Structure-function studies of recombinant murine tripeptidyl-peptidase II: the extra domain which is subject to alternative splicing is involved in complex formation

Birgitta Tomkinson[a,*], Marete Hansen[a], Wing-Fai Cheung[a,b]

[a] *Department of Veterinary Medical Chemistry, Swedish University of Agricultural Sciences, Biomedical Center, Box 575, S-751 23 Uppsala, Sweden*
[b] *Department of Medical and Physiological Chemistry, Uppsala University, Biomedical Center, Box 575, S-751 23 Uppsala, Sweden*

Received 30 January 1997

Abstract Tripeptidyl-peptidase II (TPP II) is an exopeptidase with a remarkably high native M_r ($>10^6$). Recently, an alternatively spliced, murine cDNA variant was identified which contains an additional 39 bp, encoding 13 amino acids in the C-terminal end of the protein. The two enzyme variants were expressed in human kidney 293 cells. Both types of subunit were found to form the active oligomers. In addition, subunits containing the extra 13 amino acids formed an even larger complex eluting in the void volume of a Sepharose CL-4B column. Thus, it appears that this sequence is important for aggregation of subunits.

Key words: Tripeptidyl-peptidase II; Serine protease; Subtilisin type; Structure-function relationship; Complex formation; Oligomerization

1. Introduction

Tripeptidyl-peptidase II (TPP II) (EC 3.4.14.10) removes tripeptides from a free N-terminus of longer peptides [1,2]. It has a neutral pH optimum and a high molecular mass (138 kDa for the subunit and $>10^6$ Da for the native enzyme) [1,2]. The enzyme has a widespread distribution and has been found in the cytoplasm of cells from a number of different tissues and species [2,3]. Recently, a cholecystokinin-inactivating peptidase was purified from rat brain and identified as a membrane-bound variant of TPP II [4].

The cDNAs encoding the human and murine enzymes have been cloned and the N-terminal part of the enzyme shown to be similar to serine peptidases of the subtilisin-type [5–7]. The function of the remainder of the large subunit is not known. Recently, a splicing variant of the enzyme was identified, which contains an additional 39 bp, encoding the amino acids GQSAAKRQGKFKK in the C-terminal region [7]. The present paper describes the development of an expression system and its use in an investigation of the functional importance of the 13 amino acids which result from alternative splicing.

*Corresponding author. Fax: (46) (18) 55 07 62.
E-mail: Birgitta.Tomkinson@vmk.slu.se

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; GPI, glycosyl phosphatidylinositol; PLC, phosphoinositol phospholipase C from *B. cereus*; pNA, *p*-nitroanilide; TPP II, tripeptidyl-peptidase II.

2. Materials and methods

2.1. Preparation of constructs

Murine full-length clones, with (m(+)TPP) or without (m(−)TPP) the extra 39 bp [7], were constructed in pUC19 [8] from different clones [7] by sequential subcloning [9] (Fig. 1). The construct was partially sequenced to ensure that the cloning sites were intact. Besides the complete coding sequence, the constructs also encompass 29 bp of the untranslated 5′-end and 132 bp or 140 bp of the untranslated 3′-end. The 3.9 kb *Eco*RI fragments were cloned into the pcDNA 3 expression vector (Invitrogen). Clones with the insert in both sense and antisense directions were selected and purified.

2.2. Cells and transfection

The human embryonic kidney cell line 293 (ATCC CRL 1573) was maintained in DMEM with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37°C in a humidified 5% CO_2 atmosphere. Transfection of plasmids into the cells was performed by the calcium phosphate precipitation method [10]. For transient expression of the recombinant protein, 10 μg DNA was used for transfection of 1×10^6 cells in a 60 mm culture dish. The cells were harvested and used for Western blot and activity measurements 2 days after transfection.

