The sites for fatty acylation, phosphorylation and intermolecular disulphide bond formation of influenza C virus CM2 protein

Zhu-Nan Li, Seiji Hongo, Kanetsu Sugawara, Kazuhiko Sugahara, Emi Tsuchiya, Yoko Matsuzaki and Kiyoto Nakamura

Department of Bacteriology, Yamagata University School of Medicine, Iida-Nishi, Yamagata 990-9585, Japan

The sites for fatty acylation, disulphide bond formation and phosphorylation of influenza C virus CM2 were investigated by site-specific mutagenesis. Cysteine 65 in the cytoplasmic tail was identified as the site for palmitoylation. Removal of one or more of three cysteine residues in the ectodomain showed that all of cysteines 1, 6 and 20 can participate in the formation of disulphide-linked dimers and/or tetramers, although cysteine 20 may play the most important role in tetramer formation. Furthermore, it was found that serine 78, located within the recognition motifs for mammary gland casein kinase and casein kinase I, is the predominant site for phosphorylation, although serine 103 is phosphorylated to a minor extent by proline-dependent protein kinase. The effects of acylation and phosphorylation on the formation of disulphide-linked oligomers were also studied. The results showed that, while palmitoylation has no role in oligomer formation, phosphorylation accelerates tetramer formation without influencing dimer formation. CM2 mutants defective in acylation, phosphorylation or disulphide bond formation were all transported to the cell surface, suggesting that none of these modifications is required for proper oligomerization. When proteins solubilized in detergent were analysed on sucrose gradients, however, the mutant lacking cysteines 1, 6 and 20 sedimented as monomers, raising the possibility that disulphide bond formation, although not essential for proper oligomerization, may stabilize the CM2 multimer. This was supported by the results of chemical cross-linking analysis, which showed that the triple-cysteine mutant can form multimers.

Introduction

The genome of influenza C virus consists of seven single-stranded RNA segments. RNA segment 6 (M gene) of C/Yamagata/1/88 virus is 1181 nucleotides long and contains a single open reading frame (positions 27–1148) capable of encoding a polypeptide (P42) of 374 amino acids (Hongo et al., 1994). However, the predominant mRNA transcript of this RNA segment lacks a region from nucleotides 755 to 982 and encodes a 242 amino acid matrix (M1) protein (Yamashita et al., 1988; Hongo et al., 1994). Unspliced mRNA, synthesized in very small amounts in infected cells (Hongo et al., 1994), is first translated into P42, which contains an additional 132 amino acids from the C terminus of M1 (Hongo et al., 1998). Co-translationally or immediately after completion of translation, P42 is inserted into the endoplasmic reticulum (ER) with the aid of a hydrophobic domain (amino acid residues 241–252) and is then cleaved by signal peptidase at the C-terminal side of alanine residue 259, generating the M1’ and CM2 proteins, respectively composed of the N-terminal 259 amino acids and the C-terminal 115 amino acids (Hongo et al., 1999; Pekosz & Lamb, 1998). CM2 is an integral membrane protein containing three distinct domains (see Fig. 1): a 27-residue N-terminal extracellular domain, a transmembrane domain of 32 residues and a 56-residue cytoplasmic domain (Hongo et al., 1999; Pekosz & Lamb, 1998). The protein is expressed at the plasma membranes of infected cells (Hongo et al., 1997; Pekosz & Lamb, 1997) and is also incorporated into virions (Hongo et al., 1997).

CM2 is modified post-translationally by the addition of an N-linked oligosaccharide chain (Hongo et al., 1997) and is also
modified by palmitoylation through a labile thioester-type linkage (Hongo et al., 1997) and by phosphorylation (Tada et al., 1998). In addition, the protein is modified post-translationally by the formation of intermolecular disulphide bonds, generating disulphide-linked dimers and tetramers (Hongo et al., 1997; Pekosz & Lamb, 1997). Evidence was presented by Pekosz & Lamb (1997) that N-glycosylation occurs at asparagine residue 11, located in the ectodomain of CM2, and that carbohydrate modifications are not required for either the formation of disulphide-linked multimers or transport to the cell surface. The sites of the other post-translational modifications have not yet been determined.

