Analysis of the subcellular trafficking properties of murine cytomegalovirus M78, a 7 transmembrane receptor homologue

E. L. Sharp,1 N. J. Davis-Poynter1,2 and H. E. Farrell1,2

1Infectious Diseases, Animal Health Trust, Newmarket, Suffolk CB8 7UU, UK
2The University of Queensland, Clinical Medical Virology Centre and Royal Children’s Hospital, Sir Albert Sakzewski Virus Research Centre, QLD 4072, Australia

Murine cytomegalovirus (MCMV) M78 is a member of the betaherpesvirus ‘UL78 family’ of seven transmembrane receptor (7TMR) genes. Previous studies of M78 and its counterpart in rat cytomegalovirus (RCMV) have suggested that these genes are required for efficient cell–cell spread of their respective viruses in tissue culture and demonstrated that gene knockout viruses are significantly attenuated for replication in vivo. However, in comparison with other CMV 7TMRs, relatively little is known about the basic biochemical properties and subcellular trafficking of the UL78 family members. We have characterized MCMV M78 in both transiently transfected and MCMV-infected cells to determine whether M78 exhibits features in common with cellular 7TMR. We obtained preliminary evidence that M78 formed dimers, a property that has been reported for several cellular 7TMR. M78 traffics to the cell surface, but was rapidly and constitutively endocytosed. Antibody feeding experiments demonstrated co-localization of M78 with markers for both the clathrin-dependent and lipid raft/caveolae-mediated internalization pathways. In MCMV-infected cells, the subcellular localization of M78 was modified during the course of infection, which may be related to the incorporation of M78 into the virion envelope during the course of virion maturation.

INTRODUCTION

The cytomegaloviruses (CMVs) are characterized by slow replication cycles, with periods of persistent virus shedding and latency. Virus dissemination is highly cell-associated, principally via infected leukocytes (Sinclair & Sissons, 2006). In order to maintain a stable relationship with their host, the CMVs have evolved various mechanisms to manipulate normal cellular responses, in particular innate and adaptive immune responses (reviewed by Mocarski, 2002).

Among the viral gene products that are predicted to be linked to CMV dissemination are seven transmembrane receptors (7TMR) that signal via coupling to G proteins. Leukocyte trafficking is regulated by the concerted responses of multiple cellular 7TMR, both in response to infection and during normal cellular homeostasis (Le et al., 2004) and thus, the CMV 7TMR homologues have been implicated in usurping this role during infection to promote virus dissemination. Additional roles for CMV 7TMR include chemokine sequestration, membrane fusion and production of an intracellular environment favourable for virus replication (Vischer et al., 2006).

Human cytomegalovirus (HCMV) encodes four 7TMR: UL33, UL78, US27 and US28. Of these, US28 has been the most extensively characterized, both pharmacologically and with respect to its intracellular trafficking properties. US28 has been shown to bind both CC and CX3C chemokines with high affinity, resulting in the activation of multiple intracellular activation pathways (Kuhn et al., 1995). In addition to agonist-induced signalling, US28 exhibits constitutive signalling and endocytosis, which appear to be modulated by the binding of fractalkine (Casarosa et al., 2001). Unlike most cellular chemokine receptors, US28 is located predominantly in late and recycling endosomes, rather than at the cell surface, which support the suggestion that it plays a role in sequestration of cellular chemokines (Bodaghi et al., 1998; Fraile-Ramos et al., 2001). While constitutive endocytosis of US28 has been shown to occur independently of β-arrestin proteins, it is nevertheless internalized, at least in part, via a clathrin-mediated pathway (Fraile-Ramos et al., 2003). Studies with UL33 and US27 have demonstrated that, like US28, they co-localize with endocytic vesicles. Notably, the localization of UL33 and US27 in endocytic vesicles in HCMV-infected cells has been shown to overlap with intracellular membranes containing HCMV glycoproteins important for virion maturation, consistent with the incorporation of these viral 7TMR in the virion envelope (Fraile-Ramos et al., 2002).

