Immunology Letters 190 (2017) 233–239

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

The use of BirA-BAP system to study the effect of US2 and US11 on MHC class I heavy chain in cells

S. Gauthami[a], Deepak Kumar[a], K.S.R. SivaSai[b], Nagendra R. Hegde[a,*]

[a] Ella Foundation, Genome Valley, Turkapally, Shameerpet Mandal, Hyderabad, 500078, India
[b] Sreenidhi Institute of Science & Technology, Yamnampet, Ghatkesar, Hyderabad, 501301, India

ARTICLE INFO

Keywords:
Protein turn-over
BAP tagging
Biotinylation
MHC
HCMV

ABSTRACT

Biotinylation has been extensively used for antibody tagging, affinity-based purification, and in protein/DNA-protein interaction studies. Here we describe the use of biotinylation to study the turn-over of proteins in cells. We use the prokaryotic biotin ligase (BirA) to biotinylate the human leukocyte antigen (HLA)-A2 (A2) heavy chain (HC), which was engineered to contain a biotin acceptor peptide (BAP). Controlled availability of biotin in combination with visualization using streptavidin-conjugated peroxidase made it possible to detect biotinylated BAP-A2. Further, we exploited the effects of human cytomegalovirus (HCMV) unique short (US) proteins US2 and US11 on the turn-over of BAP-A2 HC. The full-length BAP-A2 HC and its mutants lacking either the cytosolic tail (tail-less) or both the transmembrane and cytosolic regions (soluble) were expressed via recombinant adenoviruses (rAd). The effect of US2, US11 and a control HCMV protein US9, also expressed via rAd, on each of the BAP- A2 forms was assessed. Experiments using this system showed that US2 and US11 cause proteasome-mediated degradation of full-length BAP-A2 HC but only US2 could cause degradation of tail-less BAP-A2. The results demonstrate that the technique of biotinylation can be used to study protein turn-over in cells.

1. Introduction

Understanding homeostatic mechanisms has far reaching implications for illuminating pathways of disease pathogenesis and points of interdiction. The process of protein synthesis, modification, trafficking and degradation are dynamic and necessary to maintain cell homeostasis. Protein homeostatic processes are also important for obligate intracellular parasites such as viruses, which manipulate several cellular processes to their advantage. Indeed, interaction of viral proteins with cellular components have been instrumental in revealing several cell biological events and associated mechanisms [1–3].

The human cytomegalovirus (HCMV) unique short (US) proteins US2 and US11 have contributed greatly to the understanding of protein fate, especially the pathways of cellular protein quality control. Both of these proteins have been shown to direct the major histocompatibility complex (MHC) Class I heavy chain (HC) to be retrotranslocated from the endoplasmic reticulum (ER) to the cytoplasm for subsequent degradation by proteasomes [4,5]. US2 associates with the newly synthesized HC in the ER, and drives the retrotranslocation of the HC into the cytosol by utilizing the cellular translocon, ubiquitination and dislocation machineries [6–8]. The HC is then deglycosylated by the cytosolic N-glycanase before it is targeted to the proteasome for degradation, and inhibition of proteasomal activity results in the accumulation of deglycosylated intermediates of HC in the cytosol [6,9]. US11 employs a similar strategy but appears to usurp different cellular machineries than that of US2 [1,10–13]. By contrast, the HCMV US9 has been shown not to affect the MHC Class I HC biosynthesis or turnover [14].

Biosynthesis, trafficking and turn-over of proteins is typically studied using radiolabeling methods, fluorescent tags or other tags/markers. Here we describe a method based on utilization of the biotinylation system to study protein turn-over using the prokaryotic biotin ligase (BirA) and the biotin acceptor peptide (BAP) tag. BirA biotinylates the protein of interest tagged with BAP. By restricting the availability of biotin, a limited pool of the BAP-tagged protein undergoes biotinylation and the turn-over of these tagged proteins can be studied.

Abbreviations: BAP, biotin acceptor peptide; BCCP, biotin carboxyl carrier protein; CRT, calreticulin; CT, cytosolic tail; cytBirA, BirA expressed in the cytosol; DOC, sodium deoxycholate; ECL, enhanced chemiluminescence; ER, endoplasmic reticulum; ERBirA, BirA expressed in the ER; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HC, heavy chain; HCMV, human cytomegalovirus; HEK, human embryonic kidney; HLA-A2, human leukocyte antigen-A2; hpi, hours post-infection; MEM, minimum essential medium; MHC, major histocompatibility complex; MOI, multiplicity of infection; NEM, *N*-ethylmaleimide; NP-40, nonidet P-40; PEI, polyethylenimine; PMSF, phenylmethylsulfonyl fluoride; SMP, skimmed milk powder; TMD, transmembrane domain; US, unique short

* Corresponding author. Present address: National Institute of Animal Biotechnology, Miyapur, Hyderabad, 500049, India.
E-mail address: hegde@niab.org.in (N.R. Hegde).

http://dx.doi.org/10.1016/j.imlet.2017.08.027
Received 15 June 2017; Received in revised form 6 August 2017; Accepted 25 August 2017
Available online 30 August 2017

We used the well-illustrated model of HCMV US2- and US11-mediated degradation of the MHC Class I protein, Human Leukocyte Antigen-A2 (A2), to study protein turn-over in cells stably expressing BirA. In the presence of US2 or US11, BAP-A2 was degraded, and the process of degradation was proteasome-dependent. Thus, the BirA/BAP system may be used to study the turn-over of specific, tagged proteins in cells.

2. Material and methods

2.1. Plasmids and DNA constructs

The gene coding for the prokaryotic biotin ligase (BirA) was amplified from the genomic DNA of *E. coli*, fused with an HA-tag at the 3′ end and cloned into pcHis, a modified pcDNA vector. The biotin ligase thus expressed was localized to the cytosol and hence was designated as the cytBirA. BirA was localized to the ER (designated ERBirA) by including a calreticulin (CRT) signal peptide at the N-terminal end, and an ER retention signal, KDEL, at the C-terminus in addition to the HA-tag [15]. The genes coding for the wild-type A2 (A2), the tail-less A2 (A2ΔCT) and A2 lacking both the transmembrane and cytosolic domains (A2ΔTMCT) were cloned into the adenovirus shuttle plasmid pDC515 (io) (Microbix Biosystems Inc., Canada) from an earlier plasmid (a kind gift from Prof. David Johnson, Oregon Health and Science University, Portland). A sequence coding for the 16 amino acid biotin acceptor peptide (BAP) tag with glycine-serine (GS) linkers on either ends (GSGLNDIFEAQKIEWHEGSG) [16] was included after the nucleotide sequences encoding the signal sequence of each protein. The BAP-A2ΔCT mutant has a deletion of all but four amino acids in the cytosolic domain, similar to the one described earlier [17], and BAP-A2ΔTMCT was truncated at the end of the sequence coding the luminal domain. The genes coding for HCMV US proteins US2, US9 and US11 were also cloned into the plasmid pDC515 (io).

