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Original Scientific Article

Early Endosomal Retention of Murine Cytomegalovirus m06 Protein†

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Abstract. Murine cytomegalovirus (MCMV) encodes several genes which products interact with MHC class I molecules (MHC-I) in the secretory pathway to evade antigen presentation. The product of MCMV-m06 gene associates with nascent MHC-I proteins and redirects them into lysosomes for degradation. This report will demonstrate that MCMV-m06 protein is retained in perinuclear early endosomes together with internalized MHC-I molecules. Since we were not able to capture the MCMV-m06 protein on the cell surface, our data suggests that the MCMV-m06 protein is transported from the Golgi into early endosomal intermediates prior to their maturation into multivesicular endosomes and lysosomes. In NIH 3T3 cells with the transfected m06 gene, the endosomal localization of the m06 protein was not observed, suggesting that another MCMV function remodels the route of m06 trafficking from the Golgi towards late endosomes. (doi: 10.5562/cca1816)

Keywords: murine cytomegalovirus, m06/gp48, major histocompatibility class I molecules, early endosomal retention

INTRODUCTION

Upon entering the host cell, many viruses attempt to hide from detection by the host immune response, whereas infected cells activate the mechanism of antigen presentation in order to display the presence of a virus on the cell surface. For this purpose, viral peptides are presented on the cell surface in the complex with the Major Histocompatibility Class I molecules (MHC-I). Cell surface MHC-I molecules are trimoleculear complexes of 45 kDa transmembrane heavy chains, 12 kDa β2-microglobulin (β2-m) and endogenously generated viral peptides.[1] $CD8^+$ lymphocytes recognize this complex and trigger the signal of activation which ultimately destroys virus-infected cells.[2]

Murine cytomegalovirus (MCMV), a member of the herpesvirus family, encodes several proteins which target the MHC-I trafficking in the secretory pathway and therefore interfere with the antigen presentation mechanism. The MCMV-m152 protein associates with newly synthesized MHC-I proteins and arrests them within the endoplasmic reticulum (ER)/endoplasmic-reticulum-Golgi-intermediate (ERGIC) compartment.[3,4] MCMV-m06 early protein also binds the nascent and properly folded MHC-I molecules in the ER, but the m06/MHC-I complex is transported out of the ER into the Golgi and redirected from its route to the cell surface towards lysosomes for degradation.[5] The MCMV-m04 protein associates with nascent MHC-I complexes in the ER and alters their functioning at the cell surface.[6,7] These proteins are usually referred to as immunevasins.[6,7] In addition, we have recently described the MCMV function which efficiently down-regulates the cell surface resident MHC-I molecules.[8]

Transport of proteins from the biosynthetic pathway involves their sorting in the trans-Golgi network (TGN) and their delivery either directly to the cell surface or to the endosomal compartments.[9] Upon uptake, plasma membrane proteins enter into the endocytic pathway, a complex of dynamic membrane structures which represent functionally and physically distinct compartments with a number of distinct stations.[10] The central sorting station are early endosomes which either redirect internalized cargo proteins into late endosomes/lysosomes for degradation or incorporate cargo proteins into recycling tubular endosomes which transport them back to the plasma membrane.[11] Early endosomes also sort the newly synthesized proteins arriving directly from TGN, like major late endosomal and lysosomal membrane proteins (LAMPs), and their missorting to the cell surface if occur, is corrected by rapid endocytic uptake from the plasma membrane.

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Fully conformed MHC-I proteins are constitutively internalized from the cell surface into endosomal carriers via the Arf6-associated clathrin-independent pathway.[12–18] Upon internalization, MHC-I proteins travel along the endosomal system and undergo a series of sorting events at various stages. Approximately, half of internalized MHC-I is sorted into recycling tubular endosomes and returned back to the cell surface [16,19,20] whereas the rest enter the perinuclear vacuolar sorting endosomes to be delivered into the degradation route.[21]

In infected cells, several viruses interfere with functions of the vesicular system and thereby down-regulate the cell surface expression of MHC-I proteins. For instance, the HIV-1 Nef protein activates endocytosis of MHC-I[22,23] and reroutes their endosomal trafficking,[24] whereas Kaposi's sarcoma-associated herpesvirus proteins K3/K5 accelerate their internalization.[25] We recently reported on cell surface MHC-I proteins as being rapidly internalized into the MCMV infected cells due to a mechanism that occurs prior to the expression of known immunoevasins.[8] Internalized MHC-I proteins are arrested and retained in vacuolar sorting endosomes. Given that the MCMV-m06 protein associates with the nascent MHC-I in the ER and further redirects them from the Golgi into late endosomes,[5] we performed this study to test whether the m06 protein passes through the retention endosomes which collect MHC-I proteins from the plasma membrane. In this report we demonstrate that the m06 protein reaches the perinuclear endosomal compartment loaded with internalized MHC-I proteins by a route that does not involve its expression at the cell surface.

