The CD46 transmembrane domain is required for efficient formation of measles-virus-mediated syncytium

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Two phosphatidylinositol (PI)-anchored versions of a measles virus (MV) receptor membrane cofactor protein (MCP; CD46) were generated by fusing the extracellular domain of MCP to the decay-accelerating factor (DAF; CD55) or its PI anchor. The PI-anchored forms of MCP expressed on Chinese hamster ovary cells, otherwise non-permissive to MV, conferred a smaller MV cytopathic effect than a wild-type MCP, a Ser/Thr-rich domain-deletion mutant and a cytoplasmic tail-deletion mutant of MCP. Therefore the differences in MV receptor properties between the two PI-anchored and three transmembrane forms were investigated. The PI-anchored forms were predominantly expressed on microvilli as in DAF, whereas the other transmembrane forms were found on intracellular membranes. The PI-anchored forms conferred high MV-binding capacity compared with the

transmembrane versions. MV replication was, however, severely suppressed in cells expressing the PI-anchored forms, resulting in ineffective syncytium formation. In contrast, cell-to-cell fusion occurred efficiently after co-transfection of cDNA species encoding MV-H, MV-F and any version of MCP. Thus the PI-anchored forms, despite showing sufficient MV binding and cell-to-cell fusion competence together with MV-H and MV-F, mediate inefficient MV entry or replication, which causes severe suppression of the MV cytopathic effect. A biased receptor distribution on microvilli might participate in the selection of a low MV uptake pathway in the PI-anchored forms of MCP. Taken together, the transmembrane portion of MCP is a critical factor for effective virus–cell fusion and the subsequent MV replication.

INTRODUCTION

Evidence has accumulated that phosphatidylinositol (PI)-anchored proteins are predominantly distributed on the apical cell surface and are involved in endocytosis in a manner distinct from transmembrane (TM)-form proteins [1,2]. Several studies have focused on the distribution and behaviour of PI-anchored proteins in association with protein functions (reviewed in [3,4]). Association of caveolae, non-coated pits of the cell-surface compartments, with PI-anchored proteins has been proposed [5,6]. A number of signal messenger molecules such as Src family protein tyrosine kinases and heterotrimeric GTP-binding proteins are present in the inner leaflet of caveolae [6,7], although their interrelation in localization and function has not yet been clarified [8,9]. T-cell proliferation [4,10] and macrophage activation [11] have been reported as final cell responses secondary to PI-anchor signalling through decay-accelerating factor (DAF; CD55).

Replacement of TM and cytoplasmic tail (CYT) with PI anchors has been employed as a way of investigating the roles of the intracellular domains of TM proteins. This method has also been used to examine virus endocytosis via virus receptors, for example the major rhinovirus receptor ICAM-1 [12], the poliovirus receptor [13] and the HIV receptor CD4 [14,15]. After incubation with gp120 of HIV or anti-CD4 monoclonal antibody, CD4 with natural TM is rapidly internalized in transfected

Chinese hamster ovary (CHO) cells, whereas CD4 with a DAF PI anchor is internalized more slowly [15]. Their recycling profiles and localization patterns suggest that these two forms of CD4 enter cells by different pathways by using distinct populations of endosomes. In this case, PI-anchored proteins showed less potential for accelerating membrane fusion/endocytosis than the parent TM forms.

Recently, Naniche et al. [16] and Dörig et al. [17] found that CD46 (membrane cofactor protein, MCP) is a receptor for measles virus (MV), and this was subsequently confirmed by several other groups [18–21]. MV, unlike influenza virus [22], has been reported to induce pH-independent fusion without endosomal entry [23,24]. MCP is a ubiquitously expressed type 1 membrane protein with TM and CYT in its C-terminus [25–27]. The main extracellular domains are four short consensus repeats (SCRs) and a Ser/Thr-rich (ST) domain containing many glycosylation sites [25,27]. We and another group showed that SCR1 and SCR2 are the MV-H-binding sites [28,29]. Meanwhile two groups suggested that a PI-anchored form of MCP also serves as an MV receptor [29,30]. However, the differences in properties as an MV receptor between the PI-anchored MCP (MCP-PI) and the conventional MCP (MCP-TM) have been poorly explored.

To gain insight into this issue we mutated the domains adjacent to the SCRs to make PI-anchored forms, a tail-less form and a form with no ST domain. We then expressed these MCP forms

Abbreviations used: CHO, Chinese hamster ovary; CPE, cytopathic effect; DAF, decay-accelerating factor (CD55); Δ CYT, a cytoplasmic tail-deletion mutant of MCP (see Figure 1); DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; Hybrid, a PI-anchor protein generated by fusing the MCP SCR domains to DAF (see Figure 1); mAb, monoclonal antibody; MCP, membrane cofactor protein (CD46); MCP-PI, a PI-anchored protein generated by fusing the extracellular domain of MCP to the DAF PI anchor (see Figure 1); MCP-TM, a wild-type MCP (see Figure 1); MFS, mean fluorescence shift; m.o.i., multiplicity of infection; MV, measles virus; NP40, Nonidet P40; PI, phosphatidylinositol; PI-PLC, PI-specific phospholipase C; SCR, short consensus repeat; ST, a Ser/Thr-rich domain of DAF or MCP; Δ ST, a Ser/Thr-rich domain-deletion mutant of MCP; TM, transmembrane.

