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ORIGINAL PAPER

Regional Distribution of Human Trypsinogen 4 in Human Brain at mRNA and Protein Level

Júlia Tóth · Erika Siklódi · Péter Medveczky · Katalin Gallatz · Péter Németh · László Szilágyi · László Gráf · Miklós Palkovits

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Abstract Gene PRSS3 on chromosome 9 of the human genome encodes, due to alternative splicing, both mesotrypsinogen and trypsinogen 4. Mesotrypsinogen has long been known as a minor component of trypsinogens expressed in human pancreas, while the mRNA for trypsinogen 4 has recently been identified in brain and other human tissues. We measured the amount of trypsinogen 4 mRNA and the quantity of the protein as well in 17 selected areas of the human brain. Our data suggest that human trypsinogen 4 is widely but unevenly distributed in the human brain. By immunohistochemistry, here we show that this protease is localized in neurons and glial cells, predominantly in astrocytes. In addition to cellular immunoreactivity, human trypsinogen 4 immunopositive dots were detected in the extracellular matrix, supporting the view that human trypsinogen 4 might be released from the cells under special conditions.

Keywords Glia · Neuron · Human trypsinogen 4 · Human brain · Spinal cord

Júlia Tóth and Erika Siklódi contributed equally to this work.

J. Tóth · E. Siklódi · P. Medveczky · L. Szilágyi · L. Gráf (✉)
Department of Biochemistry, Eötvös Loránd University, Pázmány Street 1/C, 1117 Budapest, Hungary
e-mail: graf@elte.hu

K. Gallatz · M. Palkovits (✉)
Laboratory of Neuromorphology and Neuroendocrinology, Department of Anatomy, Semmelweis University and Hungarian Academy of Sciences, Tüzoltó utca 58, 1094 Budapest, Hungary
e-mail: palkovits@ana.sote.hu

P. Németh
Institute of Immunology and Biotechnology, Medical School, University of Pécs, Pecs, Hungary

Introduction

Serine proteases comprise a group of proteolytic enzymes in which the catalytic mechanism depends upon the hydroxyl group of the catalytic serine residue. A number of biological processes that require specific and limited proteolysis are mediated by serine proteases. There is an increasing body of evidence showing that serine proteases are widely expressed and fulfill diverse roles in the central nervous system. They contribute to structural plasticity associated with learning and memory, neurite outgrowth, regulation of neuronal survival, synaptogenesis, myelin turnover and development [1–10]. Proteases have also been implicated in the pathophysiology of neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and multiple sclerosis. The activity of these enzymes must be under strict regulation through transcription, translation, autolysis and interaction with natural inhibitors. To maintain normal physiology, a fine-tuned balance of proteolysis is required in the central nervous system, perturbation of this equilibrium may lead to serious disturbances in neuronal function.

Human trypsin 4 is an intriguing, inhibitor resistant trypsin isoform that is expressed in pancreatic and non-pancreatic tissues as well, including the central nervous system. First it was cloned from human brain [11] and was thought to be a brain-specific protease, but later its mRNA was also found in human epithelial cell lines [12]. The common gene (PRSS3) encoding human pancreatic mesotrypsinogen and human trypsinogen 4 is found on chromosome 9p13, in contrast to the genes for the two

more abundant pancreatic trypsinogens, human trypsinogen 1 and 2, that are located on chromosome 7q35 [13, 14]. Such separation of the trypsinogen loci has only been observed in the human, and recently in the chimpanzee genome. A more recent study reports that PRSS3 gene was formed by segmental duplications originating from chromosomes 7q35 and 11q24 [15]. Consequently, PRSS3 transcripts display two variants of exon 1, giving rise to two different mRNA populations (Fig. 1). Mesotrypsinogen and human trypsinogen 4 differ only in their N-terminal sequences, as a result of alternative splicing and/or alternative promoter use. Whereas the former, designated as isoform C in the Swiss–Prot data base has a typical signal sequence, isoform A and B of human trypsinogen 4 have highly charged unusual N-terminal leader sequences that do not possess the typical signal sequence characteristics. Based on genomic DNA sequences [11] the predicted isoform A may have a 72 amino acid, while isoform B possesses a 28 amino acid N-terminal leader sequence. Isoform B can be derived from isoform A via alternative translation initiation from an internal CUG codon [11, 16] (Fig. 1).

The mRNA of mesotrypsinogen is expressed in the pancreas [17, 18], and the protein itself was also detected in this tissue [19–22]. In contrast, human trypsinogen 4 mRNA was found in the brain and in epithelial cell lines originating from airway, prostate and in the colonic mucosa [12, 15]. EST sequences corresponding to isoform B were also cloned from uterus, heart and several tumor cell lines [15]. The expression of isoform B at the protein level was detected in the brain by immunoaffinity chromatography by our research group [16]. The transcription of isoform A is proven by a single EST sequence (BI823946) that was cloned from a pooled mRNA population isolated from brain, lung and testis. However, the protein for isoform A has not been detected yet. In a recent study, a monoclonal antibody (mAb) specific for the protease domain was used to detect anti-human trypsin reactivity in PC-3 and SW480 epithelial cell lines by immunostaining [12]. The antibody used reacts with all isoforms of human trypsinogen 4, thus from these data we cannot conclude which isoforms are expressed, at the protein level, in these cell lines.

