A role for the SNARE protein syntaxin 3 in human cytomegalovirus morphogenesis

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Summary

As an enveloped virus, replication of human cytomegalovirus (HCMV) is dependent on interaction with cellular membrane systems. Its final envelopment occurs into intracellular membranes prior to its secretion. However the mechanisms underlying these processes are poorly understood. Here, we show that HCMV infection induces expression of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) syntaxin 3 (STX3), a component of the cellular machinery for membrane fusion. STX3 was located at the plasma membrane and at the assembly site where it was found associated with virus wrapping membranes by immunogold labelling. Depletion of STX3 using RNA interference reduced HCMV production, while expression of a STX3 construct resistant to RNAi inhibition enhanced virus production. Ultrastructural examination of the assembly site in HCMV-infected STX3-depleted cells showed fewer mature virions and more viruses undergoing final envelopment. In contrast, silencing of STX3 did not affect herpes simplex virus type 1 production. The mechanism through which STX3 affected HCMV morphogenesis likely involved late endosomes/lysosomes since STX3 depletion reduced the expression of lysosomal membrane glycoproteins. Our results demonstrate a function for STX3 in HCMV morphogenesis, and unravel a new role for this SNARE protein in late endosomes/lysosomes compartments.

Introduction

The β-herpes virus human cytomegalovirus (HCMV) is a prevalent human pathogen that usually causes an asymptomatic infection, although it can be life threatening for immunocompromised individuals such as HIV patients, organ transplant recipients and conatally infected neonates (Mocarski et al., 2007). HCMV double-stranded DNA genome is enclosed into a capsid surrounded by the tegument proteins and a lipid envelope with embedded glycoproteins. It is thought that viral morphogenesis initiates within the cell nucleus where nucleocapsids assemble and are released into the cytoplasm through the nuclear membranes (Mettenleiter et al., 2009). In the cytoplasm, capsids acquire a set of tegument proteins and undertake final envelopment by wrapping into membranes derived from a hybrid compartment or from transport vesicles between the trans-Golgi network (TGN) and endosomes (Cepeda et al., 2010), prior to their secretion to the extracellular medium. However, the mechanisms of HCMV morphogenesis remain to be defined.

Human cytomegalovirus transcriptionally regulates expression of some cellular N-ethylmaleimide-sensitive factor soluble NSF attachment receptors (SNAREs) genes (Hertel and Mocarski, 2004) whose products accomplish membrane fusion events, among them syntaxin 3 (STX3). STX3 was first found located at the plasma membrane (Bennett et al., 1993) participating in exocytic vesicle transport from the TGN to the apical plasma membrane in polarized epithelial cells (Low et al., 1996). In addition, STX3 is involved in membrane expansion such as neurite outgrowth, elongation of neuronal processes, mediated by the exocytosis of intracellular membranes in neuroendocrine cells (Darios and Davletov, 2006), in the fusion of the tubulovesicular compartment with the apical plasma membrane upon stimulation in gastric parietal cells (Peng et al., 1997; Ammar et al., 2002; Lapierre et al., 2007), and the biogenesis and constant renewal of the rod outer segment membranes in retinal photoreceptor cells (Kwok et al., 2008).

As an enveloped virus, HCMV interacts with cellular membranes throughout its life cycle. Given that SNARE proteins drive many membrane fusion events within eukaryotic cells, it is likely that in infected cells, HCMV exploits/manipulates these cellular proteins to produce new virions. In this study we investigated the role of STX3 in HCMV morphogenesis and report that STX3 is required for infectious virus production through a mechanism that possibly involves late endosomes (LEs)/lysosomes membranes.
Results

HCMV infection induces expression of STX3

Analysis of cellular gene expression during HCMV strain AD169 infection by cDNA microarrays revealed upregulation of some SNARE proteins, involved in membrane fusion, among them STX3 (P. Kellam and V. Emery, pers. comm.; Hertel and Mocarski, 2004). To investigate the possible role of STX3 in viral morphogenesis, we first analysed its mRNA levels in HCMV-infected immortalized human foreskin fibroblast (BJ1) cells at different times post infection (Fig. 1A). STX3 mRNA is detected already 1 day post infection (dpi), is strongly enhanced at 2 dpi and maintained during the time-course of infection. Western blot (WB) assays in HCMV-infected BJ1 cells and HCMV-infected human retinal pigment epithelial cells (data not shown) showed a strong induction of STX3 from 2 dpi (Fig. 1B) consistent with the observed increase in mRNA expression.

STX3 is located at the plasma membrane and the virus assembly site, and incorporated into the HCMV viral envelope

We next investigated the subcellular distribution of STX3 by immunofluorescence (IF) microscopy. As expected, no labelling was observed in uninfected BJ1 cells (Fig. 1C), whereas in HCMV-infected cells STX3 was detected at the plasma membrane as well as intracellularly at assembly sites, where the HCMV glycoprotein H (gH) (Fig. 1C) and viral tegument protein pp28 (data not shown)
accumulate. We observed that some STX3 might be located around gH containing structures (Fig. 1C). To locate in more detail STX3 we carried out immunogold labelling of cryosections and electron microscopy (EM). Because our available antibody did not allow us to reliably detect endogenous STX3 on cryosections, we analysed the distribution of a C-terminus c-myc-tagged STX3 construct (see rescue experiments below). It has previously shown that this tag does not interfere with targeting of wild-type STX3 (Kreitzer et al., 2003) and we observed a similar staining pattern in HCMV-infected cells by IF (Fig. S1). We observed labelling at the plasma membrane (A and D), over vesicles and tubules (B and C), as well as the envelope of virions and dense bodies and the vacuole containing them (D and E). ES, extracellular space; G, Golgi cisternae; VL, vacuole; V, virion; DB, dense bodies. Scale bars, 200 nm.

