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Suppression of MHC class I surface expression by calreticulin's P-domain in a calreticulin deficient cell line

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ABSTRACT

Calreticulin (CRT) is an important chaperone protein, comprising an N-domain, P-domain and C-domain. It is involved in the folding and assembly of multi-component protein complexes in the endoplasmic reticulum, and plays a critical role in MHC class I antigen processing and presentation. To dissect the functional role and molecular basis of individual domains of the protein, we have utilized individual domains to rescue impaired protein assembly in a CRT deficient cell line. Unexpectedly, both P-domain fragment and NP domain of CRT not only failed to rescue defective cell surface expression of MHC class I molecules but further inhibited their appearance on the surface of cells. Formation of the TAP-associated peptide-loading complex and trafficking of the few detectable MHC class I molecules were not significantly impaired. Instead, this further suppression of MHC class I molecules on the cell surface appears due to the complex missing antigenic peptides, the third member of fully assembled MHC class I molecules. Therefore the P-domain of calreticulin appears to play a significant role in antigen presentation by MHC class I molecules.

1. Introduction

Peptide loading onto MHC class I heavy chain (HC)-β2-microglobulin (β2M) heterodimers is critical for both the structural stability and the function of MHC class I molecules and is assisted by the peptide-loading complex (PLC) [1]. Calreticulin (CRT) is an important member of the PLC and plays a central role in the formation of the PLC by interacting with other component of the complex. As a molecular chaperone in the endoplasmic reticulum (ER), CRT interacts with newly synthesized glycoproteins that have undergone partial trimming of their core N-linked oligosaccharides. Together with the enzymes responsible for glucose removal and a glucosyltransferase that re-glucosylates already-trimmed glycoproteins, they provide a novel mechanism for promoting folding, oligomeric assembly and quality control in the ER [2]. CRT associates with MHC class I after its heavy chain is assembled with β2M [3–6]. The association of CRT with MHC I is not dependent on either TAP or tapasin, but appears to be a prerequisite for the association of class I with TAP via tapasin. Disrupting the CRT interaction with class I HC by either mutation of the Asn-linked glycosylation site at position 86 [3,6,7], or by using the drug castanospermine to block the action of glucosidase I and II to prevent the generation of the monoglucosyl-glycan recognised by CRT [4,8] has been demonstrated. Furthermore, fewer class I molecules were incorporated into the PLC in the absence of CRT [9]. In addition to its association with class I HC, CRT has been found to interact with ERp57 through the tip of its P-domain [10,11]. It was suggested that CRT recruits ERp57, providing an "attachment" site for this enzyme to mediate disulfide bond formation in newly synthesized glycoproteins. In this context, ERp57 chaperone activity is greatly increased when complexed with CRT [12].

Functionally, CRT has been suggested to stabilize and retain the HC-β2M heterodimer in the ER until the antigenic peptide is loaded. Studies have shown that CRT preferentially binds to open conformations of MHC class I [3,4] and does not associate with the class I after peptide-induced folding [3]. Furthermore, CRT deficient cells have reduced cell surface class I expression and fail to present the majority of epitopes tested to specific T cells, which suggests that CRT is critical for MHC class I assembly and its subsequent antigen presentation [9]. We have also shown that higher concentration of antigen in the cytosol is required for specific T cell stimulation in CRT deficient cells than in CRT competent cells, whereas TAP function and protein expression level was the same [13]. Recent report [14] suggests that calreticulin recycles the sub-optimally loaded class I from the Golgi to

Abbreviations: CRT, calreticulin; ER, endoplasmic reticulum; TAP, transporter associated with antigen processing; β2M, beta-2-microglobulin; OVA, ovalbumin; TCR, T cell receptor

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the ER for loading, and the phenotype might contribute to maintain a low peptide concentration threshold required for efficient antigen presentation [13].

To learn more about how CRT contributes to MHC class I antigen presentation and how the function of CRT relates to its structure, full length CRT or one of the three structurally and functionally distinct domains; the N-domain, the central P-domain, and the C-domain, were introduced into CRT deficient cells (K42) to examine the role of CRT and its truncated forms in the formation of the peptide loading complex and MHC class I antigen presentation. An assessment was also made on the effect of such expression on different steps involved in antigen processing, such as peptide loading, TAP function, and expression of other members of the PLC.

2. Results

2.1. Loss of class I surface expression in CRT deficient cells by transfection of truncated CRT

To further dissect the role of calreticulin in MHC class I antigen processing, different domains of CRT (Fig. 1a) were amplified and cloned into the vector pEF/myc/ER (Invitrogen, UK) containing a leader signal and an ER retention sequence to make sure that all fragments has similar ER location in the cell. A set of stable cell lines (both K41 and K42) expressing the full-length human CRT or its P, PC and NP domains were established by G418 selection and limited dilution cloning. The expression of myc tagged proteins in established cell lines was confirmed by western blot using anti-myc tag antibody. As shown in Fig. 1b the anti-myc antibody reacted with a 60 kDa protein in CRT transfected K42 cells, and a 30 kDa protein in P domain transfectants, and with a 45 kDa protein in PC and NP transfectants. All truncated fragments fused with ER retention signal were seen to stay in the ER (Fig. 1c) and co-localized with calnexin, an ER resident protein.

To find out whether any of these transfectants might restore the reduced class I surface expression in calreticulin deficient cell line K42 (CRT−/−), mouse class I (H-2Kb) on transfected cells was detected with Y3, a H-2Kb conformation dependent monoclonal antibody. To our surprise although the full length CRT restored the surface MHC class I expression in CRT deficient K42 as expected, the introduction of P, PC, and NP fragments into the cells not only failed to rescue the lower surface expression caused by calreticulin deficiency, but further suppressed MHC class I surface expression on such cell lines (Fig. 2a). Among them P-domain of calreticulin almost completely abolished class I surface expression. These results might indicate that the individual fragments had a major impact on class I synthesis and folding, or caused some other damage to the transfected cells, which leads to down-regulation of surface class I