It has been suggested that dimerization/oligomerization of 7TMR influences their intracellular trafficking. Homo-
hetero-dimerization has been reported for a variety of 7TMR, and has been shown to play an important role in 7TMR biogenesis and transport to the cell surface. While some reports have suggested that dimerization/oligomerization is induced at the cell surface by the binding of ligand, others have demonstrated oligomer formation early in the biosynthetic pathway, within the endoplasmic reticulum (Bulenger et al., 2005). Higher molecular mass species consistent with 7TMR dimers have been identified for HCMV US27, US28 and for UL33, suggesting that their biogenesis may be similar to that of their cellular 7TMR counterparts (Fraile-Ramos et al., 2001; Margulies & Gibson, 2007).

In contrast to US28, UL33 and US27, little is known about the biochemical and intracellular trafficking properties of the UL78 family members, which, of the betaherpesvirus 7TMR homologues, are the least conserved with chemokine receptors. UL78 counterparts in human herpesvirus (HHV)-6 and HHV-7, encoded by U51, have been shown to bind β-chemokines, resulting in stimulation or modulation of signal transduction (Milne et al., 2000; Tadagaki et al., 2005; Fitzsimons et al., 2006). Studies with HHV-6 U51 have shown that it promotes cell fusion mediated by the G protein of vesicular stomatitis virus, consistent with a possible role for U51 in promoting cell–cell spread of virus (Zhen et al., 2005). In contrast to U51, the ligand(s) for the CMV UL78 members have not yet been identified. Clues to the contribution of the UL78 gene family to the virus life cycle have come from studies of rodent CMV homologues, M78 of MCMV and R78 of rat cytomegalovirus. Gene knockout experiments have shown that both M78 and R78 contribute to efficient cell–cell spread of these viruses in vitro and replication in target organs during acute and persistent stages of infection in vivo (Beisser et al., 1999; Oliveira & Shenk, 2001). The in vitro effects of M78 on MCMV replication were linked to an increase in immediate-early (IE) mRNA, which was observed in cells infected with wild-type MCMV, but not by a MCMV mutant with M78 deleted (Oliveira & Shenk, 2001). As M78 was identified in semi-purified virions, it was further postulated that M78 delivered to the cell upon virus entry facilitates IE mRNA accumulation (Oliveira & Shenk, 2001). Additional characterization of this phenomenon, in particular whether M78 mediates this activity as a G-protein-coupled receptor (GPCR), has not been reported.

We examined the cellular localization of MCMV M78 in both transfected and MCMV-infected cells to determine whether M78 shares features with US28 and cellular GPCRs. We produced anti-peptide antiserum specific for M78 and constructed N- and C-terminal tagged forms of M78, utilizing either green fluorescent protein (GFP) fused to the C-terminal (intracellular) domain of M78, or the influenza haemagglutinin (HA) epitope fused to the N-terminal (extracellular) domain. We demonstrated that, like US28, M78 was rapidly and constitutively endocytosed from the cell surface. Furthermore, both M78 and US28 were endocytosed by routes utilized by transferrin (Tfn) and cholera toxin subunit B (CtxB), markers for clathrin-dependent and lipid raft/caveolae-mediated pathways, respectively (Yamashiro et al., 1984; Pelkmans & Helenius, 2002). In MCMV-infected cells, the localization of M78 with markers of the secretory and early endosomal pathways at 5 h post-infection (p.i.) was markedly diminished at 16 h p.i., suggesting a temporal shift in M78 trafficking.

**METHODS**

**Cells.** All cells were grown in commercial medium obtained from Sigma. Primary mouse embryonic fibroblasts (MEF), COS-7 and HeLa cells were maintained in minimal essential medium (MEM) containing 10% fetal calf serum (MEM/10%) supplemented with 2 mM glutamine, 100 U penicillin ml⁻¹ and 0.1 mg streptomycin ml⁻¹.