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on CHO cells non-permissive for MV and studied the localization patterns of these variants, rate and efficacy for MV binding, MV replication, MV-mediated cytopathic effect (CPE) and cell-to-cell fusion-inducing capacity. We report here that PI-anchored forms possess properties distinct from those of the TM forms of MCP as a MV receptor.

MATERIALS AND METHODS

Cells, antibodies and virus

MV (Nagahata strain) was prepared as described [31]. CHO cells were maintained in Ham's F-12 medium (Nissui, Tokyo, Japan) supplemented with 10% (v/v) fetal calf serum (FCS). CHO cell transfectants were grown in the same medium containing 0.5 mg/ml G418 (Geneticin, Life Technologies, Grand Island, NY, U.S.A.) [32] or 0.7 mg/ml of hygromycin (Sigma, St. Louis, MO, U.S.A.) [33]. Vero cells for virus stock and MV titre determination were maintained in Eagle's minimal essential medium (Nissui) supplemented with 5% (v/v) FCS [34].

A panel of monoclonal antibodies (mAbs) against MCP, J48 (recognizing SCR1) [28], M177 (recognizing SCR2) [28,35] and M160 (recognizing SCR3) [28,35], and a mAb against DAF (D17) [36] were prepared as described. An mAb against MV-H was prepared as described previously [18]. Fluorescein isothiocyanate (FITC)-labelled phalloidin for detection of F-actin was from Seikagaku Kogyo (Tokyo, Japan).

MCP mutants

The cDNA encoding the MCP mutants, Δ ST (lacking amino acid residues 348–379) [26] was prepared as described previously [18]. The cDNA of Δ CYT (cytoplasmic tail-deletion) mutant was constructed from the parent MCP cDNA (ST^c/CYT2) in pUC19 (pUC-MCP) [26] so as to eliminate the sequence between the *EcoRV* site and the *BspHI* site downstream of the TM sequence. The pUC-MCP was digested with *EcoRI* and then partly digested with *EcoRV*. The 1050 bp fragment was isolated by agarose-gel electrophoresis. The parent pUC-MCP was also digested with *NcoI* and then partly with *BspHI*. After treatment with Klenow enzyme, the 2800 bp fragment was isolated by agarose-gel electrophoresis. This fragment was partly digested with *EcoRI* and then ligated with the 1050 bp fragment. The cytoplasmic amino acid sequence of this Δ CYT mutant was Arg-Ser (the Ser was substituted for Tyr).

The cDNA for the MCP-DAF hybrid was constructed as described previously [32]. The other PI-anchored form containing MCP SCR/ST domains plus the DAF PI anchor was constructed as follows: cDNA encoding amino acids 1–293 of MCP and that encoding residues 308–347 of DAF [32] were fused with PCR. Oligonucleotides 5'-GGAATACTTGACAGTTTGCATGAAACAACCA-3' (primer A) and 5'-TGGGTTGTTTCATGCAAACTGTCAAGTATTCC-3' (primer B) covering the 3' terminus of the UK region [26] of MCP and the 5' terminus of the PI-anchoring signal of DAF were synthesized and used as amplification primers. First-round PCR was performed with primers B and 5'M13-M4 and the template pUC-MCP, and with primers A and M13-RV and the template pBluescript containing the full-length DAF cDNA [32]. The two PCR products were mixed and used as a template for the second round of PCR with primers 5'M13-M4 and M13-RV. The PCR product encoding MCP-PI was digested out with *PstI* and *EcoRI*, and then placed into the *EcoRI/PstI* site of pME18S [37]. The expected protein was different from the reported PI-anchored MCP [29,30] in that our PI-anchored form possessed the ST domain of MCP, whereas the other contained that of DAF.

The sequences of all MCP mutant cDNA species were confirmed by using a DNA sequencer (373A, Perkin-Elmer Cetus, Norwalk, CT, U.S.A.).

These cDNA species were subcloned into pME18S [37] or pCAGGS [38] eukaryotic expression vectors and then transfected into CHO cells by calcium phosphate precipitation or lipofectamine methods [39] with 5–20 μ g of cDNA per plasmid. Transfected CHO cells were maintained in Ham's F12 medium/10% (v/v) FCS supplemented with 0.5 mg/ml G418 in an atmosphere of humidified air/CO₂ (19:1) at 37 °C. G418-resistant colonies were isolated with cloning cylinders and expanded in tissue-culture plates. Expression of MCP was monitored by flow cytometry with mAbs as described below [35].

Formation of the PI anchor in the MCP derivatives was monitored by enzymic cleavage with PI-specific phospholipase C (PI-PLC) [15].