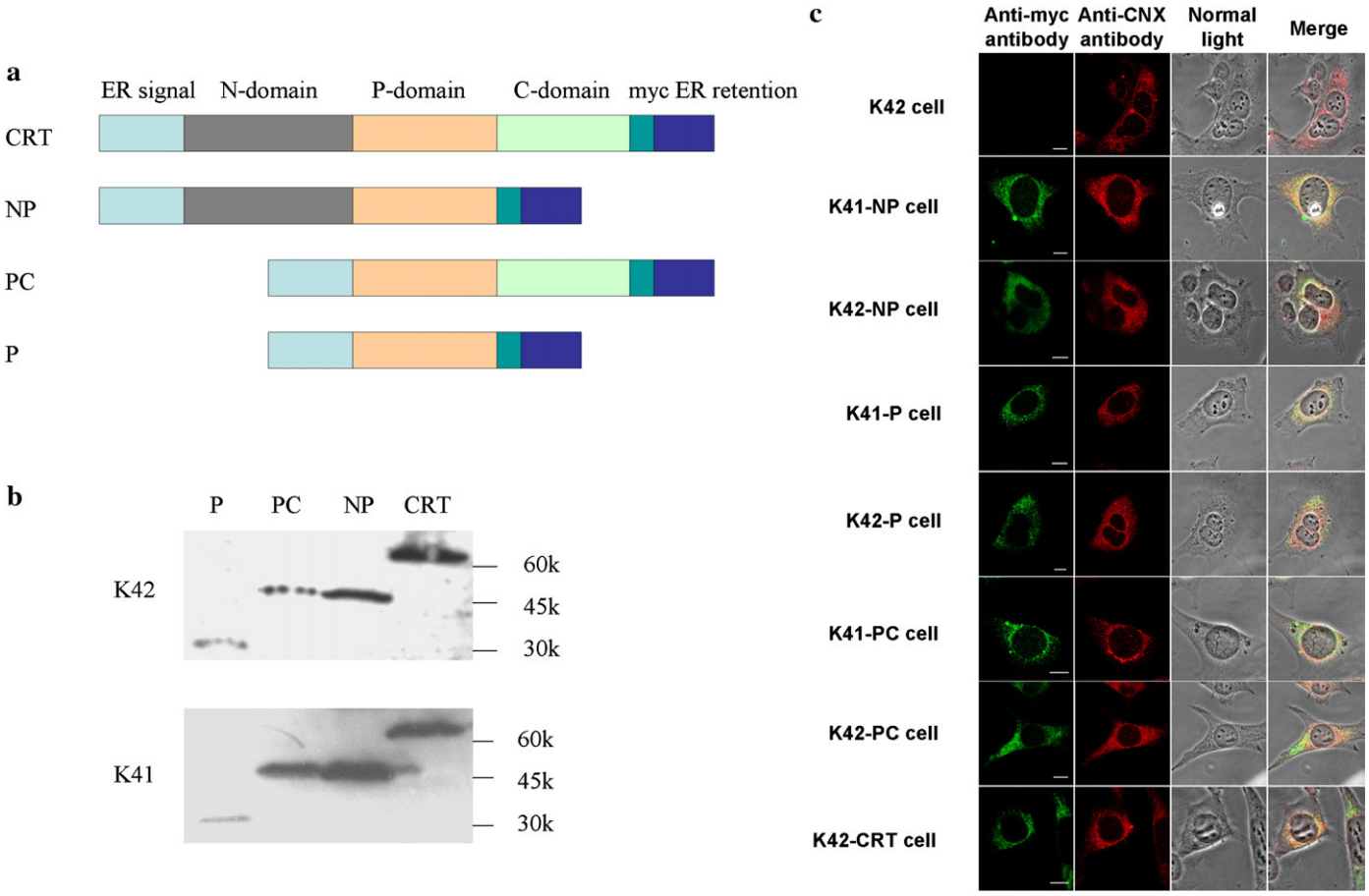


Fig. 1. ER-targeted expression of CRT and its fragments in both K42 (CRT−/−) and K41 (CRT+/+) cells. (a) Schematic representation of full length calreticulin and its NP (AA1–280), PC (AA180–400), P (AA180–280) fragments used in this study. Individual fragments and full length CRT omitting original ER retention signal were cloned into between the ER leader signal and a myc tag plus SEKDEL ER retention signal in vector pEF/myc/ER. (b) The Western blot analysis of recombinant CRT and its P, PC and NP fragments. The individual constructs were transfected onto both K42 and K41 cell lines. The stable cell lines were selected with G418 and cloned by limited dilution. The established cell lines were lysed in 1% Triton X-100, and proteins were separated by 12.5% SDS-PAGE, transferred to Hybond Extra membrane (Amersham), and probed with anti-myc mAb. Western blot with anti-myc mAb confirms transfection of P, PC, NP and full length CRT into K42 (top) and K41 (bottom) cells. (c) Intracellular ER distribution of recombinant CRT and its fragments. K42 and K41 cells were transfected with the pEF/myc/ER vectors encoding ER-targeted full length CRT (not for K41 cells) and its P, PC, NP fragments. The stable cell lines were obtained by G418 selection and limited dilution cloning. The localisation of all fragments in the cells was analysed by immunofluorescence microscopy (100×), revealing a reticular, perinuclear pattern characteristic of ER. The experiments were repeated at least three times with similar results and representative result was shown.

