US2, a Human Cytomegalovirus-encoded Type I Membrane Protein, Contains a Non-cleavable Amino-terminal Signal Peptide*§

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The human cytomegalovirus US2 gene product targets major histocompatibility class I molecules for degradation in a proteasome-dependent fashion. Degradation requires interaction between the endoplasmic reticulum (ER) luminal domains of US2 and class I. While ER insertion of US2 is essential for US2 function, US2 lacks a cleavable signal peptide. Radiosequence analysis of glycosylated US2 confirms the presence of the NH2 terminus predicted on the basis of the amino acid sequence, with no evidence for processing by signal peptidase. Despite the absence of cleavage, the US2 NH2-terminal segment constitutes its signal peptide and is sufficient to drive ER translocation of chimeric reporter proteins, again without further cleavage. The putative US2 signal peptide c-region is responsible for the absence of cleavage, despite the presence of a suitable −3, −1 amino acid motif for signal peptidase recognition. In addition, the US2 signal peptide affects the early processing events of the nascent polypeptide, altering the efficiency of ER insertion and subsequent N-linked glycosylation. To our knowledge, US2 is the first example of a membrane protein that does not contain a cleavable signal peptide, yet otherwise behaves like a type I membrane glycoprotein.

Signal peptides dictate ER insertion of integral membrane proteins and proteins destined for the secretory pathway in either co- or post-translational fashion (1–3). Signal peptides are recognized by the signal recognition particle, which directs the nascent chain and ribosome to the signal recognition particle receptor embedded within ER membrane (4–9). Upon docking with the ER membrane, many proteins are co-translationally inserted into the ER lumen via a ribosome-translocon channel that includes the heterotrimeric Sec61p complex and the translocating chain-associating membrane protein (10, 11). Once the translocon has been engaged, NH2-terminal signal peptides are cleaved from the nascent chain by signal peptidase, a serine endopeptidase present near the translocon in the ER membrane (12). Signal-anchor sequences also interact transiently with the ER translocation complex, but are not cleaved. Instead, signal-anchor sequences move laterally out of the translocon to become permanent membrane anchors (13, 14).

Signal peptides are composed of three distinct regions, n-, h-, and c-regions. The n-region consists of polar residues, often with a net positive charge, at the NH2 terminus (15). The h-region is the central hydrophobic 7–15 residue helical core that can insert into the ER membrane (13, 16). Finally, the carboxyl-terminal c-region has more polar character and contains the signal peptidase cleavage site (15). Signal peptidase recognizes a pattern that includes amino acids with small side chains in the −1 and −3 positions relative to the cleavage site, present in the c-region in extended conformation near the head groups of the inner leaflet of the ER membrane (2, 14, 16, 17).

The human cytomegalovirus (HCMV) encodes two ER-resident membrane glycoproteins, US2 and US11, that each destabilize major histocompatibility class I molecules (18). The 199-residue US2 glycoprotein contains an ER-luminal portion, a predicted single transmembrane domain, and a short cytoplasmic tail (Fig. 1). US2 recognizes class I molecules via an immunoglobulin-like fold that attaches to the class I ER-luminal domain (19) and US2 subsequently targets class I heavy chains for dislocation from the ER to the cytosol, where they are rapidly degraded by the proteasome (20). The mechanism of ER dislocation and degradation is not clearly understood.

HCMV US2 and US11 exhibit unusual characteristics concerning their ER processing events. The US11 signal peptide is cleaved from the nascent chain in delayed fashion (21). Both the signal peptide n-region and the COOH-terminal membrane anchor influence processing of the US11 signal peptide (21). Here we report several highly unusual properties of US2 ER translocation. We show that US2 behaves like a type I membrane protein that contains a non-cleavable signal sequence at its NH2 terminus.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Antibodies—**U373-MG astrocytoma cells stably transfected with US21–199 cDNA (22) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, 5% calf serum, and 0.375 μg/ml puromycin. All variants of US2 were stably transfected into U373 cells and maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, 5% calf serum, and 0.5 mg/ml genetin (Invitrogen, Frederick, MD). The human embryonic kidney cell line (HEK-293) was maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, 5% calf serum. Polyclonal antiserum generated against US2 and class I heavy chains were generated as described (23). The anti-β2m serum was generated as described (21).