SDS/PAGE and immunoblotting, and flow cytometry

MCP was solubilized from the transfectants first with 1% (v/v) Nonidet P40 (NP40) containing protease inhibitors at 0 °C as described [40]; the pellet was then recovered by centrifugation. The pellet was subsequently solubilized with 50 mM octylglycoside under conditions that will be described elsewhere (details available from the authors). PI-anchored forms of MCP were enriched in the octylglycoside-solubilized fraction. This biochemical difference was reproducibly observed, consistently with other reports [6,41], if the expression level was below a mean fluorescence shift (MFS) of 100. The samples were resolved by SDS/PAGE by the method of Laemmli [42]. After electrophoresis the samples were transblotted on nitrocellulose sheets [43]. MCP was detected with mAbs to MCP, J48, M160 or M177, HRP-labelled secondary antibody and colour reagents [44].

Flow cytometry was performed as described previously [35]. Briefly, 10⁶ cells were incubated with 1–5 μ g of murine mAbs, M177 or anti-MV-H, for 45 min at 0 °C. FITC-labelled goat anti-(mouse IgG) antibody was used as a secondary antibody. After extensive washing with ice-cold PBS/1% (w/v) BSA, cells were fixed with 0.5% paraformaldehyde. The stained cells were analysed with an EPICS Profile II flow cytometer within 2 days. MFS was evaluated on the attached computer.

Immunofluorescence and electron microscopy

The immunofluorescence method employed to stain for MCP, DAF or MV-H was as described previously [45]. Briefly, cells were cultured on Chamber-slides (Lab-Tek, Nunc, IL, U.S.A.), and the adhering cells were washed twice with PBS then fixed with 0.1% glutaraldehyde/3% (w/v) paraformaldehyde/PBS for 1 h on ice. The cells were washed extensively with PBS; the buffer was then changed to 150 mM NaCl/Tris/HCl (pH 7.4). The cells were pretreated with 2% (v/v) goat serum/0.5% BSA/0.1% gelatin/PBS (0.02% NP40 was added if the cells had been permeabilized), then the first mAb (3–5 μ g) was added. The cells were washed with PBS containing 0.01% Tween-20, 2–12 h after addition of the mAb. After two washes, cells were reacted with 20 μ g of rhodamine-conjugated goat anti-(mouse IgG) (Tago, Birmingham, CA, U.S.A.) for 1 h at 37 °C. By a similar procedure, the localization of F-actin was revealed with FITC-labelled phalloidin [46]. After extensive washing with PBS containing 0.01% Tween, cells were embedded in glycerol and photographed under a Nikon inverted microscope or an Olympus BX-60 microscope.

For electron-microscopic analysis, cells were fixed and embedded in Lowicryl K4M resin [45], ultrathin sections were cut and reacted with purified anti-(MCP M177) (0.1 μ g/ml) or anti-

(DAF D17) (0.4 µg/ml) as described for immunofluorescence, then labelled with a gold-conjugated goat anti-(mouse IgG) (0.5 µg/ml; B.B.I., Cardiff, South Glamorgan, U.K.). In some experiments the sections were stained with uranyl acetate and lead citrate, then mounted on 300-mesh copper grids. Samples were examined with a JEOL 100CX electron microscope [45].

MV-binding assay

Cells to be assayed for MV binding were detached from flasks at 80% confluency by the addition of 2 ml of PBS containing 5 mM EDTA [28]. After two washes, aliquots containing 10^6 cells were incubated for 1–2 h at 0 °C with 2 ml of a concentrated MV solution [at a multiplicity of infection (m.o.i.) of 30] in Dulbecco's modified Eagle's medium (DMEM) containing 5% (v/v) FCS. After three washes with DMEM containing 5% FCS, cells were incubated at 0 °C for 45 min with 5 µg of anti-MV-H mAb. In a chase experiment, cells were incubated for 0.5–4 h at 37 °C before the addition of anti-MV-H mAb at 0 °C. After three washes with chilled 10 ml of DMEM, cells were incubated with 5 µg of FITC-labelled goat anti-(mouse IgG) antibody at 0 °C. The levels of MV-H protein in each CHO cell strain were assessed by flow cytometry.

In some experiments the levels of MV binding were assessed with monolayers of cells by the method described above. After being labelled with the FITC-conjugated secondary antibody, cells were detached from dishes for flow-cytometric analysis.

Assays for determination of surface/intracellular MCP

M160 F(ab')₂, which does not block MV binding to MCP [28], was ¹²⁵I-labelled by the Iodogen method [35]. The specific radioactivity was approx. 2×10^5 c.p.m./µg. Cells (5×10^6) were incubated with 5 µg of M160 F(ab')₂ and 100 µg of non-immune IgG for 45 min at 0 °C. After extensive washing with ice-cold PBS, MV (m.o.i. = 30) was added and the cells were allowed to stand for 1.5 h at 0 °C. Again after washing with ice-cold PBS, cells were incubated for 0.5–4 h at 37 °C; at timed intervals surface-bound ¹²⁵I-M160 F(ab')₂ was recovered by treatment of the cells with PBS containing 1% (v/v) 2-mercaptoethanol. The supernatants (*C*_{sup}) containing the surface-bound M160 F(ab')₂ were removed after centrifugation. After one brief wash, the cells were then lysed with 0.1 M NaOH and 1% NP40; these fractions contained the internalized M160 (*C*_{pellet}). The percentage surface distribution of MCP was obtained from the formula:

$$\text{Surface distribution of MCP (\%)} = 100 \times C_{\text{sup}} / (C_{\text{sup}} + C_{\text{pellet}})$$