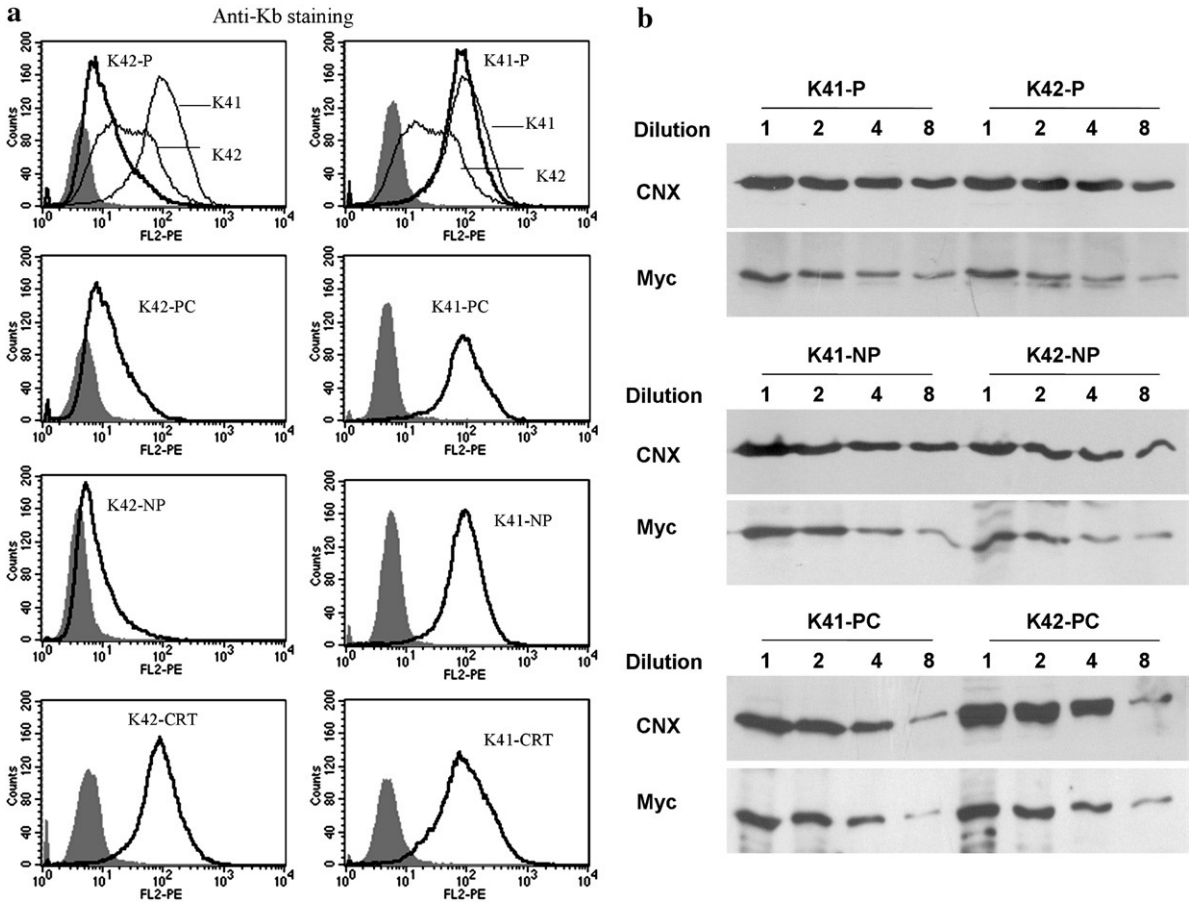


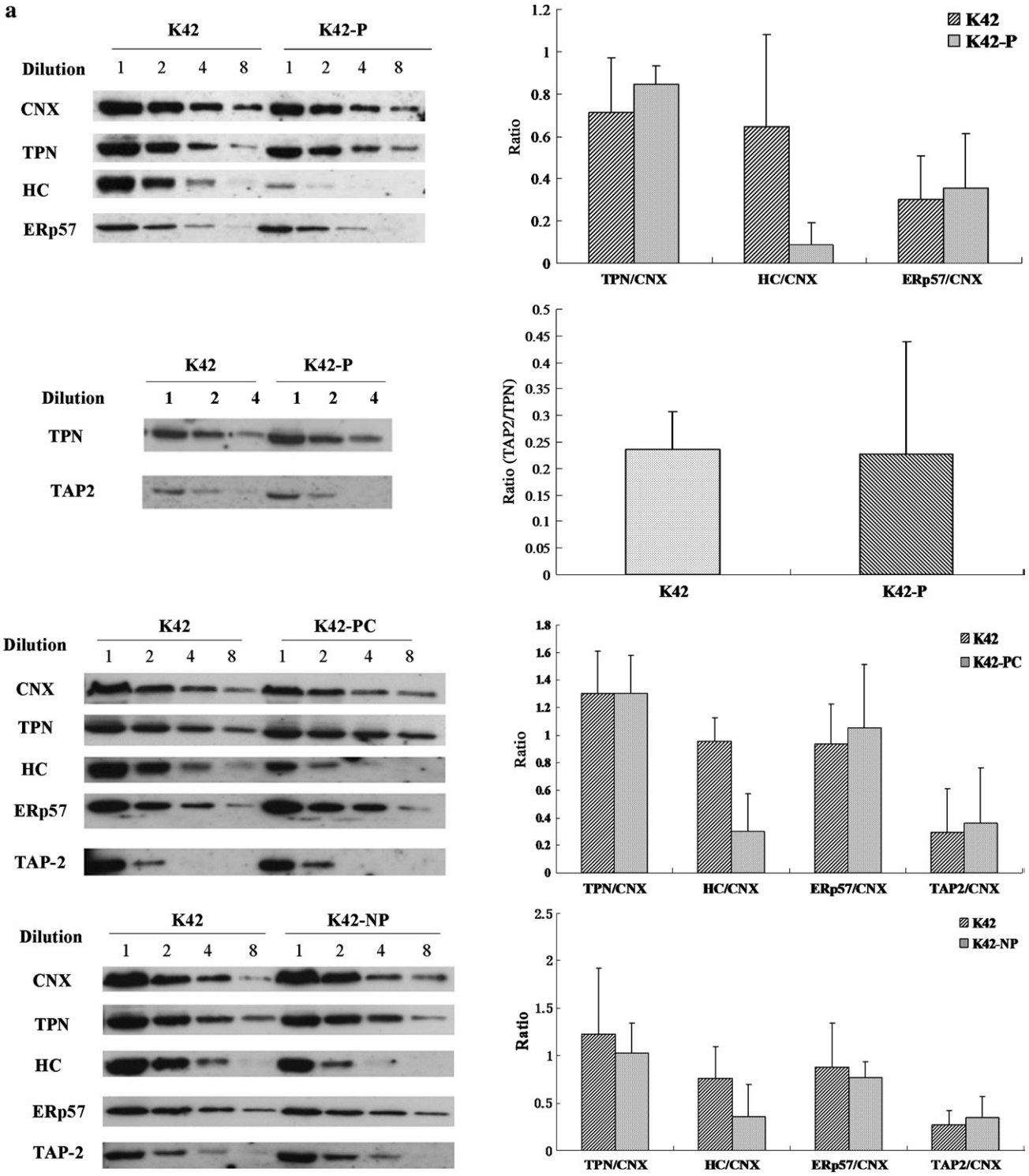
Fig. 2. Down-regulation of MHC class I by truncated CRT in the absence of calreticulin. (a) Cell-surface expression of murine MHC class I Kb on K42, K41 cells and their transfectants were detected by flow cytometry using monoclonal antibody anti H-2Kb (Y3). Left panel: the MHC class I on K42-P, K42-PC and K42-NP cells (thick line) are down-regulated compared with that on K42 cells, while full length human CRT restored MHC class I surface expression to the same level seen in wild-type cells (K41). Right panel: all K41 transfectants showed the same surface expression level of MHC class I (thick line) as that of K41. Shaded areas indicate second antibody only. (b) Similar levels of CRT fragments were expressed between K41 and K42 cell lines. 1×10^6 of individual cells were double diluted and lysed on ice in 1 ml Tris buffered saline (TBS, pH 7.4) containing 0.5% Nonidet P-40, 0.5 mM PMSF (Sigma) and 2 mg/ml IAA (Sigma). The lysates in the supernatant were run on 10% SDS-PAGE and blotted with anti-myc and anti-calnexin antibodies as loading control. The experiments were repeated at least three times with similar results and representative result was shown.

