Dependence of the localization and function of the human cytomegalovirus protein US6 on the transporter associated with antigen processing

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Human cytomegalovirus (HCMV) encodes a cohort of proteins that inhibit the MHC class I antigen-presentation pathway (Hewitt, 2003; Hewitt & Dugan, 2004). These include US6, an ER-localized type I integral membrane glycoprotein that inhibits peptide translocation by TAP (Ahn et al., 1997; Hengel et al., 1997; Lehner et al., 1997). US6 inhibits ATP binding by the cytosolic nucleotide-binding domain of the TAP1 subunit (Hewitt et al., 2001; Kyritsis et al., 2001), which is the site of the chaperone-dependent folding of MHC class I molecules (Antoniou et al., 2003; Peaper & Cresswell, 2008). The inhibition of ATP binding is an indirect conformational effect, as the ER-luminal domain of HCMV US6 is sufficient to inhibit ATP binding by the cytosolic nucleotide-binding domain of the TAP1 subunit (Hewitt et al., 2001; Kyritsis et al., 2001). Recently, we demonstrated that residues 89–108 of the US6 luminal domain are required for inhibition of TAP, whereas other parts of US6 bind to TAP and stabilize this inhibitory interaction (Dugan & Hewitt, 2008). Yet, whilst US6 binds to TAP, it is not known whether this interaction retains US6 in the ER or whether US6 can inhibit MHC class I expression by an additional, TAP-independent mechanism.

As US6 lacks classical ER-retention and retrieval motifs (Ahn et al., 1997), we investigated whether TAP is required for the ER localization of US6. The TAP-negative cell line T2 was transduced with a construct encoding US6 with a C-terminal FLAG epitope tag (US6FLAG) as described previously (Hewitt et al., 2001) to generate the cell line T2-US6FLAG. The localization of US6FLAG in these cells was probed with endoglycosidase H (Endo H). Endo H can cleave oligosaccharides from ER-resident N-linked glycoproteins, but the processing of N-linked glycans in the medial Golgi renders glycoproteins resistant to Endo H digestion. As a control, HeLa-M-US6FLAG cells, in which US6FLAG is expressed stably in the TAP-positive cervical carcinoma cell line HeLa-M (Hewitt et al., 2001), were also analysed. Cells were lysed in lysis buffer (Tris-buffered saline pH 7.4, 1% digitonin, 5 mM iodoacetamide and 1 mM PMSF). The cell lysates were digested with Endo H (New England Biolabs) and immunoblotted with the FLAG epitope-specific antibody M2 (Sigma). Endo H digestion of
US6FLAG expressed by HeLa-M and T2 cells resulted in an increase in electrophoretic mobility of the viral protein, consistent with the removal of the N-linked glycan and hence retention of US6 in the ER in both cell types (Fig. 1a). Due to their limited cytoplasm, it was difficult to corroborate the Endo H data with microscopy-based localization studies in T2 cells. We therefore used the TAP-negative small lung carcinoma cell line GLC2 (Reits et al., 2000). GLC2 cells were transiently transfected with plasmid pEGFP-N1-US6 (Dugan & Hewitt, 2008), which encodes a green fluorescent protein fusion of US6 (GFP–US6); 48 h post-transfection, the cells were stained with a calreticulin antibody (Calbiochem) and a Texas red-labelled secondary antibody (Jackson ImmunoResearch) as described previously (Dugan & Hewitt, 2008). Antibody and GFP fluorescence were visualized with an Olympus IX-70 inverted fluorescence microscope and the DeltaVision deconvolution system (Applied Precision). GFP–US6 co-localized with the ER-resident chaperone calreticulin in GLC2 cells (Fig. 1b). Together, these observations demonstrate that TAP is not required for the ER localization of US6.