The most intriguing feature of human trypsin 4 is its inhibitor resistance to proteineous canonical inhibitors [18, 20, 23–25], and also to a serpin type inhibitor, wild type α-1-antitrypsin [20, 26]. The crystal structure of human trypsin 4 complexed with benzamidine was solved [23]. This study provided the first evidence regarding the inhibitor resistance of this protease that is attributed to the presence of an arginine residue at 193 position (chymotrypsin numbering). Position 193 is highly conserved in serine proteases: in most cases it is occupied by a glycine residue. Arg193 of human trypsin 4 occupies the S2′ subsite (Schechter–Berger nomenclature) and its extended side-chain sterically clashes with the respective residues of inhibitor or substrate protein partners. Furthermore, the guanidino group of Arg193 significantly contributes to the positive charge clustering around the primary specificity pocket, thus might affect inhibitor (and substrate) binding electrostatically, as well. The dominant role of Arg193 in the inhibitor resistance of human trypsin 4 was confirmed by site directed mutagenesis studies [24, 25].

Short chromogenic and fluorogenic peptide substrates are typically cleaved by human trypsin 4 with high catalytic efficiency. Human trypsin 4 hydrolyzes oligopeptide substrates that do not make P′ sub-site contacts at comparable efficiency to conventional pancreatic trypsin isoforms [20, 23–25]. In contrast to its high activity on oligopeptide substrates, human trypsin 4 typically cleaves protein substrates poorly [25, 27], although the extent of selectivity strongly depends on the type of polypeptide

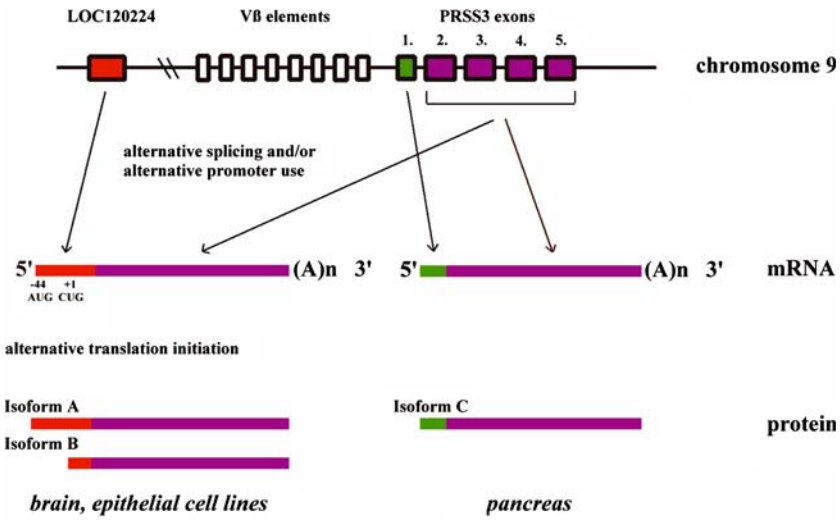

Fig. 1 Formation of human trypsinogen 4 isoforms. All three isoforms are encoded by the PRSS3 gene located on chromosome 9. These isoforms share exon 2–5 (marked *purple*) that code for the propeptide and the protease domain, but they possess different first exons as a result of alternative splicing and/or alternative promoter use (marked *red* and *green* for isoform C, A and B, respectively). As a consequence, the *N-terminal peptide* of these isoforms shows distinct characteristics

substrate [26]. This recent study revealed that human trypsin 4 exhibits a relatively high substrate specificity with a strong preference for Arg/Lys–Thr/Ser peptide bonds [26]. It was also shown that human trypsin 4 can be inhibited by the serpin mechanism if the serpin reactive site is consistent with the Arg/Lys–Thr/Ser motif.

With the aim to detect human trypsinogen 4 and determine its distribution in the brain mAbs specific for the protease domain and a synthetic peptide corresponding to the N-terminal 28-amino acid leader sequence were raised. These mAbs were utilized to measure the quantity of human trypsinogen and trypsin 4 in samples from different areas of the human brain using immunoprecipitation and sandwich ELISA method. The relative quantity of human trypsinogen 4 mRNA was also measured from these samples by real-time PCR. Furthermore, the localization of this non-conventional trypsinogen/trypsin isoform was examined by immunostainings.

Experimental procedures

Human brain samples

Tissue samples were obtained from the Human Brain Tissue Bank, Budapest. Brains were removed from the skull, rapidly frozen on dry ice, and stored at -80°C until dissection. Samples from brains with 1–12 h post mortem delay were used for immunoprecipitation and performing ELISA measurements. For immunohistochemistry, cerebral cortical and spinal cord samples were cut from brains only 2 h post mortem. Tissue harvesting occurred after written informed consent was obtained from next of kin, which included the request to consult the medical chart and to conduct neurochemical and/or biochemical analyses. The local Ethics Committee of the Semmelweis University approved analyses of such tissue samples.

Samples from 17 brain areas selected for real-time PCR and ELISA measurements were microdissected with the ''punch technique'' [28] at 0 to -10°C, and stored at -80°C. The regions to be examined were selected to represent the major anatomical and functional units of the brain.

RNA isolation

Total RNA was isolated from 30–100 mg tissue samples using TRI Reagent (Sigma-Aldrich Co., St Louis, MO, USA) according to the manufacturer's instructions. RNA pellets were dissolved in 20 μl DEPC-treated nuclease free water (Fermentas, Vilnius, Lithuania) and 20 units of RNase inhibitor (Fermentas, Vilnius, Lithuania) was added to minimize RNA degradation. The RNA samples were immediately used for cDNA synthesis.

Reverse transcription

First strand cDNA was synthesized by priming with random hexamer primer using 7.5–25 μg RNA as template. The reverse transcription was performed in 20 μl reactions using random hexamer primer according to the manufacturer's instruction (RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit, Fermentas, Vilnius, Lithuania). The cDNA mixtures were stored at -80°C until assay (usually not more than 24 h). RNA samples from a single experimental setup were usually reverse transcribed simultaneously to minimize interassay variation associated with the RT reaction.