expression. However, when the same fragments of calreticulin used for K42 cell were transfected into K41 (CRT+/+) cells, the surface class I expression in all these transfectants was found at the same level as that of parental K41 cells (Fig. 2a). One may argue that the difference of MHC class I expression between CRT positive and negative cell lines is due to different expression levels of CRT fragments. To clarify this we analyse the amount of individual CRT fragments expressed in both cell lines. The results shown in Fig. 2b demonstrate that there is almost equal amount of CRT fragments expressed in both CRT competent and deficient cell lines. Down-regulated MHC class I expression caused by P-domain only happens in the absence of CRT.

2.2. Significant reduction of class I molecule in CRT domain transfected cells while other components associated with class I assembly remains unchanged

One of the important functions of CRT is quality control for the protein trafficking and folding. Without proper involvement of chaperones the membrane bound or secreted proteins may not fold or assemble efficiently, and subsequently these misfolded or mis-assembled proteins would undergo ER-associated protein degradation [15–18]. We therefore determined if components of the MHC class I loading complex, such as calnexin, tapasin, HC and ERp57, are affected in calreticulin domain transfected cell lines, potentially affecting the folding and assembly of MHC class I molecules. We used semi-quantitative western blotting to test whether the expression of these components are affected, or failed to form the peptide loading complex. Two-fold dilutions of cell lysates, prepared in the presence of a cocktail of proteinase inhibitors, were separated by standard SDS-PAGE. The levels of calnexin, tapasin, HC and ERp57 from K42 (CRT−/−) cells transfected with truncated CRTs and the parent cell line K42 (CRT−/−) were probed with appropriate antibodies in western blot. The results shown in Fig. 3a indicate that the steady state expression of calnexin, tapasin and ERp57 were all similar for K42-P, K42-PC, K42-NP and K42 cells. (left panel). It is clearer when we normalize the expression against an internal standard, calnexin

Fig. 3. Chaperone proteins in truncated CRT transfected K42 cells are unchanged. (a) The relative amount of ER chaperons was analysed by western blot. Two fold serial dilutions of cell lysates for each cell line were subjected to 12% SDS-PAGE and transferred onto Hybond Extra membrane (Amersham), and probed with specific antibodies for calnexin, tapasin, heavy chain, ERp57 and TAP. The results shown are representative of those obtained in three separate experiments (left panel). The blots were scanned and analysed quantitatively with Software Quantity One 4.6.2 (Bio-RAD). The plots with normalization against CNX, (where CNX was not available Tpn was used) for individual CRT domain transfectants were shown in right panel. (b) The treatment of cells with proteasome inhibitors was used to analyse whether HC steady state levels can be rescued in transfectant cell lines. A mixture of MG-115 (Alexis, USA) and MG-132 (Alexis, USA) diluted in DMSO were added to cell cultures both at a final concentration of 5 μM for 6 h, whereas control plates were treated with the same amount of DMSO. Treated cells were collected and lysed on ice in 1 ml Tris buffered saline (TBS, pH 7.4) containing 0.5% Nonidet P-40 in the presence of a cocktail of proteinase inhibitors. The lysates were resolved by 15% SDS-PAGE and analysed by immunoblotting. All experiments were repeated at least three times with similar results and representative result was shown.


a
K42
K42-P
Dilution
1 2 4 8 1 2 4 8
CNX
TPN
HC
ERp57
Ratio
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HC/CNX
ERp57/CNX
K42
K42-P
Dilution
1 2 4 1 2 4
TPN
TAP2
Ratio (TAP2/TPN)
K42
K42-P
K42
K42-PC
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1 2 4 8 1 2 4 8
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HC
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Dilution
1 2 4 8 1 2 4 8
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TPN
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ERp57
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ERp57/CNX
TAP2/CNX
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Proteasome inhibitor
K42 K42-P K42 K42-P
- - + +
CNX
HC
55kD
43kD

for example. As shown in right panel on Fig. 3a all components associated with MHC class I antigen processing expressed between K42 or K42 transfected with CRT fragments appeared the same. There appeared some difference for TAP2 expression between K42 and CRT-P domain transfected K42 on the blot (left panel). But when TAP2 expression is normalized against tapasin the expression level appeared the same, indicating TAP2 expression remains the same in K42 transfected with CRT-P domain. Finally, the overall protein expression level of HC in truncated CRT transfected K42 cells was lower than that of parent K42 cells. However, the decreased MHC class I molecule in CRT-P domain transfected cells seems not to rely on proteasome function since there is no difference for HC expression between cells with or without proteasome inhibitors (Fig. 3b).

2.3. Normal TAP function in transfected cell lines

Peptide supply to the ER can regulate cell surface expression of MHC class I. Virally induced inhibition of TAP function down-regulates class I surface expression [19,20], while increased TAP levels can enhance its expression on the cell surface [20]. It is possible that the empty or sub-optimally loaded class I molecules in K42-P, K42-PC and K42-NP cells were caused by shortage of peptide supply in the ER due to the dysfunction of TAP either structurally or functionally. Without the support of other domains, individual CRT fragments might not perform their chaperone function properly, leading to misfolded or misassembled proteins, which are eventually targeted for degradation [15–18]. Such individual CRT domains might affect the expression or function of TAP directly or indirectly e.g. by modification of the environment in the ER. Although the expression of TAP seems unaffected in these cell lines, this does not prove that TAP function is maintained. To clarify this, a peptide transport assay was carried out to compare TAP function in K42-P, K42-PC and K42-NP with that of parental K42 (CRT−/−) cells. Cells were permeabilized with streptolysin O, then incubated with FITC labelled peptide RRYQNSTEL containing an N-linked glycosylation motif. In the presence of functional TAP, the peptide was translocated into the ER, where it is glycosylated and can be recovered by binding to concanavalin A-Sepharose beads [21]. The fluorescence intensity of eluted peptide from concanavalin A beads were quantitatively analysed by fluorescence spectrometer. As shown in Fig. 4, an equal amount of FITC labelled peptides were transported into the ER for all the truncated CRT K42 transfectants and K42 cells. This suggests that the ability of TAP to carry out the function of translocating peptide from cytosol into the ER remains unchanged, which is consistent with the normal synthesis of TAP in K42-P, K42-PC and K42-NP cells. In the figure, RMA, a mouse tumour cell line with H-2b background was used as positive control while its TAP deficient counterpart, RMA-S was used as negative control.