Influenza A virus M2 acts as a proton channel to allow acidification of the virion interior during virus uncoating and to regulate the pH of vesicular components of the trans-Golgi network (Sugrue & Hay, 1991; Ciampor et al., 1992; Pinto et al., 1992). This protein shares many structural features with influenza C virus CM2, including the N-terminus-C-terminus membrane orientation, the sizes of the ectodomain and the cytoplasmic tail and the ability to be modified post-translationally by fatty acylation, phosphorylation and intermolecular disulphide bond formation (Lamb et al., 1985). Holsinger et al. (1995) investigated the post-translational modification sites of M2 and revealed that cysteines 17 and 19 in the ectodomain form disulphide bonds, whereas cysteine 50 in the cytoplasmic tail is acylated, and that serine 64 is the predominant site for phosphorylation, although serines 82, 89 and 93 are also phosphorylated to minor extents. These authors also showed that none of the post-translational modifications influences the ion channel activity of M2 significantly when expressed in oocytes of Xenopus laevis. More recently, Castrucci et al. (1997) demonstrated that a virus (created by reverse genetics) in which all three of cysteine residues 17, 19 and 50 were changed to serine was not defective in replication either in cultured cells or in animals. Thomas et al. (1998) also succeeded in generating a recombinant virus mutated specifically such that M2 could no longer be phosphorylated. These observations indicate that none of acylation, disulphide bond formation or phosphorylation of M2 is essential for influenza A virus replication.

In this report, we determine the sites of palmitoylation, phosphorylation and intermolecular disulphide bond formation of influenza C virus CM2 and also present data showing that none of these modifications is essential for its transport to the cell surface.

**Methods**

- **Cells.** COS cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal calf serum.

- **Plasmid construction and site-directed mutagenesis.** A total of 14 mutated M gene cDNAs (Fig. 1) were made by PCR with different primers (whose sequences are available from the authors upon request), utilizing plasmid pCM5-5/F (Hongo et al., 1998), which contains nucleotides 1–1168 of the C/Yamagata/1/88 virus M gene and nucleotides 1169–1181 of the C/Ann Arbor/1/50 virus M gene, as a template. The PCR products were each cut with NcoI and EcoNI or Sall and the resulting DNA fragments were ligated into the NcoI and EcoNI
or S111 sites of pCM5-5'3'F. DNA molecules containing full-length copies of the altered M genes were excised by digestion with EcoRI and S111 and then subcloned into the EcoRI and XhoI sites of a transient expression vector, pME18S (Takebe et al., 1988; a gift of Y. Takebe, National Institute of Infectious Diseases, Tokyo, Japan). Nucleotide sequences of all the mutant cDNAs in pCM5-5'3'F were confirmed by deoxy-nucleotide chain-terminating sequencing.

Transfection, metabolic labelling and immunoprecipitation. Subconfluent monolayers of COS cells in 3.5 cm Petri dishes were transfected by the lipofectamine procedure with recombinant pME18S plasmid (1 µg per plate) containing the wild-type (WT) or mutated M genes and incubated at 37°C. At 48 h post-transfection, cells were labelled with [35S]methionine (30 µCi/ml, ARC) for various periods in methionine-deficient DMEM. The transfected cells were also labelled for 4 h at 48 h post-transfection with either [3H]palmitic acid (500 µCi/ml; Amersham) in DMEM supplemented with 5 mM sodium pyruvate or [32P]orthophosphate (30 µCi/ml, NEN) in DMEM lacking phosphate. Cells were then disrupted in 0.01 M Tris–HCl (pH 7.4) containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl and a cocktail of protease inhibitors (Hongo et al., 1997) (RIPA buffer) and immunoprecipitated as described previously (Sugawara et al., 1986) by using rabbit immune serum raised against a glutathione S-transferase (GST) fusion protein constructed to contain the CM2 protein (GST–CM2) (Hongo et al., 1994). The immunoprecipitates obtained were analysed by SDS–PAGE on 17.5% gels containing 4 M urea and processed for analysis by fluorography (Yokota et al., 1983). All image processing was performed on a Macintosh computer by using the public-domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Peptide-N-glycosidase F (N-glycanase) treatment. The immunoprecipitated proteins were digested with N-glycanase (Boehringer Mannheim) for 16 h at 37°C under conditions described elsewhere (Hongo et al., 1997), precipitated with acetone and analysed by SDS–PAGE.

