2b, converting them to unglycosylated $CM2_0$ (lanes G), which supports the conclusion described above that $CM2_0$ is modified by N-linked glycosylation to generate CM2a and CM2b.

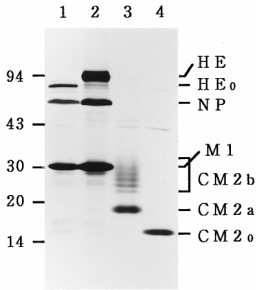

FIG. 4. Synthesis of CM2 in influenza C virus-infected cells treated with tunicamycin. C/Yamagata/1/88 virus-infected HMV-II cells were labeled with [^{35}S]methionine for 1 h at 26 h p.i. in the presence (lanes 1 and 4) or absence (lanes 2 and 3) of 5 μg of tunicamycin/ml. Cells were then immunoprecipitated with either antiviral serum (lanes 1 and 2) or GST-CM2 antiserum (lanes 3 and 4), and the resultant precipitates were analyzed by SDS-PAGE. HE_0 is a nonglycosylation counterpart of HE. Molecular weight markers (in thousands) are noted at the left.

In the lumen of the rough endoplasmic reticulum, glycoproteins acquire high-mannose carbohydrate chains that are sensitive to digestion with endo H, and after transport of vesicles to the Golgi apparatus, the carbohydrate chains are processed to a complex form that is resistant to digestion with the enzyme (21). Figure 5 (lanes H) shows that CM2a was totally sensitive to digestion with endo H whereas CM2b was resistant, suggesting that the conversion of CM2a into CM2b is accompanied by the maturation of carbohydrates from the high-mannose type to a complex one. A band which migrated slightly faster than $CM2_0$ was seen in the gel patterns of endo H-treated samples. This presumably results from proteolysis of CM2a and/or $CM2_0$ caused by contaminating protease(s). endo F cleaves both high-mannose and complex forms of N-linked carbohydrates (2), although it does not digest triantennary and tetra-antennary complex N glycans (22). endo F removed the carbohydrate chains from CM2a, causing it to migrate as unglycosylated $CM2_0$, but failed to cause a change in the mobility of CM2b (Fig. 5, lanes F). Thus, it appears that CM2b has triantennary or tetra-antennary oligosaccharide chains.

The carbohydrate structure of CM2b was further examined for the presence of the polylactosaminoglycan that is known to confer heterogeneous electrophoretic mobility on glycoproteins (3). Polylactosaminoglycan is characterized by repeating units of galactose β1-4-*N*-acetylglucosamine β1-3 attached to the core oligosaccharide component composed of $(mannose)_3(N\text{-acetylglucosamine})_2$ (3). endo β cleaves the β1-4 linkage between galactose and the *N*-acetylglucosamine of the lactosamine repeating unit (4). As shown in Fig. 5 (lanes β), the mobility of CM2b, like that of CM2a, was not affected at all by treatment with 30 mU of endo β. The same result was obtained even when the amount of endo β was increased up to 200 mU (data not shown).