**Viruses.** The K181 (Perth) strain of MCMV was used in these studies. The virus titre was quantified on MEF as described previously (Allan & Shellam, 1984).

**Receptor constructs.** M78 was PCR amplified from the HinnIII 'C' fragment of a K181 (Perth) genomic library and was cloned into pcDNA3 via introduced EcoRI flanking sites. M78 was cloned into-frame into pEGFP-N1 and pCMV-HA (Clontech) to produce M78GFP and HA-M78, respectively. The fidelity of expression constructs was confirmed by sequencing. The CD4-US28 and US28GFP plasmids were provided by M. Marsh (UCL, UK) and have been described previously (Fraile-Ramos et al., 2001, 2002). The HA-CCR5 plasmid was purchased from the UMR cDNA Resource Centre, University of Missouri-Rolla, USA.

**Antibodies.** The mouse anti-huCD63 was obtained from M. Marsh (UCL, UK) and has been described previously (Fraile-Ramos et al., 2001). Mouse monoclonal antibodies to paxillin, EEA-1 and GM130 were obtained from BD Transduction Laboratories. Polyclonal rabbit antiserum against US28 and US27, UL33 and US27, little is known about the biochemical and intracellular trafficking properties of the UL78 family members, which, of the beta-herpesvirus 7TMR homologues, are the least conserved with chemokine receptors. UL78 counterparts in human herpesvirus (HHV)-6 and HHV-7, encoded by U51, have been shown to bind β-chemokines, resulting in stimulation or modulation of signal transduction (Milne et al., 2000; Tadagaki et al., 2005; Fitzsimons et al., 2006). Studies with HHV-6 U51 have shown that it promotes cell fusion mediated by the G protein of vesicular stomatitis virus, consistent with a possible role for U51 in promoting cell–cell spread of virus (Zhen et al., 2005). In contrast to U51, the ligand(s) for the CMV UL78 members have not yet been identified. Clues to the contribution of the UL78 gene family to the virus life cycle have come from studies of rodent CMV homologues, M78 of MCMV and R78 of rat cytomegalovirus. Gene knockout experiments have shown that both M78 and R78 contribute to efficient cell–cell spread of these viruses in vitro and replication in target organs during acute and persistent stages of infection in vivo (Beisser et al., 1999; Oliveira & Shenk, 2001). The in vitro effects of M78 on MCMV replication were linked to an increase in immediate-early (IE) mRNA, which was observed in cells infected with wild-type MCMV, but not by a MCMV mutant with M78 deleted (Oliveira & Shenk, 2001). As M78 was identified in semi-purified virions, it was further postulated that M78 delivered to the cell upon virus entry facilitates IE mRNA accumulation (Oliveira & Shenk, 2001). Additional characterization of this phenomenon, in particular whether M78 mediates this activity as a G-protein-coupled receptor (GPCR), has not been reported.

We examined the cellular localization of MCMV M78 in both transfected and MCMV-infected cells to determine whether M78 shares features with US28 and cellular GPCRs. We produced anti-peptide antiserum specific for M78 and constructed N- and C-terminal tagged forms of M78, utilizing either green fluorescent protein (GFP) fused to the C-terminal (intracellular) domain of M78, or the influenza haemagglutinin (HA) epitope fused to the N-terminal (extracellular) domain. We demonstrated that, like US28, M78 was rapidly and constitutively endocytosed from the cell surface. Furthermore, both M78 and US28 were endocytosed by routes utilized by transferrin (Tfn) and cholera toxin subunit B (CtxB), markers for clathrin-dependent and lipid raft/caveolae-mediated pathways, respectively (Yamashiro et al., 1984; Pelkmans & Helenius, 2002). In MCMV-infected cells, the localization of M78 with markers of the secretory and early endosomal pathways at 5 h post-infection (p.i.) was markedly diminished at 16 h p.i., suggesting a temporal shift in M78 trafficking.