Syncytium formation assay

CHO cell clones with or without various MCP mutants were cultured at 70% confluency in 24-well plates (Corning, New York, NY, U.S.A.) and infected with MV at 0.005–3 plaque-forming units per cell. Syncytia formed by MV were then counted. Plaque-forming assays were performed simultaneously in some experiments to confirm the syncytium formation assay, and CPE characteristics of the CHO cell transfectants [18] were analysed 2–3 days after infection.

Co-transfection of MV-H and MV-F in CHO clones

CHO cells expressing each MCP mutant were grown as monolayers on plastic culture dishes (60 mm). MV-H cDNA and MV-F cDNA (5 µg of each) ligated in pCXN [21] were co-transfected into the CHO cells by the lipofectamine method [39]. The cells were allowed to react with the DNA species for 4 h and were then washed with Ham's F12 medium containing 10% (v/v) FCS. The syncytia formed were counted 4 days after transfection.

For assessment of transfection efficiency, the transfected cells were washed with ice-cold PBS before solubilization. The cell monolayers were solubilized directly with PBS/1% NP40 containing protease inhibitors as described [44]. The supernatants containing MV-H were recovered by centrifugation and resolved by SDS/PAGE and immunoblotting. The blots were subjected to densitometric analysis to quantify MV-H with a Macintosh Quadra 840 AV.

RESULTS

Construction of PI-anchored forms and other TM forms of MCP

The cDNA clones used in this study are shown schematically in Figure 1 (upper panel). The ST^C/CYT2 isoform of MCP [25–27]

Figure 1 MCP and mutants used in the study

Upper panel: structural scheme of the constructs of MCP mutants. Abbreviations: MCP-TM, a ST^C/CYT2 wild-type MCP [26]; ΔST, the ST^C/CYT2 isoform from which the ST domain (residues 282–310) was deleted; ΔCYT, the ST^C/CYT2 isoform from which the CYT (residues 348–379) was deleted; Hybrid, a fusion protein consisting of the four SCRs of MCP and a whole DAF molecule; MCP-PI, four SCRs and the ST domain of MCP (1–293) fused with the DAF sequence (308–347) for PI attachment; DAF, the control DAF molecule. Lower panel: SDS/PAGE and immunoblotting analysis of the MCP mutants expressed on CHO cells. CHO cell clones were solubilized with NP40 for the liberation of MCP-TM, ΔST and ΔCYT and the insoluble precipitates were further solubilized with octylglycoside for the liberation of DAF, Hybrid and MCP-PI. The supernatants were subjected to SDS/PAGE followed by blotting. Thus the densities of the bands in different lanes are not significant. The blot was reacted with M177, HRP-labelled secondary antibody and then enhanced chemiluminescence reagents (Amersham). The expression levels of these proteins were simultaneously evaluated by flow cytometry, shown by MFS. The positions of molecular-mass standards (in kDa) are shown at the left.

Figure 2 The localization profiles of the MCP mutants expressed on CHO cells

Top panel: immunofluorescent localization of the MCP mutants. Right images, phase-shift microscope analysis (magnification $\times 100$); left images, indirect immunofluorescence with M177 and rhodamine-conjugated goat anti-(mouse IgG) antibody. A CHO cell clone expressing a PI-anchored protein DAF was used as control, which was stained with D17 and rhodamine-conjugated second antibody. Middle panel: immunofluorescent localization of MCP-PI and F-actin. MCP-PI-expressing CHO cells on a chamber slide were fixed as described in the Materials and methods section. Cells were simultaneously stained with FITC-labelled phalloidin (right image) and M177/rhodamine-conjugated secondary antibody (left image). Representative profiles of a cell (magnification $\times 800$) are shown. Bottom panel: localization of MCP-PI on CHO cells by immunoelectron microscope. (A) The surface and intercellular portions to be enlarged for analysis are indicated by arrows; (B) the cell surface unattached to another cells; (C) the intercellular portion. The arrowheads indicate gold particles conjugated with secondary antibody recognizing anti-MCP, M177. Abbreviations: Vi, villi; M, mitochondrion; N, nucleus.

was used as a wild-type MCP-TM throughout the experiments. Δ ST and Δ CYT were constructed from this parent molecule. MCP-PI was generated by replacing the TM/CYT of MCP with the DAF PI anchor. The form Hybrid was generated by fusing the whole DAF molecule to the four SCRs of MCP. These cDNA species were all stably expressed in the CHO cells. To obtain clones expressing similar levels of MCP, we further subcloned the established clones by the limiting dilution method.