**DNA Constructs and Transfection—**The cDNA of full-length US2 (amino acids 1–199) was cloned from the AD169 HCMV genome into the eukaryotic expression vector pCDNA 3.1 (Invitrogen, Carlsbad, CA). US2 truncation mutants, US220–199 (aa 20–199) and US21–160 (aa 1–160) were subcloned from US2 (pCDNA 3.1) by PCR and inserted into
pcDNA3.1. Major histocompatibility class I (HLA-A2) heavy chain lacking a signal sequence (HC25-365) was subcloned from HLA-A2 (pcDNA3.1) by PCR and inserted into pcDNA3.1. The H-2K^b/US2 chimeras, K^b-199/US2_199 (H-2K^b (aa 1–5)/US2 (aa 7–199)) and K^b-190/US2_190 (H-2K^b (aa 1–16)/US2 (aa 17–190)), and US2/HLA-A2 chimeras US2/HIC25-365 (US2 (aa 1–20)/H2LA-A2 (aa 25–35)), US2/HC25-365 (US2 (aa 1–25)/H2LA-A2 (aa 25–35)), US2/HHC25-365 (US2 (aa 1–30)/H2LA-A2 (aa 25–35)), US2/HC25-365 (US2 (aa 1–35)/H2LA-A2 (aa 25–35)), US2_191/HC25-365 (US2 (aa 1–40)/H2LA-A2 (aa 25–365)), were generated by amplifying the appropriate fragment by PCR followed by ligation of two of the respective fragments. Using specific primers to the sense and antisense strand of the ligated product, the chimeric cDNAs were amplified by PCR, phosphorylated with T4 DNA kinase, and inserted into pcDNA 3.1. The H-2K^b/US2 chimeric mutant, K^b-199/US2_199 (H-2K^b (aa 1–21/US2 (aa 21–199)) was generated by amplifying the US2 cDNA that corresponds to aa 21–199 by PCR. This truncated US2 was ligated to the murine H2 class I heavy chain K^b signal sequence (HC 25-365), consistent with earlier proposals for the topology of US21. Lipid-mediated transient (HEK-293 cells) and stable (U373-MG cells) US2 expression systems and immunoprecipitation were performed as described (21).

Endoglycosidase H Digestion and Gel Electrophoresis—Digestion with endoglycosidase H (EndoH) (New England Biolabs, Beverly, MA) was performed on immunoprecipitated complexes according to the manufacturer’s instructions. Proteins were separated by SDS-PAGE and [35S]methionine-labeled proteins were visualized by fluorography/autoradiography (25).

In Vitro Transcription and Translation—In vitro transcription and translation of US2_199 were performed as described (26).

Subcellular Fractionation, Na_2CO_3 and Urea Treatment—The subcellular fractionation and Na_2CO_3 treatment were performed as previously described, respectively (21). Urea treatment of homogenates pre-treated with 4.5 M urea was used.

RESULTS

US2 Is a Membrane Glycoprotein—The nonpolar region present at the US2 COOH terminus likely constitutes a membrane anchor (Fig. 1A), consistent with earlier proposals for the topology of US2_199 (19, 20). To establish more directly the membrane topology of US21. Lipid-mediated transient (HEK-293 cells) and stable (U373-MG cells) US2 expression systems and immunoprecipitation were performed as described (21).

