

Establishment of mouse erythroleukemia cell lines expressing complete Influenza C virus CM2 protein or chimeric protein consisting of CM2 and Influenza A virus M2

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Summary. – The role of the Influenza C virus (ICV) CM2 protein in virus replication as well as its precise function as an ion channel remains to be elucidated. For this purpose, we established a CM2-expressing mouse erythroleukemia (MEL) cell line and determined the biochemical characteristics of the expressed CM2. The features of the expressed CM2 were similar to those of the viral CM2 synthesized in ICV-infected cells. Furthermore, we established MEL cell line expressing a chimeric protein consisting of characteristic regions of CM2 and Influenza A virus (IAV) M2 protein that could be helpful in elucidation of the specific ion conductance properties.

Keywords: Influenza C virus; Influenza A virus; CM2 protein; chimeric protein; MEL cells

The genome of ICV consists of 7 single-stranded RNA segments of negative polarity encoding proteins PB2, PB1, P3, hemagglutinin-esterase-fusion (HEF) protein, nucleoprotein (NP), matrix (M1), P42, NS1 and NS2/NEP (Palese and Shaw, 2007). RNA segment 6 containing M1 and P42 gene is 1,180 or 1,181 nts in length. Proteolytic cleavage of P42 at an internal signal peptidase cleavage site gives rise to M1' protein composed of aa 259 and CM2 protein of aa 115 (Yamashita *et al.*, 1988; Hongo *et al.*, 1994, 1998, 1999; Pekosz and Lamb, 1997).

CM2 is abundantly expressed in virus-infected cells and is incorporated into progeny virions as a second membrane protein of the virus (Hongo *et al.*, 1997). CM2 forms a Cl⁻ channel, when expressed in *Xenopus laevis* oocytes and also

has the ability to modulate the pH of the exocytic pathway (Hongo *et al.*, 2004; Betáková and Kollerová, 2006; Betakova and Hay, 2007). However, its role in the virus replication as well as its precise function as the ion channel remains to be elucidated.

The IAV M2 protein, the counterpart of CM2, has been shown to have a role in two stages of virus replication (Palese and Shaw, 2007). During endocytosis, M2 in the virus membrane acts by mediating proton transfer into the virion interior to promote an acid-induced dissociation of the matrix protein from the ribonucleoprotein (RNP). In particular avian influenza virus infections, the M2 equilibrates the pH between the lumen of trans-Golgi network and the cytoplasm, preventing the acidification of the newly synthesized hemagglutinin that is cleaved intracellularly. Recently, the cytoplasmic tail of M2 has been demonstrated to play a role in the incorporation of the RNP into virions (McCown and Pekosz, 2005; Iwatsuki-Horimoto *et al.*, 2006).

Ion permeability due to M2 measured by direct electrophysiological analyses has been reported in several studies employing *X. laevis* oocytes, CV-1 cells, phospholipid bilayers or MEL cells (Ogden *et al.*, 1998). In all studies, M2 formed an ion channel permeable to protons, which provided the basis for the putative role of M2 in the virus replication.

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Abbreviations: ACS = anti-CM2 serum; HEF = hemagglutinin-esterase-fusion; IAV = Influenza A virus; ICV = Influenza C virus; MEL = mouse erythroleukemia; TM = CM2 transmembrane domain

MEL cells were of significant use in analyzing the precise ion channel activity of M2, because they have a low intrinsic membrane ion permeability and their small size permits control of the ionic composition of cell interior (Shelton *et al.*, 1993). The M2 protein expressed in MEL cells was shown to form an ion channel that selectively transfers protons across the membrane (Chizhmakov *et al.*, 1996).

Construction of cells constitutively expressing a virus protein may also be applicable to rescuing recombinant viruses lacking the protein being expressed (McCown and Pekosz, 2005; Iwatsuki-Horimoto *et al.*, 2006). Recently, MDCK cells expressing the BM2 protein were used to complement a BM2-deficient recombinant of Influenza B virus (Imai *et al.*, 2004). In these studies, the respective roles of M2 and BM2 in the replication of the IAV and influenza B viruses were clarified.