Fig. 2. EM immunolocalization of syntaxin 3 (STX3) in HCMV-infected cells. Cryosections of BJ1 c-myc-STX3 cells HCMV infected for 5 days at 3 moi were labelled for c-myc with 12 nm gold-conjugated secondary antibodies. Gold particles were seen at the plasma membrane (A and D), over vesicles and tubules (B and C), as well as the envelope of virions and dense bodies and the vacuole containing them (D and E). ES, extracellular space; G, Golgi cisternae; VL, vacuole; V, virion; DB, dense bodies. Scale bars, 200 nm.

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Depletion of STX3 in HCMV-infected cells decreases total viral production whereas HSV-1 viral production is unaffected

To investigate the potential function of STX3 in HCMV life cycle we silenced its expression by a short-hairpin RNA (shRNA) approach. Because uninfected BJ1 cells express undetectable levels of STX3, we first evaluated shRNAs in a human melanoma cell line (MeWo) that expresses high levels of intracellular STX3. Silencing efficiency was analysed by IF and WB, and two shRNAs (STX3 #304 and STX3 #424) showed a strong reduction in STX3 levels (Fig. S3) which were used in further studies. Untransduced, non-target control shRNA- and STX3 shRNAs-expressing BJ1 cells were infected with RecCMV, a recombinant strain of HCMV AD169 that expresses green fluorescent protein (GFP) under the control of a HCMV early promoter (McSharry et al., 2001). At day 3 pi, the number of GFP-expressing cells and the mean fluorescence intensity (MFI) were analysed by flow cytometry and no differences were observed between cultures showing that STX3 does not play a role in viral entry or the early expression of viral proteins (Fig. 3A). At 4 dpi, WB analysis revealed that STX3 #304 shRNAs caused a strong reduction in STX3 levels and a slight decrease in viral protein expression compared with control shRNA transduced cells (Fig. 3B), indicating that STX3 depletion may influence the expression of viral proteins. When we analysed the number of cell-associated and extracellular infectious viruses at 5 dpi, a significant approximately fourfold decrease in total viral production...
was found in STX3-depleted cells (Fig. 3C). To distinguish whether STX3 depletion decreased the number of HCMV particles released or whether reduced viral particles infectivity, we determined the viral load in supernatants by quantitative PCR (qPCR) (Fig. 3C) and found ~2.5-fold reduction in the number of viral genomes in STX3-depleted cells supernatants.

To determine the specificity of STX3 depletion on HCMV production, we carried out rescue experiments with a STX3 c-myc-tagged recombinant construct resistant to shRNAs inhibition. BJ1 cells expressing an empty vector (non-resistant cells) or a shRNA-resistant c-myc-STX3 construct (resistant cells) were transduced with non-target control and STX3 shRNAs, infected with RecCMV at 0.3 moi and processed as above. (D) Per cent GFP-positive cells at 3 dpi. (E) Western blot of STX3 and HCMV viral proteins at 4 dpi. (F) Cell-associated and extracellular infectious viruses produced, and viral genomes secreted into the supernatants at 5 dpi. ***P < 0.001. MFI, mean fluorescence intensity; IP, infectious particles.
3 dpi, no differences in the number of GFP-expressing cells were found; however, the MFI was slightly increased in resistant cells (Fig. 3D), suggesting that exogenous STX3 expression levels may affect viral early genes expression. At 4 dpi, WB analysis showed a strong decrease in STX3 expression in non-resistant STX3 shRNA-transduced cells whereas exogenous STX3 levels were approximately eightfold higher in resistant cell lines compared with the levels induced by HCMV (Fig. 3E). Expression of viral proteins was slightly augmented in resistant cell lines (Fig. 3E), suggesting that exogenous STX3 expression may affect HCMV proteins expression. At 5 dpi, the number of infectious viruses produced was determined and a ~2.5-fold decrease in total viral production in non-resistant STX3-depleted cells was observed (Fig. 3F), whereas viral production was not only rescued in resistant cells but enhanced approximately fourfold (Fig. 3F). Analysis of viral genomes in the supernatants revealed an approximately threefold increase in the number of particles secreted from resistant cells compared with control shRNA transduced non-resistant cells (Fig. 3F).

We next assayed whether STX3 depletion affected the production of the α-herpesvirus HSV-1 with the use of MeWo cells, which support HSV-1 replication. Untransduced, non-target control and STX3 shRNAs-expressing MeWo cells were infected with HSV-1. At 24 h post infection (hpi), STX3 and HSV-1 glycoprotein G (gG) expression levels were analysed by WB (Fig. 4A). We found a strong inhibition of STX3 expression in STX3 shRNA-expressing cells meanwhile the levels of HSV-1 gG were similar between the cultures showing that STX3 depletion did not affect HSV-1 entry and expression of viral proteins. When infectious virus production was assayed a similar number of cell-associated and extracellular particles were found between cultures (Fig. 4B). These findings indicate that silencing of STX3 has no impact in HSV-1 possibly reflecting differences in the morphogenesis of HSV-1 and HCMV. In addition, these data confirm the specificity of STX3 shRNAs on HCMV production.