US2 Is Inefficiently Inserted into the ER—US2 polypeptides that differ by the presence of the single N-linked glycan are recovered from lysates of metabolically labeled US2_199 cells (Fig. 2B). To determine whether the non-glycosylated species arises as a deglycosylated degradation intermediate produced in the course of a dislocation reaction (20), pulse-chase experiments were performed on US2_199 cells in the presence and absence of the proteasome inhibitor, ZL_3VS (27). US2 molecules were recovered from cell lysates and analyzed by SDS-PAGE (Fig. 3A). The recovery of glycosylated and non-glycosylated US2 polypeptides decreases at the later chase times both in the presence or absence of ZL_3VS (Fig. 3A), but as expected, the degradation rate of non-glycosylated US2_199 is slower in the presence of proteasome inhibition (Fig. 3, compare lanes 1–4 and 5–8). We observed no evidence for a precursor-product.
relationship for glycosylated and non-glycosylated US2 polypeptides. We infer that the non-glycosylated US2 polypeptide does not arise as a degradation intermediate.

Non-glycosylated US2 could result either from incomplete ER insertion or inefficient glycosylation. To distinguish between these possibilities, the localization of non-glycosylated US2 was determined by subcellular fractionation. Metabolically labeled US2<sub>1-199</sub> cells were homogenized by mechanical disruption, followed by differential centrifugation (Fig. 3B). The 100,000 × g supernatant (S) contains cytosolic proteins, while the 100,000 × g pellet (P) contains the microsomal fraction. Glycosylated US2<sub>2</sub> is recovered exclusively from the 100,000 × g supernatant (S) (lanes 1 and 2) and from the 100,000 × g pellet (P) (lanes 3 and 6) using anti-US2 and anti-β<sub>2m</sub> sera and analyzed by SDS-PAGE (15%). B, US2<sub>1-199</sub> immunoprecipitates were treated with increasing concentrations of EndoH to reveal the number of N-linked glycans attached to US2. Glycosylated (US2<sup>+</sup>) and non-glycosylated (US2<sup>−</sup>) US2 polypeptides are indicated. CHO, carbohydrate.

US2 Lacks a Cleavable Signal Sequence—The SignalP computer algorithm (www.cbs.dtu.dk/services/SignalP/index.html) predicts a US2 NH<sub>2</sub>-terminal signal peptide with probable cleavage site between residues 20 and 21. However, most signal peptides exhibit greater hydrophobicity than that present at the US2 NH<sub>2</sub>-terminal (Fig. 4A). The 100,000 × g supernatant (S) (lanes 1 and 2) and 100,000 × g pellet (P) (lane 3) were recovered directly from cell lysates (lanes 1 and 4), from the 150,000 × g supernatant (S) (lanes 2 and 4) and from the 150,000 × g pelleted (P) (lanes 3 and 6) using anti-US2 and anti-β<sub>2m</sub> sera and analyzed by SDS-PAGE (12.5%). Glycosylated (US2<sup>+</sup>) and non-glycosylated (US2<sup>−</sup>) US2 polypeptides are indicated. B, US2 polypeptides were immunoprecipitated from non-fractionated US2<sub>1-199</sub> cells (lane 1), the 100,000 × g (100Kg) supernatant (S) (lane 2), the 100,000 × g (100Kg) pellet (P) (lane 4) of fractionated US2<sub>2</sub> cells with anti-US2 sera and analyzed by SDS-PAGE (12.5%). CHO, carbohydrate.

<sup>2</sup> B. E. Gewurz, H. L. Ploegh, and D. Tortorella, unpublished data.
**ER Insertion and Processing of HCMV US2**

**FIG. 4.** US2 lacks a cleavable signal sequence. A, US2 was recovered from stable U373 astrocytoma transfectants expressing full-length US2 (US2\_199) (lanes 1 and 2), a NH\_2-terminal deletion mutant that lacks the first 19 residues (US2\_160) (lanes 3 and 4), or a US2 chimera that contains the H2-K\_b cleavable signal sequence in place of the NH\_2-terminal 20 US2 residues (K\_b\_160/US2\_199) (lanes 5 and 6). Lysates from metabolically labeled transfectants were immunoprecipitated with anti-US2 sera and analyzed by SDS-PAGE (12.5%). Half of the immunoprecipitates were treated with EndoH as indicated. Glycosylated (+CHO) and non-glycosylated (−CHO) US2 polypeptides are indicated.