In the present study, we report the successful construction of a CM2-expressing MEL cell clone and present the biochemical characteristics of the expressed CM2 protein. Furthermore, we constructed a MEL cell line expressing chimeric protein consisting of particular regions of CM2 of ICV and M2 of IAV. The transmembrane (TM) region of M2 was replaced with that of CM2. Also, we analyzed the biochemical properties of the chimeric protein.

Initially, we attempted to establish MEL cell clones expressing the CM2 protein of the ICV strain C/YA/1/88 (Acc. No. D16261) (Hongo *et al.*, 1994). We cloned a methionine (Met)-CM2 cDNA, where an initiation codon (ATG) was added to the 5'-end of CM2-ORF, and inserted in pEV3 vector at the *EcoRI* and *BglIII* sites. The resulting plasmid was linearized and transfected into MEL cells as described previously (Chizhmakov *et al.*, 1996; Ogden *et al.*, 1998). The transfected MEL cells were cultured for 10–14 days in the presence of geneticin until the drug-resistant MEL cells became 50% confluent. Single MEL cell clones were obtained by limiting dilution in a 96-well plate and subsequently maintained in the presence of geneticin. The level of CM2 expression by individual clones was analyzed by Western blot analysis.

The MEL cell clone (MEL/CM2) expressed a considerable amount of CM2 as detected using anti-CM2 serum (ACS) (Fig. 1) (Hongo *et al.*, 1994). The optimal conditions for CM2 expression in the MEL/CM2 were determined as described previously (Ogden *et al.*, 1998). At the optimal induction concentration (2%) of dimethyl sulfoxide (DMSO), CM2 expression was comparable to that as in ICV-infected HMV-II cells, a human melanoma cell line highly susceptible to ICV (Nishimura *et al.*, 1989) (Fig. 1). The CM2 protein accumulated to a maximum level at 3–4 days after induction with DMSO (data not shown). Since expression of CM2 was comparable to that of M2 in a MEL cell clone expressing IAV M2 (MEL/M2-39) (Chizhmakov *et al.*, 1996; Ogden *et al.*, 1998), we concluded that the MEL/CM2