For permanent expression of recombinant protein, the 293 cells were transfected with 3 μg of plasmid DNA. After 2 days, transfected cells were trypsinized and transferred to two 10-cm culture dishes. Stable transformants were selected and picked after growing the cells for 10–14 days in DMEM/F-12 with 10% fetal calf serum and 600 μg/ml of Geneticin (G418, Life Technologies, Ltd).

2.3. Preparation of cell extracts

For TPP II-activity measurements and Western blot analysis 5–10×10^6 cells were lysed in 50 mM Tris buffer, pH 7.5, containing 1% Triton X-100, 100 μM bestatin (Sigma), 2 mM EDTA and 50 μg/ml soybean trypsin inhibitor (Sigma) (100 μl/10^6 cells). Bestatin was included in order to ensure that the substrate was not attacked by aminopeptidases, whereas the other peptidase inhibitors were included for protection of TPP II in the extracts. The cell extracts were immediately centrifuged for 30 min at $14\,500\times g$ at 4°C and the supernatant was collected and diluted 10-fold in 100 mM potassium phosphate buffer, pH 7.5, containing 30% glycerol and 1 mM DTT. Under these conditions the activity appeared to be stable for at least 1 week at 4°C, but did not withstand freezing at −20°C.

2.4. TPP II-activity measurements

For activity measurements, 100 μl of the diluted cell extracts (corresponding to about 10^5 cells) were incubated with 0.2 mM Ala-Ala-Phe-pNA (Bachem) in 0.1 M potassium phosphate buffer, pH 7.5, containing 15% glycerol and 2.5 mM DTT at 37°C, in a total volume of 200 μl. The change in absorbance at 405 nm was measured in an ELISA plate reader Multiscan PLUS (Labsystems).

Protein concentration was measured using the modified Bradford method [11,12] with BSA as standard.

2.5. Western blot analysis

Sample buffer was added to the diluted cell extracts (corresponding to about 10^4 cells) to give final concentrations of 2.3% SDS, 5% β-mercaptoethanol and 10% glycerol. The samples were heated for 5 min at 95°C before they were loaded onto an 8% polyacrylamide gel. The

PII S0014-5793(97)00173-7

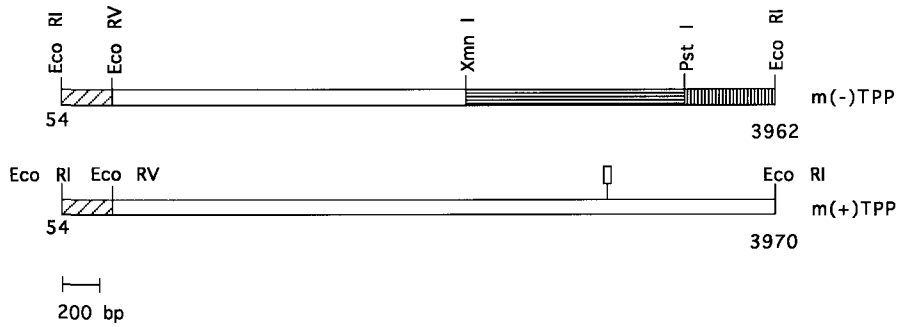

Fig. 1. Construction of murine full-length clones without (m(−)TPP) or with (m(+)TPP) the extra 39 bp insert. The different clones used for the construct are indicated by: (diagonally hatched bars) 5′ RACE; (empty bars) N10; (horizontally hatched bars) K1; and (vertically hatched bars) K8. All clones except K8 have been described previously [7]. Only relevant restriction sites are shown. The inserts were cloned into pUC19 in the antisense direction. The numbers of the first and last bp in the constructs correspond to those of the published sequence [7].

SDS-PAGE and Western blot analysis were performed essentially as described previously [13].

