Intracellular Sorting Signals for Sequential Trafficking of Human Cytomegalovirus UL37 Proteins to the Endoplasmic Reticulum and Mitochondria^{∇†}

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Human cytomegalovirus UL37 antiapoptotic proteins, including the predominant UL37 exon 1 protein (pUL37x1), traffic sequentially from the endoplasmic reticulum (ER) through the mitochondrion-associated membrane compartment to the mitochondrial outer membrane (OMM), where they inactivate the proapoptotic activity of Bax. We found that widespread mitochondrial distribution occurs within 1 h of pUL37x1 synthesis. The pUL37x1 mitochondrial targeting signal (MTS) spans its first antiapoptotic domain (residues 5 to 34) and consists of a weak hydrophobicity leader (MTS α) and proximal downstream residues (MTS β). This MTS arrangement of a hydrophobic leader and downstream proximal basic residues is similar to that of the translocase of the OMM 20, Tom20. We examined whether the UL37 MTS functions analogously to Tom20 leader. Surprisingly, lowered hydropathy of the UL37x1 MTS α , predicted to block ER translocation, still allowed dual targeting of mutant to the ER and OMM. However, increased hydropathy of the MTS leader caused exclusion of the UL37x1 high-hydropathy mutant from mitochondrial import. Conversely, UL37 MTS α replacement with the Tom20 leader did not retarget pUL37x1 exclusively to the OMM; rather, the UL37x1-Tom20 chimera retained dual trafficking. Moreover, replacement of the UL37 MTS β basic residues did not reduce OMM import. Ablation of the MTS α posttranslational modification site or of the downstream MTS proline-rich domain (PRD) increased mitochondrial import. Our results suggest that pUL37x1 sequential ER to mitochondrial trafficking requires a weakly hydrophobic leader and is regulated by MTS β sequences. Thus, HCMV pUL37x1 uses a mitochondrial importation pathway that is genetically distinguishable from that of known OMM proteins.

During infection of permissive cells, the human cytomegalovirus (HCMV) UL37 immediate-early locus encodes multiple UL37 isoforms (4, 11, 16, 22, 24, 25) (Fig. 1A). The predominant isoform, the UL37 exon 1 protein (pUL37x1), or the viral mitochondrial inhibitor of apoptosis (vMIA), is an essential HCMV gene product required for its growth in humans (17) and in cell culture (14, 20, 36, 47). pUL37x1 induces calcium efflux from the endoplasmic reticulum (ER) (40), regulates viral early gene expression (6, 12), disrupts the F-actin cytoskeleton (35, 40), binds and inactivates Bax at the mitochondrial outer membrane (OMM) (5, 32–34), and inhibits mitochondrial serine protease at late times of infection (27).

To accomplish their multiple functions in the cell, HCMV UL37 proteins sequentially traffic from the ER to mitochondria (4, 9, 17, 24–26, 45). The amino-terminal UL37x1 antiapoptotic domain serves as a mitochondrial targeting sequence (MTS) (16, 17, 24, 26). UL37 proteins first translocate into the ER, traffic through the mitochondria-associated membrane (MAM) subcompartment of the ER,

and then to the OMM (9, 11, 24–26, 45). The MAM is a lipid-rich subdomain of the ER, which directly contacts mitochondria, allowing for the transfer of lipids from the ER to the OMM and the inner mitochondrial membrane (41), and functionally provides microdomains for efficient coupling of ER to mitochondria calcium transfer (37, 42).

The HCMV UL37x1 bipartite MTS includes a weakly hydrophobic leader (MTS α , amino acids [aa] 1 to 22) that is required for ER translocation and mitochondrial import, as well as downstream sequences (MTS β , aa 23 to 34) that are additionally required for its OMM importation (24) (Fig. 2A). The HCMV UL37 MTS is conserved in the homologous primate CMV UL37x1 genes (28).

In contrast, most signal-anchored proteins of the OMM are synthesized in the cytosol as precursors with NH₂-terminal sequences that directly target them to mitochondria (31). Signal-anchored OMM proteins, such as the translocase of the OMM subunits, Tom20 and Tom70 (43, 46), are similar in topology to pUL37x1 and the NH₂-terminal cleavage product, pUL37_{NH₂}, of the UL37 glycoprotein (gpUL37) (26). Tom20 and Tom70 are anchored to the OMM by short NH₂-terminal transmembrane (TM) domains with the bulk of the polypeptides exposed to the cytosol in a type I orientation (21). The important structural elements of their signal anchor sequences are (i) moderate hydrophobicity of the TM domain and (ii) positively charged amino acids in its flanking domain (21, 43).

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FIG. 1. (A) HCMV UL37 isoforms. UL37 proteins share N-terminal UL37x1 MTS, including a moderately hydrophobic MTS α leader (aa 1 to 22, cylinder), MTS β proximal basic residues (aa 23 to 29, ++++), downstream acidic (aa 81 to 108, —) and basic (aa 134 to 151, ++++) domains. The unique C-terminal sequences encoded by UL37 exon 3 contain an N-glycosylation domain (aa 206 to 391, branches) as well as two additional TM domains (aa 178 to 196 and aa 433 to 459, cylinders). The fusion proteins carrying the full length (pUL37x1 wt₁₋₁₆₃) or MTS (wt₁₋₃₆-YFP) with C-terminal fluorophores

Tom20 is targeted from the cytosol to the OMM by a moderately hydrophobic NH₂-terminal leader (score = 1.826) with a minimal requirement for a net basic charge within one to five residues downstream of the leader (21). The juxtaposed basic residues release the Tom20 hydrophobic leader from the ER-targeting signal recognition particle (SRP) and allow for its direct targeting to the OMM. This arrangement of the Tom20 intracellular sorting signals (20, 41) is similar to that of the MTS of pUL37x1 (22), whose leader, while lower in hydropathy (score = 1.289), is nonetheless ER translocated rather than imported from the cytosol directly into the OMM (24, 26).