EXPERIMENTAL

Cell Lines and Viruses

Primary murine embryonic fibroblasts (MEFs) were generated from BALB/c mice and cultivated in the Minimal Essential Medium (MEM) supplemented with 5 % (volume fraction) foetal bovine serum (FBS), 2 mmol dm^{-3} L-glutamine, 100 mg/ml streptomycin and 100 U/ml penicillin. All reagents were obtained from Gibco (Grand Island, NY). The cells were grown in Petri dishes as adherent cell lines and used for infection after three in vitro passages when they were 90 % confluent. NIH 3T3 cells were obtained from American Typing Culture Collection (ATCC CRL1658) and NIH 3T3-m06 cells, a cell line transfected with m06,[26] were kindly provided by Dr. Ulrich H. Koszinowski. For infection we used the deletion mutant of MCMV without viral Fc receptor (Δm138-MCMV). The virus was propagated on third-passage MEFs and purified by sucrose-gradient centrifugation. MEFs were infected with 0.5–1 PFU per cell. Cells were incubated for 30 min with virus at 4 °C. To increase the efficiency of viral infection, cells were centrifuged at 500 g for 30 min.

Reagents and Antibodies

The following monoclonal antibodies (mAbs) were used: 34-5-8S (mouse IgG_{2a}, ATCC HB-102) that reacts with heavy chain (HC) of H2-D^d, MA-215 (mouse IgG_{2b}) that reacts with HC of H2-K^d,[27] R17 217.1.3 (rat IgG, ATCC TIB 219) that recognizes murine transferrin receptor (TfR) and *Croma*-229 (mouse IgG_1, produced in our laboratory) that recognizes MCMV-m06 protein.[5] MAbs were used as hybridoma culture supernatant for immunofluorescence and flow cytometry or as semi-purified ascites for immuprecipitation. MAbs to GS28, EEA1 and LAMP were from Becton Dickinson & Co (San Jose, Calif, USA), $Alexa^{555}$-cholera-toxin B subunit (CTxB), $Alexa^{555}$- or $Alexa^{488}$-conjugated anti mouse IgG_{2a}, anti mouse IgG_{2b}, anti mouse IgG_1, anti chicken IgG, anti rat IgG and FITC-conjugated anti mouse Ig were all from Molecular Probes-Invitrogen (Eugene, OR, USA). Brefeldin A (BFA) was purchased from Sigma-Aldrich Chemie GmbH (Germany). Streptavidin-peroxidase conjugate was from Roche (Indianapolis, USA). Polyclonal antibody to the θ isoform of 14-3-3 protein is an affinity purified rabbit polyclonal Ab raised against a peptide corresponding to amino acids 229-245 mapping at the carboxy-terminus of 14-3-3 θ of rat origin (C-17, Santa Cruz Biotechnology).

Cell Surface Expression and Flow Cytometry

Infected and uninfected control cells were collected by short trypsin treatment, washed in culture medium and incubated at 4 °C for 30–60 min with approx. 2 μg/ml of primary mAbs reagents in PBS containing 10 mmol dm^{-3} EDTA, Hepes pH = 7.2 and 0.1 % NaN_3 and 2 % FCS (PBS-A). Unbound Abs were removed by three washes with cold PBS-A and the cells were incubated for 30 min at 4 °C with FITC-conjugated secondary Ab reagents (2 μg/ml) in PBS-A. After three washes with PBS-A, cells were analyzed by flow cytometry using FACSCalibur flow cytometer (Becton Dickinson & Co, San Jose, CA, USA). Dead cells were excluded by propidium iodide (1 μg/ml) and a total of 5 000 cells was acquired. The fluorescence signal was determined as mean fluorescence intensity (MFI) after subtraction of background fluorescence (ΔMFI) determined on the same cells by incubation with nonreactive mAbs of the same isotype and appropriate fluorochrome-conjugated secondary reagent.

Immunofluorescence and Confocal Analysis

Adherent cells, grown in six- or twelve-well tissue culture plates on glass coverslips, were washed three times with PBS, fixed with 4 % paraformaldehyde (PFA, 20 min

at room temperature, r.t.), permeabilized with 0.5 % Triton X-100 (10–20 min at r.t.) and incubated with fluorochrome-conjugated or un-conjugated primary reagents for 60 min at 4 °C. Unbound reagents were washed by PBS-A, and cells were either embedded or incubated for 60 min at 4 °C with an appropriate fluorochrome-conjugated secondary reagent. After the three washes in PBS-A, cells were embedded in Mowiol (Fluka Chemicals, Germany)-*DABCO* (Sigma Chemical Co., Germany) in PBS containing 50 % glycerol and analyzed by Olympus Fluoview FV300 confocal microscope (Olympus Optical Co., Tokyo, Japan) with 60xPlanApo objective and either 4x or 8x zoom (z axis was 0.5 μm). Images were processed by Fluoview, Version 4.3 FV 300 (Olympus Optical Co., Tokyo, Japan) and Adobe Photoshop (San Jose, CA, USA). To visualize the level of co-localization, 8–10 cells per experimental condition were randomly selected on the same cover slip among those which were well spread, showing a well-resolved pattern. Images of single cells were acquired at the same magnification, exported in TIFF format, and processed by Fluoview, Version 4.3 FV 300 (Olympus Optical Co.).