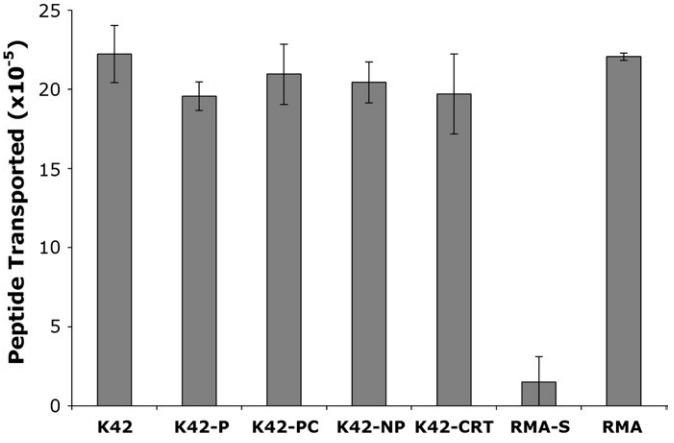

Fig. 4. TAP function is normal in truncated CRT transfected K42 cells. Cells were permeabilized by incubation with slo as described in Materials and methods. FITC-labelled peptides with a glycosylation site (RRYQNSTEL) were incubated with permeabilized and entered the cells. The peptides were subsequently transported by TAP from cytosol into the lumen of ER, where the peptides were glycosylated. Cells were then lysed, and glycosylated peptides bound to Con-A beads were eluted. The florescent intensity of FITC labelled RRYQNSTEL was quantified using a fluorescence spectrometer as described in Materials and methods. Data are presented as the percentage of transported peptides and shown as means SD of three independent experiments.

2.4. The trafficking of class I molecules and its association with peptide loading complex are unchanged in P-domain transfected calreticulin deficient cells

In certain circumstances, class I molecules are not maintained in the ER for a sufficient time to enable them to properly load with optimised peptides. They often travel to the surface of the cell faster and are degraded. For example, in a tapasin deficient cells, the association of class I MHC molecules with TAP is disrupted and ER retention of class I molecules is also lost [22]. If this were the case we would expect a fast trafficking speed of class I molecules in calreticulin domain transfected cells. Pulse-chase experiments using P-domain transfected cells and its parent cells were carried out to determine whether transport rate of class I molecules was altered in calreticulin P-domain transfected cells. However, the results shown in Fig. 5b

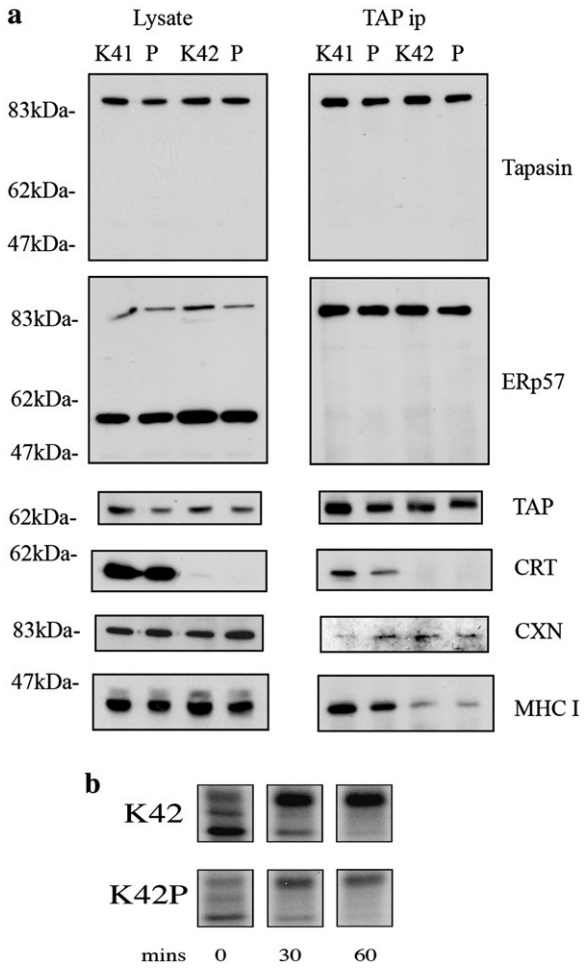

Fig. 5. Assembly of peptide-loading complex in K42, K41 and their CRT P-domain transfectants, and transport of folded molecules to the cell surface. (a) Cell lysates (left panel), and immunoprecipitates of TAP and associated proteins (right panel), were made from K41, K41-P, K42 and K42-P, fractioned by SDS-PAGE, and probed for tapasin, ERp57, TAP, CRT and MHC class I HC. (b) K42 and K42-P cells were pulse labelled and cells were removed at the indicated timepoints, followed by detergent lysis, immunoprecipitation of Kb, and digestion with endo H. The experiments were repeated at least twice with similar results and representative result was shown.

demonstrate that the trafficking speed of class I molecules in P-domain transfected cells is similar to that in its parent calreticulin negative cells, although fewer class I molecules were precipitated in P-domain transfected cells. Previous work from Gao et al. showed that in the absence of CRT (K42), MHC class I was still incorporated into the PLC, but at reduced levels [9]. Whether truncated CRT affect composition of PLC or the incorporation of class I molecules into the loading complex in the absence of calreticulin remained undetermined. Thus co-immunoprecipitation experiments combined with western blot analysis were carried out using K42-P cell paired with parental K42 cells to investigate the formation of the PLC in these cell lines. Anti-TAP antibody precipitates from digitonin extracts of the cell lines were probed with antibodies to tapasin, ERp57, TAP, CRT, calnexin and MHC class I HC. As shown in Fig. 5a similar quantities of tapasin, ERp57 and TAP were present in the TAP precipitates. As expected, CRT can be detected in both K41 cell and its CRT P-domain transfectant, but it is absent in K42 and its CRT P-domain transfectants. Similar level of class I detected in PLC of K42-P and K42 cells. Compared to the K41 series cells, densitometric analysis indicated that approximately 75% fewer MHC class I molecules were TAP associated in the K42 series cells. Overall, the results indicated that the presence of CRT P-domain did not significantly influence the formation of the PLC in either CRT deficient (K42) or competent cells (K41).

