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The insert within the catalytic domain of tripeptidyl-peptidase II is important for the formation of the active complex

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Tripeptidyl-peptidase II (TPP II) is a large ($M_r > 10^6$) tripeptide-releasing enzyme with an active site of the subtilisin-type. Compared with other subtilases, TPP II has a 200 amino-acid insertion between the catalytic Asp44 and His264 residues, and is active as an oligomeric complex. This study demonstrates that the insert is important for the formation of the active high-molecular mass complex. A recombinant human TPP II and a murine TPP II were found to display different complex-forming characteristics when over-expressed in human 293-cells; the human enzyme was mainly in a nonassociated, inactive state whereas the murine enzyme formed active oligomers. This was surprising because native human TPP II is purified from erythrocytes as an active oligomeric complex, and the amino-acid sequences of the human and murine enzymes were 96% identical. Using a combination of chimeras and a single point mutant, the amino acid responsible for this difference was identified as Arg252 in the recombinant human sequence, which corresponds to a glycine in the murine sequence. As Gly252 is conserved in all sequenced variants of TPP II, the recombinant enzyme with Arg252 is atypical. Nevertheless, as Arg252 evidently interferes with complex formation, and this residue is close to the catalytic His264, it may also explain why oligomerization influences enzyme activity. The exact mechanism for how the G252R substitution interferes with complex formation remains to be determined, but will be of importance for the understanding of the unique properties of TPP II.

Keywords: tripeptidyl-peptidase II; complex formation; association/ dissociation; exopeptidase; serine peptidase.

Tripeptidyl-peptidase II (TPP II) (EC 3.4.14.10) is an enzyme with remarkable characteristics. It was discovered 1983 as an extralysosomal peptidase in rat liver [1] and has since been extensively characterized [2–6]. It is one of only two known mammalian tripeptide-releasing enzymes (reviewed in [7]). Native TPP II is a high-molecular mass protein where the subunit (138 kDa) forms a large oligomeric complex ($M_r > 10^6$) [2,8]. The enzyme has a catalytic domain of the subtilisin-type [4], but in comparison with other subtilases, it has a 200 amino-acid insertion between the Asp and His of the catalytic triad [5,9]. In addition, TPP II has a long C-terminal extension [5,9].

The widespread distribution and conserved amino-acid sequence would suggest that TPP II plays a role in general cytosolic protein turnover, probably in association with the proteasome [7]. When TPP II was induced in proteasome-deficient cells, it appeared to compensate for the partial loss of the proteasome activity [10,11], and over-expression of TPP II protected the cells from the effect of proteasome inhibitors [12]. In addition to this general role, more specific functions have also been suggested, e.g. an involvement of a membrane-bound form of TPP II in the inactivation of the neuropeptide cholecystokinin [6], and a role upstream of caspase-1 in *Shigella*-induced apoptosis [13]. It is therefore not surprising that when an efficient proteolytic system has evolved, it will be used for specific degradation of certain targets as well as functioning in less specific processes. This appears to be the case not only for the proteasome but also for TPP II, which shows that also exopeptidases are important in protein degradation [7].

An important question is how the enzymatic activity of TPP II is regulated, because, in contrast to most other subtilases, TPP II does not appear to be synthesized as a pro-protein [9], and specific physiological inhibitors of the enzyme have not been identified as yet. The substrate specificity of TPP II is fairly broad, i.e. a variety of different tripeptides can be released, even though the enzyme apparently cannot attack peptide bonds before or after a proline residue [1,2]. TPP II is highly dependent on a free N-terminus and the recently reported endopeptidase activity of the enzyme [11] is very low compared to the exopeptidase activity. All substrates that have been identified so far are oligopeptides of 4–41 amino acids [1,2,6,11] and the cleavage of native proteins by TPP II has not been described. The substrate specificity and oligomeric structure of TPP II could indicate that it is a self-compartmentalizing peptidase, similar to the proteasome [14]. The self-compartmentalization would thus protect the cell from uncontrolled proteolysis. This agrees with the observation that the enzyme is only fully active in the oligomeric complex. Native TPP II has been shown to dissociate spontaneously, resulting in a loss of 90% of the original specific activity. The dissociated enzyme can reassociate and the activity is concomitantly restored. This reactivation is enhanced by substrates and different competitive inhibitors [15], thus suggesting the involvement of the catalytic domain. Therefore, as suggested previously [8,15], association/dissociation

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Abbreviations: pNA, para-nitroanilide; TPP II, tripeptidyl-peptidase II; DMEM, Dulbecco's modified Eagle's medium.