Colocalization Analysis

Quantitative analysis of colocalization was performed as described previously.[28] We used ImageJ software; utilizing JACoP plugin (http://rsb.info.nih.gov/ij/plugins/track/jacop.html) to calculate Manders' overlap coefficients (M1 and M2).[29] All experimental and control images were acquired using identical imaging settings. The best-fit lower threshold to eliminate most of the signal background was determined using the threshold tool and confirmed by visual inspection. Measures were made on at least 5 cells per experimental condition.

Internalization of Cell Surface Molecules

Internalization was performed on adherent cells grown on coverslips. Cells were washed three times with PBS and cell surface proteins were labeled with mAb reagents at 4 °C for 60 min. Unbound mAbs were removed by the three washes with PBS and internalization was performed at 37 °C. Internalized mAbs were visualized inside the adherent cells by immunofluorescence after short acid wash (1 min, pH = 2.2) prior fixation to remove mAbs from the cell surface.

Metabolic labeling

Cells were incubated for 30 min at 37 °C in methionine-free MEM (Gibco) and labeled with [^{35}S]methionine (Amersham Biosciences) for 2–3 hours pulse at a concentration up to 200 μCi (7.4 MBq) ml^{-1} and chased in regular MEM supplemented with nonradioactive methionine to final concentration of 1 mmol dm^{-3}. Cytoplasmic extracts of labeled cells were prepared in NP-40 lysis buffer (1 % NP-40, 50 mmol dm^{-3} Tris/HCl (pH = 7.6), 5 mmol dm^{-3} $MgCl_2$) containing 1 mmol dm^{-3} phenylmethylsulfonyl fluoride (PMSF, Sigma Chemicals Co.). Nuclei and cellular debris were removed by centrifugation at 14 000 r.p.m. for 20 min, and cellular extracts were used for immunoprecipitation.

Immunoprecipitation

Supernatants of cellular lysates were precleared with 50 % protein A–Sepharose slurry (Amersham Biosciences) at 4 °C for 30 min. Immunoprecipitation of the gp48 (m06) viral protein was performed with ascitic fluid of mAbs *Croma-229* (3 μl). Immune complexes were retrieved with protein A–Sepharose (50 μl of 50 % slurry) at 4 °C for 1 hour, eluted at 96 °C by incubation with SDS sample buffer [0.125 mol dm^{-3} Tris/HCl (pH = 6.8), 20 % glycerol, 3 % SDS, 2 % β-mercaptoethanol, 0.05 % bromphenol blue] and analyzed by SDS-PAGE (10 % gel) under reducing conditions.

Gel Electrophoresis and Autoradiography

SDS-PAGE was performed under reducing conditions. Polyacrylamide gels with metabolically labeled proteins were exposed to an autoradiography film containing scintillating emulsion (Biomax MR; Kodak) to intensifying screen.

RESULTS

The Expression Pattern of m06 Protein in MCMV-infected Cells

Expression kinetics of gp48/m06 was determined by metabolic labeling of infected fibroblasts in three hour intervals followed by immunoprecipitation of m06 protein from cellular lysates. As shown in Figure 1A, the mAb *Croma*-229 immunoprecipitated 48 kDa protein from lysates of infected cells that were labeled within first three hours after infection, indicating that the m06 protein is synthesized very early in the infection. After that, gp48 was detected in similar quantity throughout the replication cycle of MCMV. Abundant cytoplasmic protein 14-3-3 θ served as a cellular control for comparison of labeled material used for immunoprecipitation (Figure 1A, bottom).

The expression pattern was determined by immunofluorescence and confocal analysis. In the early phase of infection (up to 12 h p.i.), a majority of m06 protein was concentrated in pericentriolar tubulo-cysternal structures (Figure 1B, 8 h p.i.) representing the Golgi compartments, as verified by colocalization with a Golgi marker (Figure 4), and in perinuclear vesicular struc

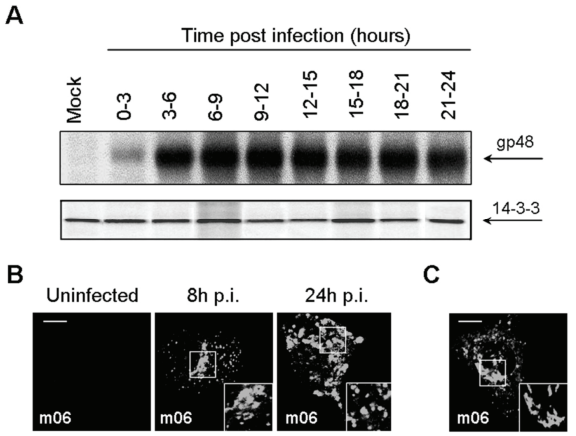

Figure 1. The expression kinetics and intracellular staining pattern of MCMV-m06 protein. Expression kinetics (A). MEFs were infected with Δm138-MCMV and pulse-labeled with [^{35}S]methionine at indicated time post-infection. Lysates were immunoprecipitated with the mAb *Croma-229* (gp48, up) or with rabbit antiserum to the cellular protein 14-3-3θ (bottom) and analyzed by reducing SDS-PAGE on 10 % gel. Confocal images of the intracellular staining pattern of m06 protein in Δm138-MCMVinfected MEFs (B) and NIH 3T3-m06 cells (C). Bars, 10 μm.

