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Characterization of the endopeptidase activity of tripeptidyl-peptidase II

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ABSTRACT

Tripeptidyl-peptidase II (TPP II) is a giant cytosolic peptidase with a proposed role in cellular protein degradation and protection against apoptosis. Beside its well-characterised exopeptidase activity, TPP II also has an endopeptidase activity. Little is known about this activity, and since it could be important for the physiological role of TPP II, we have investigated it in more detail. Two peptides, Nef_{69-87} and LL37, were incubated with wild-type murine TPP II and variants thereof as well as TPP II from human and *Drosophila melanogaster*. Two intrinsically disordered proteins were also included in the study. We conclude that the endopeptidase activity is more promiscuous than previously reported. It is also clear that TPP II can attack longer disordered peptides up to 75 amino acid residues. Using a novel FRET substrate, the catalytic efficiency of the endopeptidase activity could be determined to be 5 orders of magnitude lower than for the exopeptidase activity.

1. Introduction

Tripeptidyl-peptidase II (TPP II) is a cytosolic subtilisin-like serine peptidase with a very large (>4 MDa) homooligomeric quarternary structure [1]. It is present in most eukaryotes and as the name implies, the main activity is removal of tripeptides from the N-terminus of longer peptides. In addition to the exopeptidase activity, TPP II also exhibits an endopeptidase activity [2]. This is essential for the generation of at least one MHC class I epitope [3], although the overall role for TPP II in the generation of epitopes for MHC class I antigen presentation is disputed [4–9]. TPP II is held to be of importance in intracellular protein degradation, as it can produce easily digestible substrates for aminopeptidases from the peptides released by the proteasome (the majority of which is 5–8 amino acid residues [10]) and could thereby facilitate the release of free amino acids. Indeed, TPP II has been reported to be upregulated during conditions of accelerated protein degradation, such as in muscle tissue during sepsis [11] and serum starvation in cell cultures [12]. Cells with elevated levels of TPP II seem to be more resilient towards inhibition of the proteasome [13], and to form more rapidly growing tumours *in vitro* [12]. This is ominous with regard to the proteasome as a cancer drug target, since resistance to drugs might occur due to increased levels of TPP II [14].

Cytosolic protein degradation is usually carefully regulated, since uncontrolled proteolysis would be deleterious to the cell and a waste of energy. There has been no feedback regulation mechanism reported for TPP II to date. Instead, activity is proposed to be controlled by complex formation, since dimers have only 10% of the activity of the full-size complex [15]. In the complex, a substrate has to penetrate an intricate cavity system with two openings of 20×22 Å and 15×35 Å to reach the active site, which would sterically hinder proteins from being degraded [16]. In accordance with this, TPP II has never been reported to degrade intact proteins and the largest substrate to be cleaved by TPP II is a 41 amino acid long peptide [2].

Our previous work has demonstrated that two glutamate residues (Glu-305 and Glu-331 in murine and human TPP II) in the active site are important for exopeptidase activity in mTPP II [17]. At least one of these, Glu-331, seems to form a salt bridge to the N-terminal amino group of the substrate, positioning it for cleavage at the third peptide bond from the N-terminus [17]. Recently, a study confirmed the importance of the homologous glutamate residues in dTPP II (Glu-312 and Glu-343) for the exopeptidase activity, and proposed a role for Glu-312 in hindering peptides from being cleaved endopeptidolytically [18].

Although quite low compared to the exopeptidase activity, the endopeptidase activity is nevertheless claimed to be as efficient

Abbreviations: AAF-*p*NA, alanylalanylphenylalanyl-*para*nitroanilide; ACTR, activator for thyroid hormone and retinoid receptors; pKID, phosphorylated kinase inducible activation domain; TPP II, tripeptidyl-peptidase II, the species is indicated by a lower case letter; m, murine; h, human; d, *Drosophila melanogaster*; wt, wild-type.