Ultrastructural analysis of HCMV-infected STX3-depleted cell monolayers showed alterations in particle formation

To get insights into the step of viral morphogenesis affected in STX3-depleted cells, we examined cell monolayers embedded in situ by EM, an approach that allows the straightforward localization of viral assembly sites as previously described (Fontana et al., 2008). At first sight, we found no dramatic differences except that there might be more DBs in HCMV-infected STX3-depleted cells compared with infected control shRNAs-expressing cells (Fig. S5B and C). Thus, to further characterize the possible differences we reconstructed the assembly site of at least five cells and quantified the number of virus particles and DBs undergoing envelopment and fully enveloped.

Confirming the first sight, STX3 depletion enhanced the formation of DBs (~0.18 DBs μm−3 in STX3-depleted cells compare with ~0.05 DBs μm−3 in control shRNAs-expressing cells; Table 1). Interestingly, we found that compare with fully enveloped virus particles and DBs, the number of these structures undergoing envelopment was increased in STX3-depleted cells (Table 1), suggesting that STX3 may affect the envelopment process.

STX3 depletion reduces the expression of LEs/lysosomes markers

To gain a clue on the vesicular fusion process in which STX3 is involved and in the mechanisms underlying the role for STX3 in HCMV morphogenesis, we analysed the distribution and expression of cellular markers that we previously reported to be incorporated into the viral enve-
Control and STX3 shRNA-expressing BJ1 cell monolayers infected with RecCMV for 5 days were embedded in situ, consecutive sections were collected and analysed by EM. The entire cell area of at least five cells from each culture was imaged with each 0.5 μm at 30,000 × magnification to reconstruct the assembly site. Viral particles and dense bodies (DBs) in the process of envelopment or fully enveloped were determined. Data are means of the number of particles per cubic micrometre plus standard deviation (number of cells ≥ 5). Normalization factor was cellular volume.

lopo in STX3-depleted MeWo cells (Cepeda et al., 2010). Specifically, the TGN marker TGN46, the LE/lysosome marker CD63, the recycling endosomes marker transferrin receptor and the two mannose 6-phosphate receptors that traffic between TGN and endosomes. IF analysis showed no apparent effect among the markers analysed (Fig. S6), except a reduction in CD63 expression in STX3-depleted cells (Fig. 5A), a result confirmed by cell surface CD63 immunolabelling and flow cytometry analysis (Fig. 5B). In addition, we found an approximately fourfold reduction in CD63 levels in STX3-silenced cells (Fig. 5C) by WB. Pulse-chase experiments followed by immunoprecipitation of CD63 showed that the protein half-life decreased from ~26 h in control cells to ~13 h in STX3-depleted cells (Fig. 5D).

We next tested CD63 expression in HCMV-infected BJ1 cells and found that CD63 was reduced approximately twofold in STX3-depleted cells (Fig. 5E). Interestingly, when we analysed CD63 levels in BJ1 cells expressing a shRNA-resistant c-myc-STX3 construct, an approximately twofold increase was observed in both uninfected and HCMV-infected STX3-resistant cells (Fig. 5F).

Although we have previously shown that lysosomal membrane glycoproteins Lamp1 and Lamp2 were excluded from the HCMV assembly site (Cepeda et al., 2010) we analysed their distribution and expression in STX3-depleted MeWo cells to distinguish whether STX3 had a direct effect on CD63 or whether played a general role in the biogenesis of LEs/lysosomes. As observed for CD63, IF and WB assays showed a reduction in Lamp1 (Fig. 5A and C) and Lamp2 (data not shown) expression in STX3-depleted cells.

Together these results indicate that STX3 affects the expression of lysosomal membrane glycoproteins CD63, Lamp1 and Lamp2 and suggest a general role for this SNARE protein in LEs/lysosomes biogenesis.

Discussion

As an enveloped virus, replication of HCMV is dependent on interactions with cellular membrane systems. Intracellular membrane fusion events in eukaryotic cells are mediated by the formation of a tetrahedral bundle SNARE complex that brings opposing membranes into close proximity to initiate fusion (Jahn and Scheller, 2006). These small membrane proteins are likely key players in the interactions of enveloped viruses with their host cell; however, their role has not been explored. To gain insights into these interactions we studied the role of STX3, a SNARE protein involved in membrane expansion and plasma membrane transport, in HCMV morphogenesis.

Human cytomegalovirus induces the expression of STX3 since early times post infection what might point to a role in early stages of HCMV life cycle, consistent with our WB and FACS analysis that showed a slight reduction in viral protein expression in STX3-depleted, and an increase in c-myc-STX3-expressing cells (Fig. 3). Nevertheless, we focused our work at the cytoplasmic phase of HCMV assembly, a complex process that requires the spatiotemporally controlled interaction of a considerable number of viral proteins and largely unknown cellular factors that constitute the infectious virion. Within this phase, HCMV particles undergo final envelopment into cellular membranes the nature of which has been debated (Tooze et al., 1993; Sanchez et al., 2000; Homman-Loudiyi et al., 2003; Seo and Britt, 2006; Das et al., 2007; Fraile-Ramos et al., 2007); only recently we have shown that these membranes have characteristics of both the TGN and endosomes (Cepeda et al., 2010). However, the molecular mechanisms of HCMV morphogenesis remain undefined.