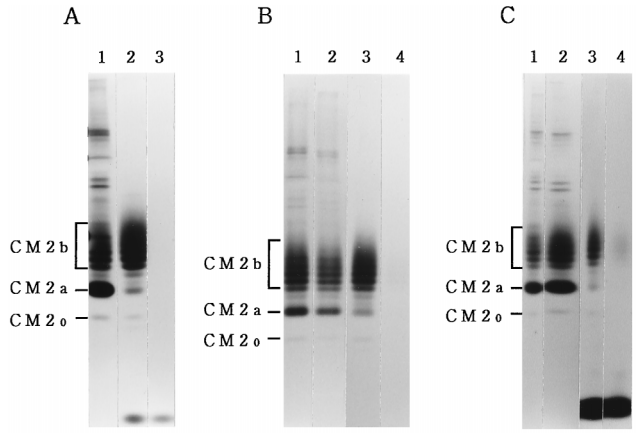

FIG. 6. CM2 is acylated with fatty acid in a thioester-type linkage. C/Yamagata/1/88 virus-infected HMV-II cells were labeled with [^{35}S]methionine or [^{3}H]palmitic acid for 1 h at 26 h p.i. and then immunoprecipitated with anti-GST–CM2 serum. (A) The [^{35}S]methionine-labeled (lane 1) or [^{3}H]palmitic acid-labeled (lanes 2 and 3) immunoprecipitates from mock-infected (lane 3) or infected (lanes 1 and 2) cells were boiled for 2 min in the presence of 1% SDS and 1% mercaptoethanol and then subjected to gel electrophoresis. (B) The immunoprecipitates labeled with [^{35}S]methionine (lanes 1 and 2) or [^{3}H]palmitic acid (lanes 3 and 4) were incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 0.25 M hydroxylamine for 1 h at 37°C and then analyzed by SDS-PAGE in the presence of 1% SDS and 1% mercaptoethanol. (C) The immunoprecipitates labeled with [^{35}S]methionine (lanes 1 and 2) or [^{3}H]palmitic acid (lanes 3 and 4) were boiled in the presence of 1% SDS and 1% (lanes 1 and 3) or 20% (lanes 2 and 4) mercaptoethanol and then subjected to gel electrophoresis.

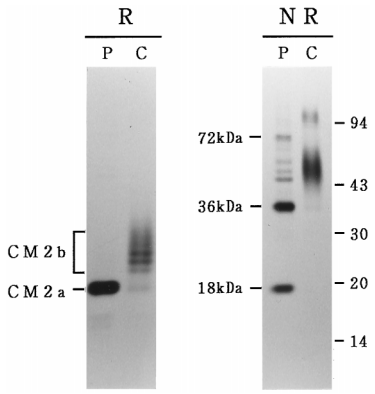

FIG. 7. Disulfide-linked multimers of CM2. C/Yamagata/1/88 virus-infected HMV-II cells were pulse-labeled with [^{35}S]methionine for 20 min and chased for 1 h. Either after a pulse (lanes P) or after a subsequent chase (lanes C), cells were disrupted and then immunoprecipitated with anti-GST–CM2 serum. The immunoprecipitated CM2 was analyzed by SDS-PAGE following dissociation in the presence (R) or absence (NR) of 1% mercaptoethanol. Molecular mass markers and molecular weight markers (in thousands) are noted at the left and right of gel NR, respectively.

Acylation of CM2. To examine if the CM2 protein, like influenza A virus M2 (18, 24), is acylated with long chain fatty acids, C/Yamagata/1/88 virus-infected cells labeled with either [^{35}S]methionine or [^{3}H]palmitic acid were analyzed by RIP with anti-GST–CM2 serum. The results (Fig. 6A) showed that both CM2a and CM2b were labeled with [^{3}H]palmitic acid. Clearly, however, the ratio of [^{3}H]palmitic acid to [^{35}S]methionine was higher in the latter form than in the former, indicating that CM2b contains a larger amount of fatty acid than CM2a. It is also to be noted that [^{3}H]palmitic acid was incorporated into unglycosylated $CM2_0$, although the amount was low.

To investigate the nature of the fatty acid bond in CM2, the immunoprecipitated CM2 protein labeled with [^{3}H]palmitic acid or [^{35}S]methionine was treated with neutral hydroxylamine and then subjected to SDS-PAGE (Fig. 6B). Hydroxylamine cleaved completely the fatty acid from any of the three forms of CM2, showing that CM2 contains the fatty acid in an ester-type linkage. Furthermore, the results of experiments shown in Fig. 6C, where the [^{3}H]palmitic acid-labeled and immunoprecipitated CM2 protein was boiled for 2 min in the presence of 20% mercaptoethanol before gel electrophoresis, revealed that the [^{3}H]palmitic acid-derived label in CM2 was highly susceptible to treatment with the reducing agent. It therefore appears that CM2 is acylated in a labile thioester-type linkage to cysteine residues rather than in a stable oxyester-type linkage to serine or threonine residues.

Oligomeric structure of CM2. C/Yamagata/1/88 virus-infected cells were pulse-labeled with [^{35}S]methionine for 20 min at 26 h p.i. and then chased for 1 h. Either after a pulse or after a subsequent chase, cells were disrupted in the presence of 50 mM iodoacetamide, immunoprecipitated with GST-CM2 antiserum, and then analyzed by SDS-PAGE under nonreducing conditions (Fig. 7). When the immunoprecipitated CM2 obtained after a 20-min pulse was analyzed, two dense bands with apparent M_rs of ~36,000 and ~72,000 were observed, in addition to the CM2a monomer with an M_r of ~18,000. The 36-kDa molecule is likely to correspond to a dimer of CM2a, and the 72-kDa molecule is likely to correspond to a tetramer. When the sample prepared after a 1-h chase was examined, two major bands were seen; one migrated more slowly than a CM2a tetramer, and the other migrated heterogeneously between the dimeric and tetrameric forms of CM2a. The former band probably represents a tetramer of CM2b, and the latter probably represents a dimer. No band corresponding to a CM2b monomer was detected. These data suggest that CM2 forms disulfide-linked dimers and tetramers and that oligomerization begins soon after the addition of core oligosaccharide and ends before CM2's maturation into complex form.