2.5. Abolition of endogenous antigen presentation to T cells in CRT fragment transfected cell lines

Cells demonstrating reduced surface expression of class I molecules are still able to process endogenous antigen and present specific epitopes to T cells, although at reduced efficiency, such as in the cases of tapasin, ERp57 and calreticulin deficient cells. However, the level of down-regulation of surface expression in these knockout cells is much less compared to that which we see in calreticulin-domain tranfected K42 cells, in which the surface expression of MHC class I was almost abolished. To determine the effect of truncated forms of CRT in K42 (CRT−/−) cells on antigen processing and presentation, their ability to process endogenous ovalbumin epitope SIINFEKL to the T cell hybridoma B3Z [23,24] was assessed. OVA protein was delivered into cells by incubation of the protein with cells treated with SLO [25]. As shown in Fig. 6, the ability of K42 (CRT−/−) cells transfected with P-domain and NP-domain to process endogenous antigen and present to specific T cells is abolished, while PC domain also significantly inhibits

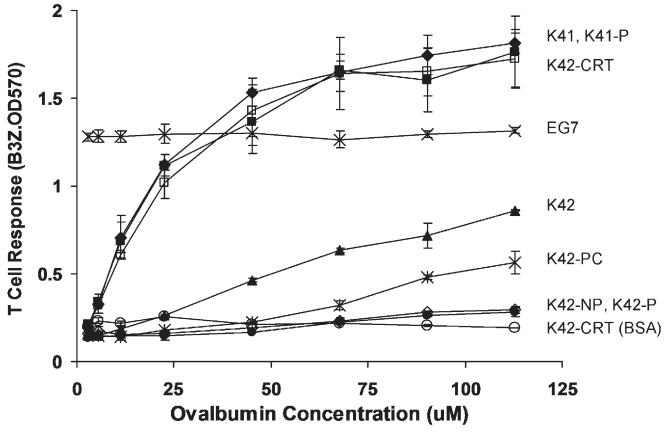

Fig. 6. MHC class I endogenous antigen presentation in calreticulin domain transfectants. Streptolysin O permeabilized cells were incubated with different concentrations of ovalbumin, and then co-cultured with the lacZ-inducible, SIINFEKL/Kb-specific B3Z T cell hybridoma overnight. The colour intensity as indication of B3Z activation was evaluated by an ELISA reader as described in Materials and methods. Experiment was carried out in triplicate each time and repeated at least three times for each cell line. Data was combined for the plot, and error bars showed with SD from the mean.

antigen presentation. As expected, the introduction of CRT into K42 (CRT−/−) cells restored the efficient processing and presentation of endogenous class I antigen presentation, comparable to the level achieved by the OVA expressing EG7 cell line. Collectively, these data suggest that CRT fragments in CRT deficient cells have a profoundly negative effect on endogenous antigen processing and presentation.

3. Discussion

There are several possible mechanisms by which truncated CRT could affect the loading of MHC class I molecules with high affinity peptide. Firstly, poor peptide supply in the ER, due to dysfunction of TAP or TAP protein folding, or impaired assembly in truncated CRT transfected K42 (CRT−/−) cells. However, our western blot and peptide transport assays do not support this, since the truncated CRT transfected K42 (CRT−/−) cells were shown to have the same level of TAP protein expression (Fig. 3a) and peptide translocation ability seen in the parental K42 (CRT−/−) cells (Fig. 4). Secondly, the presence of truncated CRT in K42 (CRT−/−) cells may affect the content of other chaperones in the MHC class I PLC, such as calnexin, tapasin and ERp57. In our experiments this was proved not to be the case either, since similar protein expression levels of these chaperones in truncated CRT transfected K42 (CRT−/−) cells to that of parental K42 cells was found by semi-quantitative western blot (Fig. 3a). Thirdly, impaired ability of PLC to load peptide onto class I molecules could affect the generation of peptide receptive class I molecules, leading to reduced surface class I expression.

Empty H-2Kb molecules could be stabilized and expressed at the cell surface by incubation with saturating K^b binding peptide. The surface expression of Kb-peptide complexes (stained by Y3) was inreased in different cell lines when the cells were pulsed with SIINFEKL peptide. The rank for the increase is as follows: K42-P>K42-NP>K42-PC>K42>K42-CRT (data not shown). Furthermore, direct delivery of SIINFEKL using ER leader/SIINFEKL vaccinia recombinant into the ER in a TAP-independent manner rescued the ability of K42-P, K42-PC and K42-NP to present epitopes at the cell surface (data not shown). It still cannot be excluded that the peptide optimisation process is impaired in these cells as the concentration of peptide expressed by vaccinia viruses is high, which could compensate for the defect in peptide loading [26]. Nevertheless, it seems that CRT deficient cells do not have a global defect in intracellular peptide loading of peptide.

To find out whether endogenous antigen presentation in the transfectants was impaired, the model antigen ovalbumin was delivered into the cytoplasm by streptolysin O, and B3Z responses were used to quantify the relative amount of surface SIINFEKL/Kb complex [22,23]. Our results showed that at the same concentrations of cytosolic ovalbumin, the truncated CRT transfected K42 (CRT−/−) cells presented SIINFEKL/Kb to B3Z less efficiently than the parental K42 (CRT−/−) cells. In contrast, expression of full length CRT in K42 cells increased the efficiency of presenting SIINFEKL in the context of Kb to T cells (Fig. 6). These data revealed that the expression of truncated CRT dramatically reduced the ability of K42 (CRT−/−) cells to present endogenous antigen, while full length CRT increased the efficiency.

Similarly it would be interesting to know whether the expression of truncated CRT in K42 (CRT−/−) cells affects the formation of the PLC resulting in the phenotype described above. Co-immunoprecipitation experiments were performed to measure the protein–protein interactions in PLC of K41, K42 and their CRT P domain transfectants (Fig. 5). Fewer MHC class I molecules were incorporated into the PLC of K42, K42-P cells compared with that of K41 and K41-P cells. Despite the reduced surface class I expression on K42-P cells however, the same amount of class I was found in the PLC of K42-P and its parental K42 cells, suggesting that the presence of the CRT P-domain did not affect the formation of PLC. Thus, in the absence of CRT, truncated CRTs, which all contain the central proline rich P domain capable of interacting with ERp57 and also possessing the lectin site of CRT

capable of binding oligosaccharide on class I HC [27], might compete with calnexin for the formation of PLC to block its function and the function of PLC, leading to the further reduced cell surface expression of MHC class I. Support for this hypothesis came from the coimmunoprecipitation results of K41 and K41-P. Less CRT proteins were incorporated into the PLC of K41-P compared with that of K41, which indicated that the expression of the CRT P-domain 14 in CRT competent K41 cells led to decreased native CRT recruitment into the PLC. The effect of P-domain on the formation of the PLC in K41 cells was not sufficient to affect MHC class I folding, assembly and peptide loading as judged by the normal cell surface class I expression on K41-P.