**FIG. 5.** The putative transmembrane domain of US2 is not required for ER insertion. A, CHO astrocytoma cell lines that express a US2 COOH-terminal deletion mutant (US2\_160) were metabolically labeled for 15 min and chased up to 30 min. The US2\_160 molecules were immunoprecipitated with anti-US2 sera and analyzed by SDS-PAGE (12.5%). Half of the immunoprecipitates were treated with EndoH (lanes 4–6). Glycosylated (+CHO) and non-glycosylated (−CHO) US2 polypeptides are indicated. CHO, carbohydrate.

**FIG. 5.** The putative transmembrane domain of US2 is not required for ER insertion. A, CHO astrocytoma cell lines that express a US2 COOH-terminal deletion mutant (US2\_160) were metabolically labeled for 15 min and chased up to 30 min. The US2\_160 molecules were immunoprecipitated with anti-US2 sera and analyzed by SDS-PAGE (12.5%). Half of the immunoprecipitates were treated with EndoH (lanes 4–6). Glycosylated (+CHO) and non-glycosylated (−CHO) US2 polypeptides are indicated. CHO, carbohydrate.
NH2 terminus (residues 1–20) and between residues 110 and 130, in addition to the putative transmembrane domain (Fig. 1). Can these hydrophobic domains act as additional membrane anchors? The crystal structure of the US2/class I complex shows that residues 110–130 comprise the F and G β-strands of the US2 Ig-like fold, indeed forming an important part of the class I binding surface (Fig. 6A) (19). Therefore, this hydrophobic segment resides within the ER lumen. Consistent with this data, an additional N-linked glycosylation sequon introduced at position 149 is efficiently utilized (supplemental Fig. 1). Since this US2 mutant acquires N-linked glycans at positions 68 and 149, the intervening segment must also be present in the ER. Together, these results demonstrate that US2 residues 110–130 do not traverse the membrane bilayer and are consistent with a type I topology for US2.

We next examined the cellular disposition of US21–160 to determine whether the uncleaved NH2-terminal signal peptide of US2 is a membrane anchor. US21–160, US21–199, and β2m were recovered from the 150,000 × g membrane pellet (P) and soluble (S) fractions of Na2CO3-treated homogenates of HEK-293-transfected cells (Fig. 6B). The membrane protein US21–199 (Fig. 6B, lanes 1–3) was recovered exclusively from the membrane fraction (P) of Na2CO3-treated homogenates (Fig. 6B, lane 3). In contrast, the soluble protein β2m (Fig. 6B, lanes 7–9) was recovered exclusively from the soluble fraction (S) of Na2CO3-treated homogenates (Fig. 6B, lane 8). In Na2CO3-treated homogenates, US21–160 is mostly recovered from the pellet fraction (P) (Fig. 6B, lane 6), while a small population of US21–160 molecules was recovered from the soluble fraction (S) (Fig. 6B, lane 5). Since protein-protein interactions can survive Na2CO3 treatment (28, 29) and could allow US2 to partition with the pellet fraction, US21–160 homogenates were treated with the weak denaturant 4.5 M urea (Fig. 6C) as an alternative to Na2CO3 treatment. Nearly all of the US21–160 molecules were recovered from the soluble (S) fraction upon urea treatment (Fig. 6C, lane 5). These results suggest that protein-protein interactions tether US21–160 to the ER membrane and that US21–160 is itself a soluble protein. Note that the membrane protein US21–199 (Fig. 6C, lanes 1–3) is not released even to the slightest extent into the soluble fraction upon urea treatment (Fig. 6C, lane 2).