Generation of external standards

Parts of the coding sequences of human trypsinogen 4 and human β-actin were amplified from the prepared human brain cDNA with the following oligonucleotides: Actin-F: 5′-ACAATGAGCTGC-GTGTGGCT-3′; Actin-R: 5′-TCT CCTTAATGTCACGCACGA-3′; Hu4-F: 5′-CGCATATG GAGCTGCACCCGCTTCTG-3′, Hu4-R: 5′-GACTGCAG AGCTC-CCGGGGGCTTT-AGC-3′ using Taq polymerase (Zenon-Bio Kft, Szeged, Hungary). The resulting PCR products were cloned into pBluescript II KS vector by TA ligation. In order to synthesize strand specific RNA sequences, the template Hu4–pKS and actin-pKS plasmids were cleaved with *Sac*I and *Hind*III, respectively. Then, 0.5 μg of these linearized plasmids were in vitro transcribed in a total volume of 50 μl by adding transcription buffer (40 mM Tris–HCl pH 7.9 at 25°C, 6 mM $MgCl_2$, 10 mM DTT, 10 mM NaCl and 2 mM spermidine), 2 mM NTP mix, 50 unit RNase inhibitor and 30 unit T7 or T3 RNA Polymerase (Fermentas, Vilnius, Lithuania), according to the direction of the insert in the plasmid. The reaction mixture was incubated at 37°C for 120 min, and 1 unit RNase free DNase I (Fermentas, Vilnius, Lithuania) was added and allowed to work at 37°C for 30 min to degrade the template DNA. The standard RNA mixtures were purified on RNA binding columns (Total RNA Mini Kit, Viogene, Sunnyvale, CA, USA) and eluted with 50 μl DEPC-treated water (Fermentas). After adding 20 units of RNase inhibitor the standard RNA solutions were stored at -80°C. Concentrations of the final purified RNA standards were determined by measuring the optical density at 260 nm (OD_{260}). cDNAs synthesized using 0.225 ng actin RNA and 0.675 ng human trypsinogen 4 RNA became the stock solutions for standards in subsequent real-time PCR experiments.

Primer design

Primers were designed using Primer Express Software v2.0 (Applied Biosystems, Foster City, CA, USA) and were

based on GenBank sequences for human trypsinogen 4 (accession no. X71345) and human β-actin (accession no. V01217). The criteria used for selecting primers for quantitative PCR are: an optimal primer length of 20 bases, a G–C content of 30–80%, melting temperature between 58–60°C, avoiding runs of an identical nucleotide and the five nucleotides at the 3′ end having no more than 2 G and/or C bases. The oligonucleotides used were the following: Q-Hu4-F: 5′-CTGCTACAAGACCCGCATCC-3′, Q-Hu4-R: 5′-GGAGAGTTTGATCAGCATGAT-GTC-3′, amplicon size 154 base pairs; Q-Actin-F: 5′-GCGAGAAGATG ACCCAGATCA-3′, Q-Actin-R: 5′-CGTAGATGGGCA-CAGTGTGG-3′, amplicon size 163 base pairs. Both forward primers span exon–exon boundaries (underlined bases in the primer sequences) to prevent amplification from genomic contamination. The selected primer pairs passed secondary structure and primer dimer test. All primers (Genodia, Budapest, Hungary) were dissolved in sterile TE (10 mM Tris–HCl, pH 8.1 mM EDTA) at a stock concentration of 100 μM, further diluted to a working concentration of 1.25 μM and stored at -20°C. The specificity of the primers was verified by cloning and sequencing of randomly chosen real-time PCR products.

Real-time quantitative PCR

Real-time PCR reactions were performed using the ABI Prism Sequence Detection System 7000 (Applied Biosystems, Foster City, CA, USA) and SYBR^{TM} Green dsDNA binding fluorescent dye. Each reaction was run in triplicate and contained 2.5 μl of cDNA template, 12.5 μl SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) along with 50 nM Q-Hu4 or Q-Actin primers in a final reaction volume of 25 μl. Cycling parameters were 95°C for 10 min to activate the chemically modified DNA polymerase, then 40 cycles of 95°C for 15 s and 60°C for 1 min. An amplification threshold value (Ct value) defined as the PCR cycle number at which the delta Rn crosses a fluorescence signal threshold, was calculated for each reaction. Standard curves were generated using serial dilutions of cDNAs corresponding to known amounts of in vitro transcribed human trypsinogen 4 and human β-actin RNA and the calculated Ct values were converted to mRNA quantities. Subsequent to the final PCR cycle melting curves were performed to verify that only a single product was amplified. Samples were also run on 2% agarose gel to confirm specificity.

Monoclonal human trypsinogen 4 antibodies

Anti-human trypsinogen 4 mAbs were raised against the protease domain (mAb 1/B1 and mAb 6B/7) and the 28-amino acid leader peptide (mAb P28) of human trypsinogen 4, respectively (details are reported in [16]). The mAbs were characterized immunoserologically using different trypsinogen and trypsin isoforms and other non-trypsin proteins as antigens. mAb 1/B1 and mAb P28 did not cross-react with any of the non-specific antigens tested, while mAb 6/B7 showed slight cross-reactivity with human trypsinogen 1, human trypsin 1 and rat trypsin and medium reactivity against human trypsinogen 2 (Table 1). For these reasons mAb 1/B1 and mAb P28 were used as capture antibodies for the immunoprecipitation and coating antibodies for the ELISA experiments, and mAb 6/B7 was used as a detection antibody for the ELISA. In a control study immunoprecipitation and sandwich ELISA experiments were carried out with bovine and rat cerebrum and rat cerebellum samples. No immunoreactivity with either antibody could be detected in these tissues that further confirms the specificity of the applied antibodies.