2.6. Phosphoinositol phospholipase C treatment of cells

Intact cell monolayers ($5–10\times10^6$ in 60-mm dishes) with a high expression of m(+)TPP or m(−)TPP were washed with PBS and then incubated with PLC from *B. cereus* (Boehringer Mannheim) (3 U/ml in 500 μl of PBS, pH 7.4). As a control, cells were also incubated with PBS alone or with 50 mM Tris buffer, pH 7.5, containing 1% Triton X-100. After incubation for 45 min at 37°C the cells were suspended in the original 500 μl of incubation buffer and the cell suspension was centrifuged for 30 min at $14\,500\times g$. The enzymatic activity in the supernatant was measured as described previously. In order to release the remaining TPP II activity, the pellet was dissolved in 500 μl 50 mM Tris buffer, pH 7.5, containing 1% Triton X-100, centrifuged as reported above, and the activity in the new supernatant was measured as described.

As a positive control, the PLC used was investigated for its ability to release alkaline phosphatase from sheep kidney slices. The enzyme (at a concentration of 20 mU/ml or 2 U/ml) released as much alkaline phosphatase during 1 h at 37°C in the PBS buffer used above as under the standard conditions normally applied [14] (data not shown).

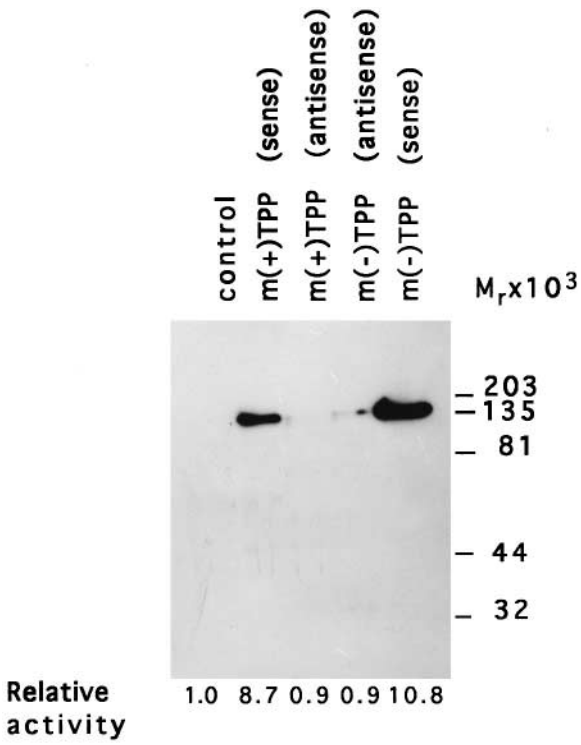

Fig. 2. Transient expression of murine full-length cDNA with (m(+)TPP) or without (m(−)TPP) the extra 39 bp. The cell extracts were analysed by Western blotting and for TPP II activity as described under Section 2. The activity in the extract from the control cells is 0.11 ΔA_{405} min^{-1} mg^{-1} protein.

3. Results

3.1. Control of expression system

The murine full-length TPP II cDNA with (m(+)TPP) or without (m(−)TPP) the extra insert was constructed as described in Fig. 1, cloned into the expression vector pcDNA 3 and used for transfection of human embryonic kidney 293 cells. Murine cDNA was chosen since the complete extra insert was cloned in this species [7] whereas only part of the corresponding human cDNA was cloned [5]. As a control, the cells were also transfected with the vector alone. The 293 cells have an endogenous expression of TPP II, which is not influenced by the transfection with vector only. Transient expression of the murine cDNAs showed that both types of subunits are active (Fig. 2). Moreover, the cells transformed with the antisense variants have as high an endogenous TPP II activity as the control cells.

In addition, stable transformants have been selected. The clones with the highest expression of the two subunit types showed 7-fold (m(−)TPP-17) and 6-fold (m(+)TPP-21) greater activity than control cells, respectively (Table 1). None of the high expressing clones showed any obvious phenotypic changes with respect to morphology or growth rate.