We first verified the proteins expressed in the CHO cells by SDS/PAGE and immunoblotting (Figure 1, lower panel). Because the procedures used for solubilization of the TM forms and PI-anchored forms were different (see the Materials and methods section), MCP protein concentrations varied. Briefly, TM forms were largely solubilized in an NP40-soluble fraction at 0 °C, whereas the PI-anchored forms were dissolved in the octyl-glycoside soluble fraction (M. Hatanaka and T. Seya, unpublished work). The MFS values of the representative clones were also assessed by flow cytometry (Figure 1, lower panel).

The levels of the two PI-anchored proteins on the cells were markedly (more than 80 %) decreased by treatment with PI-PLC compared with corresponding untreated cells (results not shown). In contrast, MCP-TM, Δ ST and Δ CYT were not liberated from the cells by PI-PLC treatment. Thus MCP-PI and Hybrid were both fixed to the CHO cell membranes via PI anchors.

Localization of MCP in the CHO cells

Immunofluorescence analysis showed that MCP-PI and Hybrid were distributed in a patch-like fashion on the CHO cell membrane, as in DAF (Figure 2, top panel). By using FITC-labelled phalloidin (Figure 2, middle panel) and an immunoelectron microscope (Figure 2, bottom panel), MCP-PI was shown to be predominantly distributed on the microvilli of CHO cells. Similar results were obtained with Hybrid-expressing cells. Note that, for unknown reasons, in some CHO cell clones MCP-PI did not show the microvilli accumulation profile. In the following experiments we used the clones exhibiting typical localization profiles as in Figure 2 (top panel).

In contrast, MCP-TM mostly showed a lateral distribution (Figure 2, top panel). Immunoelectron-microscope analysis indicated that the TM forms were largely localized on the intercellular membranes, as shown in Figure 2 (bottom panel). Δ ST and Δ CYT were also found predominantly in the intercellular spaces, similarly to MCP-TM (results not shown).

Binding of MV to the MCP mutant transfectants

Because the two PI-anchored forms and the three TM forms showed differences in membrane localization, we first tested the MV-binding capacities of these MV receptors. CHO cells expressing the mutants were detached and then incubated with MV at an m.o.i. of 30 for 2 h at 0 °C. MV binding to the cell surface was determined by flow cytometry with anti-MV-H mAb followed by FITC-labelled secondary antibody (Figure 3). MV bound to all of the transfectants regardless of mutations in MCP outside the SCRs. The levels of MV binding, however, were higher in cells with MCP-PI or Hybrid than in those with the TM forms. In particular, a broad two-peak profile was observed in CHO cells bearing Hybrid. Similar results were obtained with monolayers of cells by the method described in the previous section. The binding was specific for MCP but not CHO cell moieties including PI anchors because no binding was observed in CHO cells expressing DAF. Thus neither TM nor CYT is required for MV binding, supporting previous suggestions [29,30], but do affect the amount of virus that binds.

Figure 3 Binding of MV to cells expressing MCP mutants

Cells with similar copy numbers of the MCP mutants were incubated for 2 h at 0 °C with (solid line) or without (dotted line) MV. After extensive washing, the bound MV was detected with anti-MV-H and FITC-labelled second antibody. Flow-cytometric analysis was performed as described [35]. The value of MFS for each mutant is shown in each panel. The broad two-peak profile was observed in CHO cells bearing Hybrid, and the Δ ST mutant allowed MV binding but sometimes less shift than the other TM forms of MCP, for reasons that are unknown. Similar MV-binding profiles were observed with cells adhered to dishes. Similar experiments were performed six times; the results from one representative experiment are shown.

Internalization of MV and MCP secondary to complex formation

To determine the fate of the MV bound to MCP, we exposed transfectants to a pulse of MV at 0 °C and then a chase at 37 °C, and conducted an analysis by flow cytometry with respective mAbs that barely disturb the formation of the MV-MCP complex. MV-H disappeared from the membranes of cells expressing the TM forms in a time-dependent manner (Figure 4a, left panels; Figure 4b). In contrast, MV-H that was bound to the MCP-PI became inaccessible to the mAb within 30 min. Thereafter the density of mAb-recognizable MV-H gradually increased on the cell surface of MCP-PI-expressing cells (Figure 4a, right panels; Figure 4b). The dynamics of cell-surface MCP-TM that had been labelled with M160 F(ab')₂ and exposed to MV paralleled that of MV-H (Figure 4c), suggesting that MV or MV-H on the cell surface moves together with MCP-TM. In a similar experiment MCP-PI was minimally internalized (Figure 4c). Thus the TM and the PI-anchored forms of MCP are kinetically quite different at the cell surface and permit MV-H entry by different paths.