2.2. Cells

The human embryonic kidney cells, HEK-293 and 293IQ (Microbix Biosystems Inc., Canada), were maintained in minimum essential medium (MEM) (Gibco, USA). The ERBirA and cytBirA cells were generated by stably transfecting the HEK-293 cells with the pcHis-ERBirA or the pcHis-cytBirA plasmids respectively, using polyethylenimine (PEI) (Sigma-Aldrich, USA). The cells were re-plated 48 h post-transfection under histidinol selection. Stable transfectants were maintained in Dulbecco's MEM lacking histidine (USBiological, USA). All media were supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and penicillin-streptomycin (Gibco, USA). For propagating and maintaining ERBirA and cytBirA cells, the medium was also supplemented with 0.3 mM histidinol (Sigma-Aldrich, USA).

2.3. Antibodies and reagents

The monoclonal antibodies against an epitope of influenza virus hemagglutinin (HA-tag) and glyceraldehyde-3- phosphate dehydrogenase (GAPDH) were obtained from Sigma-Aldrich, USA. Antibody against calnexin was obtained from Cell Signaling Technology, USA. The antibodies W6/32 and BB7.2 were a kind gift from Prof. David Johnson (Oregon Health and Science University, Portland). The antibody W6/32 recognizes assembled MHC Class I HLA-A, -B, and -C molecules [18] and BB7.2 is a conformation-dependent antibody specific for HLA-A2 [19]. Horse radish peroxidase (HRPO)-conjugated secondary antibodies (anti-mouse, anti-rabbit) as well as streptavidin were obtained from Sigma-Aldrich, USA.

2.4. Recombinant adenovirus (rAd) generation

The AdMax™ Hi-IQ system (Microbix Biosystems Inc., Canada) was used to generate the various rAd viruses. Briefly, the gene of interest was cloned into the shuttle plasmid pDC515 (io) and transfected along with the genomic plasmid, pBHGfrtΔE1, E3FLP into 293IQ cells. A flippase-mediated site-specific recombination between the plasmids results in the generation of rAd vectors expressing the genes of interest. Using this system, the genes coding for BAP-A2WT, BAP-A2ΔCT, BAP-A2ΔTMCT, US2, US9 and US11 were expressed via rAd vectors. Tissue culture infective dose-50 ($TCID_{50}$) of the rAd viruses was determined using the 293IQ cells.

2.5. Cell fractionation

Fractionation of ERBirA and cytBirA cells was performed as described previously [20]. Briefly, 10^7 cells were trypsinized and washed twice with ice-cold phosphate buffered saline, pH 7.2 (PBS). Cell pellets were lysed in 0.02% digitonin (Calbiochem, USA) in 1 mL of PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, USA) and 1X protease inhibitor cocktail (Sigma-Aldrich, USA), and incubated on ice for 20 min. The lysates were then centrifuged at 18,000*g* at 4 °C for 20 min. The supernatant (cytosolic fraction) was collected and the pellet (membrane fraction) was resuspended in 1 mL of PBS containing 1% Nonidet P-40 (NP-40) (USBiological, USA) supplemented with protease inhibitors.

2.6. Infection and pulse-chase

The ERBirA and cytBirA cells were infected with a multiplicity of infection (MOI) of 5 (i.e., 5 $TCID_{50}$/cell) of the indicated rAd viruses. Eighteen hours post infection (hpi), the cells were pulsed with 50 μM biotin (Sigma-Aldrich, USA) for 2 min. Excess biotin was removed by washing the cells twice with 1X PBS and the biotinylated proteins were chased for the indicated times. Proteasome activity was inhibited by using 2 μM epoxomicin (Peptide Institute, Japan) for 2 h prior to pulse as well as throughout the chase period. Dialyzed FBS (depleted of biotin) was used during infection and pulse-chase.

For studies involving the evaluation of the turn-over of A2 and its mutant forms, the ERBirA cells were co-infected with the rAdBAP-A2 or rAd expressing mutant BAP-A2 and the rAdUS2, rAdUS11 or rAdUS9 viruses, followed by the same procedures as described above.

2.7. Immunoprecipitation, SDS-PAGE and western blotting

Following biotin pulse and chase, the cells were lysed in 50 mM Tris, 100 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate (DOC) (USBiological, USA), 1 mM PMSF, 1X protease inhibitor cocktail, and 10 mM *N*-ethyl maleimide (NEM) (Sigma-Aldrich, USA). The lysates were frozen at −80 °C until further use.

The cell lysates were thawed on ice, subjected to centrifugation at 15,600*g* for 20 min at 4 °C, and the supernatant was collected. For immunoprecipitation, the lysates were incubated with the appropriate antibodies for 1 h at 4 °C. The antigen-antibody complexes were captured by adding protein A-agarose beads (Invitrogen, USA) and incubating at 4 °C for 1 h with continuous mixing. Separately, the lysates were subjected to immunoprecipitation with streptavidin-agarose beads, and incubated at 4 °C for 1 h with continuous mixing. Then, the protein A or the streptavidin beads were washed 4–5 times by resuspending in a buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1% NP-40, 0.5% DOC, 1 mM PMSF and 1X protease inhibitor cocktail followed by brief centrifugation at 4 °C. The protein A-agarose beads were boiled in SDS-PAGE loading buffer to elute the precipitated proteins. The procedure for elution of the biotinylated proteins bound to streptavidin beads was adapted as described previously [21]. Briefly, the beads were resuspended in 1X PBS containing 8 M urea, 2% SDS and 3 mM biotin and incubated at room temperature for 15 min. This was followed by boiling the samples for 10 min at 95 °C and another 5 min after addition of the SDS-PAGE loading buffer.