3.2. Formation of the active oligomers

It was previously demonstrated by electron microscopy that TPP II is represented by large oligomeric structures, and that these structures are a prerequisite for full enzymatic activity

Table 1
TPP II activity in extracts from different stable transformants

Stable transformant	(*n*)	Activity ± S.D. (ΔA_{405} min^{-1} mg^{-1} protein)
Control cells	(9)	0.12 ± 0.02
m(+)TPP-21	(7)	0.78 ± 0.21
m(+)TPP-11	(4)	0.39 ± 0.16
m(+)TPP-14	(3)	0.36 ± 0.04
m(−)TPP-17	(8)	0.88 ± 0.29
m(−)TPP-23	(3)	0.79 ± 0.41
m(−)TPP-5	(4)	0.39 ± 0.17

Extracts were prepared and activity measured as described under Section 2. (*n*) indicates the number of measurements.

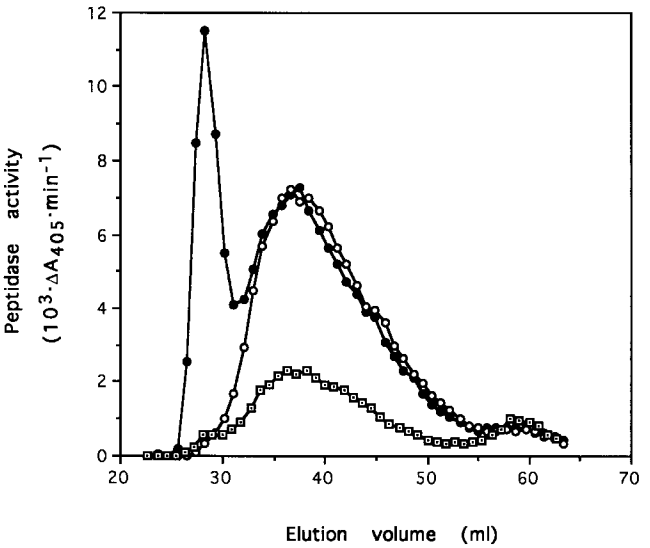

Fig. 3. Sepharose CL-4B chromatography of extracts from control cells and the high-expressing transformant cells m(+)TPP-11 and m(−)TPP-5. Extracts from 15–20×10^6 cells were prepared essentially as described under Section 2 except that the protease inhibitors were omitted and that 10 μl of lysis buffer/10^6 cells was used. The diluted supernatant was loaded onto a Sepharose CL-4B column (1×90 cm) equilibrated and eluted with 100 mM potassium phosphate buffer, pH 7.5, containing 30% glycerol and 1 mM DTT. Fractions of 0.9 ml were collected at a flow rate of 7 ml/h. The TPP II activity in the fractions was measured as described under Section 2. (□) Activity in control cells; (●) activity in m(+)TPP-11 cells; (○) activity in m(−)TPP-5 cells.

[15]. In order to investigate the degree of formation of the oligomers, extracts from control cells and high expressing clones were subjected to chromatography on a Sepharose CL-4B column (Fig. 3). The active enzyme from the control cells was eluted with a K_{av} value around 0.2, corresponding to an M_r of about 4×10^6, which is comparable with results obtained previously with TPP II from rat liver extract [1] and human erythrocytes [2]. In addition to an increase of this peak, the subunits containing the 13 extra amino acids evidently give rise to a complex which elutes in the void volume of the column, indicating that the molecular mass is $>10^7$, and thus is even larger than the normal 4×10^6 Da oligomers. Clones expressing the murine subunits lacking the 13 amino acids do not contain the large complex (Fig. 3). However, it is interesting to note that the control cells show a small, but reproducible, activity shoulder eluting in the void volume of the column. The immunoreactivity co-eluted with the activity, indicating that the main part of the protein is active (data not shown). Similar chromatographic profiles were obtained for each of two other high-expressing clones of m(+)TPP and m(−)TPP (data not shown).

The void-eluting complex is stable, i.e. the activity persists for at least 1 week at 4°C and elutes at the same position if rechromatographed on a Sepharose CL-4B column. However, the material is less stable after rechromatography.