Sucrose density gradient sedimentation. Transfected COS cells were labelled with [35S]methionine for 30 min at 48 h post-transfection and chased for 1 h. Cells were then disrupted in MNT buffer (20 mM MES, 30 mM Tris–HCl, 100 mM NaCl, pH 7.4) containing 1% Triton X-100, 50 mM iodoacetamide and 0.5% apotinin. After low-speed centrifugation to remove nuclei, the lysate was layered onto a 10–50% (w/v) sucrose gradient in MNT buffer containing 0.1% Triton X-100 and centrifuged in a Beckman SW41 rotor at 40,000 r.p.m. for 16 h at 20°C. Fourteen 0.8 ml fractions were collected dropwise from the bottom of the tube, diluted in RIPA buffer and then immunoprecipitated.

Immunofluorescent staining. For cell-surface staining, transfected COS cells grown on plastic coverslips were fixed at 48 h post-transfection with 4% paraformaldehyde in PBS, pH 7.4, for 30 min at 4°C and then stained by an indirect method. A rabbit immune serum against the GST–CM2 fusion protein (diluted 1:100 in PBS containing 1% BSA) was used as the primary antibody. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Seikagaku Kogyo) was diluted 1:50 and then used as the second antibody. Photomicroscopy was performed on an Olympus VANOX microscope. All photographic exposure times were equivalent.

Chemical cross-linking. Transfected COS cells were labelled with [35S]methionine for 30 min at 48 h post-transfection and chased for 80 min. A stock solution of 100 mM diithiobis(succinimidylpropionate) (DSP) prepared in DMSO was diluted in PBS to the concentrations of 0.5 or 2.5 mM and then added to the labelled COS monolayers. After incubation overnight at 4°C, glycine (50 mM) was added to neutralize excess cross-linker and cells were immunoprecipitated with anti-GST–CM2 serum.

Results and Discussion

Identification of the acylation site of CM2

Influenza C virus CM2 has been shown to be modified covalently with a fatty acid (probably palmitate) in a thioester-type linkage (Hongo et al., 1997). Palmitoylation is known to occur on the cytoplasmic tails of integral membrane proteins (Schmidt, 1989). The cytoplasmic tail of CM2 contains only a single cysteine residue, at position 65, which is completely conserved among all influenza C virus strains examined so far (Tada et al., 1997; Matsuzaki et al., 2000). Therefore, cysteine 65 is likely to be the site of fatty acylation. To prove this, we created mutant C65A, in which cysteine 65 was changed to alanine (see Fig. 1), and investigated the effect of this mutation on palmitoylation of CM2. COS cells transfected with the WT or mutant C65A cDNA were labelled with [35S]methionine or [3H]palmitic acid for 4 h at 48 h post-transfection and then immunoprecipitated with anti-GST–CM2 serum (Fig. 2A). The SDS–PAGE patterns of [35S]methionine-labelled immunoprecipitates showed that three forms of CM2 (CM2m, CM2a and CM2b) were present in both the WT- and C65A-transfected cells. We demonstrated previously that a mannose-rich oligosaccharide core is added to unglycosylated CM20 (16 kDa) to form CM2a (18 kDa) and that maturation of this oligosaccharide chain from the high-mannose type to the complex type converts CM2a into CM2b (22–30 kDa) (Hongo et al., 1994, 1997, 1998). Clearly, all three forms of WT CM2 were labelled with [3H]palmitic acid, whereas no form of C65A was. In order to exclude the possibility that the failure to detect incorporation of [3H]palmitic acid into C65A might be due to the heterogeneous electrophoretic mobility of CM2b (the form labelled most heavily with [3H]palmitic acid in WT), CM2b and CM2a were converted into the more homogeneously migrating CM2m by treating immunoprecipitated proteins with N-glycanase prior to SDS–PAGE (Fig. 2B). The [35S]methionine-labelling patterns showed that CM2m of both WT and C65A migrated as two distinct bands. The slower migrating CM2m but not the faster migrating one has been shown to be phosphorylated (Tada et al., 1998). It was evident that the two bands of WT CM2m were both labelled with [3H]palmitic acid, whereas neither band of C65A was labelled. Thus, we conclude that cysteine 65 is the site for fatty acylation. The protein band that migrated slightly faster than CM2m, presumably represents a degradation product of CM2m, but was not characterized further.