Importantly, it has been reported that parallel distinct trafficking pathways merge viral proteins into a single structure at the perinuclear assembly compartment (Moorman et al., 2010). IF analysis of HCMV-infected cells revealed STX3 located at the cell membrane and intracellularly at the assembly site (Fig. 1), a distribution consistent with previous data describing a plasma membrane localization of STX3 in epithelial cells (Low et al., 1996; Li et al., 2002) as well as an intracellular distribution in gastric parietal cells, normal rat kidney fibroblasts and photoreceptor cells (Peng et al., 1997; Band and Kuismanen, 2005; Kwok et al., 2008). Our immuno-EM analysis showed STX3 at the plasma membrane, in Golgi cisternae and small vesicles (Fig. 2), suggesting that the protein might traffic through these compartments or that STX3 might operate in several fusion events. Importantly,

Table 1. Quantification of viral particles in reconstructed assembly sites of HCMV-infected syntaxin 3 (STX3)-depleted cell monolayers embedded in situ.

| Control shRNA | Enveloping virus µm⁻³ | Enveloped virus µm⁻³ | Enveloping DB µm⁻³ | Enveloped DB µm⁻³ |
|---------------|------------------------|----------------------|-------------------|-------------------|
| 0.04 ± 0.01   | 0.05 ± 0.01            | 0.03 ± 0.01          | 0.02 ± 0.01       |
| 0.06 ± 0.02   | 0.03 ± 0.01            | 0.14 ± 0.07          | 0.04 ± 0.02       |

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Fig. 5. CD63 expression in syntaxin 3 (STX3) shRNA-expressing and shRNA-resistant cells.

A–C. CD63 and Lamp1 expression in MeWo cells transduced with non-target control and STX3 shRNAs. (A) Cells were fixed, permeabilized, stained with anti-STX3 (green), anti-CD63 (red) and anti-Lamp1 (red) antibodies and imaged by fluorescence microscopy. Dapi staining of nuclei in blue. Scale bars, 20 μm. (B) Intact cells were stained with anti-CD63 antibodies and analysed by flow cytometry. (C) Cells were lysed and lysates analysed by Western blot. Per cent of STX3, CD63 and Lamp1 levels, normalized to actin and clathrin heavy chain loading control, respectively, are displayed bellow each lane ($n = 3$).

D. Pulse-chase assay of CD63 half-life. MeWo cells transduced with non-target control (closed squares) and STX3 (open circles) shRNAs were pulse labelled with $[^{35}S]$-methionine for 16 h and chased for the times indicated. Cell lysates were subjected to immunoprecipitation with anti-CD63 and irrelevant control antibodies, separated by SDS-PAGE and analysed by autoradiography. Levels are expressed as a percentage of protein remaining at the various time points, with levels at time 0 designated as 100%. Data are means plus standard deviations ($n = 2$).

E. BJ1 cells untransduced, or transduced with non-target control and STX3 shRNAs, were infected with RecCMV. At 4 dpi, cells were lysed and lysates analysed as above ($n = 3$).

F. BJ1 cells expressing an empty vector (non-resistant cells) or a shRNA-resistant c-myc-STX3 construct (resistant cells) were uninfected (left blots) or infected with RecCMV (right blots). At 4 dpi, cells were lysed and lysates analysed as above ($n = 3$).
immuno-EM analysis showed STX3 associated to virus wrapping membranes and incorporated into the viral envelope (Figs 2 and S2). This association might reflect its participation in the transport of viral and cellular components to the assembly site and/or in the fusion of vesicles that ultimately generate the membranes into which virus capsid particles wrap, processes that must be interconnected. Supporting the latest proposal, a role for STX3 in the homotypic fusion of secretory granules in zymogenic acinar cells has been demonstrated (Hansen et al., 1999).

We assessed the functional involvement of STX3 in HCMV morphogenesis by knocking down and rescuing the expression of STX3. STX3 depletion reduced the number of both infectious cell-associated and extracellular viruses produced approximately fourfold (Fig. 3). This reproducible and significant partial effect could be explained by: (i) residual amounts of STX3 still present after RNA-mediated interference, (ii) another SNARE protein might functionally replace STX3, as described for SNAP25 and SNAP23 in the regulated exocytosis of chromaffin cells (Sorensen et al., 2003), and (iii) redundancy in the transport pathways of viral proteins to the assembly site, as suggested for the HCMV viral chemokine receptor US28 and the viral envelope glycoprotein B (gB), which might traffic to the assembly site via the plasma membrane or by a direct route from the TGN (Crump et al., 2003; Fraile-Ramos et al., 2003).