In order to determine quantitatively the distribution of human trypsinogen/trypsin 4 in different brain areas we used sandwich ELISA measurements combined with immunoprecipitation. Either mAb 1/B1 or mAb P28 was used as a capture antibody to precipitate different forms of the enzyme from the tissue extract. In both cases, the composition of the capture ELISA was the same: mAb 1/B1–antigen–mAb 6B/7. This arrangement can be applied for the quantitative measurement as these antibodies recognize different epitopes. The use of two antibodies with different specificities in the immunprecipitation step allowed us to measure the quantity of the zymogen and the active enzyme separately. mAb P28 reacts only with those forms that possess the 72 or 28 amino acid leader peptide, while mAb 1/B1 binds both the zymogens and the activated enzyme as well.

Immunoprecipitation and sandwich ELISA

Brain tissues (200–250 mg) were homogenized in PBS (2.5 mM NaH_2PO_4, 7.5 mM Na_2HPO_4, 145 mM NaCl, pH 7.2). After centrifugation (5,000*g* for 10 min at 4°C), the pellet was resuspended in 1 ml immunoprecipitation lysis solution (1% Triton X-100, 50 mM Tris–HCl, 145 mM NaCl, pH 7.2, containing 1 mM PMSF, 1 mM benzamidine, 1 mM cystatine, 1 mM leupeptin and 1 mM EDTA). Samples were incubated for 1 h on ice, centrifuged (14,000*g* for 30 min at 4°C) and 500 μl of the resulting supernatant was precleared with 50 μl Protein G Agarose (Sigma-Aldrich Co., St Louis, MO, USA). Following centrifugation (14,000*g* for 2 min at 4°C) 5 μg of the capture antibody (mAb 1/B1 or P28) was added to the cold precleared supernatant (the optimal concentration of antibody was determined by titration) and incubated for 1 h at 4°C. Then, 50 μl Protein G Agarose was added to the samples and incubated on a rocking platform overnight at 4°C [29]. The beads were collected with centrifugation

Table 1 Immunoreactivity of anti-human trypsin and anti-P28 antibodies detected by ELISA

Antigens	mAb 1/B1 IgG2b	mAb 6/B7 IgG1	mAb P28 IgG1
Human trypsinogen 1	-	-/+	-
Human trypsinogen 2	-	+++	-
Human trypsinogen 4 (isoform C)	++++	+++	-
Tag-P72-Hu-4 trypsinogen (isoform A)	++++	+++	++++
Rat trypsinogen	-	-/+	-
Ostrich trypsinogen	-	-	-
Bovine trypsinogen	-	-	-
Human trypsin 1	-	-/+	-
Human trypsin 2	-	-	-
Human trypsin 4	++++	+++	-
Rat trypsin 2	-	-	-
Ostrich trypsin	-	-	-
Bovine trypsin	-	-	-
Bovine chymotrypsin	-	-	-
Bovine serum albumin	-	-	-
Thyreoglobulin	-	-	-
Gelatin	-	-	-
P28 peptide	-	-	-/+
P28-BSA	-	-	++++

Different isoforms of trypsinogen and trypsin and some non-related proteins were used as antigens to characterize the cross-reactivity of the generated monoclonal antibodies by ELISA technique. The table shows the averages of three independent measurements. (Each sample applied in triplicate forms)

- represent $OD_{490} < 0.025$, -/+ represent $0.025 < OD_{490} < 0.100$, +++ represent $OD_{490} > 1.000$, ++++ represent $OD_{490} > 1.50$

(14,000*g* for 2 min at 4°C) and washed three times with 50 mM Tris–HCl, 145 mM NaCl, 1% Triton X-100, pH 7.2. The antigen was eluted by resuspending the beads in 100 mM glycine–HCl, pH 2.5. After centrifugation (14,000*g* for 2 min at 4°C) the amount of human trypsinogen/trypsin 4 in the supernatant was quantified with sandwich ELISA performed using the method of Lems-Van et al. [30] with some modifications. A 96-well ELISA plate (Costar 3590) was coated with 100 µl of antibody 1/B1 (2 µg/ml) overnight at 4°C. Remaining non-specific binding sites were blocked by the addition of 150 µl 3% bovine serum albumin in PBS for 1 h. After three washes in PBS–Tween (0.05% Tween 20 in PBS) immunoprecipitated samples in PBS were added at 100 µl/well along with appropriate positive and negative controls and incubated for 1 h. The plate was washed three times and then 100 µl of mAb 6/B7 (2 µg/ml) was added and incubated for 1 h. The wells were washed as above and 100 µl of biotin-conjugated anti-mouse IgG1 (10,000-fold dilution in PBS, A85-1 BD Bioscience Pharmingen, San Diego, CA, USA) was added to each well. After incubation for 1 h at room temperature the wells were washed as above and 100 µl peroxidase-conjugate extravidin (E-2886, Sigma-Aldrich Co., St Louis, MO, USA), 3,000-fold diluted in PBS, was added. After incubation for 1 h at room temperature, the wells were washed four times with PBS-Tween and 100 µl of *o*-phenylenediamine solution (3 mM *o*-phenylenediamine dihydrochloride, 0.1 M citrate-phosphate buffer, pH 5.0, 0.01% H_2O_2) was added. Color development was stopped by adding 100 µl 2 M H_2SO_4. Absorbance was measured at 490 and 620 nm as a reference with a Bio-Rad microplate-reader. Each data shown was determined by three parallel experiments ($n = 3$). Recombinant trypsinogen 4 was used for calibration and as positive control. The calibration curve was linear in the 0.1–1.0 ng trypsinogen 4 range.

In a control study, immunoprecipitation and sandwich ELISA experiments were carried out with bovine and rat cerebrum and rat cerebellum samples. No immunoreactivity with either antibody could be detected in these tissues.