3.3. Phospholipase C treatment

It was suggested by Rose et al. [4] that a splicing variant of TPP II could be membrane bound through a glycosyl phosphatidylinositol (GPI) anchor. Phospholipase C (PLC) from *B. cereus* can cleave such linkages [16] and was shown to release TPP II from synaptosomal membranes [4]. To determine whether the m(+)TPP splicing variant described here could be involved in membrane binding, the ability of PLC to release TPP II from cells with a high expression of m(+)TPP and m(−)TPP, respectively, was compared (Table 2). Evidently, no enzymatic activity could be released from either cells through this treatment, not even if the cells were incubated with 5 U of PLC for 4 h at 37°C (data not shown). The PLC used was active, as demonstrated by its ability to release alkaline phosphatase from sheep kidney slices, as described under Section 2.

4. Discussion

This paper describes the expression of murine TPP II in 293

Table 2
Effect of PLC treatment of intact cells

Treatment	Cells	Released activity (ΔA_{405} min^{-1} mg^{-1})	Remaining activity (ΔA_{405} min^{-1} mg^{-1})	Released activity (%)
PBS only	m(+)TPP	0.09	0.70	11
	m(−)TPP	0.12	1.07	10
PBS+PLC	m(+)TPP	0.05	0.76	6
	m(−)TPP	0.06	1.04	5
Tris buffer+Triton X-100	m(+)TPP	0.74	0.06	92
	m(−)TPP	1.02	0.05	95

Conditions are described under Section 2. The data represent one experiment out of three. Two different batches of PLC were used in the experiments.

Table 3
KEKE motif in TPP II compared with proteasome associated proteins

m(+)TPP	....KKAGQSAAKRQGKFKKDVIPVHYYLIPPPI**KIKNGSKDKEKDSEKEKDLKEE**.....
REGULATOR	....**KEKEKEERKKQQEKEDKDEKKKGEDEDK**.....
PROTEASOME C9	....**KKHEEEEAKAEREKKEKEQREKDK**.....
PROTEASOME 28.1	....**KIIEKEKEEELEKKKQK**.....
26S SUBUNIT 12	....**EKKEGQEKEESKKDRKEDKEKDKDKEKSDVKKEEKK**....

A KEKE motif (bold-face) is defined as more than 12 amino acids in length, devoid of W, Y, F, or P, where more than 60% of the amino acids are K or E/D, and where five positive or negatively charged residues do not occur in a row [18]. The TPP II sequence is from [7] and the proteasome sequences from [18]. The extra 13 amino acids which are subject to alternative splicing are underlined.

cells. Stable clones with a 3–7-fold higher enzymatic activity than that endogenously expressed have been isolated. Evidently, the over-expression is not lethal to the cells. This could indicate that the enzymatic activity can be regulated in the cells or that the substrate specificity of the enzyme, even though it is fairly broad [1,2], is still restrictive enough to prevent TPP II from damaging proteins or peptides in the cell. The establishment of an expression system for the enzyme is of great importance for further studies of structure-function relationships for TPP II.

It is demonstrated that the extra 13 amino acids, which result from alternative splicing, are involved in formation of a larger complex (Fig. 3). Whether the extra amino acids are important for aggregation of the subunits, or for the formation of complexes with other proteins or other cellular components, is not known. Neither is it known if the larger complex is functionally different. This will be the subject of future investigations. That the large complexes, eluting in the void volume of a Sepharose CL-4B column, are not artefacts is supported by three facts. Firstly, the same peak was seen in the analysis of three different, stable transformants expressing high levels of m(+)TPP. Secondly, there was a small activity shoulder in this position when the control cells were analyzed. Thirdly, a similar high-molecular weight activity shoulder is seen in the Sepharose CL-4B chromatogram when TPP II is purified from human erythrocytes (cf. Fig. 1 in [2]). It could be speculated that when both types of subunit are expressed at the same time, more of the normal oligomers and less of the high molecular weight complex are formed. Preliminary data from experiments with stable transformants which express both types of subunit simultaneously corroborate this (B. Tomkinson, unpublished).