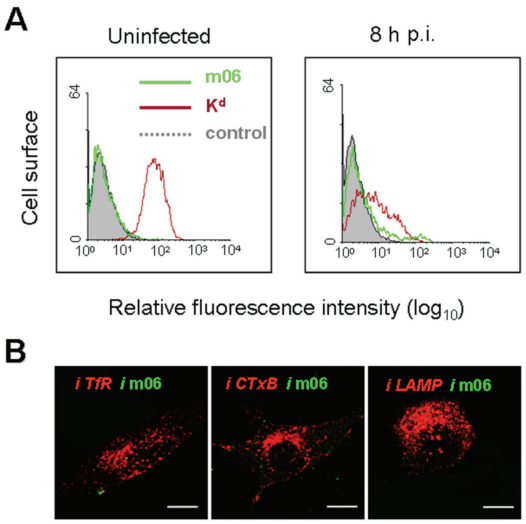

Figure 2. MCMV m06 does not reach the cell surface. (A) Cell surface expression. Flow cytometric profiles of cell surface expression of K^d and MCMV-m06 protein in uninfected and Δm138-MCMV-infected (8 h p.i.) MEFs. (B) Cointernalization of mAb captured cell surface proteins and m06. Confocal images of infected MEFs that were at 4 hours p.i. incubated for additional four hours with mAb *Croma*-229 (m06) and R17 (TfR), Alexa^{555}-cholera-toxin B-subunit (CTxB) or LAMP. After internalization, the cells were fixed and internalized mAbs stained with Alexa^{488}-anti mouse IgG$_1$ (gp48/m06, green fluorescence), and Alexa^{555}-anti rat IgG (TfR or LAMP, red fluorescence). Bars, 10 μm.

tures (Figure 1B). In the late phase of infection (14–24 h p.i.), however, m06 protein was localized in enlarged tubulo-vacuolar structures that were dispersed throughout the perinuclear area of the cell (Figure 1B), an area confined by the edges of microtubular network (data not shown). The pattern observed in the late-phase infected fibroblasts was not observed in NIH 3T3-m06 cell line (Figure 1C). In these cells the m06 expression pattern was similar to the early phase infected cells, indicating that MCMV gene products from the late phase contribute to the distribution pattern observed in late-phase infected fibroblasts.

No Evidence for Cell Surface Expression of MCMV-m06 Protein

Given that the function of m06 protein in the secretory pathway is associated with MHC-I molecules and abolishes their expression at the cell surface,[3,5,6,30,31] we next examined whether m06 reaches the cell surface. Although the cell surface K^d (Figure 2A) and D^d (data not shown) MHC-I proteins were almost completely down-regulated at 8 h p.i., very little, if any, mAb *Croma*-229 binding to the surface of infected cell was determined at 8 h p.i. (Figure 2A) and at later times of infection (data not shown). At the same time, both K^d and m06 were determined inside the cell, by permeabilization and flow cytometric analysis (data not shown).

Although, the flow cytometic analysis of surface expression is very sensitive method, many proteins that have a high turnover rate at the PM may escape detection. Thus we performed a more sensitive assay which is based on exposure of cells to mAb *Croma*-229 in culture medium. Even after 4 hours of exposure, no *Croma*-229 was detected inside the cells (Figure 2B, absence of green fluorescence), indicating that no m06 protein was exposed at the PM to be captured by mAbs. Under the same conditions, transferrin receptor (TfR) and LAMP, two membrane proteins with high PM turnover that are rapidly internalized via clathrin-dependent route, were efficiently recognized by specific mAbs which concentrated in typical intracellular localization for these proteins, the pericentriolar and perinuclear tubulo-vesicular endosomes, respectively (Figure 2B, red fluorescence). The same was observed also in NIH 3T3-m06 cells (data not shown). The fluid-phase uptake of mAbs was negligible, although another pathway of internalization which is responsible for the uptake of fluid-phase was functional in MCMV-infected cells, as demonstrated by the internalization of Alexa^{555}-cholera-toxin B-subunit (CTxB) (Figure 2B).

Perinuclear Association of MCMV-m06 Protein with MHC-I Molecules of the Endosomal Pathway

In uninfected fibroblasts, the steady-state staining pattern of intracellular MHC-I proteins hardly distinguishes

clear intracellular structures (data not shown), whereas in MCMV infected cells at 8 h p.i. the dense perinuclear accumulations of MHC-I proteins in vesicular and vesiculo-tubular structures were observed (Figure 3A).[8] These structures almost completely overlapped with the steady-state expression of m06 protein (Figure 3A), indicating that m06 protein also accumulates in the perinuclear retention endosomes of early phase infected cells.[8] In the late phase (*i.e.* 24 h p.i.), the overall amount of intracellular MHC-I proteins was reduced, as a consequence of their degradation,[8] although they were found in the clearly confined perinuclear vesicular structures (Figure 3A). In these structures, they colocalized with m06 protein (Figure 3A), although a large amount of m06 protein was also found in tubulo-vacuolar structures that were MHC-I negative (Figure 3A).