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of the oligomeric complex could be a way of regulating the enzymatic activity.

In order to study the structural basis for complex formation, a previously developed expression system for TPP II has been used [16]. It was found that recombinant human TPP II and murine TPP II displayed different association/dissociation characteristics when overexpressed in human 293-cells. The main objective of the present work was to find an explanation for this phenomenon. It is demonstrated that the formation of the active complex is profoundly influenced by a single amino acid difference, i.e. G252R, in a region within the catalytic domain. This is the first evidence that this region is involved in the formation of the active complex.

MATERIALS AND METHODS

Construction of expression clones

A 3.9-kb *Kpn*I fragment, corresponding to the complete coding sequence of human TPP II and 23 and 145 bp of the untranslated 5′ and 3′ ends, respectively [17], was cloned into the pcDNA 3 expression vector (Invitrogen, Groeningen, the Netherlands) by conventional cloning techniques [18]. Clones with the insert in the sense direction were selected and purified. Chimeras were constructed in pUC19 by sequential subcloning [18] using different clones isolated previously [5,19,20]. Full-length constructs were excised with *Kpn*I or *Eco*RI and inserted into the pcDNA3 vector. Clones with the insert in the sense direction were selected and purified.

The rat *Eco*RV–*Sac*I fragment was amplified from rat liver RNA by use of two specific primers: 5′-GGTCAC GACTGATGGGAAAC-3′ and 5′-CCATGAGCTCCTC CACTGGT-3′ and the RT-PCR kit (PerkinElmer, Boston, MA, USA), except that Advantage polymerase (Clontech, Palo Alto, CA, USA) was used. The amplified fragment was digested with *Eco*RV and *Sac*I and cloned into the pBluescript SK + vector (MBI Fermenta, Vilnius, Lithuania) and the sequence was determined by sequencing in an ABI Prism 310 automatic sequencer. The *Eco*RV–*Sac*I fragment was cloned into a chimeric construct and the full-length chimera transferred to the pcDNA3 vector.

The Dhum clone, containing the human sequence resulting in a R252G substitution, was constructed by replacing the *Eco*RV–*Sac*I fragment in clone Bhum with the *Eco*RV–*Sac*I fragment from the human F5 clone described previously [19,20].

Cells and transfection

The human embryonic kidney cell line 293 (ATCC CRL 1573) was maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Paisley, Scotland, UK) with 10% (v/v) heat-inactivated fetal bovine serum, 100 U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin, at 37 °C in a humidified 5% CO_2 atmosphere. For the preparation of stable transformants, the constructs were introduced into 293-cells by the calcium phosphate precipitation method, and stable clones were selected by growing cells in 400 μg·mL^{-1} geneticin (Duchefa, Haarlem, the Netherlands), as described previously [16]. Clones expressing murine TPP II were isolated [16]. Cells transfected with the pcDNA3 vector alone were used as controls. The expression efficiency of the constructs was determined by Western blot analysis, and the two most efficient clones of each construct were selected for further characterization.

Preparation of cell extracts

Cells from stable transformants expressing recombinant TPP II [16] were harvested and lysed with 50 mM Tris buffer, pH 7.5, containing 1% (w/v) Triton X-100 (10 μL per 10^6 cells). The lysate was centrifuged for 30 min at 4 °C and 14 500 ***g***. The supernatant was collected and diluted 10-fold with 100 mM potassium phosphate buffer, pH 7.5, containing 30% (w/v) glycerol and 1 mM dithiothreitol. Diluted supernatants were used for activity assays, Western blots and gel filtration, as indicated.