Replication of MV in CHO cells expressing MCP mutants

To clarify the above issue, we examined viral protein expression that reflects MV entry and replication competence. Cells were permeabilized 72 h after MV inoculation (m.o.i. = 0.1), and stained with anti-MV-H and rhodamine-conjugated secondary antibody. Effective MV expression was observed in CHO cells

Figure 4 The fate of MV-H bound to MCP-TM and MCP-PI on CHO cells

(a) After a pulse of MV exposure for 60 min at 0 °C, the MVs on the cells were chased for the indicated periods at 37 °C. Cells were chilled at each time point and washed with DMEM, after which anti-MV-H was added. At 45 min intervals, cells were washed twice and reacted with FITC-labelled secondary antibody. These reactions were performed at 0 °C. MV-H levels were analysed by flow cytometry. Left panels, MCP-TM; right panels, MCP-PI. Closed arrowheads indicate the peaks of control unstained cells. Numbers 1, 2 and 3 represent the ranges for gating by the flow cytometer: MFS was assessed with gate number 1; more than 95% of the control cells gathered within gate number 2; and cells distributed within gate number 3 are positive cells. (b) The MFSs obtained as in (a) are plotted as a function of the incubation period at 37 °C (indicated on the x-axis). The initial MFS is taken as 100%. The experiments were performed three times and similar results were obtained. The results of one representative experiment are shown. (c) The levels of surface-remaining MCP-TM and MCP-PI secondary to a pulse of MV (sample values) and no MV (control values) were determined as percentages with 125 I-labelled M160 F(ab')₂ as described in the Materials and methods section. The incubation period at 37 °C after MV input is shown on the x-axis as in (b). The percentage internalization of 125 I-labelled M160 F(ab')₂ due to MV input was obtained at each time point by subtracting the control values from the sample values. The assay was performed four times in duplicate; 1% corresponds to 291–408 c.p.m. The results have been subjected to a statistical analysis ($n = 6$).

bearing MCP-TM (Figure 5). The CHO cells expressing Δ ST and Δ CYT showed moderate levels of MV-H expression. However, very ineffective replication was observed in the cells with the PI-anchored forms. In the two PI-anchored forms, MV-H production was detected 5 days after MV inoculation (results not shown). No fluorescence was detected under these conditions in the CHO cells with DAF. Hence the two PI-anchored forms conferred high MV-binding capacity but inefficient MV entry and/or replication in CHO cells.

MV-mediated syncytium formation of CHO cells expressing MCP mutants

The numbers of syncytia formed were counted 72 h after inoculation with MV as described above. Cells with Δ CYT or Δ ST showed as efficient syncytium formation as those with a wild-

type MCP-TM (results not shown). In contrast, cells with MCP-PI were approx. 1/100 as sensitive to MV in terms of syncytium formation than were wild-type controls (Figure 6). No syncytia were formed with cells bearing Hybrid by incubation at an m.o.i. of up to 3 (Figure 6). These tendencies were confirmed by using independent clones with different copy numbers of MCP-TM or MCP-PI. Two other clones with Hybrid showed no syncytium formation at the m.o.i. tested, suggesting the participation of factors other than the PI anchor in CPE competence. Again, no syncytia were formed in CHO cells with DAF under our conditions. These results were consistent with those of the replication study.

Syncytium formation after transfection by MV-H and MV-F

To test whether the PI-anchored forms could induce syncytium

Figure 5 Immunofluorescence staining of MV-H in MV-infected CHO cells expressing various MCP mutants

All cells were placed on chamber slides (Lab-Tek) and infected with MV (m.o.i. = 0.1). After 72 h, cells were fixed and stained with anti-MV-H mAb and FITC-labelled second antibody. The cells were photographed under a fluorescence microscope (magnification $\times 200$). DAF-expressing CHO cells were used as controls. The types of MCP expressed on the cells are indicated at the sides. The results from one of two experiments are shown.

Figure 6 Sensitivity of the cells with MCP mutants to MV-mediated syncytium formation

CHO cells with various expression levels of MCP-PI or MCP-TM were obtained as described previously [33] and in the Materials and methods section. CHO cells with Hybrid were the same clone as those used in other experiments. The numbers of virus-dependent syncytia formed were counted and plotted as a function of the MV concentration, which is indicated on the x-axis. The results from one of three experiments are shown.

formation if the cells were permitted to express MV-H and MV-F, MV-H/F co-transfection studies were performed with CHO clones expressing MCP mutants. cDNA species of MV-H and MV-F transfected with lipofectamine induced syncytium formation in CHO cells expressing MCP regardless of the form used (Figure 7). The numbers of syncytia formed, however, differed slightly with the forms of MCP (Table 1). MCP-TM and Δ CYT, which are distributed predominantly around the intercellular region, allowed the formation of more syncytia than

Figure 7 MV-H/F co-transfection-mediated syncytia formed in CHO cell monolayers expressing the MCP mutants

Lipofectamine was used for co-transfection of MV-H/pCXN and MV-F/pCXN. CHO cells expressing DAF, which allowed no syncytium formation, were used as controls. Under the conditions employed (see the Materials and methods section), CHO cell monolayers expressing the MCP mutants allowed syncytia production as indicated in the Figure. CHO cell monolayers expressing Hybrid and Δ ST also yielded syncytia that were similar in size (results not shown) but not number (Table 1) to those with MCP-PI and MCP-TM respectively.

