Quantitative mass spectrometric analysis of tripeptidyl peptidase II (TPPII) in mouse KO CD8+ T-cells

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Overview

Purpose: Identification of proteins which are responsible for apoptosis and radio sensitivity in TPPII knockout (KO) CD8+ T cells.

Methods: SILAC and label-free quantification of proteins from total cell lysate by LC-MS using an Orbitrap™ hybrid mass spectrometer

Results: CD8+ T cell showed poor growth and therefore low label incorporation in the SILAC media. Using label-free quantitation, proteins could be identified that, although indirectly, might contribute to the cellular effects in the TPPII KO CD8+ T cell in mouse.

Introduction

Tripeptidyl peptidase II (TPPII) forms the largest known protease complex (≈6 MDa) in eukaryotic cells. It was discovered as a cytoplasmic aminopeptidase that rapidly degrades synthetic oligopeptides. A biological role for TPPII was found in a lymphoma cell line that was adapted to grow in the presence of proteasome. TPPII was up-regulated in this cell line, suggesting that it may be able to complement some of the cellular functions of the proteasome. Several other experiments confirmed important functions of TPPII in tumor cell proliferation and survival. However, the mechanism by which TPPII, as a peptidase, acts to regulate cellular proliferation and survival is not clear. The major cellular role of TPPII in normal cells resides within the downstream proteolysis of proteasome substrates, e.g. MHC class I antigen processing inhibitors (Figure 1). Whether TPPII contributes to the degradation of full-length proteins as the proteasome and/or whether there are also non-proteolytic functions has remained elusive.

In order to better understand the role of TPPII, we have generated TPPII-deficient mice and provided genetic evidence that TPPII is required for survival of proliferating cells (Figure 2). Increased apoptosis of activated CD8+ T cells was found in TPPII KO cells compared to wild type cells, suggesting that TPPII plays some role in preventing apoptosis in proliferating T cells. The apoptosis in TPPII KO CD8+ T cells was further aggravated when cells were irradiated (Figure 3), suggesting that TPPII might have some function in the DNA damage response.

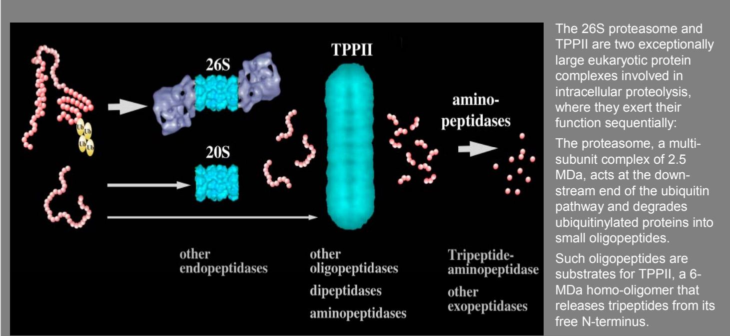
We have looked at p53, p21, phospho-ATM, gH2X (for DNA damage and cell cycle arrest), NFkB, Bax and Fas (for apoptosis) expression levels by western blot after irradiation, and found no differences except that KO CD8+ T cell exhibited higher NFkB levels when isolated from mice, and prolonged gH2X upon irradiation. We have also checked several other signal transduction pathways, but found no significant differences. In the present study, we decided to look for the missing link between TPPII and DNA damage and/or apoptosis pathways in CD8+ T cells by mass spectrometry.

Methods

Three each wild type (WT) and TPPII knockout (KO) mice were used to isolate CD8+ T cells. Splenic and lymph node CD8+ T-cells were isolated using a CD8 T-cell isolation Kit (Miltenyi Biotec), and incubated either in DMEM medium (Invitrogen) with FBS (PAA Laboratories) - label free sample, or SILAC medium with dialyzed FBS (both PAA Laboratories) and heavy lysine C^{13} (+6.02013 Da on K, Cambridge Isotope Laboratories) for WT and light lysine for KO (Sigma-Aldrich), supplemented with glutamate, NEAA and penicillin/streptomycin.

Cells were activated by phorbol 12-myristate 13-acetate (PMA: 10 ng/mL) and ionomycin (500 ng/mL) for 3 days. Total cell extracts were prepared using 100 mM ammonium bicarbonate. Same amount of protein (50 µg) were reduced and alkylated. The modified proteins were enzymatically digested and lyophilized.