Another possible mechanism by which truncated CRT could down-regulate class I molecules in K42 (CRT−/−) cells is by truncated CRTs competing with calnexin at an early stage of biogenesis of MHC class I molecules. As described before, calnexin interacts with MHC class I HC upon its co-translational insertion in the ER membrane to facilitate the folding of HC and stabilize it from aggregation as well as promote its assembly with β2M [28–32]. When the interaction of calnexin with class I HCs was prevented by treatment with the oligosaccharide processing inhibitor castanospermine, the HCs underwent a rapid degradation compared with the control cells [33]. Moreover, Vassilakos et al. have demonstrated that in the absence of calnexin interaction, the assembly of class I heavy chain (HC) and β2M was substantially impaired due to misfolding of the HCs [32]. Transfected CRT fragments, containing the central P-domain with the lectin binding site, could bind the glycan moieties on class I HC preventing HC interaction with calnexin, which could result in the accelerated intracellular degradation of the HCs without the chaperone function of calnexin. Indeed, our semiquantitative western blot result showed that the steady-state protein expression level of HCs in truncated CRT transfected K42 cells was lower than that in its parental K42 cells (Fig. 3a), indicating that the P-domain of CRT may compete with calnexin for binding to free class I HCs to block its function of facilitating the folding of HCs. In this way, the P-domain of CRT may further reduce cell surface class I expression on K42 cells.

Calreticulin might act as a bridge to link other functional components of class I assembly machinery. One domain of calreticulin may be required for interaction with a functional molecule while the other executes another unidentified function. ERp57 could be one such interaction partner in this regard. There are several reports suggesting that the P-domain of calreticulin is responsible for binding to ERp57, which is capable of interacting with the conserved cysteines in the peptide-groove of MHC class I molecules [34,35]. Thus in combination with ERp57 and PDI, calreticulin, and in particular its P-domain, appears to play a critical role in the process of MHC class I antigen processing and presentation.

4. Materials and methods

4.1. Cells, peptides and antibodies

Wild-type C57/B6 fibroblast cell line K41 and its CRT deficient counterpart K42 were kindly provided by Dr M. Michalak [36,37]. Antibodies 9E10 (anti-human c-myc), BBM1 (anti-human β2-microglobulin) and Y3 (anti-H-2Kb) were obtained from the ATCC. B3Z was a gift from Dr N. Shastri. EG7 was provided by Alain Townsend. The cell lines were grown in RPMI 1640 supplemented with 10% FCS and L-glutamine (Gibco BRL). Anti-human CRT antibodies were purchased from Stressgen and described before [9]. Rabbit serum against murine tapasin and TAP were kindly provided by Ping Wang. Ovalbumin peptides 257–284 (SIINFEKL), a H-2Kb binding peptide, flu peptide (GILGFVFTL), a HLA-A2 associated peptide and fluorescently labelled RRYQNSTEL (HLA-B27 binding peptide) were purchased from Eurogentic (Belgium). All other reagents were purchased from Sigma.

4.2. CRT and its fragment constructs

Different CRT domains were amplified using PCR. The following primers were synthesized by Sigma: Forward-F 5′-(PstI)CTG CAG-gag cct gcc gtc tac ttc aag-3′; Reverse-F 5′-(NotI)GC GGC CGC-ggc ctg gcc ggg gac atc ttc-3′; Reverse-NP 5′-(Not I)GC GGC CGC-ata ggc ata gat act ggg atc-3′ and Forward-P 5′-(PstI)CTG CAG-gat tgg gac ttc ctg cca ccc-3′. The full length of CRT (AA1–AA400) omitting the original ER retention signal was amplified with primer pair Forward-F and Reverse-F; CRT-P (AA180–AA280) domain with Forward-P; CRT-NP (AA1–AA280) domain with Forward-F and Reverse-NP and CRT-PC (AA180–AA400) domain with Forward-P respectively. Taq DNA Polymerase (Invitrogen) was used. The reagents were mixed in a total volume of 50 μl and PCR reactions were carried out for 30 cycles on a GeneAmp PCR System 9700 (PE Applied Biosystems) with the following conditions: 1, 94 °C 45 s; 2, 55 °C 45 s; 3, 72 °C 60 s and 4, 72 °C 10 min. The PCR products were gel purified and digested and then inserted between ER leader signal and myc tag followed by SEKEDL retention signal on the vector pEF/myc/ER (Invitrogen). The final constructs were sequence verified.

4.3. Stable cell lines expressing different CRT constructs

Both K41 and K42 were transfected with pEF/myc/ER constructs with human full length of CRT omitting original ER retrieve signal, P-domain (amino acids 180–280), PC (amino acids 180–400) and NP-domain (amino acids 1–280) inserted between ER leader sequence and myc tag followed by SEKDEL ER retention signal. The transfection was done using Lipofectamine kit provided by Invitrogen according to the manufacturer's instruction. The stable cell lines were selected with 6 mg/ml G418 (SIGMA) and cloned by limited dilution.

4.4. Immunofluorescence staining

The cells were seeded at a density of 1×10^5 cells/plate in a 60 mm plates containing sterile glass coverslips and cultured for 24 h. The cells on coverslips were rinsed with PBS and fixed on ice with PBS containing 4% paraformaldehyde for 15 min, and then the cells were permeabilized with 0.5% Triton-X100 in PBS for 15 min on ice. After washing with PBS and incubation for 30 min at room temperature with PBS containing 2.5% BSA, the slips were stained with a mixture of mouse monoclonal anti-myc antibody (1:400) and rabbit polyclonal anti-calnexin antibody (1:500) in PBS containing 2.5% BSA for K42 cell and transfectant cell lines, and incubated with rabbit polyclonal anti-CRT antibody in PBS containing 2.5% BSA for K41 cells. After washing with PBS, the cells were incubated with a mixture of rhodamine-labelled goat anti-rabbit IgG (1:400, Santa Cruz) and FITC-labelled goat anti-mouse IgG (1:400, Santa Cruz) in PBS containing 2.5% BSA for K42 cell and transfectant cell lines, and incubated with rhodamine-labelled goat anti-rabbit IgG (1:400, Santa Cruz) in PBS containing 2.5% BSA for K41 cell. Fluorescently labelled cells were washed three times with PBS and then mounted on slide glass with 90% glycerol. Samples were analysed by using a fluorescence confocal microscopy (Leica TCS SP2).