Identification of the phosphorylation sites of CM2

We reported previously that the CM2 protein synthesized in influenza C virus-infected HMV-II cells is modified post-
translationally by phosphorylation, which occurs exclusively on serine residues (Tada et al., 1998). Examination of the predicted amino acid sequence of C/Yamagata/1/88 virus CM2 showed that two serine residues (positions 78 and 108), both of which are located in the cytoplasmic domain, are each in a sequence context (S–X–E/D) such that they could be phosphorylated by mammary gland casein kinase (Kemp & Pearson, 1990). Serine residue 78 is also within the recognition motif for casein kinase I (X–E–X–X–S–X) (Kemp & Pearson, 1990). Furthermore, these two serine residues were found to be conserved among all influenza C virus strains analysed (Tada et al., 1997; Matsuzaki et al., 2000). In order to identify the phosphorylated residue(s) in CM2, therefore, we first created three mutants (S78A, S108A and S78–108A) in which one or both of serine 78 and serine 108 was changed to alanine (see Fig. 1). These serine-altered CM2 molecules were expressed in COS cells, labelled with [35S]methionine or [3H]palmitic acid and then immunoprecipitated with anti-GST–CM2 serum. The resulting precipitates were untreated (A) or treated with N-glycanase (B) and analysed by SDS–PAGE under reducing conditions. Numbers at the left show molecular mass (in kDa).

Fig. 2. Identification of the acylation site of CM2. COS cells, mock-transfected or transfected with WT or C65A cDNA, were labelled with [35S]methionine (lanes S) or [3H]palmitic acid (lanes H) for 4 h at 48 h post-transfection and then immunoprecipitated with anti-GST–CM2 serum. The resulting precipitates were untreated (A) or treated with N-glycanase (B) and analysed by SDS–PAGE under reducing conditions. Numbers at the left show molecular mass (in kDa).

Re-examination of the deduced amino acid sequence of CM2 showed that a proline residue is present on the C-terminal side of serine 103. The serine–proline sequence is known to be a consensus target for phosphorylation by proline-directed protein kinases (Byrappa et al., 1996; Byrappa & Gupta, 1999). In order to investigate the possibility that serine 103 is phosphorylated by this class of protein kinase, two additional serine-altered mutants (S78·103A, S78·103·108A) expressed in COS cells were examined for phosphorylation. Fig. 3(B) showed that they were not modified
Identification of cysteine residues involved in intermolecular disulphide bond formation