Membrane orientation of CM2. Examination of the predicted amino acid sequence of CM2 suggests that this protein is an integral membrane protein anchored in membranes by the hydrophobic domain consisting of residues 287 to 318 (6). Additionally, it was shown here that C/Yamagata/1/88 virus CM2, which contains only one potential site for the addition of N-linked carbohydrate at position 270 (6), is N glycosylated (Fig. 4 and 5), suggesting that the N-terminal region (residues 253 to 286) containing this glycosylation site is located on the luminal sides of intracellular membranes.

To determine whether the C-terminal region of CM2 (residues 319 to 374) is exposed on the cytoplasmic sides of intracellular membranes, microsome vesicles were isolated from infected cells labeled with [^{35}S]methionine for 20 min at 26 h p.i. The isolated vesicles were treated with TPCK-trypsin and then analyzed by RIP with either monoclonal antibody (MAb) to influenza C virus hemagglutinin-esterase (HE) glycoprotein (17) or antiserum to the GST–CM2-CTR fusion protein. As shown in Fig. 8A, the HE protein was highly resistant to trypsin digestion (lanes 2 and 3), suggesting that a large portion of this molecule is protected from proteolysis by the lipid bilayer. By contrast, trypsin treatment caused an almost complete loss of CM2 (CM2a and $CM2_0$) compared to the level in untreated microsomes without generating a protective fragment reactive with anti-GST–CM2-CTR serum, which suggests strongly that the epitope recognized by this antiserum is exposed on the cytoplasmic side and accessible to trypsin cleavage.

To further examine the topography of CM2 in membranes,

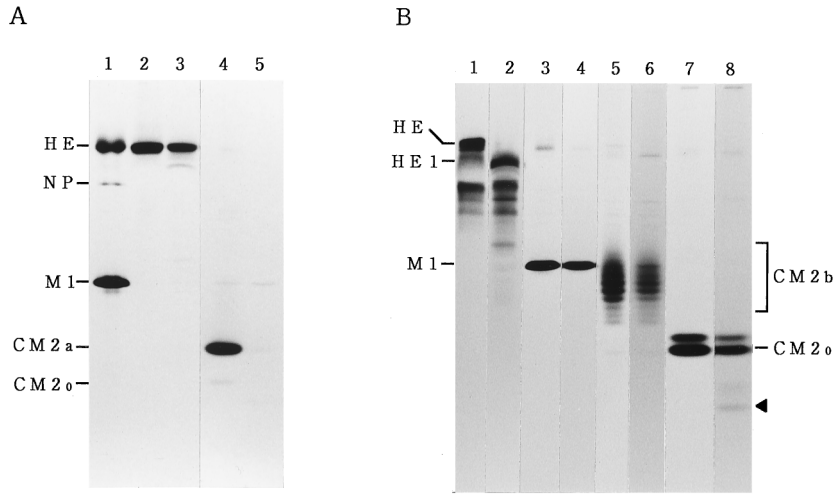

FIG. 8. Orientation of CM2 in membranes. (A) Microsome vesicles were isolated from C/Yamagata/1/88 virus-infected HMV-II cells labeled with [^{35}S]methionine for 20 min at 26 h p.i. and incubated in the absence (lanes 2 and 4) or presence (lanes 3 and 5) of 50 μg of TPCK-trypsin/ml at 37°C for 30 min. Microsomes were then pelleted and immunoprecipitated with either anti-HE MAb D37 (lanes 2 and 3) or antiserum to the GST–CM2-CTR fusion protein (lanes 4 and 5), and the resultant precipitates were analyzed by SDS-PAGE. Lane 1 shows the SDS-PAGE pattern of the immunoprecipitates obtained after treatment of infected cell lysates with antiviral rabbit serum. (B) C/Yamagata/1/88 virus-infected HMV-II cells labeled with [^{35}S]methionine for 30 min at 26 h p.i. and chased for 2 h were scraped from plates and then suspended in phosphate-buffered saline. The cells were untreated (lanes 1, 3, 5, and 7) or treated with 100 μg of TPCK-trypsin/ml at 37°C for 30 min and then immunoprecipitated with anti-HE MAb D37 (lanes 1 and 2), anti-M1 MAb L2 (lanes 3 and 4), or antiserum to the GST–CM2-CTR fusion protein (lanes 5 to 8). The resulting precipitates were untreated (lanes 1 to 6) or treated with *N*-glycanase (lanes 7 and 8) and then analyzed by SDS-PAGE. HE1 is a larger subunit of the HE glycoprotein. The arrowhead denotes the trimmed form of CM2.