Sublocalization of US2 Signal Peptide Properties—The Signal P sequence algorithm was used to predict the n-, h-, and c-regions of both the putative US2 and H-2Kb signal peptides. To determine which region of US2’s signal peptide is responsible for the observed lack of signal peptide cleavage, we replaced the predicted US2 n, n + h, or n + h + c regions with the corresponding regions of the H-2Kb signal peptide (Fig. 7A). US2 chimeras that contain the Kb n-region in place of the putative US2 n-region (Kb1–160/US21–199) are poorly inserted in the ER within these transfecants (Fig. 7B, lanes 3 and 4). Chimeras that contain the putative n + h-region of H-2Kb (Kb1–160/US21–199) are efficiently glycosylated (Fig. 7B, lanes 5 and 6). Despite efficient ER translocation and glycosylation, the hybrid Kb1–160/US21–199 signal peptide apparently remains uncleaved following ER insertion (Fig. 7B, lanes 5 and 6). In contrast, chimeras that contain the entire H-2Kb signal sequence in place of the US2 20 NH2-terminal residues (Kb1–21/US21–199) are efficiently inserted into the ER with concomitant signal peptide cleavage (Fig. 7B, lanes 7 and 8). For the Kb1–21/US21–199 chimera, its signal peptide appears to be removed, since this product migrates faster than US21–199 and other H-2Kb/US2 chimeras upon SDS-PAGE separation. Thus, elements within the putative US2 signal peptide c-region are responsible for the lack of signal peptide cleavage.

The US2 NH2 Terminus Serves as a Signal Peptide—To determine whether the US2 NH2 terminus can direct an exogenous membrane protein to the ER, we constructed chimeras comprised of US2 NH2-terminal peptides fused to a reporter type I membrane protein, the major histocompatibility class I (HLA-A2) heavy chain (HC), lacking its own signal peptide (Fig. 8). Since the length of the US2 signal peptide is not precisely known, we generated a series of chimeric proteins that contain either the NH2-terminal 20, 25, 30, 35, or 40 residues of US2 fused to the NH2-terminal end of the signal sequence-less class I heavy chain. US2/HC chimeras were transiently expressed in HEK-293 cells and ER translocation was assayed by the acquisition of the single N-linked glycan at position 86 of the class I heavy chain. Transfectants were
The terminal signal peptide is not removed upon translocation into the ER. The NH2-terminal 30 US2 residues function as a non-cleavable signal peptide, even when appended onto an unrelated type I membrane protein. Despite the hydrophobic nature of the non-cleavable US2 signal peptide, it does not appear to act as membrane anchor. A carboxyl-terminal truncation mutant that lacks its transmembrane domain, but retains its signal peptide (US21-199) remains in a soluble form upon urea extraction of microsomal membranes (Fig. 6C). Furthermore, the crystal structure of the US2-class I complex (19) makes it difficult to envision a US2-class I complex in which both the NH2- and COOH-terminal ends of US2 are anchored to the membrane bilayer (Fig. 6A). In addition, replacement of the US2 signal peptide with the cleavable murine class I H-2Kb signal peptide (Kb1-21/US21-199) (Fig. 4) does not affect the function of US2 (Supplemental Material Fig. 2). If the US2 signal peptide were tethered to the membrane, presumably the overall structure of US21-199 would be different and would likely preclude US2-induced destruction of class I molecules for the latter chimera.

There are several examples of secreted proteins whose signal peptides are not cleaved: fibroblast growth factor (FGF)-9, ovalbumin, plasminogen activator inhibitor-2 (plasminogen activator inhibitor-2), and the carp retinol-binding protein (RB). Reminiscent of US2, (FGF)-9 has a relatively weak hydrophobic NH2 terminus and the NH2-terminal 28 residues can direct ER translocation of a heterologous fusion protein (30). While the US2 NH2-terminal deletion mutant (US220-199) fails to translocate into the ER, FGF-9 deletion mutants that lack 22 NH2-terminal residues can still enter the ER (30). Furthermore, an internal sequence of considerable hydrophobicity is required for FGF-9 translocation (31). The FGF-9 internal hydrophobic region can function as a cleavable signal sequence when placed artificially at the NH2 terminus, and FGF-9 mutants that lack the NH2-terminal 90 residues are capable of ER insertion when translated in vitro in the presence of microsomes (31). In contrast, disruption of this central hydrophobic region prevents FGF-9 translocation. Taken together, FGF-9 appears to contain two separate regions that cooperate to allow ER insertion.