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as the proteasome at degrading at least some substrates [2]. The characteristics of the endopeptidase activity are still largely unknown. So far, only 10 cleavage sites in four different peptides have been reported [2,3,18]. The endopeptidase activity shows a preference for basic amino acids in the P_1 position [2,3], using the nomenclature of Schechter and Berger [19], in contrast to the exopeptidase activity, which has a preference for aliphatic and aromatic amino acid residues in the P_1 position [20,21]. In addition, it has been claimed that the endopeptidase activity is dependent on a free N-terminus in the peptide substrate, even though it does not cleave at a specific position in the peptide [22].

In this work, we have investigated the substrate specificity of the endopeptidase activity of TPP II from mouse, human and fruit fly, as well as a mutated variant of the murine enzyme. In addition, we investigated the maximum length of peptides TPP II can digest. To this effect, we incubated the enzyme with peptides and analysed the cleavage products with MALDI-TOF MS and HPLC. For further measurements of the kinetics of the endopeptidase activity, a FRET substrate was designed.

2. Materials and methods

2.1. Enzymes and peptides

Recombinant murine TPP II (mTPP II) and mutant variants thereof were expressed in *Escherichia coli* and purified as described previously [23]. Human TPP II was purchased from Enzo Life sciences (Farmingdale, USA). Recombinant TPP II from *Drosophila melanogaster* was expressed in *E. coli* and purified by polyethylene imine- and $(NH_4)_2SO_4$-precipitation followed by size exclusion chromatography on a Sepharose CL-4B column, a procedure similar to that in [24]. The Nef_{69-87} peptide (PVTPQVPLRPMTYKAAVDL) with amidated C-terminus was purchased from jpt Peptide Technologies Gmb (Berlin, Germany), while LL37 (LLGDFFRKSKEKI GKEFKRIVQRIKDFLRNLVPRTES) was synthesised as described [25]. Synthesized human pKID (residues 116–149) [27,28], with additional cysteines as the first and last residue was purchased from GL Biochem (Shanghai, China). Human ACTR (residues 1018–1088) [26] plus an N-terminal Gly-Ser-Met-Gln extension was expressed in *E. coli* as an N-terminally his-tagged lipoyl fusion protein (with a thrombin cleavage site preceding the construct). The cells were lysed by sonication and centrifuged at 70,000*g*. The supernatant was filtered and loaded onto a Ni–Sepharose fast flow column (GE Healthcare). The his-tagged fusion protein was eluted using 250 mM imidazole and dialyzed against 20 mM Tris, pH 8.0. The dialyzed sample was loaded onto an anion exchange source-30 Q (GE Healthcare) column, and eluted using a sodium chloride gradient. The lipoyl protein was cleaved off using thrombin (GE Healthcare) and separated from ACTR by loading the protein solution onto the Ni-Sepharose fast flow column. This was followed by a reversed phase chromatography step, using a C-8 column (Grace Davison Discovery Sciences). The identity of purified ACTR was verified by MALDI-TOF MS.

2.2. Endopeptidolytic cleavage assays

TPP II (1–174 nM) was incubated with 48–163 μM peptide in 17 mM potassium phosphate pH 7.5 with 5–8.3% (w/v) glycerol and 0.2 mM DTT in a total volume of 50 μl at 37 °C for 3–48 h. For hTPP II, 50 μM of the proteasome inhibitor MG-132 (Sigma–Aldrich, St. Louis, USA) was added, as per the manufacturer's recommendation. With pKID the peptide concentration was kept at 410 μM. For analysis, 10 μl of the reaction mixture was separated by reversed phase HPLC on a Reprosil C18 column (5 μm particle size, 150 × 2 mm) and a two-solvent system: (A), 0.055% (v/v) trifluoroacetic acid in water and (B), 0.05% (v/v) trifluoroacetic acid and 80% (v/v) acetonitrile in water, gradient 5–80% B in 40 min, at a flow rate of 0.25 ml/min. Peptide content was monitored at 214 nm using a Merck Hitachi L-4200 UV–VIS detector and samples were collected and further analysed by MALDI-TOF MS [29]. Alternatively, samples were diluted 10-fold in water and analysed directly by MS. Fragments were identified using FindPept [30].