In order to distinguish whether m06 protein localizes with MHC-I proteins in endosomes, we labeled cell surface K^d with mAbs at 4 h p.i. and internalized either 4 hours (early phase of infection) or 20 hours (late phase of infection). As shown in Figure 3B, internalized K^d molecules were accumulated in the perinuclear endosomes with predominant localization in the pericentriolar area of early-phase infected cells (Figure 3B, left) and cytoplasmic vacuoles of late-phase infected cells (Figure 3B, right). In these structures, internalized K^d colocalized with steady-state m06 protein (Figure 3B). Given that we labeled only cell surface K^d, we can conclude that internalized K^d molecules meet with m06 protein in endosomes. Although the pericentriolar accumulation of internalized K^d and steady-state m06 was not observed at later times of infection (Figure 3B, right), internalized K^d molecules were loaded into a part of perinuclear tubulo-vacuolar endosomes containing m06 protein, indicating that these structures are accessible to endocytosed cargo and, at least a part of them, may be of endosomal origin.

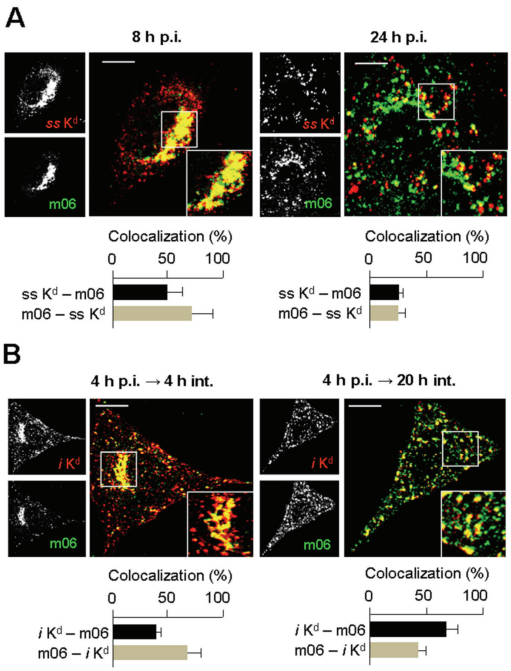

Figure 3. MCMV-m06 protein co-localizes with endosomal MHC-I molecules. (A) Confocal images of simultaneously stained steady-state (ss) MCMV-m06 protein (gp48) and K^d at 8 and 24 hours p.i. (B) Confocal images of internalized (i) K^d and steady-state MCMV-m06 protein (gp48) at 8 and 24 hours p.i. Cell surface K^d molecules of Δm138-MCMV infected cells were labeled with mAb MA215 at 4 h p.i. (at 4 °C) followed by 4 or 20 hours of internalization at 37 °C. K^d molecules were visualized with $Alexa^{555}$-anti mouse IgG_{2a} (red fluorescence) and MCMV-m06 protein with $Alexa^{488}$-anti-mouse IgG_1 (green fluorescence). Quantification of colocalization is based on Manders' coefficients M1 (*i.e.* K^d *vs.* m06) and M2 (*i.e.* m06 *vs.* K^d) representing the mean ± S.D. determined on sections of 5 cells from two independent experiments. Bars, 10 μm and 5 μm in inserts.

MCMV-m06 Protein Accumulates in Early Endosomes of MCMV-infected Cells

In order to clarify whether the m06 protein reaches early endosomal compartments, we labeled the cell surface cargo molecules of various endocytic pathways that upon endocytic uptake converge into early endosomes. MHC-I proteins using Arf6-associated endocytic route,[15] transferrin receptor (TfR) using clathrin-dependent route,[14] and cholera-toxin B-subunit using cdc42-dependent endocytic route,[32] were labeled at 4 h p.i., internalized for 4 hours (8 h p.i.) and colocalized with steady-state m06 protein (Figures 3B and 4A). After 4 hours of internalization, all three cargo proteins were retained in the perinuclear endosomal vesiculo-tubular endosomes of MCMV-infected cells, including pericentriolar tubular endosomes (Figures 3B and 4A). In these endosomes they colocalized with the m06 protein (Figures 3B and 4A, inserts) suggesting that internalized cargo from the clathrin-dependent, Arf6-associated and cdc42-dependent routes converge into early endosomal compartment that is loaded also with m06 protein. To further verify that pericentriolar endosomes which were loaded with m06 protein are of the endosomal origin, we treated the cells with brefeldin A (BFA), a chemical that disperses the Golgi compartments and causes their fusion with ER.[33] As expected, BFA treatment dispersed GS28-positive Golgi membranes and pericentriolar m06-positive tubular membranes, but not m06-positive vesicles (Figure 4B), indicating that a part of the m06-loaded membranes is BFA-resistant. These membranes were of early endosomal origin, since they remain associated with early endosomal antigen 1 (EEA1) after BFA treatment (Figure 4B). In addition, perinuclear endosomes loaded with internalized K^d, TfR and CTxB did not disperse after BFA-