Next, we investigated whether US6 utilizes a second, TAP-independent mechanism to inhibit the MHC class I pathway. Indeed, the localization of US6 in the ER, even in the absence of TAP, places the viral protein in an ideal location to exert such an effect. We therefore examined, in TAP-deficient T2-US6FLAG cells, whether US6 interacts with MHC class I molecules and/or any proteins involved in MHC class I assembly. T2 and T2-US6FLAG cells were lysed in lysis buffer and US6FLAG was immunoprecipitated with the M2 anti-FLAG epitope-tag antibody coupled to agarose beads (Sigma). As a control, US6FLAG was immunoprecipitated from HeLa-M-US6FLAG cells. Immunoblots of the immunoprecipitates were probed with the M2 antibody to detect US6FLAG, the TAP1-specific antibody R.RING4C (Oertmann et al., 1994), the tapasin-specific antibody R.gp48N (Sadasivan et al., 1996), the anti-MHC class I heavy chain antibody HC10 (Stam et al., 1986), an ERp57-specific antibody (kindly provided by Neil Bulleid, University of Manchester, UK) and antibodies specific for calreticulin and calnexin (Stressgen). When expressed in HeLa-M cells, US6FLAG co-immunoprecipitated with calnexin, TAP, tapasin, MHC class I heavy chains, calreticulin and ERp57. In contrast, only calnexin co-immunoprecipitated with US6FLAG from T2-US6FLAG cells (Fig. 2a). The co-precipitation of tapasin, MHC class I heavy chains, calreticulin and ERp57 with US6 expressed in HeLa-M cells is presumably due to the association of these proteins with TAP as part of the peptide-loading complex and not due to a direct interaction with US6. Nonetheless, in the absence of TAP, US6 interacts with calnexin, a chaperone involved in the early stages of MHC class I assembly (Antoniou et al., 2003; Jackson et al., 1994; Peaper & Cresswell, 2008).

We next examined whether US6 can block the cell-surface expression of MHC class I molecules in TAP-deficient cells. Whilst the majority of MHC class I alleles require TAP for cell-surface expression, HLA-A201 can be expressed on the surface of cells that lack TAP, as it can bind to signal sequence-derived peptides whose import into the ER is TAP-independent (Henderson et al., 1992; Wei & Cresswell, 1992). As HLA-A201 is expressed endogenously by TAP-negative T2 cells, this enabled us to ascertain whether US6 can inhibit the MHC class I pathway in a TAP-independent manner. Cell-surface HLA-A201 was detected by staining T2 and T2-US6FLAG cells with either the HLA-A2-specific antibody BB7.2 (Serotec) or an isotype control. Antibody binding was detected with a Cy5-labelled secondary antibody (Jackson ImmunoResearch) and antibody fluorescence was quantified by flow cytometry with a FACSCalibur instrument (BD Biosciences). US6FLAG did not cause any reduction in the cell-surface expression of HLA-A201 by T2 cells (Fig. 2b). As such, these data demonstrate that US6 is unable to inhibit the cell-surface expression of MHC class I molecules in the absence of TAP.

As US6 had no effect on the cell-surface expression of HLA-A201 by T2 cells, we next sought to determine whether US6 is also unable to inhibit the cell-surface expression of this MHC class I allele in cells that have the TAP transporter. We analysed the effect of US6 on cell-surface expression of HLA-A201 in the TAP-positive HeLa-M, Huh7 hepatoma and A431 epithelial carcinoma cell
lines with a transient transfection-based assay. Cells were transfected with pCDNA3.1(−)Pac-HLA-A2 (Hewitt et al., 2002), which encodes HLA-A201. Forty-eight hours post-transfection, HLA-A201 was detected by staining cells with the BB7.2 antibody and a Cy5-labelled secondary antibody; antibody fluorescence was quantified by flow cytometry. The co-transfection of the cell lines with constructs encoding GFP (pEGFP-N1; Clontech) and HLA-A201 resulted in a substantial increase in binding of the BB7.2 antibody to GFP-positive cells compared with cells transfected with GFP alone (Fig. 3a). This is consistent with the cell-surface expression of this transfected MHC allele. However, co-expression of GFP–US6 with HLA-A201 resulted in only a limited reduction in the cell-surface expression of this MHC class I molecule compared with that of cells in which HLA-A201 was co-transfected with GFP (Fig. 3a). These data demonstrate that, even in the
presence of TAP, US6 is relatively ineffective as an inhibitor of the cell-surface expression of HLA-A201.