All samples were subjected to SDS-PAGE and blotted on to

polyvinylidene fluoride (PVDF) membrane (Millipore, USA). Fish gelatin blocking reagent (Amresco, USA) in PBS containing 0.1% Tween-20 was used for blots to be probed with streptavidin-conjugated HRPO, which was also diluted in 1X PBS containing 0.1% Tween-20. For all other antibody treatments, 5% skimmed milk powder (SMP) in PBS was used as a blocking agent and the antibodies were diluted in 3% SMP in PBS. The blots were analyzed by probing with appropriate antibodies, and signals were detected using the enhanced chemiluminescence (ECL) kit (Pierce, USA).

3. Results and discussion

Both *in vitro* and *in vivo* biotinylation methods have found diverse applications in the field of protein and cell biology [22,23]. Our unsuccessful attempt with a commercially available biotinylation kit led us to modify the highly sensitive prokaryotic system described by Petris and co-workers [15] to assess the turn-over of tagged proteins in a cell. We established the system by expressing the BirA protein stably in HEK-293 cells, and by tagging the MHC Class I protein HLA-A2 with the BAP sequence. Since our aim was to study the turn-over of proteins, we used the HCMV US proteins, US2 and US11, which cause accelerated turn-over of Class I proteins by exploiting the cellular pathways of protein quality control.

The BirA-BAP system is a prokaryotic system involved in post-translational biotinylation of proteins. The BirA protein has a dual function of biotin ligase and that of the repressor of the *BirA* gene. Biotinylation of proteins is mediated by attachment of biotin to BirA, resulting in formation of a holoBirA complex. The biotin carboxyl carrier domain (BCCP) of acetyl CoA carboxylase accepts the biotin from holoBirA [24], which recognizes the BAP tag and covalently attaches the biotin molecule to the lysine residue in the tag. Biotinylation has been utilized in purification of proteins (owing to the high affinity of biotin for streptavidin), to tag antibodies [25] and to generate tagged recombinant viruses [26]. We describe the use of the biotinylation system to study turn-over of proteins in cells.

3.1. Construction of ERBirA, cytBirA and BAP-tagged A2

Based on the cellular compartment to be targeted to, the *BirA* gene was cloned with the HA-tag alone for expression in the cytosol, or with a CRT signal sequence at the N-terminus and the HA-tag and ER retention sequence, KDEL, at the C-terminus for localizing it to the ER [15]. The compartmentalization of BirA allows biotinylation of BAP-tagged proteins in specific cellular locations. For this study, we tagged the human MHC Class I molecule A2 and its tail-less and soluble mutant forms. The BirA and the BAP-tagged A2 constructs are shown in Fig. 1A and B, respectively.

3.2. Establishment and characterization of stable cell lines expressing BirA

The HEK-293 cells were transfected with pcHis-cytBirA or pcHis-ERBirA, and stable clones were derived under histidinol selection. BirA expression was confirmed by western blotting using anti-HA antibody (data not shown) and clones expressing maximum protein in the cytosol or the ER, respectively, were used for further experiments.

To determine the compartmentalization of the BirA protein expressed in ERBirA and cytBirA cell lines, equal cell numbers (10^7 cells) of each were fractionated into cytosolic and membrane fractions, and probed with various antibodies. ERBirA was observed exclusively in the membrane fraction (Fig. 2A, lane 1), which is marked by the detection of ER-resident protein calnexin (Fig. 2B, lanes 1 and 3), whereas cytBirA was localized to the cytosolic compartment (Fig. 2A, lane 4). GAPDH partitioned predominantly to the cytosolic fraction; however, a fraction of it was also associated with the membrane (Fig. 2C, compare lanes 1 and 3 with lanes 2 and 4). The detection of BirA in the membrane or cytosolic fraction was indicative of its localization to the specific cellular compartment.

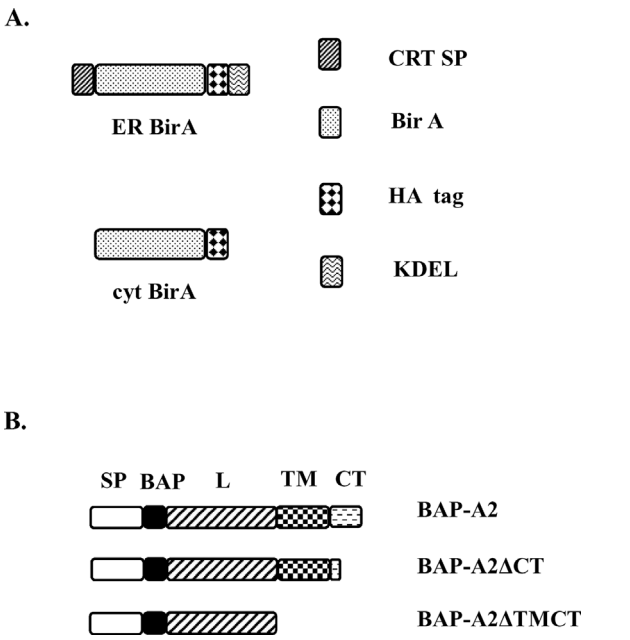

Fig. 1. Components of the BirA-BAP system. A. BirA was targeted to the ER by including an N-terminal calreticulin (CRT) signal peptide (SP) and a C-terminal KDEL sequence. BirA destined to be expressed in the cytosol was cloned as such. A C-terminal HA tag was included in both ERBirA and cytBirA for immunodetection. B. An N-terminal BAP-tag was included between the native SP and the protein coding region of A2, A2 lacking the cytosolic tail (A2ΔCT) and A2 lacking both transmembrane and cytosolic regions (A2ΔTMCT). L denotes the luminal domain of the protein.

3.3. Does BAP-A2 attain a proper conformation?

It was possible that the addition of a BAP-tag and subsequent ligation of biotin could affect the folding the A2. To test this, we infected ERBirA cells with rAdBAP-A2, and immunoprecipitated BAP-A2 by using conformation-dependent antibodies, BB7.2 and W6/32, which recognize a region within the α2 domain of HLA-A2 and the assembled HLA-A, −B or −C molecules, respectively. The immunoprecipitated species were then detected by blotting with streptavidin-HRPO. Biotinylated BAP-A2 was recognized by both W6/32 and BB7.2 (Fig. 3, lanes 2 and 3). Parallely, the biotinylated BAP-A2 was pulled down using streptavidin agarose beads. The detection of a band corresponding to the protein precipitated by W6/32 and BB7.2 indicated the presence of biotinylated BAP-A2 (Fig. 3, lane 4). These immunoprecipitation and blotting experiments showed that the inclusion of the BAP-tag at the N-terminus of A2 did not alter its conformation, and that biotinylated BAP-A2 attained native structure.