The sequence of TPP II [5,7] reveals a so-called KEKE motif [17] on the C-terminal side of the insertion site of the extra 13 amino acids (Table 3). It has been suggested that these motifs with alternating positively and negatively charged amino acids promote protein-protein interactions [17,18] and are also involved in Ca^{2+} binding [19]. It could be speculated that this motif may be involved also in the formation of the TPP II oligomers. The effect of the extra 13 amino acids (GQSAAKRQGKFKK) could then be to interact with a KEKE motif in another protein or on another subunit. Alternatively, the insertion of the extra domain could change the protein conformation and expose the KEKE motif. The understanding of the potential role of this motif will demand more extensive protein engineering studies, e.g. removing only this part or changing specific amino acids in this motif.

Finally, it was investigated whether the high molecular weight complex was lipid bound. In a recent publication, Rose et al. demonstrated that a membrane associated variant of TPP II was involved in the inactivation of cholecystokinin [4]. The authors suggested that the enzyme was bound on the outside of the plasma membrane by a GPI anchor and that a splicing variant of TPP II was involved. However, in the present work treatment of intact cells with PLC, which would break the GPI anchor [4,16], did not have any effect (Table 2). Therefore, the extra 13 amino acids in m(+)TPP does not appear to be involved in membrane binding through a GPI anchor.

Acknowledgements: The skillful technical assistance of Ann-Louise Bergefur is gratefully acknowledged. This work was supported by the Medical Research Council (project 09914), Stiftelsen Lars Hiertas Minne and Magn. Bergvalls Stiftelse.

References

[1] Bålöw, R.-M., Ragnarsson, U. and Zetterqvist, Ö. (1983) J. Biol. Chem. 258, 11622–11628.
[2] Bålöw, R.-M., Tomkinson, B., Ragnarsson, U. and Zetterqvist, Ö. (1986) J. Biol. Chem. 261, 2409–2417.
[3] Bålöw, R.-M. and Eriksson, I. (1987) Biochem. J. 241, 75–80.
[4] Rose, C., Vargas, F., Facchinetti, P., Bourgeat, P., Bambal, R.B., Bishop, P.B., Chan, S.M.T., Moore, A.N.J., Ganellin, C.R. and Schwartz, J.-C. (1996) Nature 380, 403–409.
[5] Tomkinson, B. and Jonsson, A.-K. (1991) Biochemistry 30, 168–174.
[6] Tomkinson, B. (1991) Biomed. Biochim. Acta 50, 727–729.
[7] Tomkinson, B. (1994) Biochem. J. 304, 517–523.
[8] Vieira, J. and Messing, J. (1982) Gene 19, 259–268.
[9] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning: A laboratory manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[10] Graham, F.L. and van der Eb, A.J. (1973) Virology 52, 456–461.
[11] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[12] Read, S.M. and Northcote, D.H. (1981) Anal. Biochem. 116, 53–64.
[13] Tomkinson, B. and Nyberg, F. (1995) Neurochem. Res. 20, 1443–1447.
[14] Ikezawa, H. and Taguchi, R. (1981) Meth. Enzymol. 71, 731–741.
[15] Macpherson, E., Tomkinson, B., Bålöw, R.-M., Höglund, S. and Zetterqvist, Ö. (1987) Biochem. J. 248, 259–263.
[16] Low, M.G. and Saltiel, A.R. (1988) Science 239, 268–275.
[17] Realini, C., Rogers, S.W. and Rechsteiner, M. (1994) FEBS Lett. 348, 109–113.
[18] Realini, C., Dubiel, W., Pratt, G., Ferrell, K. and Rechsteiner, M. (1994) J. Biol. Chem. 269, 20727–20732.
[19] Realini, C. and Rechsteiner, M. (1995) J. Biol. Chem. 270, 29664–29667.