Immunohistochemistry

Human brain samples from the cerebral cortex and spinal cord were subjected to immunostainings. Tissue samples were taken from suddenly died individuals (myocardial infarct, suicides and traffic accidents) with a relatively short (2 h) post-mortem delay. After removing from the skull, brains were cut into anatomically oriented small pieces and fixed by immersion in Zamboni's fixative

solution (4% paraformaldehyde, 15% picric acid in 0.1 M phosphate buffer, pH 7.4) for 7–10 h. Sections of 50 μm thickness were cut with Frigomobil (free-floating frozen sections), or Vibratome (unfrozen sections). In addition, 10 μm thick sections were cut in a cryostat, put on gelatin-coated slides and fixed with Zamboni's solution for 15 min.

Sections were immunostained by mAb 1/B1 antibody. A modification of the avidin–biotin peroxidase technique was performed as follows: sections were washed three times in 0.1 M phosphate buffer, pH 7.4 (PB) and incubated for 1 h at room temperature in 0.1 M PB containing 0.5% Triton X-100. After rinsing three times with 0.1 M PB, the endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 for 15 min. After washing again in 0.1 M PB and treating with 10% normal horse serum for 1 h at room temperature, the sections were rinsed with 0.1 M PB and incubated at 4°C in mAb 1/B1 or mAb P28 (1 μg/ml) for 24–48 h, for 1 h at room temperature with the secondary antibody (biotinylated anti-mouse IgG, Vector Laboratories, Burlingame, CA, USA) diluted 1:1,000 in 0.1 M PB) and for 1 h at room temperature with avidin-biotin horseradish peroxidase Elite kit (Vector Laboratories, Burlingame, CA, USA). Between the incubations the sections were washed three times in 0.1 M PB. The tissue bound peroxidase was visualized with diaminobenzidine (DAB, Sigma-Aldrich Co., St Louis, MO, USA) or nickel enhanced DAB. Sections were then mounted on gelatin-coated slides, dried and some of them were counterstained with kernechtrot dye.

To enhance the immunoreaction, biotin–tyramide amplification was applied for some of the sections after incubation in avidin–biotin horseradish peroxidase. Two control experiments were carried out to confirm specificity: (1) No staining was observed in the absence of the primary antibody; (2) Immunostaining was completely blocked by saturating mAb 1/B1 (1 mg/ml) with 1 mg/ml recombinant trypsinogen 4. Adjacent sections were immunostained with antibody against glial fibrillary acidic protein, GFAP (mouse monoclonal, at a dilution of 1:1,000; Novocastra, Newcastle upon Tyne, UK), a well-known marker protein for astrocytes.

Results

Quantitative real-time PCR

Figure 2 shows the relative amount of human trypsinogen 4 mRNA normalized to β-actin mRNA levels at 17 different brain regions. The mean quantity of the human trypsinogen 4 transcripts was 1.0–7.4% that of the β-actin mRNA. The highest transcript levels (above 5%) could be detected in the cerebellar cortex and the olfactory bulb. The frontopolar, somatomotor and occipital corticis, the hippocampus, putamen, periaqueductal gray matter, substantia nigra and spinal cord samples revealed intermediate mRNA quantities (1.9–3.6%). The lowest amount (below 1.7%) of human trypsinogen 4 mRNA was measured in cingulate cortex, the cortical and cerebellar white matters and in samples from the thalamus, hypothalamus, pons and the medulla oblongata.

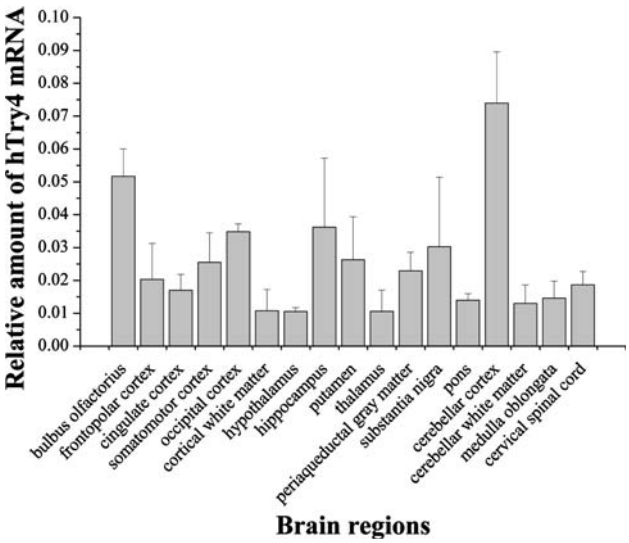

Fig. 2 The relative amount of human trypsinogen 4 mRNA in different regions of the human brain. Transcription levels were determined with quantitative real-time PCR and normalized to the amount of β-actin mRNA. The data represent mean and standard deviation of three parallel measurements

The distribution of the mRNA in different brain areas measured by real-time PCR is more or less consistent with the protein levels detected with ELISA (Fig. 3).

Regional distribution of human trypsinogen and trypsin 4 immunoreactivity in human brain—sandwich ELISA

To test the possible effect of age, gender and post-mortem delay on the amount of expressed human trypsinogen/trypsin 4 in the brain, occipital cortex samples of three males (aged 20, 50 and 71) and two females (aged 72 and 81) with post-mortem times of 1, 2, 4, 5 and 12 h, respectively, were subjected to immunoprecipitation and ELISA measurements. The amount of immunoreactive human trypsinogen/trypsin 4 as determined by capture antibody 1/B1 varied between 10 and 16 ng/g wet tissue, indicating the lack of significant correlation between the quantity of human trypsinogen/trypsin 4 and the above parameters (data not shown).