3.4. The BirA-BAP system can be used to study US2-mediated MHC protein turn-over

The wealth of information available for US2- and US11-mediated HC degradation makes this system an excellent model to study protein turn-over. To assess if the BirA protein is functional in the compartment where it is expressed, and to evaluate the effects of US2 and US11 on A2 HC using the BirA-BAP system, the ERBirA and cytBirA cells were co-infected with rAdBAP-A2 and rAdUS2 in the presence or absence of the proteasomal inhibitor epoxomicin. In the ERBirA cells, BAP-A2 was predominantly observed in its glycosylated form in both epoxomicin treated and untreated cells in the absence of US2 (Fig. 4, lanes 1- 4). Upon infection with rAdUS2, deglycosylated BAP-A2 was rescued in cells treated with epoxomicin (Fig. 4, lanes 7 and 8). The deglycosylated BAP-A2 was not detected in rAdUS2-infected cells treated with the

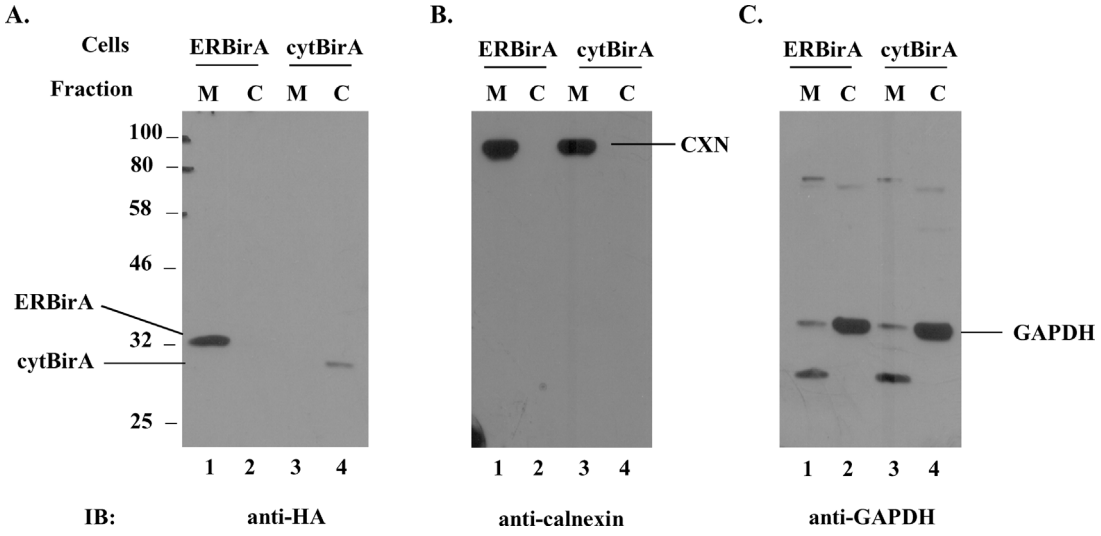

Fig. 2. ERBirA and cytBirA are localized to their respective cellular compartments. ERBirA or cytBirA cells (10^{7}) were lysed with 0.02% digitonin, and membrane (denoted M) and cytosolic (denoted C) fractions were subjected to immunoblotting for HA (panel A), calnexin (CXN, panel B) or GAPDH (panel C) with the respective antibodies. IB, immunoblot.

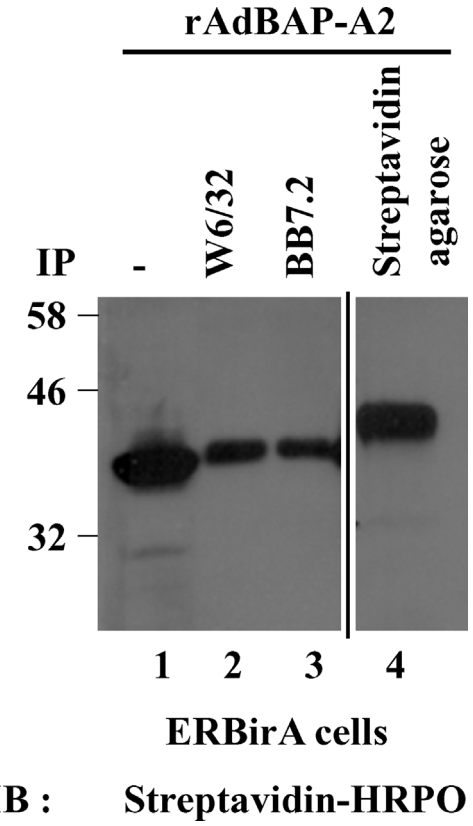

Fig. 3. BAP-tagged A2 attains native conformation. ERBirA cells were infected with rAdBAP-A2. The cells were harvested 18 h later, biotinylated for 10 min and then lysed in 1% NP-40 and 0.5% DOC. Lysates were analyzed directly (lane 1) or immunoprecipitated using the antibodies W6/32 or BB7.2 (lanes 2 and 3, respectively), or streptavidin-agarose (lane 4). Proteins eluted from beads were detected using streptavidin-HRPO. The panels separated by the line are from the same blot from which the intervening lanes containing duplicate samples were removed. IP, immunoprecipitation.

vehicle, dimethyl sulfoxide (DMSO) (Fig. 4, lanes 5 and 6), suggesting dislocation and eventual degradation of BAP-A2 in the absence of epoxomicin. In cytBirA cells, the glycosylated form of BAP-A2 was comparatively less biotinylated (Fig. 4, lanes 9 and 10) since BirA was localized to the cytosolic compartment. A faint signal corresponding to the size of deglycosylated BAP-A2 was observed in cells treated with epoxomicin, and could possibly indicate the normal turn-over of the protein (Fig. 4, lanes 11 and 12). The noteworthy increase in the signal of BAP-A2 in rAdUS2-infected cells, and more importantly the increased amount of the deglycosylated intermediate of BAP-A2 (Fig. 4 lanes 15–16) was suggestive of BAP-A2 dislocation and its subsequent biotinylation in the cytosolic compartment. Our results from cell fractionation experiments and the expression of BAP-A2 in ERBirA and cytBirA cells demonstrate that BirA was localized to and functional in the targeted compartments, and that the process of dislocation and degradation could be studied using this system.