**METHODS**

**Cells.** All cells were grown in commercial medium obtained from Sigma. Primary mouse embryonic fibroblasts (MEF), COS-7 and HeLa cells were maintained in minimal essential medium (MEM) containing 10% fetal calf serum (MEM/10%) supplemented with 2 mM glutamine, 100 U penicillin ml⁻¹ and 0.1 mg streptomycin ml⁻¹.

**Viruses.** The K181 (Perth) strain of MCMV was used in these studies. The virus titre was quantified on MEF as described previously (Allan & Shellam, 1984).

**Receptor constructs.** M78 was PCR amplified from the HinnIII 'C' fragment of a K181 (Perth) genomic library and was cloned into pcDNA3 via introduced EcoRI flanking sites. M78 was cloned int
the pellets were stored at −80 °C prior to Western blotting. Mock-infected cells were used as controls for both immunofluorescence and Western blotting.

**Cell lysis.** Cell pellets were resuspended in 200 μl solubilization buffer [20 mM Tris-HCl, 150 mM NaCl, 1% sodium deoxycholate, 1% NP-40, 2 mM EDTA, 10 mM iodoacetamide, supplemented with a protease inhibitor cocktail (Roche)] at 4 °C and incubated for 20 min on ice. Samples were centrifuged at 17,900 g for 15 min at 4 °C and the supernatants were retained.

**SDS-PAGE.** Cell lysates were mixed 1:1 with 2 × reducing sample buffer [SB-R; 125 mM Tris-HCl, 20% glycerol (v/v), 4% SDS, 0.2% bromophenol blue and 200 μM DTT] and loaded directly (without heating) onto 10% or 4–15% polyacrylamide gels (Bio-Rad). Equivalent volumes of diluted samples were loaded per lane and heating) onto 10% or 4–15% polyacrylamide gels (Bio-Rad). Following SDS-PAGE, protein was transferred to nitrocellulose, blocked with PBS-T (PBS/0.05% Tween 20) containing 5% BSA, and probed with the designated primary antibodies. Following extensive washing with PBS-T, membranes were probed with the designated horseradish peroxidase (HRP)-conjugated secondary antibodies; bound antibody was visualized with an enhanced chemiluminescence (ECL) kit, using ECL hyperfilm (Amersham Biosciences). Protein separation was visualized with rainbow pre-stained markers (Bio-Rad). Biotinylated protein markers (Bio-Rad) in conjunction with avidin-HRP (Bio-Rad) were used for size estimation on ECL blots.

**Immunoblotting.** Following SDS-PAGE, protein was transferred to nitrocellulose, blocked with PBS-T (PBS/0.05% Tween 20) containing 5% BSA, and probed with the designated primary antibodies. Following extensive washing with PBS-T, membranes were probed with the designated horseradish peroxidase (HRP)-conjugated secondary antibodies; bound antibody was visualized with an enhanced chemiluminescence (ECL) kit, using ECL hyperfilm (Amersham Biosciences). Protein separation was visualized with rainbow pre-stained markers (Bio-Rad). Biotinylated protein markers (Bio-Rad) in conjunction with avidin-HRP (Bio-Rad) were used for size estimation on ECL blots.
monoclonal antibodies to either early or late endosomal markers [EEA-1 (Patki et al., 1997) or CD63 (Metzelaar et al., 1991), respectively]. Bound primary antibodies were detected with GAR488 and GAM594. For experimental controls, transfected HeLa cells were incubated with normal rabbit serum (NRS) and isotype control mouse antibodies in place of the primary antibodies.