infected cells labeled with [^{35}S]methionine for 30 min at 26 h p.i. and chased for 2 h were scraped from tissue culture plates, incubated with 100 μg of TPCK-trypsin/ml for 30 min at 37°C, and then immunoprecipitated with either MAbs to the HE and M1 proteins (16, 17) or antiserum to the GST–CM2-CTR fusion protein. As shown in Fig. 8B, almost all of the surface HE glycoproteins were digested by trypsin to generate their larger subunit, HE1 (lane 2) whereas the internal M1 protein was largely protected from proteolysis (lane 4). No significant difference was seen in the electrophoretic mobilities of CM2b before and after trypsin treatment of the cell surfaces (lanes 5 and 6). In addition to $CM2_0$, however, a new band with an approximate M_r of 11,000 (lane 8) that presumably represents a trimmed form of $CM2_0$ could be detected when the immunoprecipitates from trypsin-treated cells were subjected to SDS-PAGE after *N*-glycanase digestion, which suggests that some of the CM2 molecules are accessible to digestion with trypsin at the cell surface. Furthermore, the finding that the peptide fragment reactive with antiserum to the GST–CM2-CTR fusion protein was protected from proteolysis supports the notion that the C-terminal region of CM2 is intracellular. More-drastic attempts to convert CM2 to the trimmed form were unsuccessful, because treatment of intact cells with higher concentrations of TPCK-trypsin resulted in unacceptable levels of cell lysis. It should be pointed out here that trypsin, which cleaves at the carboxyl side of an arginine or lysine residue, would not remove the oligosaccharide chain from CM2b since the N-terminal domain, which consists of residues 253 to 286, contained only one basic amino acid (lysine 263) at the N-terminal side of the N glycosylation site (position 270). Thus, the trimmed form of CM2b is likely to comigrate with untrimmed CM2b having the heterogeneous electrophoretic mobility, which may account for the failure to detect trimmed CM2b in lane 6.

Detection of CM2 in virions. To determine whether CM2 is incorporated into progeny virus particles, C/Yamagata/1/88 virions grown in eggs were purified and their polypeptides (5, 20 or 80 μg) were separated by SDS-PAGE. After the electrophoretic transfer of proteins to a Durapore membrane, the blots were probed with anti-GST–CM2 serum or anti-HE MAb. As demonstrated in Fig. 9B, CM2b could be detected in virions when a large amount (80 μg) of viral proteins was used for analysis. The protein amount required for detection of CM2 in virions was markedly reduced (~5 μg) when CM2b was converted into more homogeneously migrating $CM2_0$ by treating virions with *N*-glycanase prior to gel electrophoresis. Coomassie staining of a polyacrylamide gel on which purified