In addition, BAP-A2 was subjected to $EndoH_f$ treatment to ascertain its glycosylation status. We observed that biotinylated BAP-A2 was susceptible to $EndoH_f$, indicative of its translocation into the ER. Biotinylation did not affect the ability of US2 or US11 to target BAP-A2 to proteasome for degradation. However, biotinylated BAP-A2 remained sensitive to $EndoH_f$ for up to 180 min of chase (data not shown), suggesting delayed maturation. This was similar to earlier observations of impeded movement of A2 when tagged with enhanced green fluorescent protein (eGFP), dihydrofolate reductase (DHFR), hemagglutinin epitope (HA) or tandem affinity purification (TAP) tag [27–30]. The eGFP- or TAP-tagged A2 was found to be reactive to the W6/32 antibody, but degraded with delayed kinetics in comparison to endogenous HC [27,30]. On the other hand, the DHFR-tagged HC did not attain resistance to $EndoH_f$ even up to 180 min of chase, and exhibited slower dislocation in US11-expressing cells when compared to cells expressing US2 [28]. Nevertheless, these studies using tagged proteins have been crucial in elucidating some aspects of ER to cytosol dislocation of A2.

3.5. *Effect of US2 and US11 on wild-type A2*

The HCMV US2 and US11 proteins target the HC for degradation via the proteasomes [4,12]. To explore the possibility of using the BirA-BAP system to study this, the effect of HCMV US2, US11 or US9 on BAP-A2 was assessed in ERBirA cells. In rAdUS2-infected cells, the deglycosylated protein was observed in DMSO-treated cells and was rescued in the presence of the proteasome inhibitor (Fig. 5, compare lanes 1 and 2 with lanes 3 and 4). In case of rAdUS11-infected cells, deglycosylated protein was observed only when cells were treated with epoxomicin, indicative of its degradation in the absence of the inhibitor (Fig. 5, compare lanes 5 and 6 with lanes 7 and 8). The dislocation or accumulation of the deglycosylated protein was not as evident in rAdUS9-infected cells and we presume it to be the normal turn-over of BAP-A2

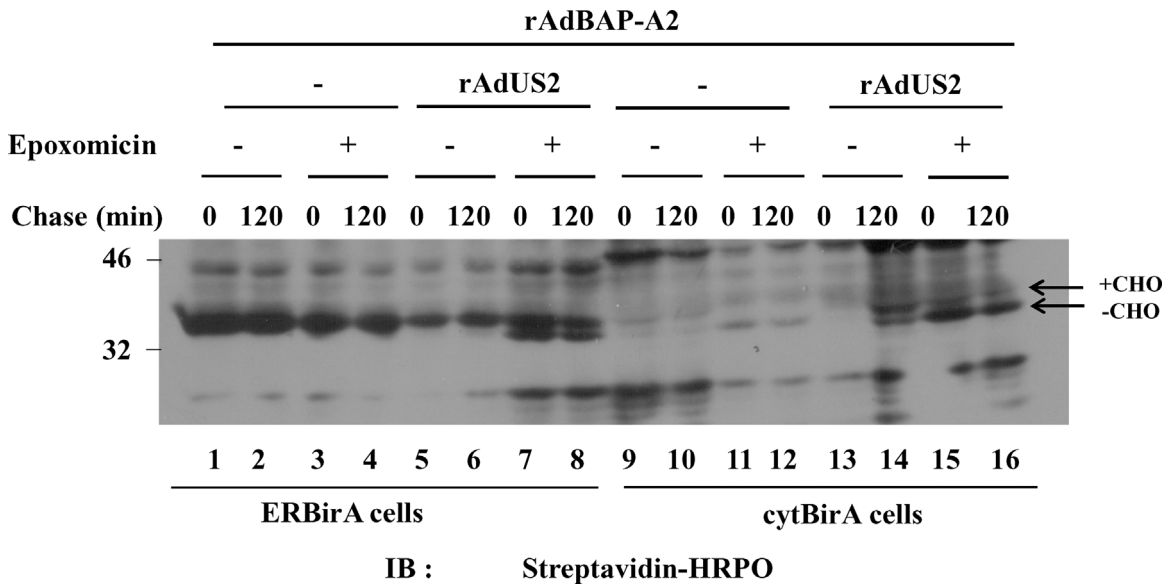

Fig. 4. ERBirA and cytBirA are functional in their targeted cellular compartments. ERBirA or cytBirA cells were infected with rAdBAP-A2 or in combination with rAdUS2. The infected cells were harvested and treated with 2 μM epoxomicin or vehicle control for 2 h. The cells were then pulsed with 50 μM biotin for 2 min, and chased for up to 120 min in the presence or absence of epoxomicin. The lysates were blotted with streptavidin-HRPO. The glycosylated (+CHO) and deglycosylated (−CHO) BAP-A2 are indicated.

(Fig. 5, lanes 9- 12). Our results support the fact that US2 and US11 mediate the dislocation of the HC from the ER to the cytosol and target it for proteasomal degradation, whereas US9 does not affect the HC turn-over. The effect of US2 and US11 on HC has been reported earlier [4,6,12], and we were able to replicate the same using the BirA-BAP system.

3.6. The differential effects of US2 and US11 on mutant forms of A2

The effect of US2 on A2 lacking the cytosolic tail (A2ΔCT) has been debatable. While it was earlier reported that the deletion of the CT made A2 impervious to the effects of US2 [17], a later study suggested that the CT deletion mutant remained a target for US2-induced dislocation and degradation [31]. We therefore generated a tail-less mutant of BAP-A2, BAP-A2ΔCT, and assessed the effect of US proteins on it using the BirA-BAP system. Lysates obtained post-infection with rAdBAP-A2ΔCT and rAdUS2, rAdUS9 or rAdUS11 were analyzed by immunoblot assay. Upon inhibition of the proteasomal activity, we observed that BAP-A2ΔCT was readily dislocated, with the appearance of the deglycosylated intermediate in the presence of US2. This was evident as visualized by the loss of deglycosylated protein in DMSO-treated cells and rescue of the same in cells treated with epoxomicin (Fig. 6, compare lanes 1 and 2 with 3 and 4). On the other hand, the effect of US11 on the mutant was similar to that reported earlier [17,32] i.e., it failed to cause dislocation of A2ΔCT (Fig. 6, compare lanes 5 and 6 with 7 and 8). US9 too did not affect the turn-over of the mutant (Fig. 6, compare lanes 9 and 10 with 11 and 12).