Similar to FGF-9, the SERPINs plasminogen activator inhibitor-2 and ovalbumin contain internal hydrophobic regions to direct ER insertion (32–34). The mildly hydrophobic NH2 termini of these serpins are not cleaved upon ER insertion. Plasminogen activator inhibitor-2 relies upon a bipartite signal sequence, composed of two internal hydrophobic domains near its NH2 terminus (32, 35). While signal recognition particle recognizes the second internal hydrophobic region (32), the first internal hydrophobic region of plasminogen activator inhibitor-2 is required for ER insertion. The first weakly hydrophobic region may allow recognition of the central hydrophobic region by the translocation machinery (32). In the case of RBP, the 17 NH2-terminal residues can direct ER insertion of a heterologous fusion protein, yet it is not known whether additional regions of RBP participate in ER insertion (36).

Elements within the US2 signal peptide itself are responsible for the lack of signal sequence cleavage. Chimeric molecules that possess portions of the murine H-2Kb class I heavy chain signal peptide in place of the corresponding US2 signal peptide regions highlight several properties of US2’s signal peptide (Fig. 3A). First, the chimeric proteins that possess the H-2Kb n-region instead of that of US2 (Kb1-21/US21-199) remain mostly cytosolic. These results could be explained by incorrect assignment of the US2 n-region by the SignalP algorithm. This possibility appears improbable. The role of the n-region in ER insertion is not fully understood, but the h-region has been implicated in directing the nascent chain to the ER membrane (1). It is possible that the n- and h-regions work together to
Alternatively, this subpopulation could represent US2 molecule suggests inefficient ER insertion for at least a fraction of US2. Non-glycosylated US2 is present both in cytosolic and microsomal and its glycosylation. Subcellular fractionation shows that non-glycosylated US2 is present in the cytosol prior to the chase, which suggests inefficient ER insertion for at least a fraction of US2. Alternatively, this subpopulation could represent US2 molecules that have docked with the ER membrane, but failed to translocate. Since US2 chimeras that contain the H-2K\(^\alpha\) signal peptide are translocated and glycosylated to a greater extent (Figs. 4 and 7), the US2 signal peptide appears to influence ER insertion and \(N\)-linked glycosylation.

Both US2 and US11, the HCMV proteins that target class I heavy chains for dislocation, contain signal sequences with unusual properties. The closely related US3 glycoprotein retains class I molecules within the ER and contains a signal peptide with the usual cleavage properties.\(^2\) It is intriguing to propose a role for the US2 and US11 signal peptides in ER dislocation of major histocompatibility class I. Do US2 and US11 signal peptides act to keep US2/US11 in close proximity to the processing of their signal sequences is remarkable. The structural and functional properties of the translocon, such as a translocon component, or perhaps the c-region possess secondary structure that impairs signal peptide cleavage activity.

The US2 NH\(_2\) terminus dictates the efficiency of US2 insertion and its glycosylation. Subcellular fractionation shows that non-glycosylated US2 is present both in cytosolic and microsomal fractions derived from US2 transfectants (Fig. 3). Non-glycosylated US2 is present in the cytosol prior to the chase, which suggests inefficient ER insertion for at least a fraction of US2. Alternatively, this subpopulation could represent US2 molecules that have docked with the ER membrane, but failed to translocate. Since US2 chimeras that contain the H-2K\(^\alpha\) signal peptide are translocated and glycosylated to a greater extent (Figs. 4 and 7), the US2 signal peptide appears to influence ER insertion and \(N\)-linked glycosylation.

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