Our studies were undertaken to define the sequence requirements for pUL37x1 sequential targeting to the ER and to the OMM and to determine whether these signals are distinct from those of other OMM proteins. We examined the potential role of conventional OMM targeting signals (leader hydrophobicity and proximal basic residues) as well as sequences conserved in the homologues of primate CMVs. Unpredictably, UL37x1 MTS β (aa 23 to 36) did not act analogously to the Tom20 mitochondrial targeting leader. Rather, HCMV UL37x1 sequences retargeted the Tom20 hydrophobic leader to sequential ER to OMM import. Moreover, mutation of conventional mitochondrial targeting basic residues did not markedly alter pUL37x1 mitochondrial import. Similarly, UL37x1 lowered hydrophobicity MTS α mutants dually trafficked to the ER and mitochondria. Conversely, pUL37x1 trafficking was altered by increased hydrophobicity, which effectively blocked mitochondrial import. From these studies, we conclude that weak hydrophobicity of the pUL37x1 MTS α and downstream residues play a role in directing translocation but involve more complex interplay than previously appreciated. Importantly, two previously unrecognized MTS signals, the consensus MTS α posttranslational modification (PTM) site (²¹SY) and a downstream MTS β proline-rich domain (PRD, aa 33 to 36), regulated pUL37x1 mitochondrial import.

are represented below. The two UL37x1 antiapoptotic domains are also shown (17). (B) Kinetics of pUL37x1 mitochondrial importation. HFFs were cotransfected with plasmids encoding pUL37x1 wt₁₋₁₆₃-YFP and DsRed1-mito (Clontech). After 2 h, anisomycin (70 μ M) was added to the medium. After 12 h, the cells were either fixed with 100% methanol (0 min) or washed with 1 $\times$ PBS and overlaid with fresh, anisomycin-free medium. The cells were incubated for the indicated times before methanol fixation and confocal imaging. The images were obtained by using comparable settings of aperture and laser power. (C) Colocalization of newly synthesized pUL37x1 with a mitochondrial marker. HFFs transiently transfected with pUL37x1 wt₁₋₁₆₃-YFP (green) were treated with anisomycin-containing medium as in panel B for 12 h. Inhibitor-containing medium was removed, and the cells were washed and overlaid with fresh, anisomycin-free medium for 45 min. At that time, 50 nM MitoTracker Red CMXRos (red, Invitrogen) was added to the medium, followed by incubation for 15 min at 37°C, prior to methanol fixation. The cells were then imaged by confocal microscopy. The panels on the left and center are grayscale. The panel on the right is the color merge of both channels. The small insets are enlargements of the indicated region of interest in the cell. (D) UL37x1 MTS is sufficient for mitochondrial import. HFFs were transiently transfected with expression vectors for wt₁₋₃₆-YFP and treated with MitoTracker Red (top row) as described above or for wt₁₋₁₆₃-YFP and DsRed1-mito (bottom). Cells were harvested 24 h later and imaged by confocal microscopy. The left and center panels are grayscale. The panels on the right show merged images of both channels.

FIG. 2. (A) Conservation of UL37x1 MTS among the primate cytomegaloviruses. The sequences of HCMV, chimpanzee CMV (CCMV), rhesus monkey CMV (RhCMV), and African green monkey (AgmCMV) are shown (top). The boxed areas enclose MTS α , the predicted alpha-helical domain, based upon HMMTOP analysis, within each leader. The MTS β spans downstream residues 23 to 36. The boldfacing and filled circles indicate identity among primate CMV UL37x1 genes. The HCMV UL37x1 hydrophobic leader was mutated to lowered hydrophobicity by replacement of nonconserved residues V4G, L8G, and L14G while maintaining the same length of the TM in the LH mutant (bottom). The predicted hydrophobicity scores (grand average of hydropathicity, GRAVY, Kyte-Doolittle scale) were calculated for the boxed residues of the wt and LH mutant using ProtParam application on the ExPASy Proteomics Server. (B) Colocalization of UL37x1 LH₁₋₃₆-YFP with MitoTracker. HFFs transiently transfected with a vector expressing pUL37x1 LH₁₋₃₆-YFP for 24 h were treated with 50 nM MitoTracker as described above and imaged by confocal microscopy. Shown on the left and middle panels are the grayscale images, while the panel on the right is the overlay both channels. The small insets are enlargements of the indicated regions of interest. (C) ER translocation and mitochondrial import of pUL37x1 LH₁₋₃₆-YFP and LH₁₋₁₆₃-YFP. HeLa cells were transfected with expression vectors of wt₁₋₃₆-YFP, LH₁₋₃₆-YFP, or YFP vector alone (top) or wt₁₋₁₆₃-YFP, LH₁₋₁₆₃-YFP, or YFP vector alone (bottom). ER and mito-

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MATERIALS AND METHODS

Cells and viruses. HeLa cells and primary human foreskin fibroblasts (HFFs; Viromed) were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; HyClone), 100 U of penicillin/ml, and 0.1 mg of streptomycin/ml (2). Life-extended HFFs (LE-HFFs), stably transfected with an expression vector encoding human telomerase (10), were grown in DMEM supplemented with 10% FBS, 4 mM L-glutamine, 0.1 mM nonessential amino acids, 100 U of penicillin/ml, and 0.1 mg of streptomycin/ml. HCMV (strain AD169) was propagated and titered as previously described in HFFs or LE-HFFs (39). Control cells were treated with uninfected cell lysates and served as mock-infected cells.

Both the parental, wild-type (wt) control and vector negative control were included in all experiments. When several mutants were analyzed in the same experiments but shown in separate figures, the corresponding controls are cited or included in the corresponding panels.

Mutagenesis. Site-specific mutations were introduced into the UL37 open reading frame by site-directed mutagenesis using QuikChange kits (Stratagene), according to the manufacturer's instructions. Table S1 in the supplemental material lists the expression vectors used for these studies.