2.3. Activity measurements

The FRET substrate PVTPQV-Lys(2-aminobenzoyl)-PMTY-KAAVDL-nitro-Tyr (jpt Peptide Technologies Gmb, Berlin, Germany) was dissolved in DMSO to a concentration of 13.8 mM. The enzyme (up to 52 nM) was incubated with the FRET substrate (at final concentrations of 1.25–20 μM) in 100 mM potassium phosphate buffer, pH 7.5, containing 15% glycerol and 2.5 mM DTT in a total volume of 100 μl. The fluorescence from excitation at 320 nm and emission at 440 nm was measured with 5 min intervals in an Infinite M200 plate reader (Tecan Group Ltd., Männedorf, Switzerland) for 10 h at 37 °C. The kinetic parameters were determined using the drc package in R [31,32] to fit the Michaelis–Menten equation for K_M and V_{max}, and the equation: $v_0 = \frac{\frac{V_{max}}{K_M} \times [S]}{1 + \frac{[S]}{K_M}}$ for V_{max}/K_M.

3. Results and discussion

3.1. Specificity of the endopeptidases activity for murine TPP II

The specificity of the endopeptidase activity was investigated using two different peptides: the HIV peptide Nef_{69-87}, which has previously been reported to be cleaved by hTPP II after Pro-72 and Lys-82 [3] and the human antimicrobial peptide LL37 [25], which has not been investigated earlier in this regard. The peptides were incubated with TPP II from different species and the products formed were analysed by HPLC and/or mass spectrometry. Identified fragments are listed in Supplementary Table 1. In order to assure that there were no confounding activities, control experiments were carried out with an inactive mutated variant of the enzyme (S449A [33]) purified using the same scheme as for wt mTPP II and the E331Q variant. In this way, any degradation products from contaminants co-purified with mTPP II wt or E331Q could be eliminated (Supplementary Table 1). Endopeptidase cleavage sites were defined from fragments that could not have been generated by the exopeptidase activity and that were detected in more than one experiment, preferably with two different enzyme preparations (Fig. 1). For Nef_{69-87}, degradation by the exopeptidase activity was prevented by proline residues in the fourth, seventh and tenth positions, since TPP II cannot cleave before or after proline at a measurable rate with the exopeptidase activity [21]. The cleavage pattern was not due to a contaminating carboxypeptidase activity, since the C-terminus of the Nef_{69-87} peptide was amidated, and no carboxypeptidase activity could be detected (data not shown).

In total, 18 unique cleavage sites were found for mTPP II in the two peptides (Fig. 1A and C). There was no highly significant deviation from a random cleavage pattern in mTPP II ($p > 0.1$, χ^2-test) in any of the ten positions closest to the scissile bond in either peptide, or when cleavage sites from both peptides were considered together (Fig. 2). This does not support earlier findings [2,3] that the endopeptidase activity of TPP II prefers basic residues in the P_1 position. However, no clear propensity for cleavage after aromatic or aliphatic amino acids could be documented either, although the exopeptidase activity displays some specificity for this type of residues [21]. Three cleavage sites after proline residues were noted, which has never been reported for the

