Molecular Immunology 46 (2009) 3198–3206

Contents lists available at ScienceDirect

Molecular Immunology

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

Calreticulin maintains the low threshold of peptide required for efficient antigen presentation

Hongmei Fu[a,1], Changzhen Liu[b,1], Barry Flutter[a], Hua Tao[b], Bin Gao[a,b,c,*]

[a] Rheumatology Unit, UCL Institute of Child Health, University College London, London WC1N 1EH, UK
[b] The Center for Molecular Immunology, CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China
[c] China–Japan Joint Laboratory of Molecular Immunology and Virology, CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China

ARTICLE INFO

Article history:
Received 11 June 2009
Received in revised form 11 August 2009
Accepted 12 August 2009
Available online 11 September 2009

Keywords:
MHC class I
Calreticulin
ERp57
Transporter associated with antigen processing

ABSTRACT

Calreticulin (CRT) plays a critical role in MHC class I antigen processing and elicits peptide-specific CD8(+) T cell responses against tumours when administered with peptides. However, how CRT contributes to class I antigen processing and the mechanism of its adjuvant effect in anti-tumour responses, remain to be elucidated. Here we show that reduced class I expression in CRT deficient cells can be restored by the direct delivery of peptides into the ER or by incubation at low temperature. CRT deficient cells exhibited a TAP-deficient phenotype in terms of class I assembly, without loss of TAP expression or functionality. Furthermore, a higher concentration of antigen in the cytosol is required for specific T cell stimulation, suggesting that CRT has a functional role in the maintenance of the low peptide concentration threshold required in the ER for efficient antigen presentation. In the absence of CRT, ERp57 is up-regulated, which indicates that they collaborate with each other in class I antigen processing.

1. Introduction

CRT is an abundant endoplasmic reticulum (ER) resident protein that has a large number of cellular functions in a variety of biological systems. Among these, two of its most important functions are intracellular calcium homeostasis and acting as a chaperone in the folding of newly synthesized glycoproteins (Krause and Michalak, 1997). CRT associates with glycoproteins when they are freshly synthesized and retains them in the ER until they are properly folded. This process of ER retention is important in quality control for the production of correctly folded glycoproteins (Hammond and Helenius, 1995; Hebert et al., 1996). CRT has also been demonstrated to be involved in class I antigen processing and presentation; it plays an important role in the formation of the peptide-receptive MHC class I complex (Sadasivan et al., 1996) and subsequently in efficient peptide loading (Gao et al., 2002).

Assembly of MHC class I molecules in the ER and their presentation to CD8(+) T cells and NK cells is a highly coordinated process involving several critical steps including: the supply of peptides, the generation of peptide-receptive class I molecules, the loading of peptide onto class I molecules and ER exit of loaded class I to the surface of cell. Peptides are generally derived from newly synthesized polypeptides located in the cytosol where they are cleaved by the proteasome (Cascio et al., 2001; Kloetzel, 2001) and other proteases (Reits et al., 2004; Saric et al., 2004; Stoltze et al., 2000). The position of cleavage generally produces peptides which are extended at the N-terminus but have the correct C-terminus (Cascio et al., 2001). Assisted by cytoplasmic chaperones (Dunn et al., 2001; Kunisawa and Shastri, 2003), these peptides are transported by TAP into the ER (Abele and Tampe, 2004; Androlewicz et al., 1993; Kelly et al., 1992; Lankat-Buttgereit and Tampe, 1999; Powis et al., 1991) and the extra N-terminal residues are removed by an aminopeptidase in the ER (ERAAP) (Saric et al., 2002; Serwold et al., 2001, 2002; York et al., 2002). In the ER peptides are loaded onto the class I complex through collaborative work of molecules of the peptide-loading complex (Cresswell et al., 1999). CRT is closely associated with MHC class I–β2M dimers via its lectin-like binding site and acts as an important member of peptide-loading complex (PLC) (Sadasivan et al., 1996). In the absence of CRT, MHC class I molecules are still able to appear on the surface of cell, but the majority of class I molecules are either empty or loaded with sub-optimal peptides, demonstrating that CRT is indispensable for efficient class I

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

* Corresponding author at: 1, Beichen Xilu Rd, Chaoyang District, Beijing 100101, China. Tel.: +86 10 64807338; fax: +86 10 64807338.
E-mail address: bgao2004@gmail.com (B. Gao).

[1] These authors contributed equally.

0161-5890/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2009.08.012

assembly and antigen presentation. The CRT deficient cell line also fails to present the majority of epitopes tested to specific corresponding T cells (Gao et al., 2002). In some respects the phenotype appears similar to the T134K heavy chain mutant, which exits the ER rapidly as an 'empty' heterodimer; in contrast empty wild type molecules are normally retained in the ER and degraded and the association of the T134K mutant class I molecules with TAP and CRT is disrupted (Lewis et al., 1996; Peace-Brewer et al., 1996) and this could explain the similarity in the phenotype of the T134K mutant and class I molecules in CRT deficient cells.

