Human High Temperature Requirement Serine Protease A1 (HTRA1) Degrades Tau Protein Aggregates

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Background: Protein quality control proteases degrade damaged proteins and protein fragments. Results: The human serine protease HTRA1 degrades tau aggregates and is induced by its substrates. Conclusion: A member of the widely conserved HtrA family is involved in protein quality control in mammalian cells. Significance: HTRA1 might function as a tau protease in vivo.

Protective proteases are key elements of protein quality control pathways that are up-regulated, for example, under various protein folding stresses. These proteases are employed to prevent the accumulation and aggregation of misfolded proteins that can impose severe damage to cells. The high temperature requirement A (HtrA) family of serine proteases has evolved to perform important aspects of ATP-independent protein quality control. So far, however, no HtrA protease is known that degrades protein aggregates. We show here that human HTRA1 degrades aggregated and fibrillar tau, a protein that is critically involved in various neurological disorders. Neuronal cells and patient brains accumulate less tau, neurofibrillary tangles, and neuritic plaques, respectively, when HTRA1 is expressed at elevated levels. Furthermore, HTRA1 mRNA and HTRA1 activity are up-regulated in response to elevated tau concentrations. These data suggest that HTRA1 is performing regulated proteolysis during protein quality control, the implications of which are discussed.

Human HTRA1 belongs to the widely conserved high temperature requirement A (HtrA) family of homo-oligomeric and ATP-independent serine proteases (1, 2). HtrAs of pro- and eukaryotes are implicated in protein quality control. They can act as key stress sensors and regulators of unfolded protein response signaling pathways and can mediate the repair and assembly or the removal of damaged, fragmented, and mislocalized proteins (3–9). Defining features of HtrA proteases are their homo-oligomeric architecture and the presence of C-terminal PDZ domains that can be involved in substrate processing, sensing of misfolded proteins, mediating allosteric and cooperative regulation of the proteolytic activity, and in the switch between various oligomeric states (2).

Among the four human HTRAs, HTRA1–4, the ubiquitously expressed HTRA1 consists of a signal sequence for secretion, partial insulin-like growth factor-binding protein-7 domain, serine protease domain resembling classic serine proteases such as trypsin and one C-terminal PDZ domain. Like Escherichia coli DegP, a prototypic HtrA protease involved in protein quality control, HTRA1 is activated by oligomerization. Substrate binding triggers the switch between the resting and the active conformations and between various oligomeric states (10, 11).

HTRA1 has at least three cellular localizations and a multitude of functions. Extracellular HTRA1 is involved in the homeostasis of the extracellular matrix, and elastin, fibulin 5,

The abbreviations used are: HtrA, high temperature requirement A; AD, Alzheimer disease; AFM, atomic force microscopy; Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; PHP, pseudohyperphosphorylated; pNA, p-nitroaniline; qRT-PCR, quantitative RT-PCR; ThT, thioflavin T.

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Recent studies suggest that substrate specificity and processing of individual HtrA proteases can differ significantly. Whereas bacterial DegS is a regulatory protease that cleaves its single substrate at one defined position, other HtrAs such as E. coli DegP digest a great many of un- or misfolded proteins into small peptides (8, 9, 27, 28). However, these and other studies suggested that HtrA proteases do not degrade protein aggregates. This model was supported, for example, by the precise understanding of the proteolytic mechanism of DegP, requiring concurrent binding of substrates to its PDZ domain 1 and the active site for both activation and proteolysis (28–30). However, the recent elucidation of crystal structures of HTRA1 and complementing mechanistic studies indicating that proteolysis and activation of HTRA1 occur in a PDZ domain-independent manner prompted us to address the question of whether human HTRA1 is able to use protein aggregates as substrates (10).

To test whether HTRA1 degrades protein aggregates we used tau as a model substrate because tau is, like HTRA1, associated with microtubules. Furthermore, tau is of important clinical relevance, and its aggregation is widely studied. It is therefore well established that the tau protein can aggregate into intracellular neurofibrillar tangles that are specific pathological features of AD and other tauopathies. Normal tau, which is abundant in axons, is thought to regulate microtubule dynamics. Interaction of tau with microtubules is mediated by its microtubule binding domain consisting of three or four repeats sharing the consensus sequence VXS/S/QXX(N/L/I)XXHPGGG. Posttranslational modifications, such as phosphorylation, can decrease the affinity of tau to microtubules leading to dissociation and polymerization into straight or paired helical filaments, ribbons, and other conformations. Progressive intramolecular interactions of fibrils composed of paired helical filaments of tau lead to the formation of neurofibrillary tangles. A core domain of paired helical filaments consists of three or four repeats of tau involved in stable tau-tau interactions (Fig. 1A). Within this core domain, one short stretch, the self-assembly region VQI(I/V)/XK, seems of critical importance. Proteolytic processing of tau stimulates the assembly of tau into fibrils. Such assembly can capture further full-length tau proteins for processing leading to progressive growth of the toxic protein aggregates eventually causing cell death (for review see Refs. 31, 32).