FIGURE 1. Role of TPPII in proteolysis

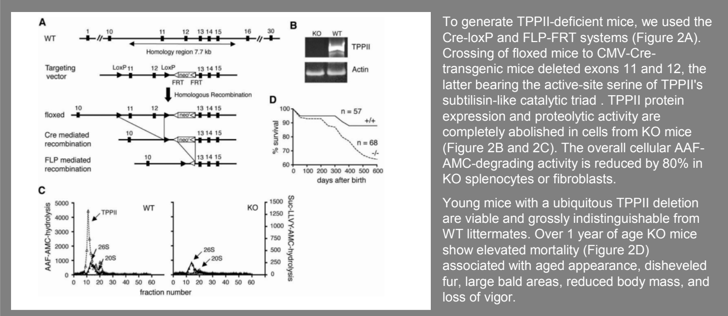


The 26S proteasome and TPPII are two exceptionally large eukaryotic protein complexes involved in intracellular proteolysis, where they exert their function sequentially:

The proteasome, a multi-subunit complex of 2.5 MDa, acts at the down-stream end of the ubiquitin pathway and degrades ubiquitinylated proteins into small oligopeptides.

Such oligopeptides are substrates for TPPII, a 6-MDa homo-oligomer that releases tripeptides from its free N-terminus.

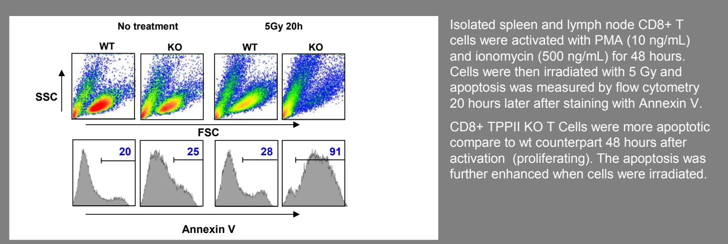
FIGURE 2. Generation and conformation of TPPII-deficient mice



To generate TPPII-deficient mice, we used the Cre-loxP and FLP-FRT systems (Figure 2A). Crossing of floxed mice to CMV-Cre-transgenic mice deleted exons 11 and 12, the latter bearing the active-site serine of TPPII's subtilisin-like catalytic triad . TPPII protein expression and proteolytic activity are completely abolished in cells from KO mice (Figure 2B and 2C). The overall cellular AAF-AMC-degrading activity is reduced by 80% in KO splenocytes or fibroblasts.

Young mice with a ubiquitous TPPII deletion are viable and grossly indistinguishable from WT littermates. Over 1 year of age KO mice show elevated mortality (Figure 2D) associated with aged appearance, disheveled fur, large bald areas, reduced body mass, and loss of vigor.

FIGURE 3. Increased apoptosis of CD8+ TPPII KO T cells after γ-irradiation analyzed by FACS



Isolated spleen and lymph node CD8+ T cells were activated with PMA (10 ng/mL) and ionomycin (500 ng/mL) for 48 hours. Cells were then irradiated with 5 Gy and apoptosis was measured by flow cytometry 20 hours later after staining with Annexin V.

CD8+ TPPII KO T Cells were more apoptotic compare to wt counterpart 48 hours after activation (proliferating). The apoptosis was further enhanced when cells were irradiated.

The protein lysates were resuspended in 5% acetonitrile (CAN) in water with 0.1% formic acid and subjected to the LC-MS analysis. LC-MS analysis was performed on a Thermo Scientific LTQ Orbitrap XL mass spectrometer coupled to a Thermo Scientific Surveyor LC using a Magic C18 (5 µm, ID 100 µm, Spectronex) column at a flow rate of ≈200 nL/min. After injection of the sample, the column was washed at 5% ACN for 20 min, then the peptides were separated ramping the LC gradient to 40% ACN within 70 min. The full MS scans were acquired in the Orbitrap mass analyzer and the ten most abundant ions of each full scan were fragmentated in the LTQ ion trap using collision-induced dissociation (CID).

For the SILAC quantitation, WT and KO samples (samples no. 226 and 313) were mixed and each biological replicate was analyzed using three technical replicates. The identity and quantity of the proteins were determined using Thermo Scientific Proteome Discoverer software. The UniProt mouse database was searched using the SEQUEST® algorithm. The label incorporation was checked with heavy-labeled WT sample only.