Transient transfection. For isolation of ER and mitochondria, HeLa cells were used since they provide sufficient material for fractionation and detection of transiently expressed wt and mutant UL37 proteins. pUL37x1 trafficking in HeLa cells is experimentally indistinguishable from that observed in transfected HFFs or HCMV-infected HFFs (9, 24, 26). HeLa cells (ca. 6×10^6 to 8×10^6) were transfected by using Lipofectamine 2000 (Invitrogen) suspended in Opti-MEM (Gibco) according to the manufacturer's protocols as previously described (9, 26). DNA (μ g)/lipid (μ l) ratios for transfection were performed at 1:1.75, with $\sim 20 \mu$ g of DNA and 35 μ l of Lipofectamine 2000 used per T-175 flask. At 24 h after transfection, adherent cells were harvested and either stored on ice for fractionation or stored at -80°C for subsequent fractionation as previously described (8, 9).

Subcellular fractionations. Protocols for subcellular fractionations were as previously described (8, 9). Briefly, for isolation of ER and mitochondrial fractionations, pellets of transfected cells were resuspended in 2 ml of MTE buffer (270 mM mannitol, 10 mM Tris-HCl, 0.1 mM EDTA [pH 7.4]) supplemented with complete protease inhibitor cocktail (Roche) and 1 mM phenylmethylsulfonyl fluoride (Sigma) and lysed by sonication (an output power of 3.5). Cellular debris and intact cells were removed by centrifugation at $700 \times g$ for 10 min (min) at 4°C . Crude mitochondria were obtained by centrifugation at $15,000 \times g$ for 10 min at 4°C , and the postmitochondrial supernatant was used for purification of the ER fractions. Purified mitochondria were obtained by banding in discontinuous sucrose gradients consisting of 1.0 and 1.7 M sucrose steps in 10 mM Tris-HCl (pH 7.6), followed by centrifugation at $40,000 \times g$ for 22 min at 4°C . Postmitochondrial supernatants were layered onto discontinuous sucrose gradients (1.3, 1.5, and 2.0 M sucrose in 10 mM Tris-HCl [pH 7]) and banded by centrifugation at $100,000 \times g$ for 45 min at 4°C . The purified ER and mitochondrial pellets were resuspended in $1 \times$ phosphate-buffered saline ($1 \times$ PBS) and stored at -20°C until use.

Protein concentration determination. Protein concentrations of isolated subcellular fractions were determined by using a BCA assay kit (Pierce), according to the manufacturer's recommendations.

Western analyses. Unless otherwise stated, 20 μ g of total lysate or subcellular fractions were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in 10% Bis-Tris NuPage gels (Invitrogen) and transferred onto nitrocellulose membranes in Tris-glycine buffer with 20% methanol (50 V, 60 min) as described previously (9, 26). Membranes were blocked for 2 h at room temperature in $1 \times$ PBS containing 5% milk powder (Carnation) and

chondrial fractions were isolated as described previously (8, 9). (Top) 10 μ g (wt₁₋₃₆-YFP and YFP vector alone) or 40 μ g (LH₁₋₃₆-YFP) of each fraction was analyzed by Westerns with anti-GFP (1:200) antibody. (Bottom) 20 μ g of each fraction was analyzed by Western analysis with anti-UL37x1 (DC35, 1:2,500) or Grp75 (1:1,000) antibodies.

0.1% Tween 20 (Sigma) and then probed with primary antibody for 1 h at room temperature in 1× PBS containing 1% bovine serum albumin (BSA; Sigma). Primary antibodies used for these studies included anti-pUL37x1 (DC35; 1:2,500), anti-green fluorescent protein (anti-GFP; 1:200; Santa Cruz Biotechnology), and anti-Grp75 (1:1,000; Stressgen). Horseradish peroxidase-conjugated secondary antibodies (1:2,500; Santa Cruz) were incubated for 1 h at room temperature in 1× PBS with 1% (wt/vol) BSA. Protein bands were detected by using an ECL detection kit (Pierce). Each blot was then stripped in 62.5 mM Tris buffer containing 2% SDS and 100 mM 2-mercaptoethanol for 1 h at 50°C, with shaking. Stripped blots were washed seven times with 1× PBS, blocked, and reprobed for the detection of the specific protein organelle markers. Digital images were generated by using Scan Wizard Pro version 1.21 and processed in Adobe Photoshop CS3. Western blot films were scanned with a GS-800 Bio-Rad calibrated densitometer, and bands quantified with Quantity One v4.6.6 (Bio-Rad) Basic software. Adjusted volume outputs, using local background subtraction, from ER or mitochondria lanes were utilized to compare trafficking efficiency of pUL37 mutant proteins. Ratios of band intensities were calculated and designated as ER/mitochondrial abundance (denoting that the ER band quantification output was divided by the mitochondrial band output) or mitochondrial/ER abundance (denoting the inverse calculation).

Indirect immunofluorescence assay (IFA). HFFs were seeded at 20 to 30% confluence ($\sim 4 \times 10^5$ cells/cm²) on sterile coverslips in six-well plates (9.72 cm² per well). After 24 h, the cells were transiently transfected using Lipofectamine 2000 suspended in Opti-MEM, according to the manufacturer's protocols. DNA (μ g)/lipid (μ l) ratios for transfection were at 1:1.75, with ~ 0.5 μ g of total DNA used per cm² of available plating surface area. Cells were fixed with ice-cold 100% methanol for 10 min. Fixed cells were washed with 1× PBS. The cells were then sequentially probed with primary antibodies as described previously (9, 11, 26). In addition, mouse anti-Golgin 97 (Molecular Probes; 1:250) was used as a primary antibody with Texas Red-tagged goat anti-mouse IgG secondary antibody (1:250).