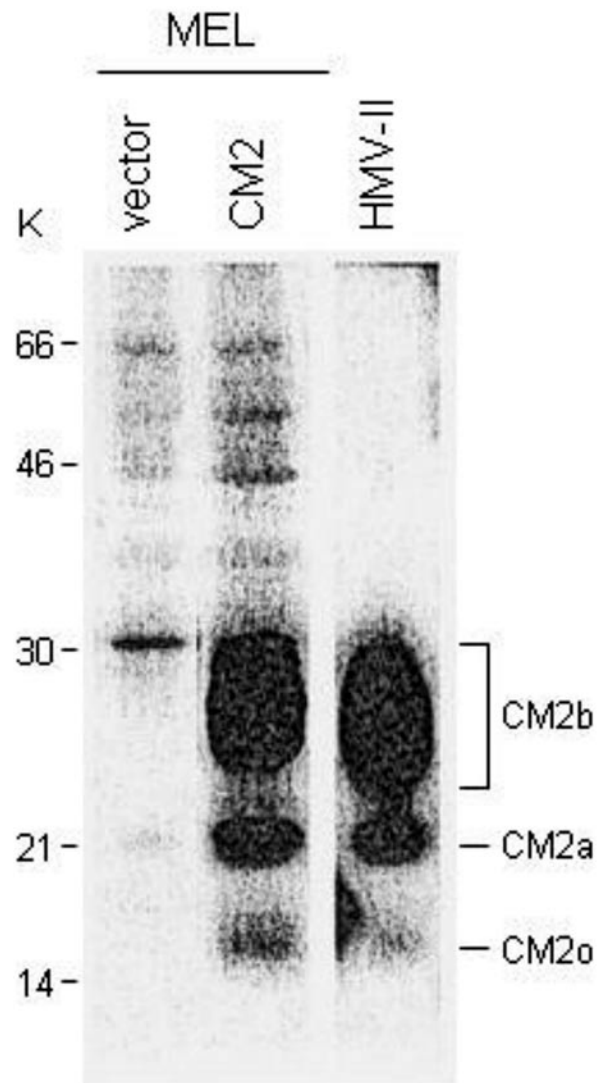


Fig. 1

Western blot analysis of CM2 expression in MEL cells

MEL cells transfected with an empty pEV3 vector (lane vector), with the pEV3 vector with a Met-CM2-ORF insertion (lane CM2), and HMV-II cells infected with ICV (lane HMV-II). CM2o = non-glycosylated form (16 K), CM2a = N-linked glycosylated form (high mannose type) (18 K), CM2b = N-linked glycosylated form (complex type) (22–30 K). M_r markers on the left (K).

was suitable for the subsequent analyses of the biochemical properties of CM2.

We examined glycosylation, phosphorylation, acylation, and tetramer formation of the CM2 expressed in the MEL cells. At day 4 post induction, the MEL/CM2 cells were labeled with [³⁵S]-methionine for 30 mins and then chased for 2 hrs. Cell lysates were prepared either immediately after the pulse or after the chase and immunoprecipitated with

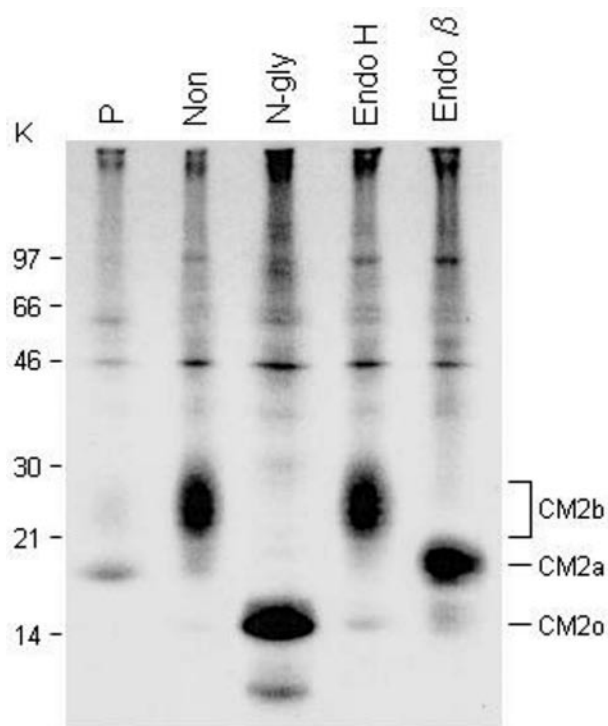


Fig. 2

Pulse-chase study of MEL cells expressing CM2

SDS-PAGE and autoradiography of [³⁵S]-methionine pulse-chased and precipitated proteins extracted from MEL cells. The precipitated proteins were treated with N-glycanase (lane N-gly), endo H (lane Endo H), endo-β-galactosidase (lane Endo β) or mock-treated (lane Non). Pulse without chase (lane P). For description of bands see legend to the Fig. 1.