EXPERIMENTAL PROCEDURES

Plasmids

Bacterial expression vectors of human tau and the GFP-tau construct pEGFP-C3–3Rtau were described earlier (33, 34). Expression plasmid pHTRA1 was generated by PCR and sub-cloning of HTRA1 into pBabe (35). HTRA1-mCherry constructs were derivatives of pmCherry-N1 (Clontech). Expression of WT-tau, Ala-tau, and pseudohyperphosphorylated (PHP)-tau was done as described (36).

Purification of HTRA1, HTRA1ΔPDZ Domain, WT-Tau, Ala-Tau, and PHP-Tau

For all in vitro protease assays, HTRA1 and HTRA1ΔPDZ domain were purified and used as described (24) except that an additional hydroxyapatite column (Bio-Rad) was added. Recombinant tau variants were isolated by boiling of cleared bacterial cell extracts (lysis buffer: 33 mM Tris-HCl, pH 8, 100 mM KCl) in the water bath for 30 min. Tau remains soluble, whereas the precipitated bacterial proteins were cleared from the lysate by centrifugation (35,000 × g, 40 min). For further purification of the 3R tau variants (0N3R, 352 amino acids), the supernatant was incubated with ammonium sulfate at 30% saturation for 30 min, 4 °C, followed by centrifugation at 20,000 × g, 30 min. Tau was precipitated from the supernatant with 40% saturated ammonium sulfate. Purified tau was suspended in 80 mM PIPES/KOH, pH 6.8, 1 mM EGTA, 1 mM MgCl2. For the isolation of insoluble and soluble fractions of tau, protein reconstituted from ammonium sulfate pellets was incubated at 25 °C for 20 min to ensure complete dis-solving of precipitated tau. Ultracentrifugation (100,000 × g, for 1 h) was used to separate insoluble pellet (3RP) and soluble fractions (3RS). The insoluble protein was resuspended from the resulting pellet and the protein concentration determined by Bradford quantitation.

Purification of 441-Amino Acid WT-Tau Protein

The 4R WT isoform of human tau (2N4R, 441 amino acids) used for heparin-induced fibrillization was purified in the same way as the 3R variants described above except that instead of ammonium sulfate precipitation the boiled lysate was subjected to hydroxyapatite chromatography (Bio-Rad). 4R tau was eluted with an NaCl gradient in 100 mM HEPES, 10 mM KPO4, 2 mM DTT, pH 7.6. Subsequently, monomeric and dimeric 4R tau was isolated using size exclusion chromatography (Superdex HiLoad 200 26/60; GE Healthcare).

Heparin-induced 4R Tau Fibrillization, Degradation of 4R Tau Fibrils

The in vitro formation of PHF-like tau filaments was performed as described (37). Briefly, 20 μM 4R tau was incubated at 55 °C, 10 min in aggregation buffer (100 mM sodium acetate, pH 7.0, 2 mM DTT) before addition of 50 μM heparin (Sigma-Aldrich) and incubation at 37 °C, 1,000 rpm, for the time points indicated. Proteolytic digests of the tau aggregates were performed as described above except for the following modifications. A 5-fold molar excess of tau over the protease was used based on the molecular mass of monomeric tau, 5 mM reducing
agent Tris(2-carboxyethyl)phosphine was added to the reactions and 50 mM NaH$_2$PO$_4$, pH 8, was used for proteolysis by HTRA1.

**Atomic Force Microscopy (AFM)**

Tau protein samples (1 $\mu$M tau in PBS, pH 7.5) were deposited on a freshly cleaved mica surface (Plano GmbH) without any previous treatment and adsorbed for 3 min at room temperature. After addition of 15 $\mu$L of 1 x TAEM (40 mM Tris, 20 mM acetic acid, 2 mM EDTA, 12.5 mM Mg acetate, pH 8.0), the sample was scanned in tapping mode using a MultiMode$^\text{TM}$ microscope (Veeco Metrology, Santa Barbara, CA) equipped with a Nanoscope IV controller. 0.58 N/m force constant cantilevers with sharpened pyramidal tips (SNL-10 tips, Veeco Metrology) were used for scanning. After engagement, the tapping amplitude set point was typically less than 1 volt, and the scan rates ranged between 1 and 2 Hz. Multiple AFM images were recorded from different locations of the mica surface to ensure reproducibility of the results. All images were analyzed with the Nanoscope 6.14R1 and ImageJ software (38).