Enzyme assay

Enzyme aliquots were incubated with 0.2 mM Ala-Ala-Phe-*p*NA (Bachem, Bubendorf, Switzerland) in 0.1 M potassium phosphate buffer, pH 7.5, containing 15% (w/v) glycerol and 2.5 mM dithiothreitol at 37 °C, in a total volume of 200 μL. The rate of change in absorbance at 405 nm was measured in a Multiscan PLUS ELISA plate reader (Labsystems, Helsinki, Finland) [21]. A molar absorbance of 9600 M^{-1}·cm^{-1} for *p*NA was used [22]. The activity was related to the total amount of protein in the sample, determined with a modified Bradford method [23,24], using BSA as the standard.

Gel filtration

Cell extracts were prepared as described above. The diluted supernatant (1.8 mL, corresponding to 1–2 × 10^7 cells) was loaded onto a Sepharose CL-4B (AP Biotech, Uppsala, Sweden) column (≈ 1 × 90 cm, several columns being used for the experiments). The column was equilibrated and eluted with 0.1 M potassium phosphate buffer, pH 7.5, containing 30% (w/v) glycerol and 1 mM dithiothreitol, at a flow rate of 6 mL·h^{-1}. Fractions of 1 mL were collected. The void-volume (V_o) and total volume (V_t) of the column were determined from the elution positions of Blue dextran (AP Biotech, Uppsala, Sweden) and dinitrophenol-β-Ala (Sigma), respectively. K_{av} values for different elution volumes (V_e) were calculated from $K_{av} = V_e - V_o/V_t - V_o$. Individual fractions were investigated through activity measurements and Western blot analysis.

Western blot analysis

Aliquots from fractions of the chromatography were mixed with SDS/PAGE sample buffer to give final concentrations of 2.3% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 10% (w/v) glycerol. The samples were heated for five minutes at 95 °C before they were loaded onto an 8% polyacrylamide gel. The SDS/PAGE and Western blot analysis were performed as described previously using affinity purified polyclonal chicken anti-(human TPP II) Ig [25]. The immunoreactivity was quantitated from scanned X-ray films by use of the MOLECULAR ANALYST software (Bio-Rad, Hercules, CA, USA).

RESULTS AND DISCUSSION

Complex-forming characteristics of recombinant human and murine TPP II

Expression of recombinant human TPP II, encoded by full-length cDNA, in 293-cells indicated that only part of the expressed protein was active. Although there was 8- to 10-fold more immunoreactive material in the high-expressing clones than in the control, according to densitometer scanning of a Western blot of cell lysates, the enzyme activity increased only threefold (data not shown). Investigation of the cell lysate by gel filtration demonstrated that a substantial part of the immunoreactive protein from the extract of an individual clone with a high expression of human TPP II eluted with a K_{av} of 0.55 and was virtually inactive (Fig. 1A). The M_r of this protein was $2–3 \times 10^5$ as determined through chromatography on a calibrated Sepharose CL-6B column (cf. [15]; data not shown). The experiment was repeated with two other high-expressing human clones with the same result. Evidently, only a fraction of the expressed protein had formed the large, active oligomers, which eluted at a K_{av} of 0.26. This was in contrast to stable transformants expressing the murine enzyme, where activity increased about eightfold, compared to the control cells. The majority of the protein was in the oligomeric form and coeluted with the activity upon gel filtration (Fig. 1B; [16]). The 293-cells used for the experiments have an endogenous expression of TPP II [16], and the activity in control cells, untransfected or transfected with vector alone, were used as a comparison (Fig. 1). In the control cells, the immunoreactivity followed the activity (data not shown).