Co-localization of endocytosed HA-M78 and CD4-US28 with Tfn and CTxB, was determined by incubating HeLa cells with Tfn594 (50 μg ml⁻¹) or CTxB594 (8 μg ml⁻¹) together with either mouse anti-HA (for HA-M78) or mouse anti-CD4 (for CD4-US28) for 1 h at 37 °C prior to fixation and permeabilization. To deplete intracellular Tfn levels prior to the addition of Tfn594, cells were incubated for 30 min at 37 °C in BM. Anti-HA or anti-CD4 antibodies were detected with GAM488. For control samples, normal mouse serum was used in place of the primary antibody.

Co-localization of M78 in MCMV-infected MEF (3 p.f.u. per cell) was assessed with markers of the cis-Golgi (GM130; Nakamura et al., 1995) or early endosomes (EEA-1) on glass coverslip cultures at 5 or 16 h p.i. Cells were co-incubated with the rabbit anti-M78 and mouse monoclonal antibodies to either GM130 or EEA-1 for 1 h at 37 °C. Bound primary antibodies were detected with GAR488 and GAM594. To assess co-localization of M78 with Tfn594 or CTxB594 the infected cells were labelled with Tfn594 or CTxB594 as described above. The

![Image](https://via.placeholder.com/150)

**Fig. 2.** Detection of M78GFP (a), HA-M78 (b) and untagged M78 (c) in transfected HeLa cells and of M78 in MCMV-infected MEF (d). The distribution of M78 was compared with US28 using US28GFP-transfected HeLa cells (e) and US28GFP-transfected MEF (f). HeLa and MEF cells were fixed and permeabilized at 24 or 48 h p.i., respectively. HA-M78 was detected with mouse anti-HA followed by GAM488, untagged M78 and M78 expressed in MCMV-infected MEF were detected with rabbit anti-M78 serum followed by GAR488. Antibody specificities were confirmed using normal mouse/rabbit antibodies as appropriate (results not shown).

**RESULUTS AND DISCUSSION**

**Detection of tagged and untagged forms of M78 in transfected and infected cells**

The specificity of the polyclonal rabbit antisera was examined against native or tagged M78 by immunoblotting of lysates from transiently transfected COS-7 cells. In order to minimize non-specific aggregation, samples were not heated following addition of SDS/DTT sample buffer. Lysates were analysed by Western blotting (Fig. 1a) and the duplicate set was stained with Coomassie blue to verify that similar total protein amounts were obtained from each set of transfected cells (data not shown). The predicted molecular masses of M78, HA-M78 and M78GFP, are 51.5, 52.8 and 78.4 kDa, respectively. The Western blots detected a major band for untagged M78 corresponding approximately to the predicted monomeric molecular mass. The HA-M78 band ran at a slightly slower mobility than the untagged form, consistent

![Image](https://via.placeholder.com/150)

**Fig. 3.** Detection of HA-M78 in permeabilized (left panel) and non-permeabilized (right panel) HeLa cells at 24 h post-transfection (c, d) or in untransfected controls (a, b). Cells were incubated with rabbit anti-HA followed by GAR488 to detect total or cell-surface HA-M78 expression. An intracellular protein marker (mouse anti-paxillin, followed by GAM594) was used to identify cells with either permeabilized or ruptured cell membranes.
with the addition of the small 9 aa tag. In the case of M78GFP, the putative monomer ran at higher mobility than expected, corresponding to a molecular mass of ~60–65 kDa. It should be noted, however, that since the samples were not heated prior to loading, they may not have been fully denatured, which may have resulted in anomalous migration of particular protein species. Indeed, additional minor protein bands were detected on some blots, in particular for the M78GFP construct, which may have been due to incomplete denaturation or degradation products.