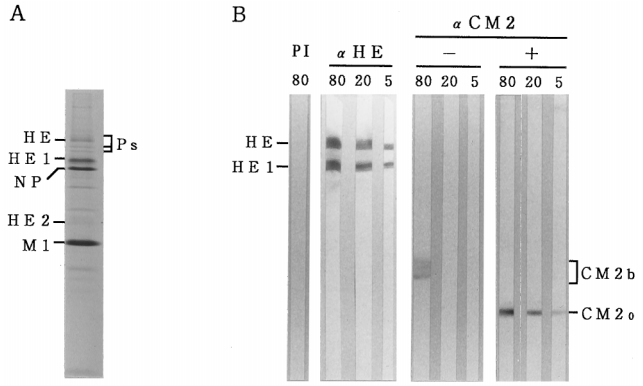

FIG. 9. Detection of CM2 in virions by Western blotting. (A) C/Yamagata/1/88 virions grown in eggs were pelleted from the harvested amniotic fluids and purified by banding in a potassium tartrate gradient as described previously (14). After SDS-PAGE of the virions (25 μg), the gel was stained with Coomassie blue. HE2 is a smaller subunit of the HE glycoprotein. Ps indicates three polymerase proteins: PB1, PB2, and P3. (B) Purified C/Yamagata/1/88 virions (5, 20, or 80 μg of total protein) that had been treated (+) or not treated (−) with *N*-glycanase were subjected to SDS-PAGE. Their polypeptides were then electrophoretically transferred to a Durapore membrane and allowed to react with preimmune rabbit serum (PI), anti-HE MAb D37 (αHE), or rabbit antiserum to the GST-CM2 fusion protein (αCM2).

virions (25 μg) were analyzed confirms that the amounts of host cellular proteins copurified were very low, if existent (Fig. 9A).

DISCUSSION

The data presented here revealed that the CM2 protein of influenza C/Yamagata/1/88 virus is modified by N glycosylation (Fig. 4 and 5). Examination of the predicted amino acid sequence of the CM2 of this strain (Fig. 1) indicates that there is only one potential site (residue 270) for the addition of an N-linked carbohydrate, suggesting that this site is used. We reported previously that the CM2 protein of C/Ann Arbor/1/50, unlike those of C/Yamagata/64, C/Aomori/74, C/Aichi/1/81, and C/Yamagata/1/88, contains two potential N glycosylation sites, one at position 270 and the other at position 281 (6). It is unlikely that the latter site is used, since the CM2 of C/Ann Arbor/1/50 was indistinguishable in electrophoretic mobility from those of C/Yamagata/64, C/Aomori/74, and C/Aichi/1/81, although the CM2s of these four strains migrated differently from that of C/Yamagata/1/88 (Fig. 2). This finding is reminiscent of the previous observation that the potential N-linked glycosylation site on influenza A virus M2 is not used (29). The potential glycosylation site in M2 is located 5 residues away from the membrane-spanning segment (8, 29), and asparagine residue 281 in C/Ann Arbor/1/50 virus CM2 is similarly spaced at 6 residues away from the transmembrane domain (6, 27). The proximity of these sites to the lipid bilayer may hinder access by the glycosylation enzyme complexes.

Three forms of CM2 ($CM2_0$, CM2a, and CM2b) were identified in influenza C virus-infected cells. The data obtained (Fig. 3 to 5) suggest that a mannose-rich core oligosaccharide is added to unglycosylated $CM2_0$ (M_r, ~16,000) to form CM2a (M_r, ~18,000) and that the processing of an oligosaccharide chain from a high-mannose type to a complex type converts CM2a into CM2b with heterogeneous electrophoretic mobility (M_r, ~22,000 to 30,000). The complex carbohydrate chain associated with CM2b is likely to be triantennary or tetra-antennary, since it was totally resistant to digestion with endo F, an enzyme which hydrolyzes N glycans from the high-mannose type and biantennary complex type but does not digest tri- and tetra-antennary complex N glycans (22). The heterogeneous electrophoretic mobility of CM2b reminded us of the heterogeneously migrating NB glycoprotein of influenza B virus, which has been shown to possess the carbohydrate chains modified to contain polylactosaminoglycan (26). However, it was found that CM2b, unlike NB, was not digested at all by endo β (Fig. 5). This result does not necessarily indicate that CM2b lacks polylactosaminoglycan chains, because highly branched or terminally modified regions and the short polylactosamine chains are digested with endo β with difficulty (3). Thus, the carbohydrate structure of CM2b must be further examined for the presence of polylactosaminoglycan by different approaches.

Unglycosylated $CM2_0$ was detected, although in a small amount, in infected cells grown in the absence of tunicamycin (Fig. 4). A similar observation has been made with influenza B virus NB protein (26). This result raises the possibility that the CM2 protein may be capable of integrating posttranslationally into endoplasmic reticulum membranes, as has been reported for the human glucose transporter protein (9).