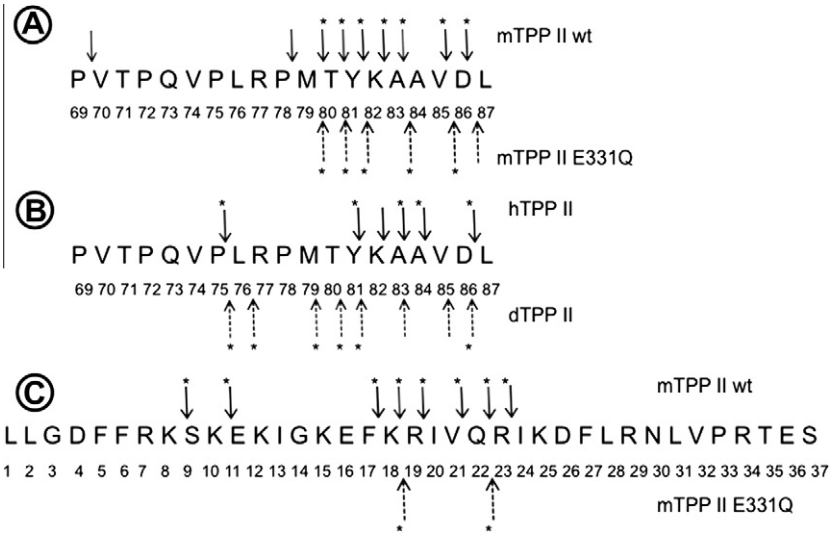

Fig. 1. Cleavage sites detected in $Nef_{69–87}$ and LL37. The peptide was incubated with the indicated enzyme for 24 h, and the mixture was analysed by MALDI-TOF-MS. Asterisk (∗) indicates cleavage sites found in the presence of butabindide. (A) Cleavage sites in $Nef_{69–87}$ incubated with mTPP II wt (solid arrows) and the mutant variant E331Q (dotted arrows). (B) Cleavage sites in $Nef_{69–87}$ incubated with TPP II from hTPP II (solid arrows) and dTPP II (dotted arrows). (C) Cleavage sites in LL37 incubated with mTPP II wt (solid arrows) and the mutant variant E331Q (dotted arrows).

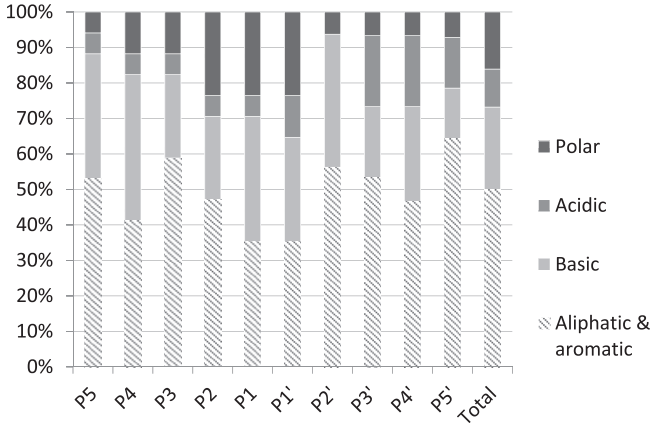

Fig. 2. Diagrams over endopeptidolytic cleavage sites found in $Nef_{69–87}$ and LL37 using mTPP II. The diagrams are compilations of amino acid type propensity in each position for each of the cleavage sites noted in Fig. 1. The nomenclature for cleavage sites by Schechter and Berger [19] is used. The distribution of amino acids in general in the two peptides is denoted by "Total".

exopeptidase activity. Hence, the endopeptidase activity is more promiscuous than previously reported [2], and certainly different from the specificity of the exopeptidase activity.

Comparisons between the cleavage sites for murine, human and *Drosophila* TPP II reveals that the sites are mostly overlapping, with some exceptions (Fig. 1B, Supplementary Table 1). Since $Nef_{69–87}$ was included as a positive control in this investigation it was curious to note that this peptide did not give the cleavage products earlier reported [3], not even with hTPP II. The C-terminal trimming of this epitope precursor was observed when $Nef_{69–87}$ was incubated with TPP II from all three species, as well as several longer and shorter cleavage products, but no trace of cleavage after Pro-72 could be detected. Hence, we cannot confirm the involvement of TPP II in the formation of the N-terminal end of the $Nef_{73–82}$ epitope.