ACS. The precipitates were digested with various endoglycosidases and then analyzed by SDS-PAGE and processed for fluorography (Yokota *et al.*, 1983; Hongo *et al.*, 1997). N-glycanase converted CM2a and CM2b to non-glycosylated form of CM2 (CM2o) (Fig. 2). CM2a was sensitive to digestion with endo H, whereas CM2b was resistant. On the other hand, CM2b was sensitive to endo-β-galactosidase treatment. Taken together, these data indicated that CM2 in MEL cells was modified with polylactosaminoglycans. Previously, Pekosz *et al.* (1997) reported that CM2b expressed in both MDCK and HeLa-T4 cells was sensitive to endo-β-galactosidase treatment, whereas Hongo *et al.* (1997) reported that CM2b in ICV-infected HMV-II cells was resistant to that treatment. Thus, the glycosylation machinery on CM2 appears to vary according to the cell line used.

The induced MEL/CM2 cells were labeled also with [³²P]-orthophosphate or [³H]-palmitic acid and immunoprecipitated with ACS or the cell lysates were analyzed by Western blot analysis under non-reducing conditions (Hongo *et al.*, 1997; Tada *et al.*, 1998). The results showed that CM2 was

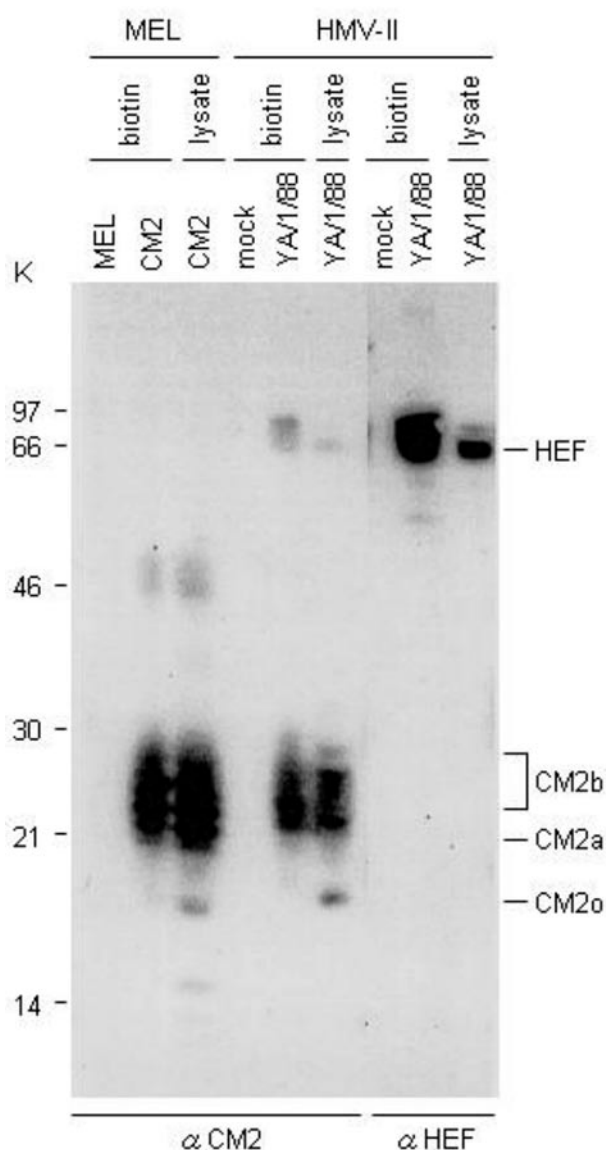


Fig. 3

Surface expression of CM2 on MEL cells

Western blot analysis of cell surface proteins labeled with Sulfo-NHS-biotin. For detection of CM2 and HEF protein, anti-CM2 serum (αCM2) or anti-HEF monoclonal antibody (αHEF), respectively, were used. MEL cells (lane MEL), MEL/CM2 cells (lanes CM2), mock-infected HMV-II cells (lane mock), HMV-II cells infected with ICV (lanes YA/1/88), biotinylated protein (biotin), and cell lysate (lysate). M_r markers on the left (K). For description of bands see legend to the Fig. 1.

phosphorylated, acylated, and formed tetramers (data not shown). Thus, except for glycosylation in some cell lines, the posttranslational modifications of CM2 in the MEL cells were similar to those in ICV-infected cells.