To confirm that US6 was able to inhibit TAP in these cell lines, the experiments were repeated with HLA-B2705, an MHC class I allele that requires TAP for peptide binding and cell-surface expression (Daniel et al., 1998; Smith & Lutz, 1996). The co-transfection of GFP with pCR3-HLA-B2705, a construct that encodes HLA-B2705 (Dugan & Hewitt, 2008), resulted in a substantial increase in binding to GFP-positive HeLa-M and Huh7 cells by the antibody ME1 (Ellis et al., 1982) (a kind gift from Simon Powis, University of St Andrews, UK), which recognizes HLA-B2705 (Fig. 3b). Unfortunately, we were unable to detect expression of transfected HLA-B2705 in A431 cells, as they express an endogenous molecule recognized by the ME1 antibody (data not shown). Co-transfection of HeLa-M and Huh7 cells with GFP–US6 and HLA-B2705 resulted in a substantial reduction in cell-surface expression of this MHC class I molecule compared with that of cells co-transfected with HLA-B2705 and GFP (Fig. 3b). As such, these data corroborate our previous observations, in which US6 inhibited the cell-surface expression of HLA-B2705 (Dugan & Hewitt, 2008), and are in marked contrast to the limited reduction in the cell-surface expression of HLA-A201 induced by US6 in the same cells.

In summary, we demonstrate that the ER localization of US6 does not require interaction with TAP, but that the inhibition of MHC class I expression by US6 is TAP-dependent. How US6 is retained in the ER in TAP-negative cells is unclear, as the viral protein has no canonical ER-retention or retrieval motifs (Ahn et al., 1997), although the interaction with calnexin may contribute to the ER localization of US6. Yet, despite binding to calnexin, a chaperone involved in the folding of MHC class I molecules (Antoniou et al., 2003; Jackson et al., 1994; Peaper & Cresswell, 2008), US6 did not inhibit cell-surface expression of HLA-A201 in TAP-negative T2 cells. Likewise, US6 only had a marginal effect on the cell-surface expression of HLA-A201 in TAP-positive cell lines, whereas US6 was an efficient inhibitor of the cell-surface expression of HLA-B2705. This difference is presumably because HLA-A201 can circumvent the loss of TAP activity by binding signal sequence-derived peptides (Henderson et al., 1992; Wei & Cresswell, 1992), whereas HLA-B2705 is dependent on TAP for peptides (Daniel et al., 1998; Smith & Lutz, 1996). Due to the inability of US6 to mediate efficient inhibition of the cell-surface expression of the TAP-independent MHC class I molecule HLA-A201, these data are consistent with TAP being the sole target for US6 in the MHC class I pathway.

Although we demonstrated that US6 can induce only a marginal reduction of HLA-A201 expression in three different cell lines, our observations differ from those of Oosten et al. (2007), who found that US6 was able to cause a more substantial reduction of HLA-A2 expression in lymphoblastoid cell lines. This difference may reflect an increased abundance of signal sequence-derived peptides in HeLa-M, Huh7 and A431 cells compared with lymphoblastoid cell lines. Nonetheless, our data demonstrate that US6 is an ineffective inhibitor of HLA-A201 expression in a variety of different cell types. Furthermore, by only targeting TAP for inhibition, we predict that US6 will also be unable to prevent other MHC class I alleles from binding and presenting peptides that do not require TAP for ER import. This will have important implications for HCMV, as the virus cannot rely on TAP inhibition by US6 for comprehensive inhibition of the MHC class I pathway. This may explain why HCMV encodes multiple proteins that inhibit MHC class I expression, including US2 and US11, both of which target HLA-A2 for degradation (Gewurz et al., 2001; Schust et al., 1998). Indeed, by encoding a cohort of inhibitors with different mechanisms of action, HCMV will ensure that, irrespective of the host’s profile of MHC class I allele expression, infected cells evade CTL lysis.

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References


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