The CM2 protein synthesized in infected cells has been shown to form disulphide-linked dimers and tetramers (Hongo et al., 1997; Pekosz & Lamb, 1997). This protein contains three cysteine residues in its extracellular domain (positions 1, 6 and 20), all of which are conserved among all analyzed influenza C virus strains (Tada et al., 1997; Matsuzaki et al., 2000). In order to identify cysteine residues involved in intermolecular disulphide bond formation, seven different mutant proteins were created in which one or more of the three cysteine residues was converted to alanine (see Fig. 1). COS cells expressing the WT or mutant CM2 were pulse-labelled for 30 min with [35S]methionine at 48 h post-transfection and chased for 1 h. Cells were then immunoprecipitated with anti-GST–CM2 serum and analysed by SDS–PAGE under non-reducing conditions. As shown in Fig. 4, WT CM2 formed a mixture of disulphide-linked dimers (~ 32 kDa, 58%) and disulphide-linked tetramers (~ 64 kDa, 34%), although a small amount of the monomeric form (~ 16 kDa, 8%) was also detected. A protein band that migrated slightly faster than the 64 kDa tetramer presumably represents a CM2 tetramer that possessed a disulphide bond(s) formed between cysteine residues different from those used for formation of the 64 kDa tetramer. Replacement of all three cysteine residues in the ectodomain eliminated the formation of disulphide bonds and the mutant protein (C1·6·20A) migrated exclusively as a 16 kDa monomer, as expected. Clearly, the efficiency of mutants C1A and C6A to form disulphide-linked oligomers was low compared with that of WT (> 50% of these mutant proteins were present as monomers), suggesting that cysteines 1 and 6 are both involved in intermolecular disulphide bond formation. Interestingly, mutant C20A did not form a CM2 tetramer at all, although it produced a dimeric form efficiently, which suggests strongly that cysteine 20 plays a critical role in the formation of the disulphide-linked tetramer. In the gel patterns of C1A and C6A, a band was detected with an apparent molecular mass of ~ 48 kDa, corresponding to that expected for a CM2 trimer. It is possible, therefore, that the trimeric intermediate may be formed in the absence of cysteine at residues 1 or 6.

As was expected, the three double mutants (C1·6A, C1·20A and C6·20A) failed to form disulphide-linked tetramers. It was also evident that the efficiency of C6·20A (whose ectodomain contains only one cysteine residue, at position 1) in forming a disulphide-linked dimer was very low compared with C1·6A (with cysteine at position 20) and C1·20A (with cysteine at position 6), suggesting that cysteine 6 may play a more important role in the formation of the disulphide-linked dimer than cysteine 1 and that cysteine 20 can participate in dimer formation (in addition to tetramer formation), especially in the absence of cysteine at residues 1 and 6. However, the cysteine-to-alanine change introduced into each of the mutant CM2 proteins is likely to have created a situation where the remaining cysteine residues now undergo formation of disulphide bonds that are not present in the WT protein. Thus, the only conclusion we can draw from the data shown in Fig. 4 may be that all three cysteine residues located in the ectodomain can participate in the formation of disulphide-linked dimers and/or tetramers.
Effects of acylation and phosphorylation on the formation of disulphide-linked CM2 oligomers

As shown above, fatty acylation and phosphorylation respectively do not occur in mutants C65A and S78·103·108A. In order to study the effects of these modifications on disulphide-linked oligomer formation, pulse–chase experiments were done with COS cells expressing C65A or S78·103·108A according to the procedures described above. As demonstrated in Fig. 5, the amounts of tetramer relative to monomers and dimers were comparable between WT and C65A (about 55%), but were very low in S78·103·108A (26%). These results suggest that, while palmitoylation has no role in disulphide-linked oligomer formation, phosphorylation accelerates tetramer formation without influencing dimer formation. Undoubtedly, this effect of phosphorylation is caused mostly, if not entirely, by casein kinase-mediated phosphorylation at serine 78, since a marked reduction in the amount of tetramer was also observed with the single mutant S78A (data not shown). Thomas et al. (1998) investigated the role of phosphorylation of influenza A virus M2 by using an exogenous incorporation assay. The authors first established a stable and clonal cell line that expressed A/PR/8/34 virus, they monitored the M2 species in the released virions and found efficient incorporation of tetrameric forms of unphosphorylated A/WSN/33 M2 into virions, a result that indicated that incorporation of M2 tetramers into virus particles does not require phosphorylation of the cytoplasmic tail. However, the data presented in their report also showed that, in cells stably expressing unphosphorylated mutant M2, the amount of tetramer relative to dimer was fairly small compared with that in cells expressing phosphorylated WT M2, suggesting that phosphorylation of M2, like that of CM2, may influence the formation of disulphide-linked tetramers.