The two forms of the enzyme, eluting at a K_{av} of 0.26 and a K_{av} of 0.55, respectively, will be referred to as 'associated' and 'nonassociated' throughout this work. It is not possible, however, to know whether the human enzyme never associates or whether it transiently associates and then dissociates. In general, the total amount of immunoreactive protein obtained from the human clone was lower than from the murine clone (Fig. 1). This may be due to the fact that nonassociated enzyme is more sensitive to proteolytic digestion than enzyme associated into the complex, as has been seen previously for purified human TPP II [26].

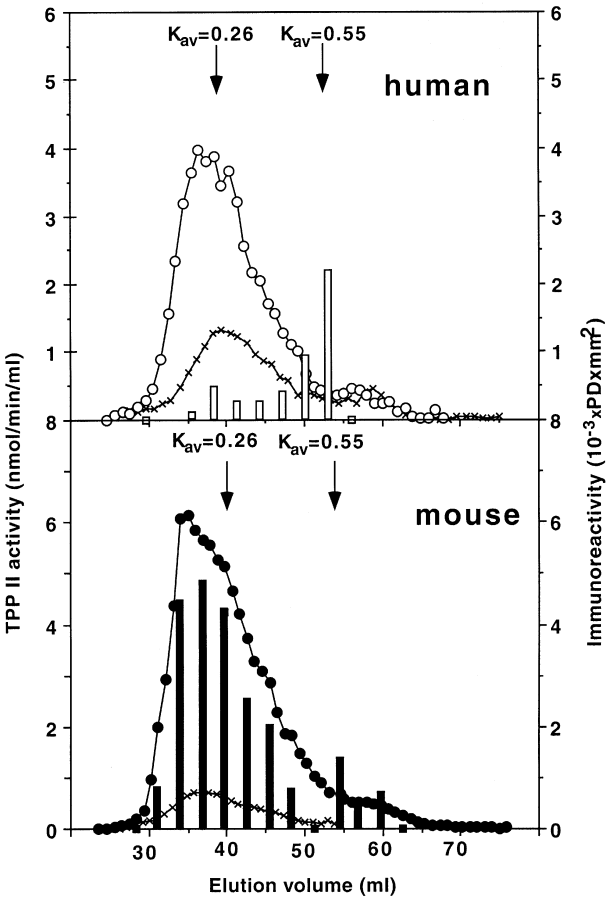

Fig. 1. Gel filtration of extracts of cells expressing recombinant human or murine TPP II. Cell lysates (corresponding to $1–2 \times 10^7$ cells) from stable transformants or control cells were loaded onto a Sepharose CL-4B column and chromatography was performed as described in Materials and methods. Enzyme activity was analysed by the standard assay and the immunoreactivity was detected by Western blot analysis and quantitated as described in Materials and methods. Open and filled circles indicate the activity, and open and filled bars the immunoreactivity (PD, pixel density) for human and murine TPP II, respectively. The enzyme activity in control cells is indicated (×). (A) Human TPP II and control cells ($V_0 = 27.5$ mL; $V_t = 76.7$ mL). (B) Murine TPP II and control cells ($V_0 = 26.5$ mL; $V_t = 74.7$ mL).

Identification of the region causing different association characteristics

The difference in association characteristics of the enzyme from the two sources was surprising because the sequence is extremely well conserved between the two species, i.e. 96% of the amino acids are identical and a number of the amino-acid differences are conservative [5]. A comparison shows that there is a cluster of amino-acid differences in the C-terminal part of the enzyme (Fig. 2A) where 13 of 44 amino acids are different. Therefore, chimeric enzymes with the N-terminal part from the human and the C-terminal part from the murine enzyme and *vice versa* were constructed by use of an *Xmn*I site. When stable transformants expressing these chimeric constructs were studied, it was evident that the sequence difference responsible for the lack of association of the human enzyme resided in the N-terminal part of the human enzyme (Figs 2B,C), not in the hypervariable C-terminal part. As 23 amino acids differ between the N-terminal part of the human and mouse enzyme, new chimeras were constructed by use of the *Eco*RV and *Sac*I sites in the cDNA and were then used to transform 293-cells. The region responsible for the different degree of association of the human and murine enzyme could be defined as being within the *Eco*RV–*Sac*I fragment of the enzyme (Figs 2B,C). This 591-bp fragment corresponds to 197 amino acids located between the Asp and His of the catalytic triad. Most other subtilases have about 20 amino acids in this region and the large insertion is a special feature of TPP II and pyrolysin [9,21]. There are, in total, 12 amino-acid differences between the human and mouse sequences in this region, and a number of them are conservative changes (e.g. Val → Ile) (Fig. 3).