3.2. Kinetics of the endopeptidase activity

The kinetics of the endopeptidase activity of mTPP II with $Nef_{69–87}$ and LL37 was investigated using HPLC. The specific activity was 0.45 ± 0.074 μmol s^{-1} g^{-1} for $Nef_{69–87}$ and 33 ± 20 nmol s^{-1} g^{-1} for LL37 (average ± SD for two experiments).

For a further kinetic analysis of the endopeptidase activity, a FRET substrate based on the $Nef_{69–87}$ peptide, i.e. PVTPQV-Lys(Abz)-PMTYKAAVDL-nitro-Tyr was used. With this substrate, kinetic parameters could be determined for the endopeptidase activity of wt mTPP II (Fig. 3). These parameters were $k_{cat}^{app} = 8 \pm 1 \times 10^{-4}$ s^{-1} and $k_{cat}^{app}/K_M = 63 \pm 9$ s^{-1} M^{-1}, using the assumption that each enzyme subunit is fully active. These values are 4-5 orders of magnitude lower compared to those for the exopeptidase activity of mTPP II measured with the substrate AAF-*p*NA,

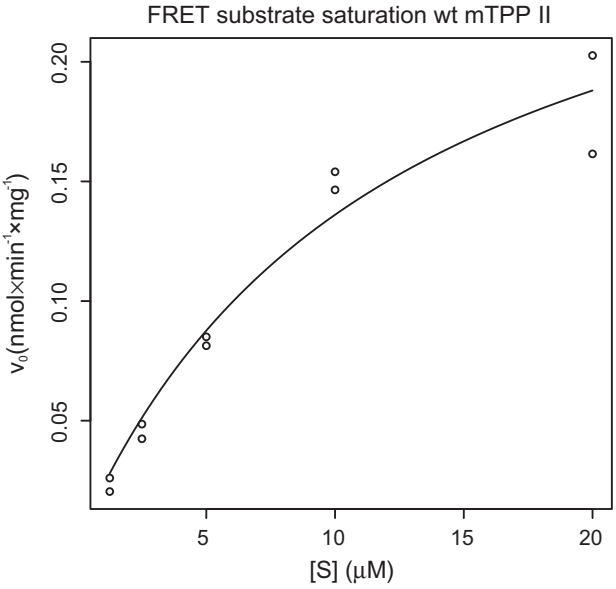

Fig. 3. Saturation curve for wt mTPP II using the FRET-substrate PVTPQV-Lys(Abz)-PMTYKAAVDL-nitro-Tyr. Enzyme, at a concentration of 52 nM, was incubated with substrate at the indicated concentrations for 10 h at 37 °C. Fluorescence change was monitored with 5 min intervals. The linear phase was used for determination of v_o.

which has a $k_{cat}^{app} = 96 \pm 0.9\ s^{-1}$, and a $k_{cat}^{app}/K_M = 3.5 \times 10^6 \pm 2 \times 10^5\ s^{-1}\ M^{-1}$ under similar conditions [23].

Surprisingly, K_M for the FRET substrate was estimated to be as low as 12 ± 3 μM, i.e. in the same range as K_M for the exopeptidase substrate AAF-*p*NA. This could be because of extensive interactions with the peptide, since peptide inhibitors have been shown to bind with higher affinity the longer they are [20,34].

3.3. Inhibition experiments

Inhibition experiments conducted in the presence of the specific inhibitor of the exopeptidase activity of TPP II, butabindide [35] resulted in essentially the same cleavage pattern as for the uninhibited wt enzyme (Fig. 1). Butabindide did not have any effect on the cleavage rate with Nef_{69-87}, but with LL37 a significant decrease in cleavage rate could be noted (20–40% inhibition, data not shown), probably due to an inhibition of the exopeptidase activity on this substrate. It has previously been noted that butabindide and other competitive inhibitors facilitate complex formation and therefore could have a stabilizing effect [15]. To see if butabindide had a stabilizing effect on TPP II during the long incubation period, two samples were incubated with mTPP II and Nef_{69-87} peptide with or without 4 μM butabindide, and diluted hundred-fold before exopeptidase activity was measured using the substrate AAF-*p*NA as described [36]. The results showed that although the activity had decreased by 90% after 24 h at 37 °C in the sample incubated with butabindide, the activity was lower by another order of magnitude for the sample incubated without butabindide. We concluded that butabindide indeed had a stabilizing effect on mTPP II, and that this might be the reason for the observed activity in the presence of butabindide.