Next, the cell surface expression of the CM2 was investigated. CM2 was previously shown to be transported

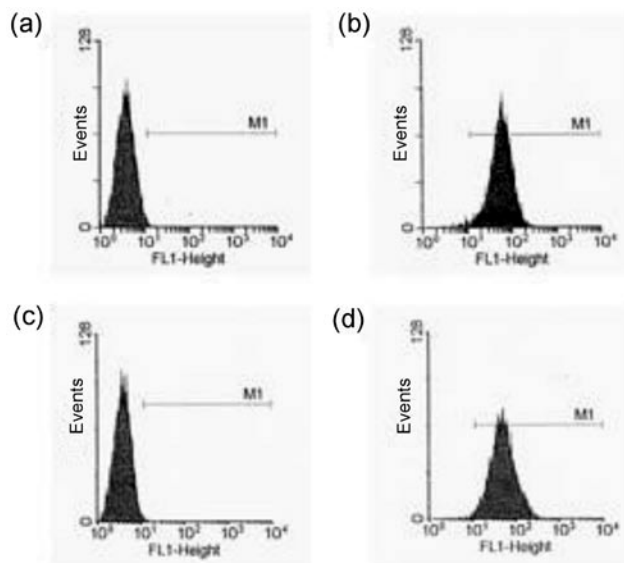


Fig. 4

Surface expression of chimeric M2/CM2 and M2 proteins on MEL cells detected by flow cytometry

Cells expressing M2/CM2 protein (b) and M2 protein (d) treated with anti-M2 serum. Control cells expressing M2/CM2 protein (a) and M2 protein (c) without treatment with anti-M2 serum.

to the cell surface using immunofluorescence techniques (Hongo *et al.*, 1997; Pekosz *et al.*, 1997; Li *et al.*, 2001). In the present study, we labeled cell surface proteins with Sulfo-NHS-Biotin (Pierce) and detected the labeled CM2 protein by Western blot analysis, since in the immunofluorescence analysis of MEL cells we detected a high background signals using ACS (data not shown). Induced MEL/CM2 cells or C/YA/1/88-infected HMV-II cells were subjected to Western blot analysis using ACS or anti-HEF monoclonal antibody S16 (Sugawara *et al.*, 1993; Muraki *et al.*, 1999). Both CM2b and HEF protein were detected on the surface of C/YA/1/88-infected HMV-II cells (Fig. 3). Furthermore, a 22–30 K protein was detected on the surface of the MEL cells indicating that CM2b was transported to the plasma membrane (Fig. 3).

Lastly, we established a MEL cell clone (MEL/M2/CM2) expressing an M2/CM2 chimeric protein. The chimeric protein was composed of the M2 extracellular domain (24 aa), the CM2 TM domain (23 aa) and the M2 cytoplasmic domain (54 aa). The expressed chimeric protein was shown to be phosphorylated, acylated, and oligomerized as a homotetramer (data not shown). Flow cytometry analysis using antiserum against the M2 extracellular domain revealed that the protein was transported to the cell surface at a similar level as M2 in the MEL/M2-39 cells (Smith *et al.*, 2002) (Fig. 4).

Preliminary electrophysiological studies of CM2-expressing MEL/CM2 cells have identified Na⁺-activated proton permeability in addition to the low pH-activated Cl[−] permeability (I.V. Chizhnikov, D.C. Ogden, A. Hay, personal communications). The H⁺-activated H⁺-selective permeability of the M2 channel is mainly attributable to the TM domain. Studies of the chimeric protein could help in the elucidation if both activities are intrinsic properties of CM2 or are due to the activation of an endogenous channel.

In conclusion, we reported here the successful establishment of MEL cell clones expressing CM2 and M2/CM2 proteins, which could be used in detailed studies to define the properties of the CM2 ion channel and to rescue CM2-deficient mutants of ICV.

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