Table 1 Syncytia formation on the transfectants after co-transfection with MV-H/F cDNA

Results for Δ ST are not shown because the expression of the Δ ST level decreased gradually during passage. Syncytia were formed with Δ ST-expressing cells to a similar extent to MCP-TM-expressing cells even when the MFS reflecting the level of Δ ST was 8.6. The numbers of syncytia formed are representative of three experiments. For the MFS results, the MFS (0.87) of DAF was taken as 0 and subtracted from each MFS value of the other MCP mutants. The levels of the expressed MV-H protein were estimated by SDS/PAGE and immunoblotting with anti-MV-H followed by densitometry. The background level (that of DAF) was taken as 0 and subtracted from each densitometric value of the MV-H bands. The relative densitometric value of the MV-H protein assessed in CHO cells with MCP-TM was defined as 1.0.

CHO cells expressing:	MFS with M177	Number of syncytia formed	Densitometric levels of MV-H
DAF	0	0	0
MCP-TM	69	86	1.0
Δ CYT	32	121	1.3
MCP-PI	79	22	1.1
Hybrid	41	38	0.96

MCP-PI and Hybrid, which are distributed predominantly on the microvilli. Again, no syncytia were observed in control CHO cells or those expressing DAF under the conditions employed. Transfection efficiency seemed to be similar in these clones because the amounts of the expressed MV-H protein, assessed by densitometric analysis of immunoblots, were not very different (Table 1). The syncytia that formed in monolayers of CHO cells with the PI-anchored forms tended to be smaller than those in

monolayers of cells with the TM forms (Figure 7). We interpret the results in Figures 6 and 7 as indicating that cell-to-cell fusion can occur efficiently only if sufficient MV-H and MV-F proteins are supplied, even in the cells with the PI-anchored forms of MCP.

DISCUSSION

The results in the present study demonstrated the following (summarized in Figure 8): (1) the ST, TM and CYT domains of MCP are not required for MV binding or fusion; (2) TM and probably a proximal part of CYT are required for efficient virus entry and/or replication; (3) the predominant localization of MCP on microvilli is induced by replacing the TM and CYT with a PI anchor; (4) co-transfection of three isolated cDNA species of MV-H, MV-F and human MCP, regardless of version, confers fusogenic competence on non-primate cells.

The first piece of evidence described above supports previous reports, including our own, that SCR1 and SCR2 essentially constitute the functional domain of the MV receptor [28]. In contrast, the second point is inconsistent with a previous report [29] that suggested that a PI-anchored version of MCP, which has the DAF ST domain instead of the MCP ST domain, conferred fusion competence on CHO cells. The MV strains used in those experiments differed from our strain. The major difference between the results of that previous and our present study can be explained by the levels of MV-H and MV-F expression. Their experimental conditions in which the cells were infected with MV at an m.o.i. of more than 1 and incubated for 6–8 days were entirely different from ours (inoculation of MV at an m.o.i. of 0.1 with an incubation period of 2–3 days). Such an artificially high MV dose and long-term incubation conditions could have allowed a yield of MV-H and MV-F sufficient for fusion. On the basis of these conditions and CPE criteria, our PI-anchored MCP also served as an MV receptor (see Figure 6), which is consistent with the previous reports [29,30]. In contrast, similarly to our results, a study in the murine cell line M12 showed that the expression of human MCP-PI [30] was ineffective

in the induction of CPE. In summary, CHO cells are non-permissive under the conditions we employed unless any version of MCP is expressed; the major differences between the previous and our present conclusions were probably due to the different MV strains and/or doses used, the different PI-anchored forms of MCP, and the different cells used for transfection.

With regard to point (3), the modes of membrane anchorage and the matter of the receptor itself can cause a biased localization. The mechanism whereby the TM and PI-anchored forms of MCP confer distinct localization profiles, however, remains unknown. An intriguing notion is that the patch-like localization profile is common in other endogenous PI-anchored proteins in polarized cells [2] and the PI-anchored forms of MCP expressed here on CHO cells, even though they are non-polarized. The previous finding that CHO cells possess caveolae on their membranes [41] seems to support the observation that the two PI-anchored forms were selectively transported to the apical surface. These microenvironmental factors might refer to the high MV-binding capacity and unique kinetic profiles of the two PI-anchored forms.

An intriguing point in this study is that the PI-anchored forms barely allow MV replication and subsequent syncytium formation at an m.o.i. sufficient to induce CPE in the cells with TM forms. In contrast, the MV-H/F co-transfection experiment showed that all mutants were competent to undergo MV-mediated cell-to-cell fusion (Figure 7, Table 1). Thus MV-mediated cell fusion is exclusively a result of the interaction of MV-H, MV-F and MCP. Because the only structural difference between the receptors lies in the membrane-anchoring region, the intracellular portion of MCP must influence the steps from MV entry to replication. On the basis of these results, the major means by which the PI-anchored forms render the cells 100-fold less permissive to MV by CPE criteria than the TM forms (Figure 6) is in their differences in virus entry/replication competence but not in fusion competence. As shown in CD4-mediated HIV entry studies [14,15], the localization properties of the PI-anchored forms are likely to affect virus-to-cell fusion, i.e. viral entry as well. These tendencies are also observed for other virus receptors with PI anchors [47,48]. Taking these points together, we favour the interpretation that selection of the different entry pathway results in different MV uptake and replication, as shown in Figures 4 and 5.