The gray columns in Fig. 3 show the regional distribution of the total amount of human trypsin and trypsinogen 4

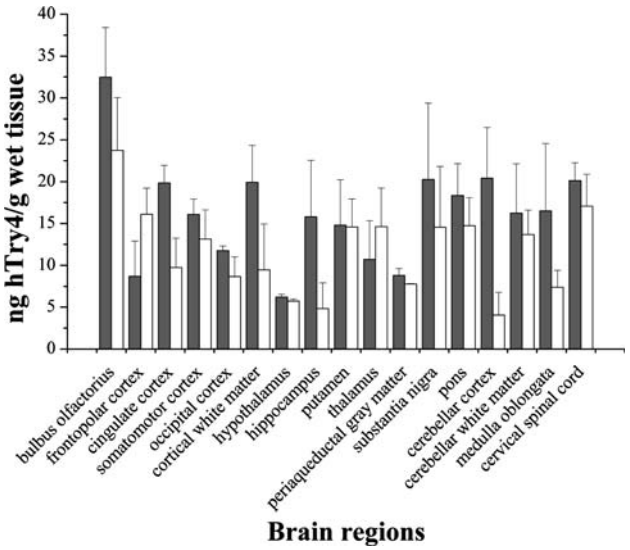

Fig. 3 The amount of different forms of human trypsinogen 4 in different areas of the human brain. Immunoprecipitation from brain extracts was carried out with 1/B1 (*gray columns*) or P28 (*white columns*) capture mAb's. In both cases the sandwich ELISA construction mAb 1/B1-antigen–mAb 6/B7 was used for quantification. Each extraction was performed in triplicates. The data shown represent mean and standard deviation of five independent experiments

in 17 selected areas of the human brain. In the examined brain areas, the amount of total protein is in the range of 4–33 ng/g wet tissue. In ten areas (olfactory bulb, somatomotor cortex, occipital cortex, hypothalamus, putamen, periaqueductal gray matter, substantia nigra, pons, cerebellar white matter and cervical spinal cord) the total amount is roughly equal to the amount of the leader sequence carrying forms (the ratio of active to total amount is below 30%). It should be noted that in two brain areas, namely frontopolar cortex and thalamus the amount of zymogen was higher than that of the total amount. As the differences are within experimental error, we assume that the amount of the zymogen and the total protein is approximately equal in these areas. In contrast, in five areas (cingulate cortex, cortical white matter, hippocampus, cerebellar cortex and medulla oblongata) the amount of zymogen was substantially less than the total amount (the ratio of active to total amount is above 50%). Consequently, the extent of the activation appears to depend on the brain area. The highest ratio of activated to total amount was found in cerebellar cortex and hippocampus where this value is 80.0 and 69.4%, respectively.

Amino terminal sequencing of trypsin 4 isolated from human brain indicates that it is correctly processed, i.e., its N-terminal amino acid sequence is Ile-Val-Gly-Gly [16]. This sequence is identical with that obtained by enterokinase cleavage of the zymogen. At the moment, however, the nature of the activating enzyme is unknown.

Immunohistochemistry

Human trypsinogen 4-like immunoreactivity was localized in human cerebral cortex and spinal cord using both types of mAbs. In the spinal cord, glia cells showed human trypsinogen 4-like immunoreactivity in the white matter (funiculi) (Fig. 4a). Since the distribution, shape and size of the immunostained cells were similar with those in GFAP-stained sections (Fig. 4b), the majority of human trypsinogen 4 immunostained cells in the spinal white matter are most probably astrocytes. Some of the neuronal perikarya in the ventral horn (Fig. 5) and the central gray matter of the spinal cord were also immunostained for human trypsinogen 4 (Fig. 6a).

Human trypsinogen 4-like immunopositive neuronal perikarya and glial cells were observed in the cerebral cortex, especially in cortical layers III through V (Fig. 6b). Like in the spinal cord, glial cells, predominantly astrocytes were observed in the cortical white matter (Fig. 7).

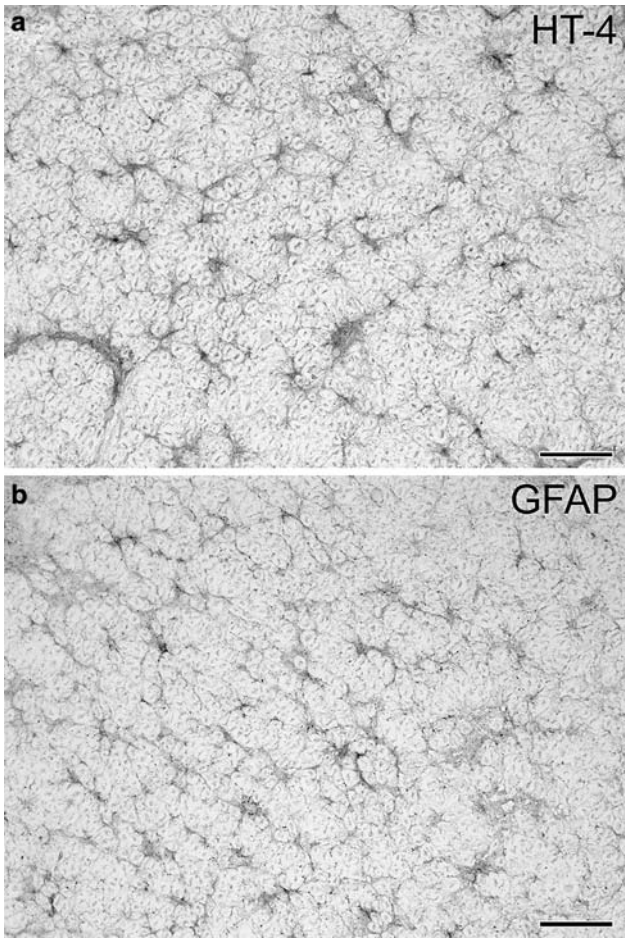

Fig. 4 Human trypsinogen 4-like immunopositive glial cells (astrocytes) in the spinal cord. Dorsal funiculus. tangentional sections: **a** immunostaining with mAb 1/B1 for human trypsinogen 4, **b** GFAP immunostaining. Biotin–tyramide amplification. *Scale bars*: 50 μm