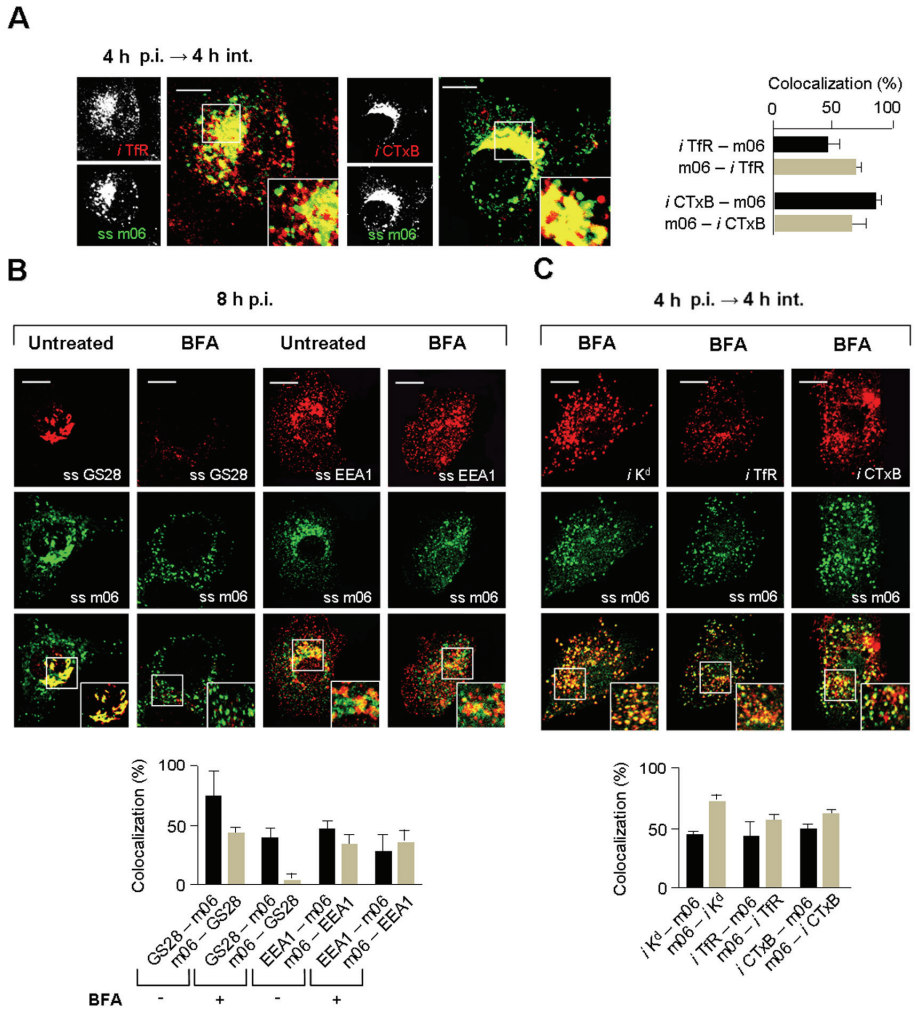

Figure 4. Localization of MCMV-m06 protein in early endosomes of Δm138-MCMV-infected cells. (A) Colocalization of steady-state (ss) MCMV-m06 protein (gp48) with internalized transferrin receptor (TfR) and cholera-toxin B-subunit (CTxB). Infected MEFs were at 4 hours p.i. incubated with mAbs to TfR (R17) or Alexa^{555}-CTxB (at 4 °C). After 4 hours of internalization at 37 °C, the cells were fixed and stained with mAb *Croma*-229 and Alexa^{488}-anti-mouse IgG_1 to visualize ss m06 and Alexa^{555}-anti-rat IgG to visualize internalized TfR. (B) Infected MEFs were untreated or brefeldin A (BFA) treated (10 μg/ml, 60 min) at 8 hours p.i. Confocal images of ss m06 (gp48) simultaneously stained with either the Golgi marker (GS28) or early endosomal marker (EEA1). (C) Infected MEFs were at 4 h p.i. incubated with mAb MA-215, mAb R17 or Alexa555-CTxB (at 4 °C) and after four hours of internalization treated with 10 μg/ml of BFA for 60 min. Steady-state m06 protein and internalized cargo molecules were visualized as described above. Quantification of colocalization is based on Manders' coefficients M1 and M2 representing the mean ± S.D. determined on sections of 5 cells from two independent experiments. Bars, 10 μm and 5 μm in inserts.

treatment and remain associated with m06 protein (Figure 4C). Taken together, this data indicates that pericentriolar tubular accumulation of m06 protein resembles its Golgi localization and that m06 protein accumulates in perinuclear endosomes but not in pericentriolar tubular recycling endosomes of MCMV infected cells.