Reconstruction and ultrastructural examination of the assembly site showed an increase in the number of uninfected DBs in STX3-depleted cells (Table 1 and Fig. S5). This suggests that there might be differences in virions and DBs morphogenesis pathways, being the latest stimulated in STX3-silenced cells. Indeed, proteomic analysis has revealed preferential incorporation of certain viral proteins into virions rather than into DBs (Varnum et al., 2004), such as the tegument phosphoprotein pp150, which is essential for the maturation and secretion of virions but may be expendable for the release of DBs (AuCoin et al., 2006). In addition, these studies showed an increase in the number of viral particles undergoing envelopment, suggesting that final envelopment may be retarded which would lead to a reduction in the number of viruses produced. This delay could be due to a defect in STX3-dependent delivery of viral proteins and/or cellular components to the assembly site required for this process. It could also be possible that in STX3-depleted cells the available short cisternae where virions wrap are diminished, decelerating the process. Concomitantly, virus production was increased in c-myc-STX3-expressing cells where these cisternae might be augmented, allowing a larger number of viruses to wrap into these membranes. Interesting in this respect is that STX3 has been involved in cellular processes that require high membrane demand such as neurite outgrowth (Darios and Davletov, 2006) or the continual renewal of the rod outer segment disc membranes, the light-sensing organelle, of photoreceptor cells (Chuang et al., 2007).

Following our demonstration of a role for STX3 in HCMV morphogenesis, we wanted to get insights into the mechanism that underlies these phenomena. Our immuno-labelling survey discovered that expression of lysosomal membrane glycoproteins CD63, Lamp1 and Lamp2 was reduced in STX3-depleted cells (Fig. 5) likely due to an enhanced degradation of these proteins in silenced cells (Fig. 5D). These data suggest a general role for this SNARE protein in LEs/lysosomes biogenesis. How STX3 influences trafficking and degradation of lysosomal membrane glycoproteins and/or lysosomes biogenesis will require future studies. Although lysosomal proteins Lamp1 and Lamp2, and lysosomal enzyme cathepsin D have been found to be excluded from the virus assembly site (Homman-Loudiyi et al., 2003; Krzyzaniak et al., 2009; Cepeda et al., 2010) we cannot rule out a possible contribution of lysosomes to HCMV morphogenesis. Interestingly, knocking down and rescuing the expression of STX3 affected the levels of CD63 in HCMV-infected cells, a cellular component that has been shown to be recruited to the assembly site and incorporated into the viral envelope (Krzyzaniak et al., 2009; Cepeda et al., 2010). These data suggest that the role of STX3 in HCMV morphogenesis might be mediated by LE/lysosome CD63-positive membranes.

Future goals include the identification of viral and cellular protein cargo transported in STX3 vesicles, the SNARE proteins that partner in HCMV-infected cells with STX3, and the Rab small GTPase and fusion-accessory proteins that regulate the STX3-mediated fusion event. It will be of interest to learn whether the Rab proteins required for HCMV morphogenesis, Rab27a (Fraile-Ramos et al., 2010) or Rab11 (Krzyzaniak et al., 2009), interact with STX3.

In conclusion, our results demonstrate a function for the SNARE protein STX3 in HCMV morphogenesis that likely involved LE/lysosome membranes. To our knowledge this is the first report on the interactions between an enveloped virus with a component of SNARE cellular machinery for membrane fusion. In addition this study unravels a novel interplay between STX3-mediated transport and lysosomal membrane glycoproteins.

**Experimental procedures**

**Reagents and antibodies**

Tissue culture reagents and culture plastic were from LabClinics S.A. (Barcelona, Spain) and chemicals from Sigma Aldrich (Madrid, Spain).
Antibodies were obtained from the following sources: anti-STX3 (Synamtic Systems, SYSY, Göttingen, Germany); anti-β-actin clone AC-15 (Sigma Aldrich); anti-HCMV gH (Cranage et al., 1988), and anti-HSV-1 gG LP10 (Richman et al., 1986), and gD (Minson et al., 1986) (Dr Helena Browne, University of Cambridge, Cambridge, UK); anti-HCMV tegument protein pp28 (Silva et al., 2003) (Dr Thomas Shenk, Princeton University, Princeton, NJ, USA); anti-HCMV UL33 (Marguelles et al., 1996) (Dr Wade Gibson, The Johns Hopkins University School of Medicine, Baltimore, MA, USA); anti-c-myc-Tag 9B11 (Cell Signalling, Massachusetts, USA); anti-HSV-2 gB and gD (Domingo et al., 2003) (Dr Enrique Tabarés, Universidad Autónoma de Madrid, Madrid, Spain); anti-c-CD63 1B (Fraile-Ramos et al., 2001) (Dr Mark Marsh, MRC, LMCB, London, UK); anti-c-CD63 TEA3/18 (Yanez-Mo et al., 1999) (Dr Francisco Sanchez-Madrid, Servicio de Inmunología, Hospital de la Princesa, Universidad Autónoma de Madrid, Madrid, Spain); anti-Lamp1 and Lamp2 (Dahlgren et al., 1995) (Dr Sven Carlsson, University of Umeå, Sweden); anti-clathrin clone 23 (BD Bioscience, San Jose, California, USA); anti-MPR46, 22d4 (Messner, 1993), developed by Dr Donald Messner, and anti-Lamp1 and Lamp2, HA3 and HA4B (Chen et al., 1985), developed by Dr Thomas August were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, IA, USA); anti-HCMV antibody-negative and -positive sera from anonymous healthy donors (Comunidad de Madrid, Blood Transfusion Centre, Spain) were provided by Dr Hugh Reyburn (CNB, Madrid, Spain); Fluorescent, HRP- and gold-conjugated secondary antibodies were from Invitrogen S.A. (Barcelona, Spain), Thermo Scientific (Madrid, Spain) and BioCell International (Cardiff, UK) respectively.