For each of the constructs, in addition to the putative monomeric form, a lower mobility band was detected at approximately double the apparent molecular mass of the monomeric form. Replicate blots probed with anti-HA or anti-GFP confirmed detection of the putative monomeric and dimeric species for each tagged construct (data not shown). We hypothesized that these higher molecular mass bands are dimers of M78, a feature that has been described for other 7TMR. To investigate this further, we analysed lysates from cells transfected singly or in combination with M78 and M78GFP by Western blotting, to determine whether a species corresponding to M78–M78GFP dimers was detected. As a control, lysates from singly transfected cultures were also mixed prior to analysis. To aid the resolution of dimeric species, the lysates were separated on gradient 4–15 % polyacrylamide gels (Fig. 1b). Bands corresponding to the putative monomers and dimers were observed in each of the singly transfected lysates. In the co-transfected lysate, an additional band, migrating at a position consistent with the expected mobility of M78–M78GFP dimers, was detected. This intermediate species was absent from the mixed lysate sample, suggesting that the M78–M78GFP dimers formed within the co-transfected cells, rather than as an artefact during sample preparation and SDS-PAGE. In the co-transfected sample, the band corresponding to the M78GFP–M78GFP dimer was only weakly detected, but whether this was a consequence of the formation, stability or detection of this species being less efficient than that of the M78–M78GFP dimer is not known. Western blot analysis of M78 from MCMV-infected cells demonstrated the detection of putative M78 monomers and dimers from MEF harvested as early as 6 h p.i. using the rabbit anti-M78 sera (Fig. 1c). These studies confirmed that the putative dimers were present in MCMV-infected cell lysates and therefore unlikely to be an artefact attributable to high level expression in transfected COS-7 cells. M78 was also detected in MEF infected with MCMV in the presence of PAA, confirming previous reports that M78 is expressed with early kinetics (Oliveira & Shenk, 2001). Further studies are required to confirm that M78 dimerization occurs within the cell and, if so, whether this has functional significance. The mobilities of the M78 protein species detected by Western blotting (transfected cells) were unaffected in the presence of tunicamycin, consistent with the absence of potential N-linked glycosylation sites for M78 (data not shown).

Intracellular localization of M78 in transfected cells

Most functional G protein-coupled 7TMR are located at the cell surface. Generally, following binding of ligand by the extracellular face and triggering of G-protein-mediated signalling, the GPCR is endocytosed and dissociated from ligand prior to recycling to the cell surface (Hanyaloglu & von Zastrow, 2008). Studies of several viral 7TMR have demonstrated variable degrees of cell-surface versus intracellular distribution; for example, in the absence of ligand...
Fig. 5. Dual detection of endocytosed HA-M78 or CD4-US28 (green) and EEA-1, Tfn, CD63 or CTxB (red) in transfected HeLa cells. For co-localization with EEA-1 (early endosomes) and CD63 (late endosomes), HeLa cells expressing HA-M78 were incubated with rabbit anti-HA to allow internalization. Following fixation and permeabilization, EEA-1 and CD63 were detected with specific mouse antibodies. Primary antibodies were detected with GAM$^{594}$ and GAR$^{488}$. For co-localization with Tfn (clathrin-mediated endocytosis) and CTxB (caveolae-mediated endocytosis), cells were incubated with either Tfn$^{594}$ or CTxB$^{594}$ together with mouse anti-HA (for HA-M78) or mouse anti-CD4 (for CD4-US28) during internalization. Following fixation and permeabilization, bound anti-HA/CD4 antibodies were detected with GAM$^{488}$. Cell nuclei were counterstained with Hoechst. The figure shows merged low-power images (top row) with selected enlargements of merged (row 2) and single-filter images (rows 3 and 4). Bar, 10 μm.
HCMV US28 displays a predominantly intracellular distribution within endocytic organelles, in contrast to most cellular chemokine receptors (Fraile-Ramos et al., 2001).