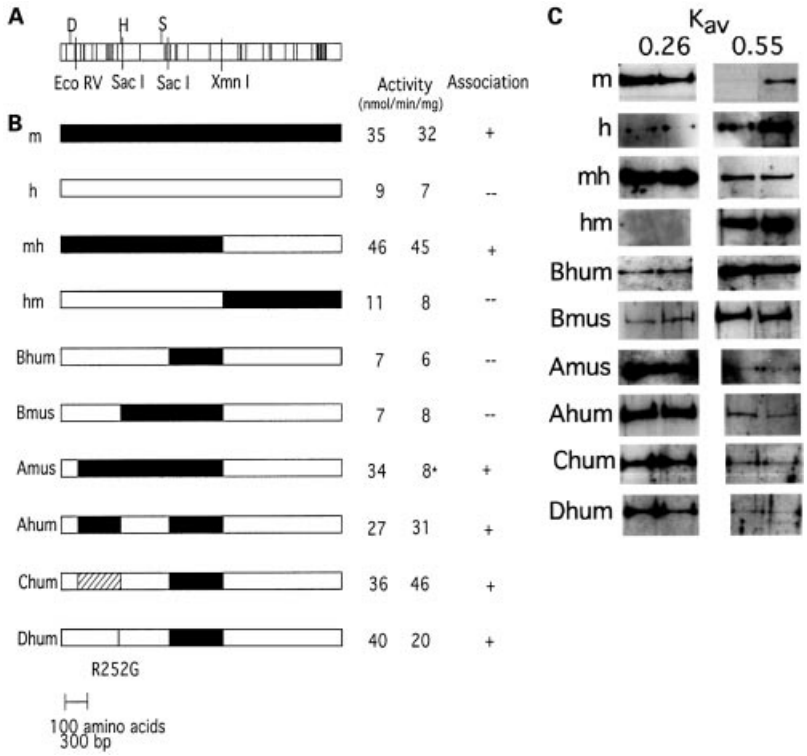

Fig. 2. Comparison of human and murine TPP II and properties of chimeric constructs. (A) Black vertical lines indicate amino-acid differences between human and murine TPP II. D, H, and S denote the catalytic triad (Asp44, His264 and Ser449, respectively). The restriction sites used for creation of the chimeras are shown. (B) Murine and human fragments in the constructs are indicated by filled and open bars, respectively. The fragment originating from the rat gene is indicated by a hatched bar. The activity in cell extracts of stable transformants was measured as described in Materials and methods. The values represent means of two to five measurements each of two individual clones with the highest expression of each of the chimeras. The activity in control cells transformed with vector alone is 4 nmol·min^{-1}·mg^{-1}. Association was investigated by gel filtration of cell extracts on a Sepharose CL-4B column, as described in Materials and methods. At least two individual clones of each chimera were investigated (except Bhum), and both clones displayed the same result. +, the immunoreactivity at $K_{av} = 0.26$ > the immunoreactivity at $K_{av} = 0.55$; –, the immunoreactivity at $K_{av} = 0.55$ > the immunoreactivity at $K_{av} = 0.26$ (cf. Figure 2C). *, indicates a clone with a relatively low expression rate. (c) Western blot analysis of fractions from gel chromatography (compare to Figure 1) was performed as described in Materials and methods. For each construct, one of the clones with the highest expression was selected. Two fractions eluting at a K_{av} of about 0.26, and two fractions eluting at a K_{av} of about 0.55 are shown.