Cells and viruses

Immortalized human foreskin fibroblast (BJ1) cells were from Clontech (California, USA), human melanoma MeWo cells were a gift from Dr Lluis Montoliu (CNB, Madrid, Spain). Cells were maintained as recommended by suppliers and transduced with lentiviral vectors expressing shRNAs. MeWo and BJ1 transduced cells were selected in media containing 10 and 2 μg ml⁻¹ puromycin, respectively, and STX3 silencing analysed by WB. BJ1 cells were transduced with lentiviruses expressing an empty construct or a c-myc-STX3 construct resistant to shRNA inhibition. shRNA-resistant c-myc-STX3-expressing cells were sorted with an ALTRA HyPerSort flow cytometer (Beckman Coulter, Palo Alto, USA). Kidney epithelial cells of African Green Monkey (Vero) cells were a gift from Dr Cristina Risco (CNB, Madrid, Spain).

The Towne strain of HCMV and a recombinant strain of HCMV AD169 expressing GFP under the control of the HCMV early promoter beta 2.7 gene that is expressed from 8 hpi, RecCMV (McSharry et al., 2001), were propagated on BJ1 cells and titrated as previously described (Cepeda et al., 2010). HSV-1 was propagated in Vero cells and titrated by immunostaining with a mix of anti-HSV-2 gB and gD antibodies that cross-react with their homologues in HSV-1.

Lentiviral vectors for shRNA-mediated gene silencing were prepared with pMDG, p8.91 and retroviral expression plasmids encoding non-target control (SHC002) and STX3 shRNAs TRCN0000065013, TRCN0000065014, TRCN0000065015 and TRCN0000065016 (Mission® TRC-Hs shRNA libraries, Sigma Aldrich) (Moffat et al., 2006) as described (Naldini et al., 1996).

shRNA-resistant c-myc-STX3 construct (GenScript, New Jersey, USA) was extended with Gateway® recombination sequences and transferred via pDONR201 (Invitrogen) to the lentiviral plasmid pLNT-SFFV-WPRE-Gateway® (obtained through Dr Peter van der Sluijs from Dr Adrian Thrasher, University College London, UK). Recombinant lentiviruses were prepared as described above.

Quantitative real-time PCR

To evaluate STX3 mRNAs expression in infected cells, BJ1 cells growing in 60 mm plates were uninfected or infected with HCMV Towne strain at a moi of 3 infectious particles per cell. Total RNA was extracted at time 0 in uninfected cells and at 1, 2, 3, 4 and 5 dpi using the RNeasy mini kit (Quiagen, supplied by Izasa S.A., Barcelona, Spain). The analysis of the relative gene expression of STX3 by qPCR was carried out as previously described (Cepeda et al., 2010) at the Genomics Core Facility at the Centro de Biología Molecular ‘Severo Ochoa’ CSIC-UAM, Madrid. 18S rRNA was used for normalization.

Human cytomegalovirus genomes number was analysed by qPCR as previously described (Fraile-Ramos et al., 2007). Experimental duplicates of supernatants containing viruses were diluted 1:10 and 2 μl used for amplification using primers specific for gB as previously described (Fox et al., 1992) 5′-GAGGACAACGAATACTCTGTTGGGCA-3′ (gB1) and 5′-TCGACGGTGGAGACTGCTGAGG-3′ (gB2). A standard curve was created using the cloned HCMV gB DNA and the 149 bp product was detected using SYBR Green® reagent (Applied Biosystems, California, USA). The number of viral genomes was calculated using the software provided with the ABI 7900HT platform (Applied Biosystems).

IF assays

To analyse the subcellular distribution of STX3, BJ1 cells growing on glass coverslips were uninfected or infected with HCMV Towne strain at a moi of 0.5, fixed at 5 dpi and processed for IF microscopy as described (Cepeda et al., 2010). To compare the distribution and expression of cellular markers between non-target control and STX3 shRNAs-expressing MeWo cell cultures, cells growing on glass coverslips were fixed and processed for IF. Cells were examined with a Leica DMI6000 microscope equipped with a Leica TCS-SP5 multispectral confocal laser scanning system or with a Leica DMRXA microscope equipped with a CCD camera DC300F. To assemble the figures, digital images were transferred to Adobe Photoshop and adjusted so that intensity values extended over the full measurable range (0–255 grey levels).