Early Endosomal Loading of the m06 Requires Other MCMV Proteins

Given that the MCMV infection reorganizes early endosomal system of the cell and remodels endosomal routes,[8] we next examined whether m06 protein distributes into early endosomes of NIH 3T3-m06 transfectants, in the absence of other MCMV functions. In these cells most of m06 protein was found in the membranous structures in the pericentriolar area which was also crammed with GS28-positive Golgi membranes (Figure 5A, left), and EEA1-positive (Figure 5A, middle) and TfR-loaded (Figure 5A, right) endosomes. Thus, the level of colocalization of m06 with these markers was rather high in untreated cells (Figure 5C). In order to distinguish in which membranous structure m06 proteins localize, we treated the cells with BFA in

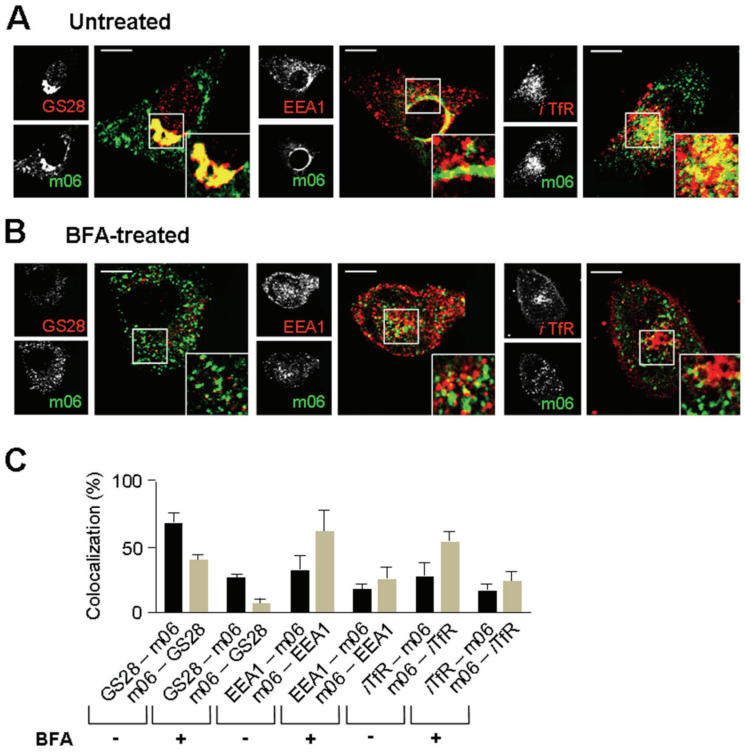

Figure 5. Intracellular distribution of MCMV-m06 protein in the absence of other MCMV proteins. Co-distribution of steady-state (*ss*) m06 protein (gp48) with *ss* GS28 (Golgi marker), *ss* EEA1 (early endosomal marker) and internalized (*i*) TfR in (A) untreated and (B) brefeldin A (BFA)-treated (10 μg/ml, 60 min) NIH 3T3-m06 cells. (C) Quantitative analysis of colocalization based on Manders' coefficients. Data represent the mean ± S.D.

concentration that dispersed Golgi in normal fibroblasts (Figure 4). As expected, BFA treatment dispersed the m06 protein together with the Golgi but a large fraction of m06 remained in the vesicular form (Figure 5B, left), indicating that it localized in a compartment which lacks capacity to fuse with membranes of the ER. BFA treatment did not disperse EEA1- positive endosomes and TfR-loaded juxtanuclear endosomes which remained in the pericentriolar area (Figure 5B, middle and right), but dispersed the m06 protein into vesicles which were largely EEA1-negative (Figure 5B, middle) and TfR-negative (Figure 5B, right). Altogether, this data indicates that in the absence of other viral functions the m06 protein localizes in the Golgi compartments and in perinuclear vesicular structures which are not early endosomes, suggesting that the m06 protein does not reach classical early endosomes in the absence of other MCMV proteins.

DISCUSSION

In this report we demonstrate that the MCMV-m06 protein accumulates in the endosomal system of MCMV-infected cells together with internalized MHC-I proteins, clathrin-independent (TfR) and cdc42-dependent (CTxB) cargo.

The MCMV-m06 protein associates with nascent MHC-I proteins in the ER into relatively stable complex,[5] which is transported into the Golgi and from this localization is rerouted towards late endosomes for degradation.[5] This viral activity is combined with the effect of MCMV-m152 protein, which retains MHC-I proteins in the ER/ERGIC compartment,[3,4] and the MCMV-m04 protein which associates with MHC-I proteins in the ER into the stable complex that reaches the cell surface.[6] The consequence of the activity of m06 and m152 is prevented export of nascent MHC-I proteins to the cell surface and their loss,[3,4] and the consequence of the activity of m04 is the export of masked MHC-I complexes with an important immunoregulatory function.[6,7] In addition to these viral functions which interfere with MHC-I trafficking in the secretory pathway, MCMV also encodes an early function that remodels the early endosomal system and early endosomal routes.[8] This remodeling affects cell surface resident MHC-I proteins which are, upon constitutive endocytic uptake, retained in perinuclear vacuolar early endosomes and inhibited of entry into the recycling and degradative route.[8] Similar mechanism of down-regulation of cell surface MHC-I follows also HIV Nef.[34]