Sucrose density gradient analysis of CM2 mutant proteins

In order to investigate further the effects of acylation, phosphorylation and disulphide bond formation on the oligomeric structure of CM2, COS cells transfected with WT or mutant protein genes were disrupted after 30 min pulse-labelling with $^{35}$S-methionine followed by a 1 h chase and the resulting lysates were analysed on sucrose gradients. Fig. 6(A) shows the results of an experiment with WT CM2 in which proteins present in each gradient fraction were immunoprecipitated and electrophoresed (after N-glycanase treatment) under non-reducing conditions. Disulphide-linked tetramers and dimers of WT sedimented as peaks in fractions 8–10 and fractions 10–11, respectively, and monomers sedimented with a peak forming in fractions 11 and 12. The observation that the monomeric, dimeric and tetrameric forms of WT sedimented differently from each other on sucrose gradients indicates that the CM2 molecules do not undergo the formation of homooligomers (dimers, trimers or tetramers) composed of monomers or disulphide-linked dimers held together by non-covalent interactions or, alternatively, that WT CM2 can oligomerize to form such oligomers but that non-covalent interactions are dissociated during detergent solubilization, as has been observed with influenza A virus M2 (Holsinger & Lamb, 1991).

In Fig. 6(B), the sedimentation patterns of mutants C65A (defective in acylation), S78·103·108A (defective in phosphorylation) and C1·6·20A (defective in disulphide bond formation) are compared with that of WT. In these experiments, immunoprecipitated proteins obtained from each of the gradient fractions were analysed by SDS–PAGE (without N-glycanase treatment) under reducing conditions. C65A had sedimentation properties indistinguishable from those of WT, being recovered mostly in fractions 9–11, which contained a mixture of disulphide-linked dimers and tetramers. S78·103·108A showed a reproducible difference in its sedimentation patterns compared with WT, the amounts of proteins recovered in fractions 9 and 10 decreased, with an increase in that recovered in fractions 11 and 12, a result compatible with the finding described above that this mutant had a decreased ability to form disulphide-linked tetramers (see Fig. 5). Furthermore, it was found that C1·6·20A sedimention
Influenza C virus CM2 protein

Fig. 6. Sucrose density gradient analysis of the oligomeric forms of the WT and mutant CM2s. COS cells expressing WT CM2 or mutant CM2 defective in acylation (C65A), phosphorylation (S78\textsuperscript{103}S\textsuperscript{108}A) or disulphide bond formation (C1\textsuperscript{6}C\textsuperscript{20}A) were disrupted after 30 min pulse-labelling with \(^{35}\text{S}\)methionine at 48 h post-transfection followed by a 1 h chase and the resulting lysates were subjected to sucrose velocity sedimentation on 5–15% sucrose gradients. Fractions were collected and immunoprecipitated with anti-GST–CM2 serum. (A) Immunoprecipitated WT CM2 protein was treated with N-glycanase and analysed by SDS-PAGE under non-reducing conditions. (B) Proteins were analysed directly by SDS–PAGE under reducing conditions. Experiments with the different proteins are shown as follows: (a) WT, (b) C65A, (c) S78\textsuperscript{103}S\textsuperscript{108}A and (d) C1\textsuperscript{6}C\textsuperscript{20}A. BSA (67 kDa; filled arrowheads) and trypsin inhibitor (20 kDa; open arrowheads) were used as sedimentation markers.