To analyse the expression of CD63 at the cell surface in MeWo cells, 2 × 10⁵ cells detached in phosphate buffer containing 10 mM EDTA were stained with anti-CD63 antibodies TEA3/18 and a mouse antiserum conjugated with phycoerythrin (PE). CD63 expression was quantified by PE detection by flow cytometry with an Epics XL-MCL flow cytometer (Beckman Coulter). As a control, cells incubated without primary antibodies were included.
**Immunolabelling of cryosections and of isolated viruses for EM**

BJ1 cells expressing c-myc-tagged STX3 were infected with the Towne strain of HCMV at a moi of 3. At 5 dpi, supernatants were harvested to immunogold isolated viruses and cells were first fixed for 30 min at room temperature with a solution of 4% paraformaldehyde (PFA) plus 0.25% glutaraldehyde (GA) in PHEM buffer 0.4 M (240 mM PIPES, 100 mM Hepes, 40 mM EGTA, 8 mM MgCl₂, pH 6.9) and next fixed for 90 min at 4°C with 2% PFA plus 0.125% GA in PHEM buffer and processed for EM analysis. Ultrathin cryosections of 50 nm thick were stained with anti-c-myc antibody, followed by a mouse antiserum conjugated with 12 nm gold (BritishBioCell, Cardiff, UK) in the presence of 10% human serum (Fraile-Ramos et al., 2007). Sections were examined with a JEOL 1011 transmission electron microscope. Images were recorded onto electron image film (SO-163; Kodak), negatives were scanned and images were transferred to Adobe Photoshop and assembled.

For isolated virus immunogold labelling, viruses secreted into the supernatants from HCMV-infected parental and c-myc-STX3-expressing BJ1 cells were adsorbed onto Formvar coated copper grids. Viruses were left intact or permeabilized with saponin prior staining to gain access to antigens sited at the inner leaflet of the viral envelope, and processed as previously described (Cepeda et al., 2010). The specificity of the labelling was tested with an anti-HSV-1 gD antibody or by omitting the primary antibody. Samples were examined with a JEOL 1011 transmission electron microscope and virions were identified according to their size, ~200 nm in diameter. A minimum of 20 viral particles was examined for the control samples, while at least 100 virions were analysed and the number of gold particles associated to each virion was determined.

**HCMV infection in shRNAs-expressing and in shRNA-resistant BJ1 cells**

For HCMV infection assays, 2.5 × 10⁵ BJ1 cells transduced with non-target control or STX3 shRNAs were infected with RecCMV at a moi of 0.3. Puromycin was added to the culture to select transduced cells. At 3 dpi, a fraction of the cells were fixed, analysed by FACS and the number of infected cells was quantified by GFP expression. At 4 dpi, a fraction of the cells were lysed to quantify the expression of STX3 and HCMV proteins by WB, whereas the remainder of the cells was washed and fresh medium was added to collect virions secreted into the supernatants. At 5 dpi supernatants and cells were harvested and cell-associated and extracellular infectious particles were titrated on fresh BJ1 cells as previously described (Fraile-Ramos et al., 2007). In these assays cells were fixed at 56 hpi, analysed by flow cytometry and the number of infectious cells, i.e. number of infectious virus particles, was assayed by GFP expression.

For HCMV infection rescue assays, BJ1 cells expressing an empty construct (non-resistant cells) or a shRNA-resistant c-myc-STX3 construct (resistant cells) were plated at 2.5 × 10⁵ BJ1 cells per well, transduced with non-target control or STX3 shRNAs, infected with RecCMV and processed as above.

**HSV-1 infection in shRNAs-expressing MeWo cells**

For HSV-1 infection assays, 6 × 10⁵ untransduced, non-target control and STX3 shRNA-expressing MeWo cells were infected with HSV-1 at a moi of 0.5. After 24 hpi, a fraction of the cells were lysed to analyse the levels of STX3 and HSV-1 gG by WB, and the rest of the cells and supernatants were harvested to determine the number of cell-associated and extracellular infectious particles on Vero cells. In these assays, cell monolayers were fixed in a mix of methanol : acetone (1:1) and the number of infected cells, i.e. viral foci, was detected by immunostaining as described above.

**Western blotting**

For the analysis of STX3 protein expression during HCMV infection, BJ1 cells growing in 60 mm tissue culture plates were uninfected or infected with HCMV Towne strain at a moi of 3. Uninfected cells at time 0 and HCMV-infected cells at 1, 2, 3, 4 and 5 dpi were lysed in 100 μl of non-reducing SDS-PAGE sample buffer, separated on 10% SDS-PAA gels under non-reducing conditions and transferred to PVDF membranes. Blots were analysed as described (Cepeda et al., 2010). Autoradiography films were scanned and bands were quantified with the public domain ImageJ 1.33 program. Protein loading was normalized to either actin or clathrin heavy chain expression levels. Images were cropped and assembled with Adobe Photoshop.

For the analysis of STX3 expression in functional studies, BJ1 or MeWo untransduced, non-target control and STX3 shRNAs cells RecCMV or HSV-1 infected at moi 1 were lysed. The expression levels of STX3 and viral proteins were assayed as above. The expression of HCMV proteins was examined with a HCMV antibody-positive serum since it has been reported that serum from CMV-infected patients reacts with a large number of structural virion proteins (Nowak et al., 1984). A blot with BJ1 uninfected cell lysates incubated with HCMV-positive serum (Fig. S4) and a blot with HCMV-negative serum (data not shown) were included as controls and no reactivity was observed.