As seen in Fig. 3, the corresponding rat sequence [6] is more or less a mix between the human and the murine sequence. Therefore, the *Eco*RV–*Sac*I fragment was amplified from rat RNA by use of PCR, as described in Materials and methods. This fragment was used to create a human–murine–rat chimera, as outlined in Fig. 2; the chimera was used for transfecting 293 cells. This chimera behaved like the murine enzyme (Fig. 2B), demonstrating that seven amino-acid substitutions of potential importance for the different association remained (Fig. 3).

It is important to note that there is a single nucleotide difference between the sequences of two human clones reported, one encoding a Gly at position 252 [19], and another an Arg [20]. The Arg252-encoding cDNA clone was employed for construction of the human full-length cDNA-clone used for expression [17]. Currently available sequence information indicates that the Arg252 variant is atypical, as all hitherto sequenced variants of TPP II (i.e. rat, mouse, fruit fly, *Arabidopsis thaliana*, *Caenorhabditis elegans* and *Schizosaccharomyces pombe*), and at least three human EST-clones covering this area (GenBank accession numbers AU118610, AW452455, BF511874) encode a Gly in this position. In order to test the consequence of this single amino-acid difference, a construct containing the human N-terminal part with an R252G substitution was made. This construct associated and had a high activity (Fig. 2B, Dhum), which was in contrast to the construct Bhum. The only difference between these two clones is the amino acid in position 252. Evidently, changing Gly252 to an Arg was critical for the association properties of the enzyme.

The nonassociated form is inactive

For purified human TPP II and recombinant murine TPP II, it has been shown that the smallest active form of TPP II appears to be dimers, which have about one tenth of the specific activity of the oligomeric complex [15]. For the recombinant human enzyme the nonassociated form also appeared to be dimers of the 138 kDa subunit, since their M_r was determined to be 2–3 × 10^5. However, no activity peak eluting at a K_{av} of 0.55 could be detected, indicating that they were inactive (Fig. 1). This nonassociated form of the recombinant human enzyme has been isolated after gel filtration and a variety of experiments have been performed

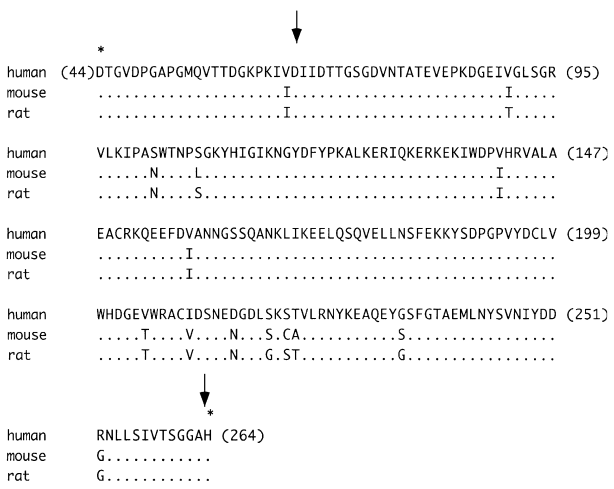

Fig. 3. Alignment of the amino acid sequences between the catalytic Asp44 and His264 residues from human, murine and rat TPP II. A dot indicates that the amino acid is identical to that in the human sequence. The arrows indicate the part corresponding to the *Eco*RV–*Sac*I fragment. The GenBank accession numbers for the sequence data are M73047, X81323 and U50194. The catalytic Asp44 and His264 are indicated by asterisks.

to activate the material, as previously described [15]. However, all attempts so far to associate this material have failed. Thus, it appears that the isolated Arg252-containing dimers cannot form the active oligomers.