CM2 was shown here to be modified covalently with a fatty acid (probably palmitate) in a thioester-type linkage (Fig. 6). Palmitoylation is known to occur on the cytoplasmic tails of integral membrane proteins (13). The cytoplasmic domain of CM2 contains only a single cysteine residue (position 324) (Fig. 1), which is conserved among all of the 27 influenza C virus strains examined (19a). It is likely, therefore, that this cysteine residue is the site for fatty acylation, which must be confirmed by site-directed mutagenesis followed by expression of the mutated gene in eukaryotic cells. Further studies are also needed to verify that CM2 is certainly acylated with palmitic acid; the HE glycoprotein has been shown to possess mainly stearic acid as a protein-bound acyl chain, irrespective of the fatty acid used for labeling (23). Three forms of CM2 ($CM2_0$, CM2a, and CM2b) were labeled with [^{3}H]palmitic acid to different degrees, with CM2b being labeled most heavily (Fig. 6), an observation which suggests that fatty acylation of CM2 begins in the rough endoplasmic reticulum but occurs more extensively after its migration into the Golgi apparatus.

On SDS-polyacrylamide gels under nonreducing conditions, both the influenza A virus M2 and influenza B virus NB proteins form disulfide-linked dimers and tetramers (5, 19, 25). This was also the case with influenza C virus CM2 protein (Fig. 7). As has been observed with M2 (5), however, the CM2 tetramers formed by hydrostatic interaction of disulfide-linked dimers may exist in infected cells, in addition to the disulfide-linked tetramers. In the case of M2, cysteine residues 17 and 19 located in its extracellular domain have been shown to participate in the formation of disulfide-linked multimers (5). The predicted amino acid sequence of CM2 indicates that it contains three cysteine residues (positions 260, 265, and 279) in the N-terminal region, which consists of residues 253 to 286 (Fig. 1), all of which are conserved among the 27 influenza C virus isolates analyzed (19a). The roles of these cysteine residues in the formation of disulfide-linked oligomers will be clarified by site-directed mutagenesis and expression of the mutants in eukaryotic cells.

We proposed previously that the CM2 hydrophobic domain spanning residues 287 to 318 that has a hydropathy index of >2 would anchor this protein in the lipid bilayer (6). This study found that the C-terminal domain consisting of residues 319 to 374 was digested by trypsin treatment of infected cell microsomes (Fig. 8A) but remained undigested after treatment of infected cell surfaces with trypsin (Fig. 8B), suggesting that this domain is cytoplasmic. It is also likely that the N-terminal region, composed of residues 253 to 286, is extracellular since the potential N-glycosylation site present in this domain (residue 270) is presumably used, as discussed above. This is supported by the observation that a peptide fragment with an M_r of ~11,000 that was not removed by trypsin digestion of intact cells was reactive with antiserum to the C-terminal domain of CM2 (Fig. 8B). We suggest therefore that CM2 is an integral membrane protein with an orientation in membranes similar to those of influenza A virus M2 and influenza B virus NB (8, 25). However, it remains to be determined whether the hydrophobic region composed of residue 236 to 252, which has no counterpart in either M2 or NB, acts as a cleavable signal sequence in translocation across the membrane.

Influenza A virus M2 and influenza B virus NB are both expressed abundantly on the infected cell surface (8, 25). It was also demonstrated here that some of the CM2 molecules were accessible to digestion with trypsin at the cell surface (Fig. 8B). However, no fluorescent staining was seen on the surfaces of influenza C virus-infected cells when unfixed cells were subjected to indirect immunofluorescence with antiserum to the GST-CM2 fusion protein (data not shown). This unexpected result could be accounted for if the antiserum did not contain antibodies reactive with the extracellular domain of CM2. In fact, immunization of rabbit with the GST fusion protein constructed to contain residues 253 to 286 (GST–CM2-NTR) failed to generate antibodies that recognize the CM2 protein synthesized in infected cells (data not shown). The poor rec-

ognition of the native CM2 by antiserum to the GST–CM2-NTR fusion protein might be due to hindrance of antigenic sites by the complex carbohydrate chain linked to asparagine residue 270.

It has been reported that 14 to 68 molecules of M2 are associated with influenza A virus particles (30). CM2 was also detected in purified influenza C virions (Fig. 9), although the number of the CM2 molecules per virion remains to be determined. This is not the result of contamination of copurified intracellular membranes, because CM2b but not CM2a or $CM2_0$ was found in our virion preparations. Hereafter, it will be important to determine whether virion-associated CM2, like influenza A virus M2 (12, 19), has ion channel activity and plays a role in virus uncoating.

ACKNOWLEDGMENTS

We thank Akira Suzuki and Yoko Shida (students of Yamagata University School of Medicine) for excellent technical assistance.

This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture, Japan, and the Ichiro Kanehara Foundation.

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