We consistently observed the deglycosylated intermediate in the absence of epoxomicin as well (data not shown). This could possibly suggest a higher turn-over of the mutant in the presence of US2. It is also possible that the higher sensitivity of the biotinylation system enabled us to detect the deglycosylated protein in the absence of the

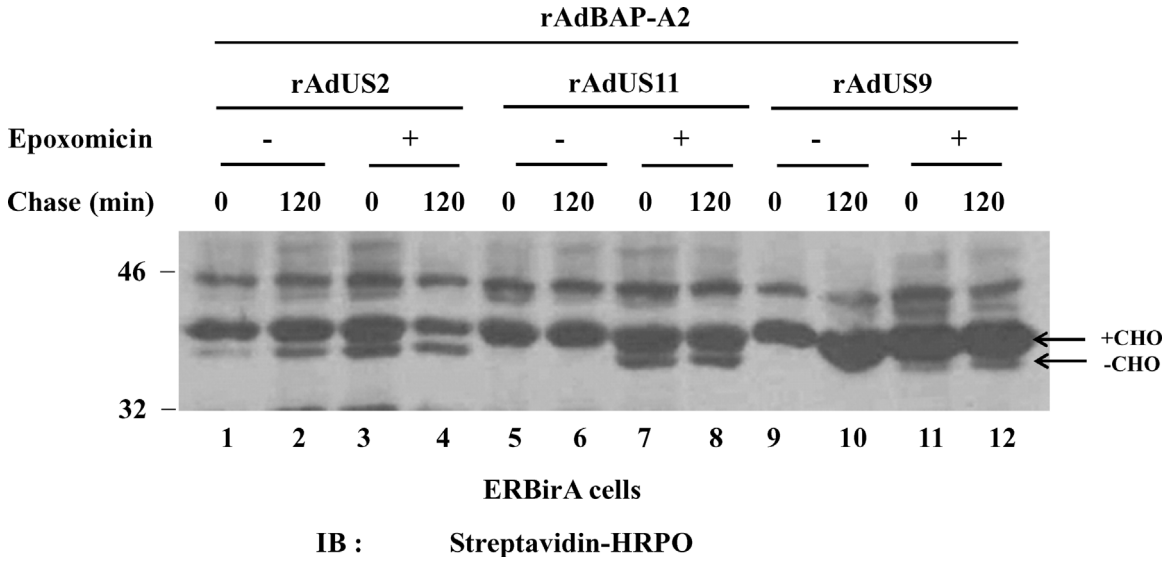

Fig. 5. BAP-A2 is targeted for proteasomal degradation by US2 and US11. ERBirA cells were infected with rAdBAP-A2 in combination with rAdUS2, rAdUS9 or rAdUS11, and biotin pulse-chase was performed as in Fig. 4. The lysates were probed with streptavidin-HRPO to detect biotinylated BAP-A2. The glycosylated (+CHO) and deglycosylated (−CHO) BAP-A2 are indicated.

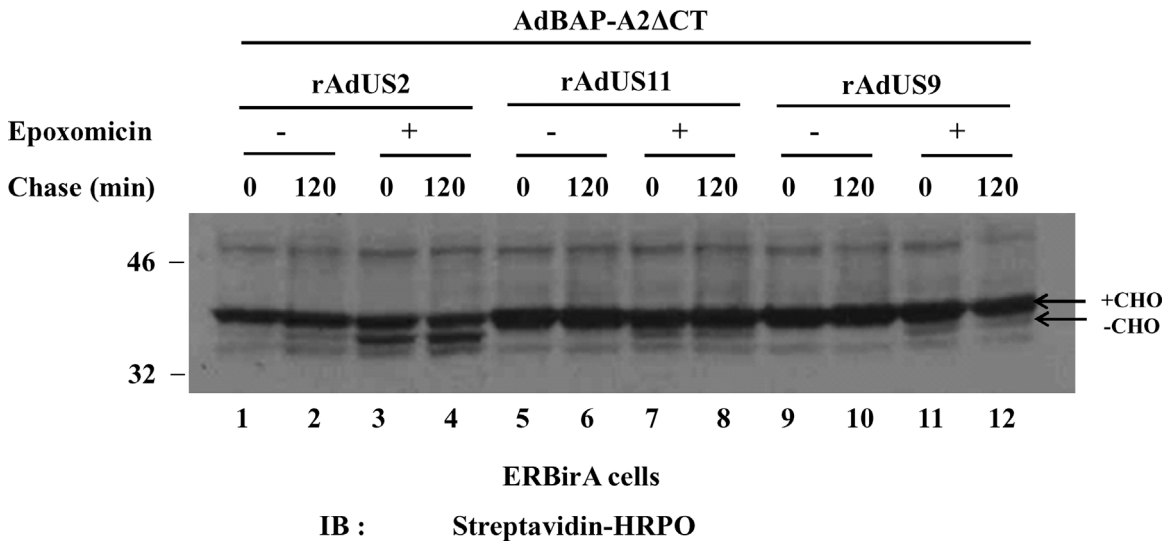

Fig. 6. BAP-A2ΔCT is susceptible to proteasome-mediated degradation by US2 but not US11. ER BirA cells were co-infected with rAdBAP-A2ΔCT and rAdUS2, rAdUS9 or rAdUS11. Biotinylation assay and immunoblotting were performed as described in Fig. 5. The glycosylated (+CHO) and deglycosylated (−CHO) BAP-A2 are indicated.

inhibitor. Thus, our study supports the observations of Barel et al. [31] which showed that A2 lacking the cytosolic tail was degraded in a proteasome-dependent manner by US2.

The effect of US proteins on BAP-A2ΔTMCT was also studied. The expression levels of BAP-A2ΔTMCT did not differ among rAdUS2-, rAdUS11- or rAdUS9-infected cells, thus indicating its failure to be targeted for degradation by US2 or US11 (Fig. 7). The inability of US2 to degrade BAP-A2ΔTMCT is probably not due to the lack of interaction between the proteins, since a bacterially expressed soluble A2 and US2 lacking the predicted signal peptide and also the TMD have been shown to be associated *in vitro* [33]. This supports the argument for the requirement of critical residues of the TM domain of A2 for the recruitment of membrane-associated dislocation factors by US2 [8].