Confocal microscopy. Images were acquired with a Zeiss LSM 510 confocal microscope (Intellectual and Developmental Disabilities Research Center) as described previously (9, 45). The excitation wavelengths for the Zeiss LSM 510 microscope were 488 nm (for GFP), 514 nm (for yellow fluorescent protein [YFP]; emission collected through a 530- to 600-nm filter), and 561 nm (for Texas Red-conjugated secondary antibodies, DsRed1-mito). Images were acquired by sequential excitation through $\times 63$ (NA = 1.4) objective lens. Post-acquisition processing was performed by using Adobe Photoshop CS3.

RESULTS

HCMV encodes multiple UL37 isoforms (Fig. 1A) that are ER translocated and traffic from there to mitochondria (1, 11, 16, 22, 24, 25). The predominant UL37 protein isoforms share their amino-terminal sequences, including two antiapoptotic domains, but differ in their C-terminal sequences.

Kinetics of mitochondrial import of pUL37x1. To gain insight into pUL37x1 sequential ER to mitochondrial trafficking, we first examined the kinetics of mitochondrial import of the predominant pUL37x1 (Fig. 1B). Protein synthesis in HFFs, transiently cotransfected with vectors expressing pUL37x1 wt₁₋₁₆₃-YFP and DsRed-mito (a mitochondrial marker), was temporally blocked by anisomycin to allow for accumulation of the encoding transcripts. After removal of the inhibitor, pUL37x1 wt₁₋₁₆₃-YFP production was rapidly detected (within 15 min) in a broadly distributed reticular pattern and progressively increased through the last time point tested (2 h). In contrast, the mitochondrial marker, DsRed1-mito, was not detected within 2 h after release, although it was strongly detected in control cells not treated with anisomycin (C. D. Williamson and A. M. Colberg-Poley, data not shown).

To verify whether pUL37x1 wt₁₋₁₆₃-YFP trafficked into the OMM within the timeframe tested, we treated cells expressing pUL37x1 wt₁₋₁₆₃-YFP with MitoTracker. Within an hour of release, pUL37x1 wt₁₋₁₆₃-YFP was partially detected in the mitochondrial compartment (Fig. 1C), suggesting its import

into mitochondria within that timeframe. Consistent with these results, pUL37x1 wt₁₋₁₆₃-YFP colocalized with MitoTracker throughout the cell, suggesting its synthesis at widely distributed ER-mitochondrial contact sites.

Trafficking of pUL37x1 requires its first antiapoptotic domain (aa 1 to 34), a bipartite MTS with a weakly hydrophobic leader (MTS α) and proximal downstream residues (MTS β) (Fig. 2A) (16, 17, 22, 24). We next determined whether the UL37x1 MTS is sufficient to recapitulate its authentic trafficking (Fig. 1D). pUL37x1 wt₁₋₃₆-YFP colocalized with a mitochondrial marker in a manner similar to that observed for the full-length parental construct, pUL37x1 wt₁₋₁₆₃-YFP, indicating the sufficiency of the UL37x1 MTS leader to target pUL37x1 to mitochondria.

Conserved UL37x1 MTS sequences. Multiple residues within the UL37x1 MTS are conserved among the primate CMV pUL37x1 homologues (Fig. 2A) (28). These residues are located within the NH₂-terminal leader (⁵Y, ⁶V, ¹⁰G, ¹³G, and ¹⁷F) and a consensus PTM, possibly phosphorylation, site at ²¹SY, a downstream ²⁴WI motif, a basic domain (²⁷RKR) and a PRD (³³PLPP) (Fig. 2A). Because the UL37x1 PRD includes two additional well-conserved prolines (³⁵PP), we included these in the MTS β signal.

Hydropathy of the UL37x1 hydrophobic leader. It is thought that moderate hydropathy of leader sequences facilitates re-targeting of proteins from SRP-dependent ER translocation machinery to mitochondrial import pathways (30). Even though it is ER translocated, hydropathy of the pUL37x1 uncleaved leader is weak (Fig. 2A). In an attempt to block its ER translocation, we generated a UL37x1 lowered hydropathy (LH) leader mutant with the same leader length but with markedly reduced hydropathy (score = 0.605) by triple substitution of nonconserved residues. The UL37x1 LH mutant leader has a hydropathy score about half of that of the UL37x1 wt leader (1.289) and should dramatically reduce binding to SRP and predictably inhibit ER translocation.

To verify the anticipated targeting by the pUL37x1 LH leader, the LH leader was fused to YFP and examined for its subcellular targeting by confocal imaging. pUL37x1 LH₁₋₃₆ leader-YFP was, as expected, predominantly cytosolic with some well-circumscribed punctate bodies (Fig. 2B). Nonetheless, some minor colocalization with MitoTracker was detected by IFA.

To examine more quantitatively the relative efficiency of ER versus mitochondrial import of the LH mutant, HeLa cells expressing wt₁₋₃₆-YFP, the LH mutant leader (LH₁₋₃₆-YFP), or control YFP were fractionated (Fig. 2C, top). Although lower in abundance, ER translocation and mitochondrial import of LH₁₋₃₆-YFP were observed, comparable in relative proportions with those of the corresponding wt₁₋₃₆-YFP control. Both the LH₁₋₃₆-YFP and the LH₁₋₁₆₃-YFP mutants showed an ER/mitochondrial distribution of ~ 1.0 -fold compared to their corresponding controls of wt₁₋₃₆-YFP (1.0-fold) and wt₁₋₁₆₃-YFP (1.1-fold). This result suggests that the LH mutation does not block ER translocation and mitochondrial import of pUL37x1.

To determine whether this is indeed the case, HeLa cells expressing full-length pUL37x1 wt₁₋₁₆₃-YFP, the LH mutant (LH₁₋₁₆₃-YFP), or control YFP vector were fractionated (Fig. 2C, bottom). Although less abundant, the LH mutant

(LH₁₋₁₆₃-YFP) protein was detected to similar levels in fractionated ER and mitochondria. These relative abundances were comparable to those of the corresponding wt₁₋₁₆₃-YFP parent. Grp75 verified that ER localization was not due to mitochondrial contamination of the purified ER fraction. These results suggest that the LH mutant in the context of the full-length pUL37x1 dually traffics to both ER and mitochondria, analogously to pUL37x1.