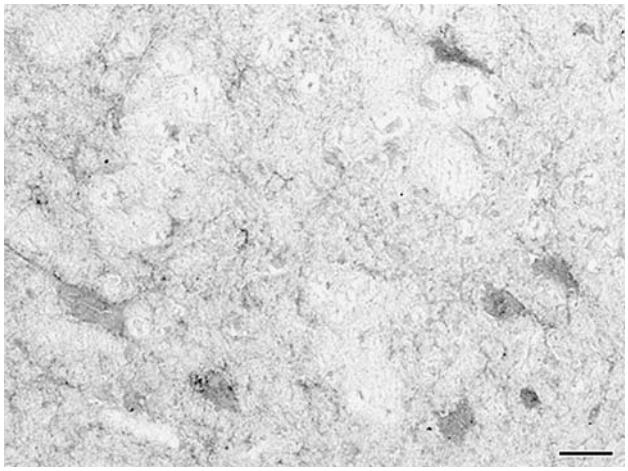

Fig. 5 Human trypsinogen 4-like immunostaining by mAb 1/B1. Immunoreactive motoneurons in the ventral horn of the spinal cord. Biotin–tyramide amplification. *Scale bars*: 50 μm

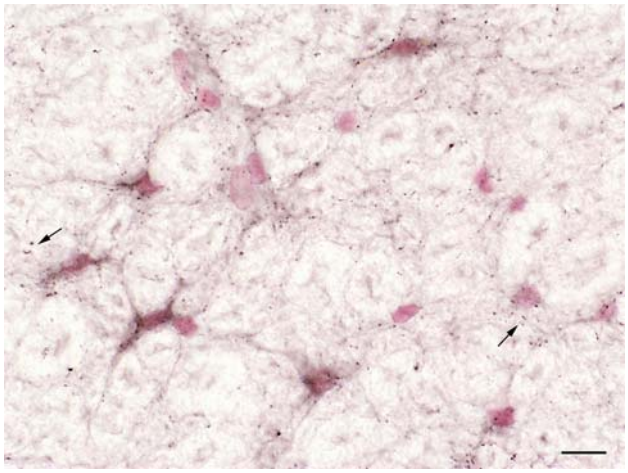

Fig. 7 Dot-like human trypsinogen 4-like immunoreactivity is present on membranes and major processes of glial cells, as well as in the extracellular space (*arrows*). Monoclonal 1/B1 was used for immunostaining. Spinal cord, dorsal funiculus. Biotin–tyramide amplification. *Scale bar:* 10 μm

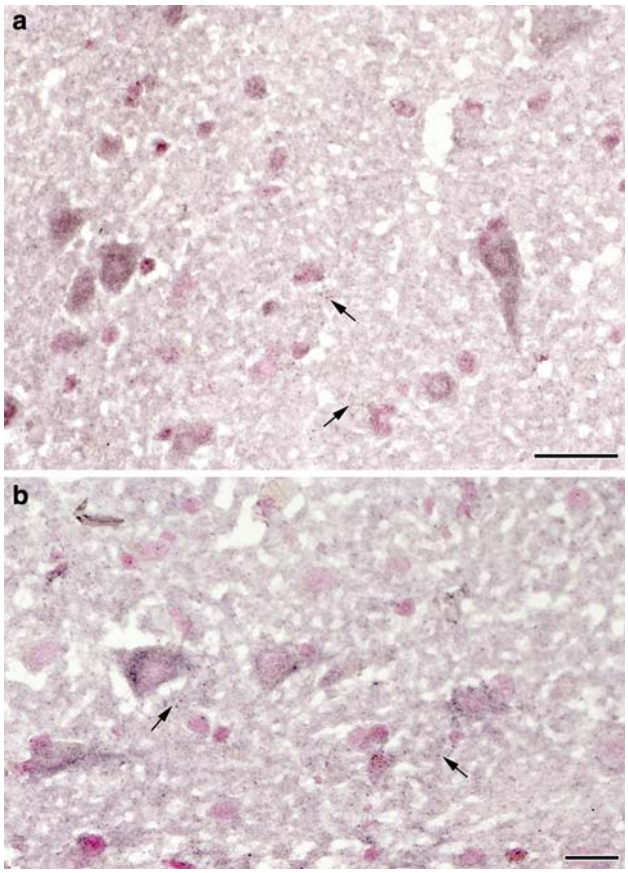

Fig. 6 Human trypsinogen 4-like immunostaining with mAb 1/B1. **a** Immunoreactive large and small neuronal perikarya in the *central gray* of the spinal cord. **b** Frontal cortex, *layer V*. Granular immunostaining in neuronal perikarya and moderately intense homogenous staining throughout the extracellular space (*arrows*). *Scale bars*: 20 μm

Here, some small cells with variable shape and size were also immunostained for human trypsinogen 4. These cells may represent oligodendrocytes and microglia. In addition to cellular immunostainings, low to moderate levels of homogenous, dot-like human trypsin/trypsinogen 4-like immunostaining were present in the gray matter of the cerebral cortex (Fig. 7), and the granule cell layers of the cerebellum (not shown). This type of immunostaining may indicate the presence of trypsinogen 4/trypsin 4 (like most other serine proteases) in the neuronal matrix, i.e., in the extracellular space of the central nervous system.