Analysis of M78 expressed in either transfected HeLa or MCMV-infected MEF indicated that the majority of the M78 protein was located intracellularly within the perinuclear region of permeabilized cells with punctate staining that is consistent with a vesicular distribution (Fig. 2a–d). Notably, a similar intracellular distribution was observed for untagged M78 (Fig. 2c, d) as for the N- or C-terminally tagged counterparts (Fig. 2a, b), suggesting that the tags did not interfere with the normal distribution of M78. A similar intracellular distribution was observed for US28GFP in transfected HeLa and MEF cells (Fig. 2e, f). In permeabilized cells, there was little detection of M78 near the cell-surface of transfected or infected cells (Fig. 2a–d and Fig. 3c). Nevertheless, HA-M78 was readily detected in non-permeabilized cells, which confirmed that M78 was competent for trafficking to the cell surface (Fig. 3d).

To determine whether the predominantly intracellular distribution of M78 was due to constitutive endocytosis, HeLa cells transfected with HA-M78 were incubated with mouse anti-HA monoclonal antibody (HA-7) at 4 °C for 2 h and shifted to 37 °C and endocytosis was monitored over a time course from 5 to 60 min. In these studies HA-CCR5, which is only endocytosed following agonist stimulation, was included for comparison (Mack et al., 1998; Signoret et al., 2000). As expected for HA-CCR5, internalization was negligible across the full incubation period (Fig. 4). This contrasted with the observations for HA-M78, where punctate intracellular staining of M78 was observed within 5 min of the 37 °C shift and by 10 min it appeared that most of the labelled surface pool was internalized. No staining was observed for untransfected controls (results not shown).

Fig. 6. Dual detection of M78 (green) and either GM130 or EEA-1 (red) in MCMV-infected MEF (3 p.f.u. per cell) at 5 and 16 h p.i. Fixed and permeabilized MEF were incubated with rabbit anti-M78 and either mouse anti-EEA-1 (early endosomes) or mouse anti-GM130 (cis-Golgi), followed by GAR 488 and GAM 594. Cell nuclei were counterstained with Hoechst reagent. The figure shows low-power merged images (top row), with selected enlargements of merged (second row) and single-filter images (rows 3 and 4). Bars, 10 μm.
To investigate where M78 was located following endocytosis, double-label immunofluorescence was performed on HA-M78-transfected HeLa cells that were fixed and permeabilized following 1 h of antibody feeding at 37 °C. In previous studies of US28, co-localization with endocytic vesicles, including late endosomes/lysosomes has been demonstrated (Fraile-Ramos et al., 2001). While M78 was shown to co-localize with the early endosomal marker EEA-1, co-localization with the late endocytic marker CD63, was not observed, suggesting that there was negligible transfer of M78 to late endosomes/lysosomes during the 1 h antibody feeding period (Fig. 5). Alternatively, it is possible that the HA-tagged M78 protein is masked in lysosomes, resulting in the lack of detection. Endocytosed M78 and US28 co-localized with Tfn594 consistent with the use of the clathrin-mediated pathway. However, both M78 and US28 also co-localized with CTxB594, suggestive of internalization via clathrin-independent mechanisms, although both M78+ and US28+ vesicles lacking the CTxB marker were also evident (Fig. 5). While co-localization with CTxB594 provides some indication that receptor internalization occurs through lipid raft/caveolae-mediated mechanisms, a degree of overlap of this marker with endosomes associated with clathrin-mediated endocytosis has been reported. It should be noted that internalization of CTxB has been shown to be mediated by lipid raft/caveolae in HeLa cells that bind high, rather than low levels of CTxB (Pang et al., 2004). For this reason, observations of co-localization with this marker were restricted to HeLa cells with high levels of CTxB594. For US28, inhibition of clathrin-mediated endocytosis using small interfering RNA (siRNA) has been shown to inhibit US28 internalization (Fraile-Ramos et al., 2003), although the isolation of US28 from detergent-resistant cellular membranes is suggestive of an endocytic route normally associated with lipid rafts or the caveolae-dependent pathway (Droese et al., 2004). While the results of the M78 and US28