MTS α high hydropathy blocks pUL37x1 mitochondrial import. Because low hydropathy did not effectively block ER translocation of pUL37x1, we tested whether increased MTS α hydropathy could exclude pUL37x1 from mitochondria as it does with other signal-anchored OMM proteins (18, 21). MTS α hydropathy was increased by missense mutations to levels (HH A, 2.232; HH B, 2.600) well above that of the wt parent (1.289) to levels comparable with secretory proteins (21) (Fig. 3A). Confocal imaging of HFFs expressing pUL37x1-CFP carrying the HH MTS alone (HH A₁₋₃₆ or HH B₁₋₃₆) or full-length HH mutant (HH A₁₋₁₆₃ or HH B₁₋₁₆₃) showed that increased hydropathies of MTS markedly reduced pUL37x1 colocalization with mitochondrial marker (Fig. 3B and C). These results suggest that, similar to other OMM proteins, pUL37x1 can be excluded from mitochondria by elevated hydropathy of its leader sequence, underscoring the importance of low hydropathy for UL37x1 sequential trafficking.

To verify that HH was targeted to the secretory apparatus, colocalization with a *trans*-Golgi marker (Golgin 97) was per-

FIG. 3. High hydropathy of the UL37x1 leader blocks its mitochondrial import. (A) Sequences in UL37x1 MTS high hydropathy mutants. The leader residues mutated to generate HH A or HH B mutant are encircled. The conserved residues are in boldface. The hydropathy scores for the wt and mutant proteins, calculated by using the ProtParam application, are indicated on the right. (B) Lack of colocalization of MTS HH A mutants with mitochondrial marker. HFFs transiently expressing pUL37x1 HH A₁₋₃₆-CFP (green, top) or pUL37x1 HH A₁₋₁₆₃-CFP (green, bottom) and DsRed1-mito (red) were fixed 24 h after transfection and examined by confocal microscopy for colocalization of CFP with DsRed1-mito. Shown in the left and middle panels are the grayscale images, while the panels on the right are the merged images of both channels. The small insets are enlargements of the indicated regions of interest. (C) Lack of colocalization of MTS HH B mutants with mitochondrial marker. HFFs transiently expressing pUL37x1 HH B₁₋₃₆-CFP or pUL37x1 HH B₁₋₁₆₃-CFP and DsRed1-mito were fixed 24 h after transfection and examined by confocal microscopy for colocalization of CFP with DsRed1-mito. Shown in the left and middle panels are the grayscale images, while the panels on the right are the overlays of both channels. The small insets are enlargements of the indicated regions of interest. (D) UL37x1 HH mutants traffic to the secretory apparatus. HFF cells expressing HH A₁₋₁₆₃-CFP, HH B₁₋₁₆₃-CFP, or pUL37x1 wt₁₋₁₆₃-YFP (green) were stained with anti-Golgin 97 antibody (red) and imaged by confocal microscopy. Shown in the left and middle panels are the grayscale images and the panels on the right are overlays of both channels. The small insets are enlargements of the indicated regions of interest. (E) UL37x1 HH mutants are excluded from mitochondria. HeLa cells were transfected with expression vectors for HH A₁₋₃₆-CFP, HH A₁₋₁₆₃-CFP, or vector (left panels) or pUL37x1 wt₁₋₃₆-YFP, HH B₁₋₃₆-CFP, HH B₁₋₁₆₃-CFP. Cells were harvested 24 h after transfection and fractionated into ER and mitochondrial fractions. Portions (20 μ g) of protein were resolved by Western analyses with anti-UL37x1 (DC35, 1:2,500) and anti-Grp75 (1:1,000) antibodies.

formed (Fig. 3D). The two HH full-length mutants (HH A₁₋₁₆₃ and HH B₁₋₁₆₃) showed substantial colocalization with Golgin 97, while the corresponding parent pUL37x1 wt₁₋₁₆₃ did not. These results verify that the increased hydropathy leader efficiently targets the mutant protein to the secretory apparatus.

To more quantitatively assess the relative trafficking of the HH mutant to the ER and mitochondria, Western analyses were performed on cells expressing either HH mutant or parent vectors (Fig. 3E). Consistent with the imaging results above, mitochondrial import of HH A and B mutants, either MTS alone or full length, was markedly reduced to trace levels compared to the wt parent. The levels of HH A₁₋₃₆ and HH A₁₋₁₆₃ showed increased ER/mitochondrial abundances of 3.3- and 17.2-fold, respectively, compared to a wt₁₋₃₆ abundance of 0.7-fold. Similarly, the HH B₁₋₃₆ and HH B₁₋₁₆₃ mutants had ER/mitochondrial abundances of 3.6- and 20.6-fold, respectively. Markers for the ER (DPM1) and mitochondria (Grp75) were used to verify the purity and identity of the fractions. The markers suggest that trace amounts of HH A or B mutants detected in mitochondrial fractions could be attributed primarily to contamination of the mitochondrial fractions with trace levels of ER/MAM. Thus, these results verify that the elevated hydropathy leader efficiently targets the secretory apparatus and consequently causes exclusion of pUL37x1 from mitochondrial import. Moreover, this exclusive HH targeting of the secretory apparatus is not overcome by downstream UL37x1 sequences.