Formation of active heterocomplexes

Even if the recombinant human enzyme appeared to form inactive dimers, the total activity in cells overexpressing recombinant human TPP II or different chimeras was at least twice as high as the endogenous TPP II-activity in control cells (Fig. 2B). The active enzyme eluted at a K_{av} of about 0.26 (Fig. 1), which shows that the expressed subunits can, in fact, be part of an active complex. It appears that complex formation involves molecular interactions on at least two levels, dimerization and oligomerization, where the oligomeric complexes have a 10-fold higher specific activity than the dimers [15]. Even though inactive dimers are formed when over-expressing the Arg252-variant, these dimers may contribute to the formation of active oligomers in the presence of the endogenously expressed Gly252-containing subunits. The exact composition of the hetero-complexes could not be established, i.e. if heterodimers were formed by endogenous and recombinant monomers or if the active complexes were assembled from the two types of homodimers.

The insert within the catalytic domain is of importance for complex formation

No functional significance has previously been ascribed to the insert between Asp and His of the catalytic domain of TPP II. We can now report that the region surrounding Arg252 is of importance for the formation of the oligomeric enzyme complex, which is a prerequisite for obtaining a fully active enzyme [8,15]. Upon removal of this entire region (amino acids 68–255), no protein of the expected size could be detected, although mRNA was expressed in transformed cells (data not shown). One interpretation of this finding is that the protein did not oligomerize properly, with the consequence that the subunits were prone to degradation by proteases. With such a large deletion, it is also possible that the enzyme was not folded correctly and therefore more easily subjected to proteolysis.

Part of the subtilisin-like catalytic N-terminal part of TPP II has been modeled on the structure of subtilisin BPN′ (http://biospace.stanford.edu) [27]. In this model (Nr 0381678/1), residues 211–507 of human TPP II were aligned with residues 18–273 of subtilisin BPN′. The catalytic His264 and Ser449 residues were aligned correctly, whereas the catalytic Asp44 of TPP II was not aligned to the active Asp36 of subtilisin, probably due to the large insertion between the catalytic Asp and His in TPP II. This region would, of course, be difficult to model, but as Arg252 is so close to His264, where the structure is conserved, the model is still expected to be useful. In this model, Arg252 is predicted to be on the surface of the enzyme where it could be directly involved in a subunit–subunit interaction. By substituting Gly252 with Arg, this interaction could be disturbed by electrostatic or steric interference. Moreover, the relative short distance to the active site may explain the effect of complex formation on activity [8,15]. Further studies with a number of different Gly252 mutants and other amino-acid changes in this region will be required to fully elucidate the role of this interaction for oligomerization and catalytic activity.

Although the data presented here suggests that the region surrounding residue 252 is directly involved in complex formation, it may instead have a more indirect function. For example, this region may function as an intramolecular chaperone. By promoting the folding of the protein itself, it would have a similar role as that of pro-peptides in other proteases [28,29]. Incorrect folding could also explain the reduced amount of immunoreactive protein observed for all enzyme forms with Arg252 (Fig. 2C), as this protein would be more susceptible to proteolytic degradation. However, the enzyme activity in cells overexpressing all the Arg252 variants still increases twofold to threefold (Fig. 2), indicating that these Arg-containing subunits may be part of an active complex. This suggests that the subunits could still adapt to the three-dimensional fold required for interaction with endogenously expressed subunits. Alternatively, the region surrounding Arg252 may be of importance for interaction with a chaperone or other factors influencing the formation of the active complex. For example, it is possible that a protein in the 293-cells sequesters the Arg-containing subunits, thereby preventing complex formation. This could explain why the nonassociated form, isolated by gel filtration, cannot be made to associate [cf. 15]. The recombinant protein incorporated into the active enzyme complex together with endogenous TPP II would then be protected from sequestration. However, additional data is required to show whether the G252R substitution interferes with activity and/or structure of the dimer or with the oligomerization, and whether this effect is direct or indirect.

CONCLUSIONS

We have shown that a single amino-acid difference, G252R, is critical for formation of the TPP II complex.

This amino acid is located in the insert within the catalytic domain, close to the catalytic His264, and the proximity to the active site may explain the effect of oligomerization on enzyme activity. Even though the exact mechanism for complex formation and activation of the enzyme remains to be determined, it can be concluded that the insert within the catalytic domain is of importance for oligomerization.

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