4.5. Immunoblotting

1×10^6 exponentially growing cells were lysed on ice in 1 ml Tris buffered saline (TBS, pH 7.4) containing 0.5% Nonidet P-40, 0.5 mM PMSF (Sigma) and 2 mg/ml IAA (Sigma). The lysates were resolved by 12% SDS-PAGE and transferred onto Hybond Extra-C membrane (Amersham). After blocking with 2.5% skimmed milk (Marvel) in phosphate buffered saline (PBS, pH 7.4), the membrane was incubated with antibody in PBS containing 0.05% Tween 20 (PBST, pH 7.4) containing 2.5% skimmed milk (Marvel) for 1 h at room temperature. The membrane was washed with PBST twice and

incubated with anti-rabbit antibody conjugated with peroxidase (Sigma) in PBS with 2.5% skimmed milk. The membrane was thoroughly washed three times with PBST and visualised with an enhanced chemiluminescence detection system according to the manufacturer's instructions (Pierce). The treatment of cells with proteasome inhibitors was used to analyse whether HC steady state levels can be rescued in transfectant cell lines. A mixture of MG-115 (Alexis, USA) and MG-132 (Alexis, USA) diluted in DMSO were added to cell cultures both at a final concentration of 5 μM for 6 h, whereas control plates were treated with the same amount of DMSO. Treated cells were collected and lysed on ice in 1 ml Tris buffered saline (TBS, pH 7.4) containing 0.5% Nonidet P-40 in the presence of a cocktail of proteinase inhibitors. The lysates were resolved by 15% SDS-PAGE and analysed by immunoblotting. Software Quantity One 4.6.2 (Bio-RAD) was used to analyse the scanned blot quantitatively.

4.6. Co-immunoprecipitation

1.5×10^7 cells were lysed in digitonin (Wako) lysis buffer (1% digitonin, 150 mM NaCl, 10 mM Tris pH 7.6, 1 mM PMSF, 10 mM N-ethylmaleimide) on ice for 15 min. Lysates were spun at 14,000 rpm to remove insoluble debris, and precleared with 50 μl Protein G-Sepharose beads for 30 min. Lysates were then immunoprecipitated with sheep anti-rat TAP1 and TAP2 sera (which also recognises mouse TAP), or monoclonal anti-Kb antibody, and Protein G-Sepharose beads. Beads were washed 3 times in lysis buffer and heated in non-reducing sample buffer. Samples were run on 8% SDS-PAGE, immunoblotted and probed with relevant antibodies.

4.7. Flow cytometry analysis

Cells were harvested and re-suspended to a concentration of 10^6 cells/ml in cold FACS buffer (1×PBS, 1% fetal calf serum, and 0.1% sodium azide) and 100 μl aliquots were added to wells in a round-bottom 96-well plate (Merck). The plate was centrifuged at 300×*g* for 5 min, the supernatant flicked off gently and the plate vortexed to lose the cells. The appropriate antibody in 50 μl of FACS buffer was added to the wells and incubated in the dark at 4 °C for 30 min. If required cells were further stained with the secondary antibody as above and fixed (2% Paraformaldehyde in PBS, pH 7.4) and analysis was carried out on a Becton Dickinson FACScan running CellQuest software. Typically 20,000–50,000 events were collected for each measurement.

4.8. Antigen presentation assay with B3Z

1×10^6 cells were washed twice with warm RPMI 1640, resuspended in 200 μl RPMI 1640 in a 24-well plate. The cells were permeabilized by adding Streptolysin-O (SLO, SIGMA) (10 U/10^6 cells for K41, K42 and K42-CRT; 45 U/10^6 cells for RMA and RMA-S) to the wells. Ovalbumin or BSA as control were added and incubated at 37 °C with 5% CO_2 for 10 min. 500 μl of media with 10% FCS was added and incubated for further 30 min. The cells were resuspended at 1×10^6/ml. 100 μl of cell were cultured with 100 μl of 1×10^6/ml B3Z cells overnight. The cells were washed once with 200 μl PBS and lysed by addition of 100 μl Z buffer (0.12 mM Chlorophenolred-beta-D-galactopyranoside (CPRG, Calbiochem), 100 mM 2-mercaptoethanol, 9 mM MgCl2, 0.125% NP-40 in PBS). After 4 h of incubation at 37 °C, 50 μl stop buffer (300 mM glycine and 15 mM EDTA in water) was added and the signal was read in an ELISA reader at 570 nm referenced at 630 nm [26].

4.9. Peptide transport assay

3×10^6 cells were washed twice with RPMI 1640 media and permeabilized for 10 min at 37 °C with streptolysin-O described as above. Cells were then washed once with $MgCl_2$ buffer (0.005 M $MgCl_2$ in PBS) and resuspended in 200 μl warm translocation buffer (78 mM KCl, 4 mM $MgCl_2$, 8.4 mM $CaCl_2$, 10 mM EGTA, 1 mM DTT, 10 mM ATP, 0.1%BSA, 50 mM HEPES, PH7.0). Fluorescein (FITC) tagged RRYQNSTEL peptide [35] was added to the cells at a final concentration of 10 μM and the cells were incubated at 37 °C for 10 min. 1 ml ice-cold lysis buffer (1%NP-40 in PBS) was added followed by incubation on ice for 15 min. Insoluble proteins were removed by centrifugation at 20,000×*g* for 15 min and glycosylated peptides were bound to 50 μl concanavalin A Sepharose beads (50%) by overnight incubation at 4 °C with vigorous agitation. The beads were washed three times with 0.1% NP-40 in PBS and then bound peptides were eluted by a 60 min vigorous agitation in 500 μl of 200 mmol/l methyl-α-D-mannopyranoside in PBS, in the dark at room temperature. The fluorescence intensity of supernatants was quantified using a fluorescence spectrometer with excitation and emission wavelengths at 484 nm and 535 nm, respectively. The reading was calculated as the proportion of the total fluorescent peptide added that was transported into the ER.

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