UL37x1 MTSβ basic residues. Basic residues immediately downstream of moderately hydrophobic leaders reduce retention of nascent polypeptides by signal recognition particle and ER targeting of mitochondrial proteins (21). UL37x1 MTSβ contains four basic residues, two of which are suitably positioned (Fig. 2A) to predictably reduce SRP retention of nascent pUL37x1. To determine whether the UL37x1 MTSβ basic residues are required for mitochondrial importation, we mutated these to neutral (basic I) or acidic residues (basic II) (Fig. 4A). Surprisingly, basic I and basic II mutants were efficiently imported into mitochondria (Fig. 4B). Basic I and basic II mutants had ER/mitochondrial abundances of 1.1- and 0.8-fold, respectively. Thus, ablation of UL37x1 MTSβ basic residues did not markedly reduce pUL37x1 import into mitochondria. These results establish that UL37x1^{23R, 27R, 28K, and 29R} do not play dominant roles for pUL37x1 import into the OMM, in contrast to other OMM proteins such as Tom20. Thus, the OMM import pathway used by pUL37x1 is genetically distinguishable from that of known OMM proteins.

The UL37x1 MTSβ retargets the Tom20 leader to dual trafficking. The Tom20 hydrophobic leader combined with its downstream basic residues efficiently targets Tom20 directly from the cytosol to the OMM (21). We therefore tested the ability of the Tom20 leader to target downstream pUL37x1 MTS to mitochondria (Fig. 5). A chimeric construct carrying the Tom20 leader (UL37x1-Tom20 B) and downstream UL37x1 MTSβ sequences, including two suitably spaced UL37x1 basic residues (+1 and +5) was examined by IFA (Fig. 5A). Both the leader (UL37x1-Tom20 B₁₋₃₆) and the full-length (UL37x1-Tom20 B₁₋₁₆₃) chimeras partially colocalized with a mitochondrial marker, whereas control full-length mouse Tom20 (mTom20) protein colocalized completely with the mitochondrial marker (Fig. 5B).

FIG. 4. Mutation of UL37x1 MTSβ basic residues do not alter pUL37x1 trafficking. (A) The basic mutant constructions are represented. The mutated residues are encircled, while the retained, conserved residues are in boldface. Basic I mutant contains R23A, R27A, K28A, and R29A substitutions, whereas basic II contains mutations to negatively charged glutamic acid, R23E, R27E, K28E, and R29E. (B) HeLa cells transiently expressing basic I₁₋₁₆₃-YFP or basic II₁₋₁₆₃-YFP were fractionated and ER and mitochondrial fractions isolated. Portions (20 μg) were resolved by SDS-PAGE and analyzed by Western analysis with anti-UL37x1 (DC35) and anti-Grp75.

To confirm that these UL37x1-Tom20 chimeras trafficked through the secretory apparatus, colocalization with Golgin 97 was performed (Fig. 5C). Despite the presence of mitochondrial targeting Tom20 leader and suitably positioned MTSβ basic residues, the UL37x1-Tom20 B₁₋₃₆ chimeras detectably trafficked to the secretory apparatus, partially colocalizing with Golgin 97 more than the corresponding parent (UL37x1 wt₁₋₃₆). Together, these results establish the dual trafficking of UL37x1-Tom20 chimeras to the secretory and mitochondrial compartments.

The imaging results were verified by Western analyses (Fig. 5D). UL37x1-Tom20 B₁₋₁₆₃ chimera and the corresponding wt₁₋₁₆₃ trafficked efficiently into the ER, as well as to the OMM. The UL37x1-Tom20 B₁₋₁₆₃ chimera showed an ER/mitochondrial abundance of 0.6-fold compared to the corresponding wt₁₋₁₆₃ control's abundance of 1.1. In contrast, control mTom20 protein was detected exclusively in mitochondrial fractions. mTom20 showed an ER/mitochondrial distribution of 0.002. Together, these results further suggest that UL37x1 dual targeting signals are dominant over the Tom20 mitochondrial targeting leader.

Conserved PTM site. Conserved within the primate UL37x1 leader sequences are two residues identified in pUL37x1 as consensus PTM site, possibly phosphorylation sites for mammalian protein kinase C (²¹S) and epidermal growth factor receptor (²²Y), as detected by NetPhosK 1.0 software (7) and PROSITE motif scanning software (15). We therefore tested whether these sequences or their modification play a role in pUL37x1 sequential trafficking to the ER or mitochondria. To that end, we mutated the residues to phosphoablative alanine (S21A and Y22A) or to phosphomimetic (S21D) (Fig. 6A). The phosphomimetic mutant, pUL37x1 S21D-YFP, was more abundant in the ER than in the mitochondrial fraction (Fig. 6B). pUL37x1 S21D₁₋₁₆₃-YFP had an ER/mitochondrial abundance of 4.7-fold compared to its corresponding parent wt₁₋₁₆₃

FIG. 5. A chimeric UL37x1-Tom20 leader dually targets the ER and mitochondria. (A) The sequences in the wt UL37x1 and in the Tom20 leader (red)-UL37x1 MTS β (black) (UL37x1-Tom20 B₁₋₃₆) chimera. Their corresponding hydropathy scores, calculated as in Fig. 2, are shown. The conserved UL37x1 residues are in boldface. (B) Colocalization of chimeric UL37x1-Tom20 with MitoTracker. HFF cells were transiently transfected with expression vectors for UL37x1-Tom20 B₁₋₃₆-YFP, UL37x1-Tom20 B₁₋₁₆₃-YFP, or mTom20-YFP and 24 h later stained with MitoTracker Red and imaged by confocal microscopy. Shown are the grayscale images (left and middle panels) and the overlays of both channels (right panels). The small insets are enlargements of the indicated regions of interest. (C) Colocalization of UL37x1-Tom20 B₁₋₃₆-YFP with Golgin 97. HFFs, expressing UL37x1-Tom20 B₁₋₃₆-YFP or the corresponding control (UL37x1 wt₁₋₃₆-YFP) were harvested at 24 h after transient transfection and stained with anti-Golgin 97 antibody and imaged by confocal microscopy. Shown are the grayscale images (left and middle panels) and the overlays of both channels (right panels). The small insets are enlargements of the indicated regions of interest. (D) Dual ER and mitochondrial traffic-

(2.4-fold). Conversely, the phosphoablative mutant (S21A, Y22A-YFP) increased the relative abundance of pUL37x1 detected in mitochondrial fractions compared to the corresponding parental wt₁₋₁₆₃ protein. pUL37x1 S21A, Y22A₁₋₁₆₃-YFP had an ER/mitochondrial abundance of 1.0-fold compared to its corresponding parent wt₁₋₁₆₃ (1.5-fold). These results suggest that modification of consensus PTM (²¹S) favors ER retention, while the absence of posttranslational modification favors pUL37x1 mitochondrial import.