3.7. Summary

To establish the *in cyto* biotinylation system, we generated 293 cells stably expressing bacterial BirA. The BAP-tagged protein HLA-A2 expressed via a rAd virus was stable and attained native conformation as assessed by immunoprecipitation with epitope-specific antibodies. Further, the BAP-tagged A2 was susceptible to the effects of both US2 and US11 as observed by the degradation or rescue of the deglycosylated intermediate in the absence or presence, respectively, of the proteasomal inhibitor. The cytosolic domain truncation mutant was also susceptible to the effect of US2 but not US11. The A2 mutant lacking both the cytosolic and TM domains remained unaffected by both US2 and US11.

4. Conclusion

The effect of US proteins on MHC Class I HLA-A2 using the BirA-BAP system demonstrates that the system is simple and yet highly sensitive to study turn-over of proteins in cells. We believe that the BirA-BAP system could be further modified in combination with the use of protein synthesis inhibitors to study the turn-over of nascent proteins. The sensitivity of the biotinylation system combined with the ease of

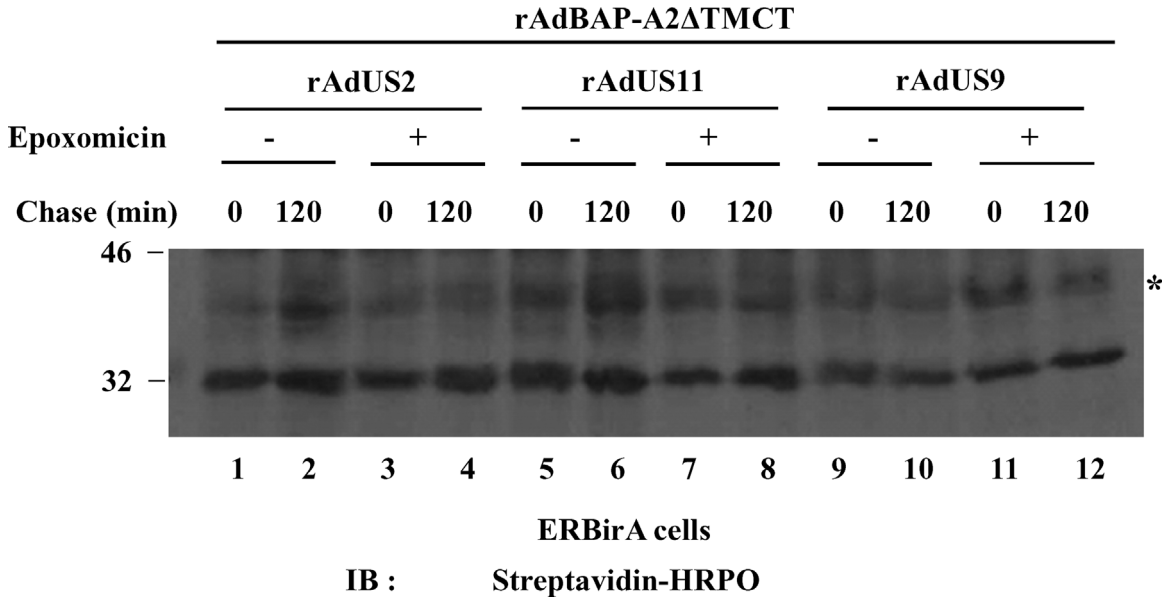

Fig. 7. US2 and US11 do not alter the stability of BAP-A2ΔTMCT. ERBirA cells were infected with rAdBAP-A2ΔTMCT and rAdUS2, rAdUS11 or rAdUS9. Biotinylation assay and immunoblotting were performed as described in Fig. 5. * denotes non-specific band.

handling should make it an attractive method to study protein turnover.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank Umakantha Madala (Ella Foundation, Hyderabad) for providing the pcHis plasmid and other members of the lab for discussion and help. We also thank Prof. David Johnson, Oregon Health & Science University, Portland, Oregon, USA, for providing us the MHC Class I and HCMV genes, and the antibodies W6/32 and BB7.2.