CRT has also been shown to bind to the peptides transported into the ER by the TAP (Spee and Neefjes, 1997). A number of experiments now indicate that binding of CRT to peptide may contribute to the production of specific immunity. For instance, immune responses have been generated against tumours by immunising with CRT extracted from them. The immunogenicity is attributed to peptides associated with the extracted CRT and not to the CRT molecule itself (Basu and Srivastava, 1999). It has also been shown that CRT molecules can be complexed in vitro to unglycosylated peptides and used to elicit peptide-specific CD8(+) T cell response by exogenous administration (Basu and Srivastava, 1999). CRT conjugated with tumour antigen at DNA level produced a protective immunity against the tumour accompanied with a significant increase in tumour antigen specific CD8(+) T cell precursors (Cheng et al., 2001). Furthermore, CRT and other members of HSP family when associated with peptides can provide a necessary and sufficient source of antigen for cross-priming of CD8(+) T cells (Binder and Srivastava, 2005).

To further understand how CRT is involved in proper peptide loading onto the class I complex and how it contributes to efficient anti-tumour responses by its adjuvant effect, we investigate the involvement of CRT in TAP function, its direct or indirect affect on peptide loading, the expression and folding of members of peptide-loading complex and the threshold of peptide required for proper antigen presentation.

2. Materials and methods

2.1. Cells, peptides and antibodies

K42 is a CRT deficient fibroblast cell line derived from mouse embryos with a targeted knockout of the CRT gene. Wild type (K41) fibroblasts were derived from a normal mouse embryo. Both cell lines were kindly provided by Dr M. Michalak (Mesaeli et al., 1999; Nakamura et al., 2000). K42 was transfected with human CRT and selected with 6 mg/ml G418 (Sigma) to produce K42-CRT. Hybridoma 9E10 (anti-human c-myc), BBM1 (anti-human β2-microglobulin) and Y3 (anti-H-2K^b) were obtained from the ATCC. B3Z is a T cell hybridoma specific for K^b-SIINFEKL, which upon activation expresses LacZ, described in detail previously and was originally from the laboratory of Dr N. Shastri (Karttunen et al., 1992). 25-D1.16 is a monoclonal antibody specifically against K^b-SIINFEKL complex (Porgador et al., 1997) and originally provided by Dr R. Germain. EG7-OVA is a transfected clone of EL4, which produces chicken ovalbumin, (OVA) kindly provided by Alain Townsend. The cell lines described above were grown in RPMI 1640 supplemented with 10% FCS and L-glutamine (Gibco BRL). Antibodies Y3, 9E10, BBM1 and 25-D1.16 were affinity purified from respective cell culture supernatants using Protein G columns (ImmunoPure, Pierce). Anti-human CRT antibody was purchased from Stressgen. Anti-rat CRT rabbit serum cross-reacts with both human and mouse CRT and has been described before (Gao et al., 2002). Rabbit serum against murine tapasin and TAP were kindly provided by Ping Wang. OVA peptide 257–284 (SIINFEKL), flu matrix protein peptide (GILGFVFTL) and fluorescently labeled RRYQNSTEL used as a TAP transport substrate were purchased from Eurogentic (Belgium), received as lyophilised powder and resuspended in water to a stock concentration of 5 mg/ml. For routine use the stock was further diluted 1/10,000 (final concentration 0.5 μg/ml). All other reagents were purchased from Sigma.

2.2. 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 manufacture's instructions (Pierce).

2.3. Flow cytometry analysis

Cells were harvested and resuspended to a concentration of 1×10^6/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 bottomed 96-well plate (Merck). The plate was centrifuged at 300 × *g* for 5 min, the supernatant flicked off gently and the plate vortexed to loose 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. Cells were then centrifuged at 300 × *g* for 5 min and the supernatant was discarded. The cells were then washed two times with FACS buffer. If required cells were then further incubated with the secondary antibody in 50 μl of FACS buffer as above and then washed three times in FACS buffer. After washing cells were resuspended in 100 μl of fixation buffer (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.

2.4. Antigen presentation assay with TCR-like antibody

0.5×10^6 target cells were infected with 25 μl recombinant vaccinia viruses expressing either a minigene encoding SIINFEKL with leader sequence to the ER or matrix protein of Influenza as control in 200 μl PBS solution supplemented with 0.1% (w/v) bovine serum albumin at 37 °C for 1 h. The infected cells were transferred into a T25 flask containing 2.5 ml normal growth medium and incubated at 37 °C in a 5% CO_2 incubator overnight. The cells were washed and stained as above with 25-D1.16, a mAb with TCR-like specificity that recognizes SIINFEKL in the context of H-2K^b. Anti-mouse IgG-PE was used as secondary antibody and cells were analysed by FACS.

2.5. Antigen presentation assay with B3Z

1×10^6 cells were washed twice with warm RPMI 1640, resuspended in 200 μl RPMI 1640 and added to the wells of a 24-well plate. The cells were permeabilised by adding an appropriate concentration of 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. Different concentrations of OVA or BSA as control were added to each well and the plate was incubated in a 37 °C incubator with 5% CO_2 for 10 min, and agitated twice during this period.