To investigate if the endopeptidase activity is sensitive to other inhibitors, murine, human and *D. melanogaster* TPP II was incubated with Nef_{69-87} or LL37 in the presence of 2 mM Z-GLA (a molecule reported to inhibit TPP II with nanomolar affinity [37], although this has been questioned [38]), 1 mM PMSF (covalent inhibitor of serine peptidases) or 10 mM EDTA (chelating agent known to inhibit metallopeptidases). The cleavage reactions were analysed by both HPLC and MALDI-TOF MS. Based on the HPLC results, only PMSF seemed to give consistent inhibition with all enzymes tested, with LL37 as the substrate (data not shown). When considering the MS data, however, the only molecule to display consistent inhibition as deduced from the reduced peak intensities and number of detected cleavage products was EDTA, the metalloprotease inhibitor. This is not the expected result since TPP II is a serine peptidase, and the exopeptidase activity is not affected at the concentrations of EDTA used in this experiment. However, when the same inhibitors were assessed using the FRET substrate and mTPP II, PMSF and Z-GLA gave 60–70% inhibition and EDTA merely 28%, and it would thus seem that the endopeptidase activity is dependent on a serine peptidase active site.

3.4. TPP II can degrade intrinsically disordered protein domains

The longest peptide chain known to be degraded by TPP II is a 41-mer fragment of ovalbumin, Ova_{37-77} [2]. To test whether TPP II can degrade even longer peptides provided no secondary structure elements prevents access to the active site, mTPP II was incubated with two intrinsically disordered protein domains, ACTR (75 amino acid residues) and pKID (35 residues), and cleavage products were detected by MS after 24–48 h incubation. It is clear that both the exo- and endopeptidase activity of TPP II can attack these peptides, mTPP II more efficiently than dTPP II. However, the cleavage pattern is not reproducible with different enzyme preparations (data not shown).

3.5. No interaction with Glu-331

Fewer degradation products were found when Nef_{69-87} was incubated with the mutant variant E331Q than when incubated with wt mTPP II (Fig. 1). Although this could indicate a somewhat decreased binding affinity for the substrate, it is evident from kinetic data that this mutant variant had similar specific activity to the wt mTPP II enzyme (0.49 ± 0.08 vs. 0.22 ± 0.01 nmol min^{-1} mg^{-1} for the E331Q variant and wt, respectively, at 20 μM FRET substrate). Thus, if the endopeptidase activity is dependent on an interaction with the free N-terminus of the substrate, as has been proposed [22], this activity does not depend on Glu-331 to the same extent as the exopeptidase activity, where the K_M value increases by 3 orders of magnitude upon mutation to Gln [17]. This is consistent with findings for dTPP II, where mutation of the homologous residue, Glu-343, to Gln only had a minor effect on the endopeptidase activity [18].

3.6. Potential physiological importance

Endopeptidase activities are common among aminopeptidases, as this has been demonstrated for the lysosomal tripeptidyl-peptidase I, dipeptidyl-peptidase IV, and bleomycin hydrolase [39–41]. However, in the case of TPP II the rates of cleavage are so low that the physiological relevance of the endopeptidase activity is questionable. Under normal conditions, the proteasome degrades proteins to peptide products 3–24 amino acid residues long [10]. During conditions of hampered proteasome activity in combination with increased TPP II levels, such as in cells adapted to proteasome inhibitors [2] or in certain tumour cells [12], these products would likely increase in size and under these circumstances it is possible that the endopeptidase activity of TPP II could be important.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.144.

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