**EM analysis of HCMV-infected cell monolayers**

Confluent control and STX3 shRNAs-expressing BJ1 cells growing on Thermox® (NUNC) coverslips were infected with RecCMV at a moi of 0.5 infectious particles per cell. At 4 dpi some cells were lysed to examine STX3 depletion by WB. At 5 dpi, supernatants were harvested to determine infectious virus production and cell monolayers were fixed for 1 h in 1% glutaraldehyde/0.5% tannic acid in phosphate buffer and processed by embedding in situ in the epoxy-resin EML-812 (Taab Laboratories, Adermaston, Berkshire, UK) as described (Fontana et al., 2008). Ultrathin consecutive sections of 70 nm thick parallel to the thermox coverslip surface from the base to the top of the cells were collected on formvar-coated parallel-bar copper grids each 0.5 μm and analysed with a JEOL 1011 transmission electron microscope. We found that the assembly site surround the microtubule-organizing centre (MTOC), as previously described (Sanchez et al., 2000), and was located from 2 μm up to 5 μm from the base of the cell. We reconstructed the assembly site throughout the series by imaging the entire cell area from selected sections each 0.5 μm. In each section ~40 micrographs at 30 000× magnification were taken to cover the cell with...
enough detail to quantify intracellular viral particles. At least five control or STX3-depleted cells were analysed. All images were recorded with an ES1000W Erlangshen CCD Camera (Gatan, California, USA) transferred to Adobe Photoshop, printed and assembled to recreate the assembly site. Because analysed cells had different sizes, we normalized in function of the cellular volume that was calculated measuring the cell area with the use of the public software ImageJ 1.33. For each cell, viral particles in the process of envelopment or fully enveloped particles were counted throughout the recreated assembly site.

**Pulse-chase analysis**

A total of 8 x 10^6 MeWo cells expressing non-target control or STX3 shRNAs growing in 60 mm tissue culture plates were incubated in methionine and cysteine-free media for 30 min followed by metabolic labelling of the cells in 200 μCi ml⁻¹ [35S]-methionine for 16 h and either lysed immediately or chased in fresh complete medium containing excess unlabelled methionine for various times. CD63 was immunoprecipitated with an anti-CD63 TEA 3/18 antibody and analysed by SDS-PAGE and autoradiography. As a non-specific immunoprecipitation control, cell lysates at time 0 were immunoprecipitated with an anti-h-HCMV tegument protein pp28 antibody. Autoradiography films were scanned and bands were quantified as above.

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**Supporting information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Subcellular localization of c-myc-syntaxin 3 (STX3) in HCMV-infected cells. BJ1 c-myc-STX3 cells HCMV infected for 5 days at 0.5 moi were fixed, permeabilized, stained with anti-c-myc (green) and anti-HCMV glycoprotein UL33 (red) antibodies and imaged by confocal microscopy (panels show two single optical sections ~0.4 μm thick of the same field). Dapi staining of nuclei in blue. Higher-magnification views of the boxed areas are shown in the insets. Scale bars, 20 μm.

**Fig. S2.** Immunogold localization of syntaxin 3 (STX3) on the viral envelope of isolated HCMV viral particles. A and B. Secreted viruses from HCMV-infected parental and c-myc-STX3-expressing BJ1 cells were harvested at 5 dpi, permeabilized with saponin or directly labelled with antibodies against STX3 (A) or c-myc (B) and 10 nm gold-coupled protein-A. C. General structure of HCMV virions stained with 2% uranyl acetate; when viral membranes were partially disrupted, uranyl acetate revealed the nucleocapsids. Scale bars, 50 nm.

**Fig. S3.** Syntaxin 3 (STX3) expression in STX3 shRNA MeWo cells. MeWo cells untransduced, or transduced with non-target control and STX3 shRNAs were grown on glass coverslip for immunofluorescence analysis or lysed. A. Cells were fixed, permeabilized and stained with anti-STX3 (red) antibodies. Dapi staining of nuclei in blue. Scale bars, 20 μm.

B. Cell lysates were analysed by Western blotting and per cent STX3 levels, normalized to actin loading control, are displayed bellow each lane (n = 2).

**Fig. S4.** Analysis of viral proteins with HCMV-positive human serum. BJ1 cells uninfected or infected with HCMV at 3 moi were lysed at 4 dpi. Cell lysates were analysed by Western blotting with HCMV antibody-positive sera.

**Fig. S5.** EM analysis of HCMV-infected syntaxin 3 (STX3) shRNA-expressing cell monolayers embedded in situ. BJ1 cells transduced with non-target control and STX3 shRNAs growing on Theranox® coverslips were infected with RecCMV at 0.5 moi. At 5 dpi, cells were fixed and embedded in situ. A. Diagram depicts sectioning parallel to the Theranox® surface. Low-magnification EM micrographs of a cell from 2 to 5 μm selected serial sections. Scale bars, 5 μm. B and C. High-magnification EM micrographs of HCMV-infected non-target control (B) and STX3 (C) shRNA-expressing cells. MTOC, microtubule-organizing centre; V, virion; DB, dense bodies. Scale bars, 200 nm.

**Fig. S6.** Subcellular localization of TGN46, transferrin receptor and cation-dependent mannose 6-phosphate receptor in STX3 shRNA MeWo cells. MeWo cells untransduced, or transduced with non-target control and STX3 shRNAs were grown on glass coverslip for immunofluorescence analysis or lysed. A. Cells were fixed, permeabilized and stained with anti-STX3 (red) antibodies. Dapi staining of nuclei in blue. Scale bars, 20 μm.

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