![Fig. 7. (a) Detection of the uptake of Tfn594 and CTxB594 in uninfected MEF (top panels) or in MCMV-infected MEF (3 p.f.u. per cell) at 16 h p.i. (bottom panels). (b) Dual detection of M78 (green) with either Tfn594 or CTxB594 (red) in MCMV-infected MEF. Following fixation and permeabilization at 16 h p.i., Tfn594- and CTxB594-labelled cells were incubated with rabbit anti-M78 followed by GAR488. (b) Shows merged low-power images (top row) with selected enlargements of merged (row 2) and single-filter images (rows 3 and 4). Cell nuclei were counterstained with Hoechst reagent. Bars, 10 μm.](https://www.microbiologyresearch.org/journal-of-general-virology)
co-localization studies presented here are consistent with the usage of both endocytic pathways, further studies using specific inhibitors of clathrin-dependent and -independent pathways are required to determine the endocytic itineraries of these receptors.

**Intracellular localization of M78 in MCMV-infected cells**

We next investigated whether the distribution of M78 observed in transfected HeLa cells was similar in MCMV-infected cells and furthermore, whether the pattern of M78 distribution was maintained at early (5 h p.i.) and late (16 h p.i.) stages in the infectious cycle. M78 has been detected in semi-purified virion preparations (Oliveira & Shenk, 2001) and may be delivered to cells upon virus entry. Any such virion-associated M78 was below the threshold for detection in our studies, since infected cells were negative for M78 immunofluorescence at 2 h p.i. (results not shown). At early times p.i., the cytoplasmic punctate distribution of M78 was accompanied by prominent perinuclear co-localization with GM130, consistent with high levels of M78 expression via the secretory pathway (Fig. 6). However, at late times p.i., the perinuclear distribution of M78 was much reduced. Similarly, co-localization of M78 with EEA-1 was prominent at 5 h p.i., although M78-containing vesicles that lacked these markers were also observed. However, at 16 h p.i., co-localization of M78 with EEA-1 was negligible. The reactivity of control rabbit serum or mouse isotype control localization of M78 with EEA-1 was negligible. The markers were also observed. However, at 16 h p.i., co-localization of M78 with EEA-1 was prominent at 5 h p.i.) or as an indirect consequence of potential perturbations to these organelles at late times in MCMV infection.

To investigate the co-localization of M78 with Tfn594 and CTxB594 MCMV-infected or uninfected MEF were pulse-fed with the Alexa Fluor594-labelled protein conjugates for 1 h prior to detection of M78 with rabbit anti-M78 peptide antiserum/goat anti-rabbit488 antiserum. Notably, Tfn594 uptake was observed only in infected MEF at late times p.i.; uptake in uninfected MEF (Fig. 7a) or in MEF infected for 5 h (results not shown) was negligible. At late times p.i. some co-localization of Tfn594 with M78 was observed (Fig. 7b). For CTxB594, uptake was observed in both infected and uninfected MEF (Fig. 7a) and minor co-localization with M78 was observed at both early (results not shown) and late times p.i. (Fig. 7b). These results demonstrate that the level of recycling endosomes was increased following MCMV-infection, and that M78 co-localized, at least in part, with these organelles. In comparison, CTxB594 uptake was unaffected by MCMV infection and M78 trafficking via vesicles positive for CTxB594 was demonstrated.

In conclusion, this study has shown that M78 is located predominantly within cytoplasmic vesicles, due to rapid, constitutive endocytosis from the cell surface. As for HCMV US28 and a number of other cellular chemokine 7TMR, our data are consistent with M78 being endocytosed via both clathrin-dependent and -independent pathways. Subcellular distribution of M78 was shown to alter during MCMV infection; at late times p.i. the trafficking of M78 may be directed towards sites of virion maturation and assembly, and studies are in progress to address this question.

**ACKNOWLEDGEMENTS**

This study was supported by a University of Queensland ‘New Research Start-up’ grant and a Queensland Health Research Grant. We thank Dr M. Marsh for generous provision of antibodies and advice.

**REFERENCES**