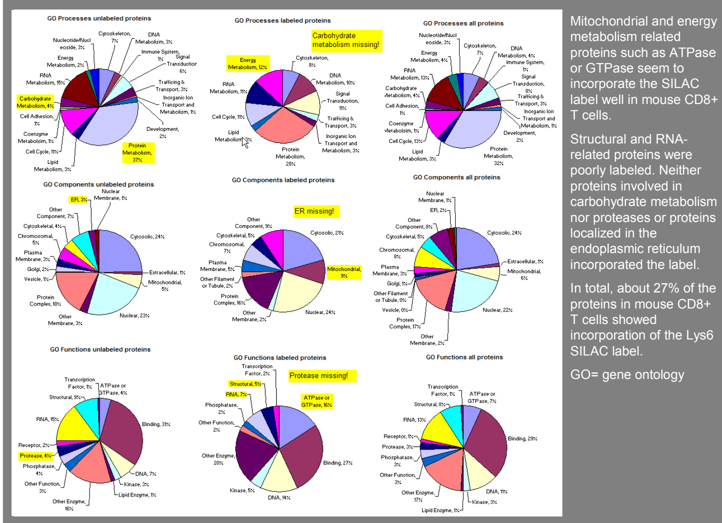
For the label-free quantitation of two biological replicates (samples no. 226 and 313) of each WT and KO cells were analyzed, each of them with at least three technical replicates. Quantitation of the proteins was done with Thermo Scientific SIEVE 1.2 label-free quantitation software and protein identity was determined using a database search with the SEQUEST search algorithm.

Results

The corporation of the of the SILAC label (K+6.02013) in the WT CD8+ cells was surprisingly low. Only about 27% of the proteins had Lys6 labeled peptides. The label incorporation seems to be related to the protein function and localization as shown in the GO (gene ontology) map in Figure 4.

Isolated primary T cells were stimulated by PMA and ionomycin, and incubated for 2-3 days, resulting in 3-6 cell divisions. Due to the excessively fast proliferation, however, almost as many cells died via apoptosis, thus yielding approximately the same to double number of cells as originally seeded for wild-type cells. KO cells, however, yielded a little less than original number and 90% of the cell died after irradiation where more than 70% of WT cell survived. Originally, we planned to compare WT and KO cells via the SILAC labeling method.

FIGURE 4. Incorporation of Lys6 SILAC label in CD8+ T cells depends on protein function and localization



Mitochondrial and energy metabolism related proteins such as ATPase or GTPase seem to incorporate the SILAC label well in mouse CD8+ T cells.

Structural and RNA-related proteins were poorly labeled. Neither proteins involved in carbohydrate metabolism nor proteases or proteins localized in the endoplasmic reticulum incorporated the label.

In total, about 27% of the proteins in mouse CD8+ T cells showed incorporation of the Lys6 SILAC label.

GO= gene ontology

However, growth of these primary cells in the SILAC medium was very poor resulting in a recovery of only ~10% of number of cells compared to our standard DMEM. This problem appeared to be cell-type specific, as a colon carcinoma cell line used as a control grew normally. Since SILAC medium uses dialyzed FCS, the growth of primary T cells in DMEM containing either normal FCS or 10K cut-off dialyzed FCS was investigated. The use of dialyzed FCS reduced growth by 20%. We then checked whether the use of non-dialyzed FCS would improve the growth rate in SILAC media. However, the recovery was still only 20% of live cells from WT and 4% from KO mice compared to our standard DMEM. We decided to use a label-free approach in order to identify and quantify proteins differentially expressed between WT and TPPII KO primary T cells.

The label-free quantitation workflow, as shown in Figure 5, does not rely on incoporation of heavy labeled amino acids, but on the reproducibility of the LC-MS runs. With this approach we could quantifiy several hundreds of proteins, most of them high-abundance proteins. Many proteins were down-regulated in TPPII KO T-cells. Compared to WT-cells, few proteins were up-regulated in TPPII KO T cells.