### Transport of mutant CM2 proteins to the cell surface

Not only CM2a (endoglycosidase H-sensitive) but also CM2b (endoglycosidase H-resistant) could be detected in COS cells transfected with any of the mutant protein genes studied here (Figs 2A and 6B and data not shown), showing that none of acylation, phosphorylation and disulphide bond formation is essential for intracellular transport of CM2 from the ER to the Golgi apparatus. In order to determine whether these modifications are required for cell-surface expression of CM2, COS cells transfected with WT or each of three mutants (C65A, S78\textsuperscript{103}S\textsuperscript{108}A and C1\textsuperscript{6}C\textsuperscript{20}A) were fixed with paraformaldehyde and incubated with anti-GST–CM2 rabbit serum followed by incubation with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG.

We reported previously that indirect immunofluorescence with the anti-GST–CM2 serum used here failed to stain the surfaces of C\textsuperscript{Yamagata/1/88} virus-infected HMV-II cells that were unfixed or fixed with paraformaldehyde (Hongo et al., 1997). This unexpected observation led us to postulate that the rabbit immune serum might not contain antibodies reactive with the CM2 ectodomain or, alternatively, the complex carbohydrate chain linked to asparagine residue 11 (Pekosz & Lamb, 1997) might mask antigenic sites present in the ectodomain (Hongo et al., 1997). As seen in Fig. 7, however, COS cells transfected with the WT gene showed bright surface staining. The discrepancy between these two observations may be attributable to differences in the cell type used or may be due to hindrance of antigenic epitopes by the haemagglutinin–esterase glycoprotein molecules expressed abundantly in infected cells but not in transfected cells.

Fig. 7 shows that all three mutants tested, like WT, could be transported to the cell surface. Although the intensity of the fluorescence was not quantified, the level of surface expression did not appear to differ significantly between the WT and mutant CM2 proteins, since electronic metering indicated equivalent exposure times. Thus, we conclude that CM2 can be transported to the cell surface in the absence of acylation, phosphorylation and intermolecular disulphide bond formation. It has generally been accepted that proper oligomerization is a prerequisite for integral membrane proteins to as monomers, peaking in fractions 11 and 12. Thus, no evidence was obtained from sucrose density gradient analysis that the mutant CM2 lacking cysteine residues in the ectodomain has the potential to form non-covalently linked multimers.

*Influenza C virus CM2 protein*
be transported out of the ER (for review, see Doms et al., 1993). This notion, together with the results of Fig. 7, leads us to hypothesize that CM2 molecules oligomerize properly in the absence of any of these post-translational modifications, including the formation of intermolecular disulphide bonds, although sucrose density gradient analysis failed to detect non-covalently linked multimers of mutant C1·6·20A (see Fig. 6B).

**Chemical cross-linking analysis of a CM2 mutant defective in disulphide bond formation**

In order to examine the possibility that mutant C1·6·20A may form a multimer that is held together weakly by non-covalent forces that are sensitive to disruption by detergent, COS cells expressing WT or C1·6·20A were pulse-labelled with [35S]methionine for 30 min at 48 h post-transfection and then chased for 80 min. Monolayers of the labelled cells were incubated with DSP (a homobifunctional cross-linking reagent), immunoprecipitated and analysed (after N-glycanase treatment) by SDS–PAGE under non-reducing conditions (Fig. 8). It was clear that C1·6·20A formed cross-linked dimers. A small amount of the 48 kDa species (probably representing a trimer) was also detected, in addition to a trace amount of tetramer. A small amount of the dimeric C1·6·20A molecule was even found in the absence of DSP. This species might have arisen by intermolecular disulphide bonding after cell disruption through linkage of cysteines at residue 65, although iodoacetamide was included in the disruption buffer. The data shown in Figs 6–8, taken together, suggest that, although the formation of disulphide bonds is not a prerequisite for oligomeric assembly, it stabilizes the CM2 multimer (perhaps tetramer) against dissociation by detergent solubilization, as is the case with influenza A virus M2 (Holsinger & Lamb, 1991).

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

**References**


Influenza C virus CM2 protein


Received 15 November 2000; Accepted 18 January 2001

1093