We demonstrated the localization of m06 protein in early endosomes that collect internalized MHC-I

proteins, TfR and CTxB. It is well known that MHC-I molecules constitutively internalize via Arf6-associated endosomal route[14,15,35] into nascent endocytic carriers free of clathrin-dependent cargo (*i.e.* TfR).[14] These carriers fuse with the early acceptor compartment which collects nascent endocytic carriers derived in clathrin-dependent route.[14,15] Additionally, this compartment collects also endocytic carriers known as CLICs, which carry CTxB and dextran, in a phosphatydil-inositiol-3-kinase (PI3K)-dependent manner.[15] Thus, the early acceptor compartment represents "classical" early endosomes[33] in which internalized MHC-I proteins, TfR and CTxB are mixed and transported towards the cell center. During transport in uninfected cells early endosomes mature. TfR and a fraction of MHC-I proteins are sorted into tubular recycling endosomes,[28,35,36] the rest of MHC-I proteins into late endosomes and CTxB into the Golgi.[37] In MCMV infected cells, however, the segregation into recycling and late endosomal route is inhibited, resulting in vacuolization of early endosomes and their arrest around the pericentiolar area, including retention of internalized cargo proteins.[8] Given that the m06 protein was found together with internalized cargo from three different routes, we concluded that the m06 is capable of reaching early endosomes in MCMV infected cells. In contrast to MHC-I proteins and TfR, m06 was dispersed from the pericentriolar tubular endosomes after BFA treatment (Figure 4), suggesting that m06 does not enter into the recycling route but rather progresses towards late endosomes into the degradative route. In MCMV-infected cells, however, exit from early endosomes into the degradative route is slow,[8] resulting also in the retention of m06 protein.

The data presented in this report, demonstrates that m06 protein does not reach the cell surface and that the MCMV effect on cell-surface and endosomal MHC-I is not associated with the activity of this protein. Nevertheless, m06 protein was found in the retention endosomes. This indicates either that m06-MHC-I complexes from the ER are transported from the Golgi into early endosomes prior to their entry into late endosomes, or that a fraction of the m06 protein not associating with nascent MHC-I proteins is rerouted from the trans-Golgi network into early endosomes. This pathway of the m06 protein was not observed in NIH 3T3-m06 cells, a transfectant cell line, suggesting that other viral functions rearrange the Golgi-endosomal route in MCMV infected cells (Figure 5). In the transfectant, m06 was retained outside the early endosomes, in a vacuolo-tubular compartment. The distribution pattern of this compartment, which occupies perinuclear area, disperses after BFA treatment and relies on the microtubular network, suggests that it can be either of ERGIC or of late endosomal origin. Biochemical analysis revealed two populations of m06 expressed in the cell with different fate.[26] Complexes of m06 with nascent MHC-I proteins are transported into lysosomes for degradation, whereas the unassembled m06 is degraded by cytosolic proteasomes in the ER-associated degradation (ERAD).[26] Thus, it cannot be excluded that vacuolar accumulations of the m06 protein outside early endosomes of transfected cells represent an excess of m06 which is unassociated with MHC-I and thereby cannot be rerouted into late endosomes but rather remains in the ER/ERGIC on its way towards proteasomal degradation.

MCMV, like other members of the herpesvirus family, possess a large collection of genes that target almost every step of MHC-I biogenesis in the secretory pathway and their cell surface localization.[38] In contrast, HIV-1 uses single early gene Nef to down-regulate MHC-I.[39] According to the "signaling" model, Nef down-regulates MHC-I by multistep interaction with cellular proteins which leading to the recruitment of PI3K,[40] a key regulator of endosomal shaping, and internalization of MHC-I proteins through clathrin-independent Arf6-associated pathway.[40,41,42] After internalization, Nef sequesters MHC-I molecules into paranuclear endosomes.[40,41] In contrast, according to the "stoichiometric" model, Nef down-regulates MHC-I by diverting newly synthesized MHC-I to lysosomes by an M20- and AP1-dependent mechanism.[43,44] In the recent report, Dikeakos *et al.* demonstrated that Nef orchestrates MHC-I down-regulation by sequential actions of the signaling mode followed by the stoichiometric mode.[34] It appears that the similar sequence operates also in MCMV infected cells. Thus, the role of the m06 protein in this sequence remains to be determined.

CONCLUSION

Summing up, we can conclude that the MCMV-m06 protein reaches early endosomes of MCMV-infected cells without export to the cell surface. As such, m06 is not essential for accelerated down-regulation of cell surface resident MHC-I proteins in MCMV-infected cells. However, it appears that m06 associates with MHC-I in the retention endosomes formed by a separate viral function. The function of these endosomes remains to be elucidated.

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