UL37x1 MTS β PRD. The downstream PRD (aa 33 to 36) within the UL37x1 MTS β is conserved in primate CMV sequences (Fig. 2A). We mutated the HCMV PRD and tested its effects on pUL37x1 trafficking (Fig. 7A). The pUL37x1 PRD mutant carrying the MTS (PRD₁₋₃₆) or the full-length pUL37x1 open reading frame (PRD₁₋₁₆₃) showed partial colocalization with mitochondrial marker (Fig. 7B).

Subcellular trafficking of the PRD mutant was further examined by using Western analyses (Fig. 7C). The mutation affects the epitope used to generate DC35 and reduced its detection by this polyclonal antibody. Using anti-GFP antibody, which reacts with YFP, the fusion protein was detected predominantly in the mitochondrial fraction. The PRD₁₋₁₆₃ mutant had a mitochondrial/ER abundance of 5.9-fold compared to its corresponding parent wt₁₋₁₆₃ (1.4-fold). Thus, although the abundance of the PRD₁₋₁₆₃ mutant was reduced, its relative efficiency of importation into mitochondria was increased above that of the parent control. These results suggest that the MTS β PRD plays a critical role in regulating dual trafficking of pUL37x1.

DISCUSSION

To our knowledge, this study provides the first comprehensive genetic analyses of the signals required for sequential ER to mitochondrial trafficking of a herpesvirus protein. Moreover, these studies establish differences between direct mitochondrial import used by most signal-anchored OMM proteins and the sequential ER to OMM import used by HCMV pUL37x1.

We first examined the synthesis and sequential trafficking of pUL37x1 using the YFP fusion protein for confocal imaging. Within 15 min, pUL37x1-YFP is detectably synthesized in multiple broadly distributed sites in the cell. The broad distribution of pUL37x1 synthetic sites is similar to that observed by us for an MAM marker (9). Although confocal microscopy did not allow accurate quantification of the abundance of the newly synthesized pUL37x1 wt₁₋₁₆₃-YFP, it is noteworthy that the new synthesized protein is dispersed to mitochondria throughout the cell within 1 h of synthesis. The timing of its mitochondrial import may result from its translation in close proximity to mitochondria. As pUL37x1 traffics through the MAM and

ing of UL37x1-Tom20 chimeras. HeLa cells were transfected with expression vectors for UL37x1-Tom20 B₁₋₁₆₃-YFP or with mTom20-YFP (23). Cells were fractionated into ER and mitochondria as described previously (8, 9). Portions (20 μ g) of proteins were resolved by SDS-PAGE and examined by Western analyses with anti-UL37x1 (DC35) or Grp75 antibodies as described above.

FIG. 6. Increased mitochondrial import of pUL37x1 phospho-ablative mutant. (A) PTM site mutants. Phospho-ablative (S21A, Y22A) and phospho-mimetic (S21D) mutations of the consensus MTS α PTM sites are indicated by the encircled residues. The conserved residues are in bold font. (B) HeLa cells were transfected with expression vectors for pUL37x1 wt₁₋₁₆₃-YFP, pUL37x1 S21D₁₋₁₆₃-YFP, pUL37x1 S21A, Y22A₁₋₁₆₃-YFP, or YFP alone. Fractions were isolated 24 h later, 20 μ g were resolved by SDS-PAGE and analyzed by Western blotting with anti-UL37x1 (DC35) and Grp75 antibodies.

these are contact sites between the ER and mitochondria, pUL37x1 may be translated in the MAM or sites proximal to the MAM. Consistent with this possibility is the visualization of ribosomes at or proximal to ER and mitochondrial contact points (13). The second possibility is that diffusion within mitochondria is rapid. However, pUL37x1 causes discontinuity in mitochondrial networks (29, 32), suggesting that diffusion throughout an intact mitochondrial network is less likely following its synthesis. In our experiments, both reticular and punctate mitochondria were detected within one to two h of detectable pUL37x1 synthesis. This rapid disruption of mitochondrial morphology suggests relatively low levels of pUL37x1/vMIA may be sufficient to alter mitochondrial morphology throughout the cell. Because there was no MitoTracker in the time course in the first series, this disruption could not result from MitoTracker treatment. Moreover, the lack of observable ER saturation with pUL37x1 wt₁₋₁₆₃-YFP during kinetic studies and the predictably rate-limiting steps of ER to mitochondrial protein transfer lead us to favor the first possibility of pUL37x1 synthesis at numerous sites close to or at the MAM for directed transfer to mitochondria. The ability to detect YFP within 15 min of translation release is remarkable and potentially provides a useful tool for examining trafficking kinetics. Even though the YFP tag is on the C-terminal cytosolic tail of pUL37x1, this efficient fluorescent detection likely resulted from its N-terminal anchoring to cellular membranes (26), thereby concentrating the optimized YFP signal, which some groups have utilized for the detection of single molecules (48).

Although pUL37x1 has a similar sequence arrangement and topology as Tom20, another OMM protein, it traffics sequentially from the ER to the OMM. Because of its uncommon trafficking to the ER prior to mitochondrial import, we set out to determine the key sorting signals within its MTS required for UL37 sequential trafficking. Surprisingly, we found that only three features of the pUL37x1 MTS markedly affect its dual trafficking: the hydropathy of its leader, the consensus

PTM site (²¹SY), and the MTS β PRD (³³PLPP). Mutation of MTS β basic residues, potentially critical for mitochondrial import, did not alter pUL37x1 trafficking. Neither did the conserved ²⁴WI, anticipated to affect its positioning in membranes, detectably alter pUL37x1 trafficking to the ER and mitochondria (see Fig. S1 in the supplemental material).