References

[1] B.N. Lilley, H.L. Ploegh, A membrane protein required for dislocation of misfolded proteins from the ER, Nature 429 (6994) (2004) 834–840.
[2] B. Mueller, B.N. Lilley, H.L. Ploegh, SEL1L, the homologue of yeast Hrd3p, is involved in protein dislocation from the mammalian ER, J. Cell Biol. 175 (2) (2006) 261–270.
[3] Y. Ye, Y. Shibata, C. Yun, D. Ron, T.A. Rapoport, A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol, Nature 429 (6994) (2004) 841–847.
[4] T.R. Jones, L. Sun, Human cytomegalovirus US2 destabilizes major histocompatibility complex class I heavy chains, J. Virol. 71 (4) (1997) 2970–2979.
[5] T.R. Jones, L.K. Hanson, L. Sun, J.S. Slater, R.M. Stenberg, A.E. Campbell, Multiple independent loci within the human cytomegalovirus unique short region down-regulate expression of major histocompatibility complex class I heavy chains, J. Virol. 69 (8) (1995) 4830–4841.
[6] E.J. Wiertz, D. Tortorella, M. Bogyo, J. Yu, W. Mothes, T.R. Jones, T.A. Rapoport, H.L. Ploegh, Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction, Nature 384 (6608) (1996) 432–438.
[7] H.R. Stagg, M. Thomas, D. van den Boomen, E.J. Wiertz, H.A. Drabkin, R.M. Gemmill, P.J. Lehner, The TRC8 E3 ligase ubiquitinates MHC class I molecules before dislocation from the ER, J. Cell Biol. 186 (5) (2009) 685–692.
[8] N. Soetandyo, Y. Ye, The p97 ATPase dislocates MHC class I heavy chain in US2-expressing cells via a Ufd1-Npl4-independent mechanism, J. Biol. Chem. 285 (42) (2010) 32352–32359.
[9] D. Tortorella, C.M. Story, J.B. Huppa, E.J. Wiertz, T.R. Jones, I. Bacik, J.R. Bennink, J.W. Yewdell, H.L. Ploegh, Dislocation of type I membrane proteins from the ER to the cytosol is sensitive to changes in redox potential, J. Cell Biol. 142 (2) (1998) 365–376.
[10] A. Ruggiano, O. Foresti, P. Carvalho, Quality control: ER-associated degradation: protein quality control and beyond, J. Cell Biol. 204 (6) (2014) 869–879.
[11] D.J. van den Boomen, R.T. Timms, G.L. Grice, H.R. Stagg, K. Skodt, G. Dougan, J.A. Nathan, P.J. Lehner, TMEM129 is a Derlin-1 associated ERAD E3 ligase essential for virus-induced degradation of MHC-I, Proc. Natl. Acad. Sci. U. S. A. 111 (31) (2014) 11425–11430.
[12] E.J. Wiertz, T.R. Jones, L. Sun, M. Bogyo, H.J. Geuze, H.L. Ploegh, The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol, Cell 84 (5) (1996) 769–779.
[13] Y. Ye, H.H. Meyer, T.A. Rapoport, The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol, Nature 414 (6864) (2001) 652–656.
[14] N.R. Hegde, R.A. Tomazin, T.W. Wisner, C. Dunn, J.M. Boname, D.M. Lewinsohn, D.C. Johnson, Inhibition of HLA-DR assembly, transport, and loading by human cytomegalovirus glycoprotein US3: a novel mechanism for evading major histocompatibility complex class II antigen presentation, J. Virol. 76 (21) (2002) 10929–10941.
[15] G. Petris, L. Vecchi, M. Bestagno, O.R. Burrone, Efficient detection of proteins retro-translocated from the ER to the cytosol by in vivo biotinylation, PLoS One 6 (8) (2011) e23712.
[16] P.J. Schatz, Use of peptide libraries to map the substrate specificity of a peptide-modifying enzyme: a 13 residue consensus peptide specifies biotinylation in Escherichia coli, Biotechnology (N Y) 11 (10) (1993) 1138–1143.
[17] C.M. Story, M.H. Furman, H.L. Ploegh, The cytosolic tail of class I MHC heavy chain is required for its dislocation by the human cytomegalovirus US2 and US11 gene products, Proc. Natl. Acad. Sci. U. S. A. 96 (15) (1999) 8516–8521.
[18] C.J. Barnstable, W.F. Bodmer, G. Brown, G. Galfre, C. Milstein, A.F. Williams, A. Ziegler, Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens-new tools for genetic analysis, Cell 14 (1) (1978) 9–20.
[19] P. Parham, F.M. Brodsky, Partial purification and some properties of BB7.2. A cytotoxic monoclonal antibody with specificity for HLA-A2 and a variant of HLA-A28, Hum. Immunol. 3 (4) (1981) 277–299.
[20] X. Wang, Y.Y. Yu, N. Myers, T.H. Hansen, Decoupling the role of ubiquitination for the dislocation versus degradation of major histocompatibility complex (MHC) class I proteins during endoplasmic reticulum-associated degradation (ERAD), J. Biol. Chem. 288 (32) (2013) 23295–23306.
[21] J.N. Rybak, S.B. Scheurer, D. Neri, G. Elia, Purification of biotinylated proteins on streptavidin resin: a protocol for quantitative elution, Proteomics 4 (8) (2004) 2296–2299.
[22] M. Fairhead, M. Howarth, Site-specific biotinylation of purified proteins using BirA, Methods Mol. Biol. 1266 (2015) 171–184.
[23] E. de Boer, P. Rodriguez, E. Bonte, J. Krijgsveld, E. Katsantoni, A. Heck, F. Grosveld, J. Strouboulis, Efficient biotinylation and single-step purification of tagged transcription factors in mammalian cells and transgenic mice, Proc. Natl. Acad. Sci. U. S. A. 100 (13) (2003) 7480–7485.
[24] J.R. Knowles, The mechanism of biotin-dependent enzymes, Annu. Rev. Biochem 58 (1989) 195–221.
[25] E. Strachan, A.K. Mallia, J.M. Cox, B. Antharavally, S. Desai, L. Sykaluk, V. O'Sullivan, P.A. Bell, Solid-phase biotinylation of antibodies, J. Mol. Recognit. 17 (3) (2004) 268–276.
[26] M.B. Parrott, K.E. Adams, G.T. Mercier, H. Mok, S.K. Campos, M.A. Barry, Metabolically biotinylated adenovirus for cell targeting, ligand screening, and vector purification, Mol. Ther. 8 (4) (2003) 688–700.
[27] E. Fiebiger, C. Story, H.L. Ploegh, D. Tortorella, Visualization of the ER-to-cytosol dislocation reaction of a type I membrane protein, EMBO J. 21 (5) (2002) 1041–1053.
[28] B. Tirosh, M.H. Furman, D. Tortorella, H.L. Ploegh, Protein unfolding is not a prerequisite for endoplasmic reticulum-to-cytosol dislocation, J. Biol. Chem. 278 (9) (2003) 6664–6672.
[29] M.H. Furman, J. Loureiro, H.L. Ploegh, D. Tortorella, Ubiquitinylation of the cytosolic domain of a type I membrane protein is not required to initiate its dislocation from the endoplasmic reticulum, J. Biol. Chem. 278 (37) (2003) 34804–34811.
[30] K. Oresic, D. Tortorella, Endoplasmic reticulum chaperones participate in human cytomegalovirus US2-mediated degradation of class I major histocompatibility complex molecules, J. Gen. Virol. 89 (Pt 5) (2008) 1122–1130.
[31] M.T. Barel, M. Ressing, N. Pizzato, D. van Leeuwen, P. Le Bouteiller, F. Lenfant, E.J. Wiertz, Human cytomegalovirus-encoded US2 differentially affects surface expression of MHC class I locus products and targets membrane-bound, but not soluble HLA-G1 for degradation, J. Immunol. 171 (12) (2003) 6757–6765.
[32] M.T. Barel, N. Pizzato, D. van Leeuwen, P.L. Bouteiller, E.J. Wiertz, F. Lenfant, Amino acid composition of alpha1/alpha2 domains and cytoplasmic tail of MHC class I molecules determine their susceptibility to human cytomegalovirus US11-mediated down-regulation, Eur. J. Immunol. 33 (6) (2003) 1707–1716.
[33] B.E. Gewurz, E.W. Wang, D. Tortorella, D.J. Schust, H.L. Ploegh, Human cytomegalovirus US2 endoplasmic reticulum-lumenal domain dictates association with major histocompatibility complex class I in a locus-specific manner, J. Virol. 75 (11) (2001) 5197–5204.