Discussion

Studies over the past few years have been carried out aiming to understand the potential role of human trypsinogen 4 in the central nervous system. In astrocytes of transgenic mice expressing human trypsinogen 4 enhanced GFAP expression was found that is indicative of increased activation of these cells which is typical in Alzheimer's disease and other type of brain injuries [31]. However, no sign of neurodegeneration was found in these mice. On the other hand, increased levels of intracellular β-amyloid were observed, although extracellular deposits or plaques could not be detected. The significance of these observations remains to be elucidated.

Extrapancreatic human trypsin 4 is a potential agonist of protease-activated receptors (PARs), a G-protein coupled receptor family that activate signaling pathways upon being cleaved by serine proteases [32, 33]. The tethered ligand domains become exposed by the proteolytic step and

bind to the cleaved receptor that, in turn, gets activated. It was also suggested that human trypsin 4 might function as an agonist of PARs, PAR-2 and PAR-4 [12]. Later, these findings were questioned and evidence was provided that high concentrations of human trypsin 4 induce a relatively weak Ca^{2+} response through PAR-1 cleavage in human 1321N1 astrocytoma cells, suggesting that it can be considered as a potential activator of PAR-1 in the brain, and thus, human trypsin 4 might play a role in protection, degradation and plasticity related processes in the central nervous system [34]. A very recent study shows that human trypsin 4 selectively activates PAR-1 but not PAR-2 in primary rat astrocytes mediating a pronounced transient Ca^{2+} mobilization in these cells [35].

Recently it was recognized that trypsin 4 activates PAR-1 in human astrocytoma cells [34]. PAR-1 localization and function was also explored in the human brain [36]. This study revealed that PAR-1 is expressed intensively in astrocytes of white and gray matter, and although to a lesser extent, it is also expressed in neurons. Since the expression patterns of human trypsinogen 4 and PAR-1 overlap, these data suggest that the activated protease could be considered as a signaling molecule in the central nervous system, acting via PAR-1. The distribution of human trypsinogen 4 in different brain areas and its immunohistochemical localization indicates that this serine protease might be involved in multiple physiological/pathological processes in the brain.

Consistent with its S1′ specificity, human trypsin 4 selectively processes myelin basic protein at the Arg79-Thr80 and Arg97-Thr98 peptide bonds in vitro. Upon cleavage this protease generates a peptide fragment containing the sequence that is recognized by major antibodies isolated from patients with multiple sclerosis [37]. These results indicate that human trypsin 4 might be one of the candidate proteases involved in the pathogenesis of this neurodegenerative disease.

The aim of the present study was to determine the regional distribution of human trypsinogen/trypsin 4 in the human brain both at the protein and the mRNA level. Monoclonal antibodies were raised against the recombinant protease (mAbs 1/B1 and 6/B7) and against the synthetic N-terminal 28 amino acid leader peptide (mAb P28). The specificity of our antibodies was tested on trypsinogens of different origin. Immobilized mAb 1/B1 and mAb P28 were used to immunoprecipitate different forms of the enzyme from tissue extracts. With these antibodies we were able to measure either the total amount of trypsinogen and activated trypsin 4 as both captured by mAb 1/B1, or separately, that of the sum of the different zymogen forms only retrieved by mAb P28 with sandwich ELISA technique. Our data suggest that the extent of the activation depends on the brain area. The greatest extent of activation was observed in the cerebellar cortex and hippocampus. We also measured the relative quantity of the mRNA of this protease. Although human trypsinogen 4 mRNA showed an uneven distribution in the brain, in either region, its mRNA proved to be a relatively abundant message.

As shown in the present study, the abundance of human trypsinogen/trypsin 4 immunoreactivity in the white matter of the cerebral cortex and the spinal cord, and their topographical overlap with the distribution pattern of GFAP-immunopositive cells indicates that human trypsinogen 4 in these brain regions is expressed predominantly by astrocytes. The cellular localization or extracellular transport of a protein is determined by its N-terminal sequence. In the case of human trypsinogen 4, the dominant (or exclusive) expressed variant is *isoform B* in the brain [16]. This isoform lacks a typical signal sequence that is required for proteins to be directed to the secretory pathway. Rather, *isoform B* possesses a unique N-terminal peptide, the function of which in the cellular/extracellular localization of this protease is yet unknown. In our immunohistochemical studies the membrane association and extracellular localization of human trypsinogen/trypsin 4 was also observed. Most of our immunohistochemistry data are consistent with the intracellular localization of trypsinogen 4 (Figs. 4, 5, 6). Dot-like immunoreactivity appeared on the surface of the perikarya and processes of glial cells as shown in Fig. 7. Futhermore, human trypsinogen 4 immunoreactivity was also demonstrated in the neuronal matrix of the cortical gray matter and in the spinal cord (Fig. 7). This may raise the possibility that trypsinogen 4 may have been released from the cells and then associated with the cell membranes. As to the structural basis of this putative accumulation of trypsinogen 4 in the extracellular matrix we propose that the 28-amino acid N-terminal propeptide of *isoform B* may mediate this process. In this context it is interesting to note that both the synthetic peptide corresponding to the 28-amino acid N-terminal leader sequence and recombinant *isoform B* of trypsinogen 4 possess significant affinity toward artificial lipid monolayers. Furthermore, these species (but not the active protease) bind to the membranes of microsomes isolated from rat brain (J. Tóth et al., unpublished data). The proposed biological functions of this enigmatic, primate specific protease, such as generation of β-amyloid fragments [31] and the activation of PARs, PAR-1 [34], PAR-2 and -4 [12] all require some kind of membrane association. Further studies are in progress to provide evidence for the functional association of trypsinogen 4 with cell membranes and a specific biological role of this association in some physiological or pathological processes in human brain.

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