In contrast, in the cells with the TM-forms, MV-H is internalized rather slowly. Evidence accumulated by Gerlier and co-workers [30,49] indicates that MV must be partly endocytosed in a chloroquine-sensitive manner, allowing H- and N-antigen presentation by MHC class II molecules. Thus it is a prerequisite for MV-H to be embedded in the plasma membrane after virus-to-cell fusion. This is not always in accordance with the currently accepted concept that MV fuses directly with the outer membrane in a pH-independent fashion without endosome formation [23,24]. Our results (Figure 4) might support the MV endocytosis theory in that the complex consisting of MCP-TM and MV-H gradually disappears on effective replication. Yet this is not true of unusual receptors, the PI-anchored forms of MCP.

A PI-anchored form of CD4 binds and internalizes gp120 of HIV, although less effectively than the conventional TM form of CD4 [15]. In the HIV receptor CD4, TM and CYT are required for efficient virus internalization, as in the MV receptor MCP [15,48]. The CD4-TM is endocytosed via clathrin-coated vesicles and is connected with the inner plasma membrane Src family proteins [15]. In contrast, virtually no information is available about the association of other molecules with the TM or CYT of MCP, although there are several isoforms with different tails [25,27,50] that possess putative targeting sequences and affect

	Wild	ΔST	MCP-PI	MCP/DAF-PI	ΔCYT
Binding	+	+	++	++	+
Entry	+	ND	+	+	+
Repl.	++	++	+/-	+/-	+
CPE	++	++	+/-	-	++
Fusion	++	++	+	+	++

Figure 8 Summary of the properties of wild-type MCP and mutants

All constructs were expressed on CHO cells, non-permissive to MV under the conditions employed. MV binding was more effective in the cells with the two PI-anchored forms of MCP than in those with the three TM forms, whereas MV replication and CPE formation were severely suppressed in the cells with the two PI-anchored forms. Nevertheless cell-to-cell fusion could be induced in the cells with the two PI-anchored forms via co-transfection of MV-H and MV-F cDNA species. Abbreviation: Repl., replication. Entries of +/– indicate that MV replication and CPE were observed only in cells treated with high titres of MV.

MCP trafficking [51]. Furthermore the tail-less form Δ CYT (retaining the two residues Arg-Ser beneath the TM) behaves like the MCP-TM, with the ability to induce syncytia indistinguishable from that of MCP-TM. Thus the TM, but not a large distal part of the CYT of MCP, should be a critical factor in determining the efficiency of MV CPE induction. In this regard, as virus receptors MCP would differ from CD4 [15,48] in their behaviour and properties for virus internalization.

The basic sequence Arg-Arg-Lys-Lys-Lys beneath the TM domain of MCP has been reported to be functionally important. First, this basic sequence seems to participate in the targeting for endoplasmic reticulum [52], which may in part explain the large intracellular pool of MCP. Secondly, the cluster of basic amino acids near the TM domain has also been observed in ICAM-1 [53] and CD44 [54], which are important for association with the moesin family proteins [54]. As suggested previously [55], MCP might actually interact with moesin at this sequence. Deletion of these basic sequence from the TM, however, did not result in a failure of syncytium formation (Figure 8). Thus our results did not confirm the importance of this basic sequence, at least for MV-mediated syncytium formation. As the properties of the TM and PI-anchored forms of MCP are different (Figures 4–7), again the divergent pathways in the TM and PI-anchored forms might result from the function of the TM domain. A determination of which molecules bind the TM of MCP will be crucial in resolving the nature of the signalling pathway.

Membrane fusion is physiologically important for a variety of cellular functions, including synaptic transmission, fertilization and intracellular membrane traffic. Characteristic membrane fusion is induced in viral infection. In addition to a function for the MV receptor, MCP serves as a sperm-adhesion molecule for oocytes contributing to fertilization [56], and as a complement regulatory protein/receptor for complement C3b/C4b, which covalently binds membrane hydroxy or amino residues [33,40]. Hence MCP is a multifunctional protein. The implication of MCP in viral- and sperm-mediated membrane fusion suggests that the primary role of MCP involves a fusogenic modulator of cellular functions [27]. Because augmentation of C3b deposition in MV-infected cells has been reported (reviewed in [27]), MCP might also be related to the internalization of C3b/C4b deposited on the same cell membrane in a similar fashion to that of MV. Our interest is now directed at discerning which molecules are responsible for intracellular signalling for MV internalization and the machinery resulting in cell fusion. MV and our MCP mutants will be good tools with which to investigate these issues.

After the submission of this paper, Bucholz et al. [57] published data showing that the length between the SCR and the TM domains is also a crucial factor in determining the efficiency of MV binding and entry.

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