Tom20 sorting requires its hydrophobic leader and a net positive charge within five residues of the leader to target correctly the OMM (21). It is thought that the juxtaposed basic residues cause dissociation of the nascent Tom20 polypeptide from the signal recognition particle, association with chaperones and targeting of mitochondrial import pathway. Despite the presence of two UL37x1 basic residues, analogously positioned at +1 and +5, the chimeric pUL37x1-Tom20 MTS was still ER translocated. These results suggest that either the MTS β basic residues are insufficient to block pUL37x1 signals for ER translocation or that other UL37x1 MTS β sequences override the dissociation of the nascent strand from signal recognition particle complex. Consistent with this finding, ablation of the UL37x1 basic residues did not detectably alter pUL37x1 trafficking.

Weak hydropathy of the UL37x1 leader appears to be critical to targeting its OMM trafficking. The requirement for weak hydropathy of UL37x1 leader was demonstrated by aberrant trafficking of the UL37x1 high hydropathy mutant, while the lowered hydropathy mutant was still able to dually traffic. However, we do not yet know whether the UL37x1 LH mutant is integrally associated with membranes or peripherally associated. The planar residue at ²⁴W may serve to associate the weakly hydrophobic UL37x1 LH leader to membranes. Nonetheless, we note that the WI mutation did not adversely affect ER translocation. Alternatively, the UL37x1 C-terminal sequences, which contain a consensus myristoylation site (⁵⁹GVIDGE⁶⁴), might allow for its continued membrane association despite reduced hydropathy of the leader sequence.

In contrast, both the leader and the full-length HH mutants trafficked almost exclusively to the secretory apparatus. This re-

FIG. 7. Increased mitochondrial import of a pUL37x1 MTS β PRD mutant. (A) Sequence of the UL37x1 PRD mutant. The substitutions of UL37x1 PRD residues (aa 31 to 36) are circled. The conserved residues are in boldface. (B) Colocalization of UL37x1 PRD₁₋₃₆-YFP and PRD₁₋₁₆₃-YFP with MitoTracker. HFFs transiently expressing PRD₁₋₃₆-YFP or PRD₁₋₁₆₃-YFP were treated with MitoTracker and imaged by confocal microscopy 24 h later. Shown are grayscale images (left and middle) and the overlay of both channels (right). The insets show enlargements of indicated regions of interest of the cells. (C) Efficient mitochondrial import of UL37x1 PRD mutant. HeLa cells expressing pUL37x1 wt₁₋₁₆₃-YFP or the PRD₁₋₁₆₃-YFP were fractionated and fractions analyzed by Western analysis with anti-UL37x1 (DC35), anti-GFP or anti-Grp75.

sult suggests that the downstream UL37x1 sequences could not reverse the dominant secretory pathway targeted by the high hydrophathy mutation and allow mitochondrial import. This finding suggests that the UL37x1 MTS selectively targets an ER subdomain, possibly the MAM, different from that targeted by most secretory proteins using the ER translocon (Sec61).

Two additional signals affected pUL37x1 sequential trafficking. Mutation of the UL37x1 MTS α consensus PTM site suggests that posttranslational modification, possibly phosphorylation, of the ²¹S and/or ²²Y regulate pUL37x1 trafficking. Prediction of phosphorylation sites is imperfect and

newer prediction programs are being developed (19). Nonetheless, the UL37x1 consensus MTS phosphorylation site was reproducibly predicted by two programs (NetPhosK v1.0 and PROSITE motif scanning). Tellingly, the consensus site (²¹SY) is conserved in all of sequenced UL37x1 genes in primate cytomegaloviruses (28). Further, mutation of the consensus phosphorylation site resulted in detectable phenotypes unlike most of the UL37x1 mutants generated. In complementary approaches, we independently found that pUL37x1 appears to be posttranslationally modified, as detected by the presence of multiple discrete species differing slightly in molecular mass during HCMV infection (24, 25). Abrogation of the predicted phosphorylation sites resulted in faster migration of the mutant proteins. Replacement with ²¹SY that cannot be posttranslationally modified improved mitochondrial import. Consistent with the SY mutant phenotypes, we observed that the smaller, less-modified pUL37x1 is relatively more abundant in mitochondria than the higher mass species. Although we have not directly detected phosphorylation of this site, we have consistent results establishing that this sequence is likely posttranslationally modified and affects pUL37x1 dual trafficking.

Ablation of UL37x1 MTS β PRD enhanced its mitochondrial importation. PRDs have been documented to play roles in trafficking through the secretory apparatus (38, 44). The UL37x1 MTS β PRD may be involved in retaining a subset of the protein to the secretory apparatus. Consistent with this possibility is the finding that a small fraction of pUL37x1 traffics through the secretory apparatus as is evidenced by partial colocalization with a *cis*-Golgi marker (11). However, in the present study we did not find a detectable fraction of pUL37x1 in the *trans*-Golgi network.

Together, our studies provide evidence that pUL37x1 targets mitochondrial import by a genetically distinguishable pathway from that of Tom20. The differences in sorting signals for pUL37x1 in contrast to Tom20 highlight its use of an alternative pathway or modification of the known mitochondrial pathway. Some mitochondrial precursors have been found to be imported by the TOM complex using pathways distinct from those followed by most mitochondrial precursors (3). Signal anchored OMM proteins, such as Tom20, are thought to be inserted into the OMM at the interface between the TOM core complex and the lipid phase of the membrane (3). Trafficking and sorting signals of pUL37x1 may have evolved to make use of high lipid environment of the MAM to ensure efficient trafficking of pUL37x1 to the OMM. In support of this, we detected pUL37x1 in the lipid-rich MAM subcompartment (9).

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