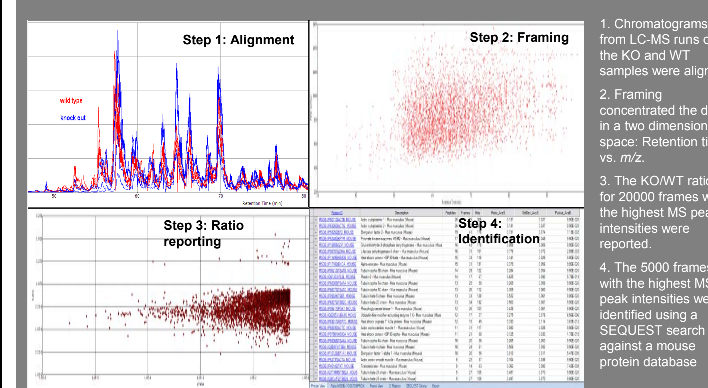
However, among the down-regulated several interesting proteins could be identified. T-complex protein (TCP) 1, a cytosolic chaperonin-containing t-complex protein 1 (CCT), is a large (approximately 900 KDa) protein consisting of eight subunits, that plays an important role in the folding of proteins in the eukaryotic cytosol as shown in Figure 6. CCT preferentially recognizes quasi-native (or partially folded) intermediates, especially those displaying hydrophobic surfaces. Actin, tubulin, and several other proteins are known to be folded by CCT, and about 15% of newly translated proteins in mammalian cells are folded by CCT.

We found alpha, beta, gamma, delta, epsilon, eta and zeta subunits down regulated in our biological replicas. Actin and several tubulin isoforms, i.e. client proteins of CCT, were also found down regulated in TPPII KO T cells. Although the connection between the aminopeptidase TPPII deficiency and CCT down-regulation it is not clear, they do share a common role in antigen presentation. CCT is the cytosolic component that binds post-proteasomal cleavage products, protects them from degradation and transfers them to the endoplasmic reticulum for antigen presentation on MHC class I molecules.

Conclusions

Primary mouse CD8+ T cells grew poorly in the SILAC medium used in this study. Only about 27% of the proteins incorporated Lys6 within 3 days of culture. The label incorporation seems to be related to protein function and localization. An improved quantitative comparison might require either an optimized medium formulation or a total animal SILAC approach.

FIGURE 5. Workflow for label free quantitation of protein samples



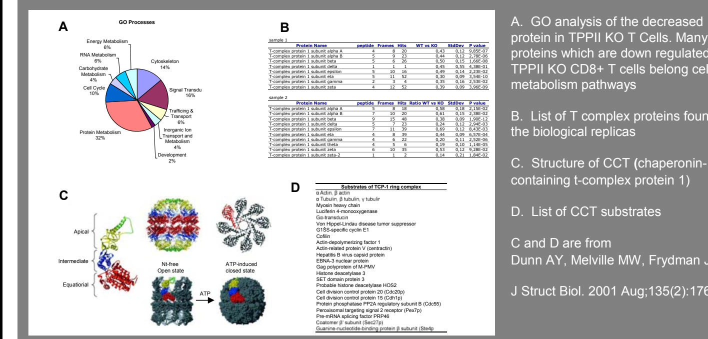
1. Chromatograms from LC-MS runs of the KO and WT samples were aligned.

2. Framing concentrated the data in a two dimensional space: Retention time vs. *m/z*.

3. The KO/WT ratios for 20000 frames with the highest MS peak intensities were reported.

4. The 5000 frames with the highest MS peak intensities were identified using a SEQUEST search against a mouse protein database

FIGURE 6. Proteins down-regulated in TPPII KO CD8+T cells - CCT as a possible down-stream mediator of enhanced apoptosis in TPPII KO CD8+T cells



A. GO analysis of the decreased protein in TPPII KO T Cells. Many proteins which are down regulated in TPPII KO CD8+ T cells belong cell metabolism pathways

B. List of T complex proteins found in the biological replicas

C. Structure of CCT (chaperonin-containing t-complex protein 1)

D. List of CCT substrates

C and D are from
Dunn AY, Melville MW, Frydman J.

J Struct Biol. 2001 Aug;135(2):17684.

Label-free quantitation of the samples revealed several interesting proteins and pathways, which are down regulated in TPPII knock-out primary T cells during activation.

- T-complex protein (TCP) 1, a cytosolic chaperonin-containing T-complex protein 1 (down regulated)
- Proteins from the glycolysis pathway (down regulated)

We did not find proteins which are directly related to apoptosis pathways. However, the down-regulation of actin and tubulin, possibly due to the lack of TCP1, might negatively affect fast-dividing cells. Also many abundant housekeeping proteins (e.g. elongation factor, HSP, and proteasome subunits) were found down regulated.

A depletion of high-abundance proteins of the cell lysate prior to LC-MS analysis might help to identify less-abundant proteins.

No proteins directly linked to TPPII could be found. A comparison of the peptide level between WT and KO cells by MS might give further information whether certain peptides accumulate in the absence of TPPII in the cell.

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