**Thioflavin T (ThT) Fluorescence**

To characterize the aggregation state of tau, 10-$\mu$L duplicates of the samples at a tau concentration of 20 $\mu$M were added to 90 $\mu$L of 12 $\mu$M ThT (Sigma-Aldrich) in 50 mM glycine, pH 8.5, using black clear-bottom 96-well microtiter plates. After incubation at 37 °C, 900 rpm, the fluorescence was measured with a SpectraMax M5 Microplate Reader (Molecular Devices). For emission spectra, the excitation wavelength was kept constant at 440 nm with emission wavelengths ranging from 450 to 520 nm.
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nm in 5-nm intervals. Single measurements were performed at an excitation and emission wavelength of 440 and 480 nm, respectively. The cutoff filter was set to 450 nm in all cases.

**Protease Assays and Mass Spectrometry**

Protease assays were performed at 37 °C in 50 mM Tris-HCl, pH 8.0. For mass spectrometry, 1 μg of HTRA1 was incubated with 10 μg of WT-tau, Ala-tau, or PHP-tau in a final volume of 300 μl. After 4 h at 37 °C, the pellets containing the insoluble tau protein were precipitated with acetone. MALDI-TOF mass spectra were obtained as described (24). Samples were loaded onto 10% SDS-PAGE and analyzed by Western blotting using a specific antibody against the tau396 phosphorylation site.

**HTRA1 Assay**—The proteolytic activity of human recombinant HTRA1s was measured using PGGGNKETHKL-p-nitroaniline (pNA) (tau fragment (Pro363-Leu376)) and VEN-TLPMMGKASPV-pNA as substrates that were synthesized following standard procedures (10, 39, 40). 1.35 μM HTRA1s were incubated with 500 μM respective substrate and the concentrations of WT- or PHP-tau indicated in 50 mM Tris-HCl, pH 8, at 37 °C. The release of nitroaniline was monitored continuously by measuring the absorption at λ = 405 nm every minute for 90 min. The reactions were performed in 96-well microplates using a SpectraMax M5 Microplate Reader. For calculation of the specific activity, a time segment of linear increase in absorption was used to quantify the turnover of the substrate by employing the specific molar absorption coefficient of nitroaniline, 8,800 M⁻¹ cm⁻¹.

**Caspase 3 Assay**—The proteolytic activity of human recombinant caspase 3 was assayed using 200 μM Ac-DEVD-pNA (AAT Bioquest) as a substrate and 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol, 10 mM DTT, as the reaction buffer.

**Fluorometric Calpain Assay**—Human calpain 1 (Calbiotech) activity was determined at 23 °C using Suc-LLVY-AMC (Sigma) as a substrate in 50 mM Tris-HCl, 100 mM NaCl, 2 mM CaCl₂, 1 mM DTT, pH 7.5. Substrate and calpain 1 concentrations were 200 μM and 0.52 μM, respectively. Fluorescence was monitored continuously by performing single measurements every minute for 90 min with an excitation wavelength of 380 nm and an emission wavelength of 450 nm using a SpectraMax M5 Microplate Reader, and the cutoff filter was set to 435 nm. From the initial linear increase in fluorescence, the concentration of free AMC was quantified using an AMC standard curve generated. The specific activity of calpain 1 was calculated as the ratio of substrate turnover and amount of enzyme.

**Proteolysis of Insoluble and Soluble Tau Fractions**—Purified WT-3R tau samples were centrifuged at 100,000 × g, 4 °C, for 1 h to separate the soluble and insoluble fractions using an Optima MAX-XP Benchtop Ultracentrifuge (Beckman Coulter). The pellets containing the insoluble tau protein were resuspended with 50 mM Tris-HCl, pH 8. The protein samples were diluted to the assay concentration with the respective assay buffers, which were 50 mM Tris-HCl, pH 8, for HTRA1, 2 mM CaCl₂, 1 mM EDTA, for calpain 1 and 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol, 10 mM DTT for caspase 3. Before addition of the protease, tau was diluted in assay buffer to the final concentration, which was for calpain 1 and HTRA1 20 ng/μl and for caspase 3 1.8 ng/μl. After incubation of the tau solutions at the assay temperature for 2 min, protease was added at the molar ratio of protease to substrate of 1:10. The samples were incubated at 37 °C (HTRA1 and caspase 3) or 23 °C (calpain 1) with agitation, aliquots were taken at the time points indicated. Aliquots were mixed with SDS loading dye and a final concentration of 40 mM reducing agent TCEP and immediately frozen with liquid N₂. Prior to SDS-PAGE using Novex NuPage 10% Bis-Tris gels (Invitrogen) and MES running buffer, the samples were heat-treated at 75 °C for 10 min. Protein bands were visualized by silver staining.

**Transfection**

Stable transfection and transduction of PC12 cells with tau constructs or HTRA1 were carried out as described (36, 41).

**Microscopy**

To analyze the localization of HTRA1 in U373 cells, cells were transiently transfected. Cells were plated in a 35-mm poly-D-lysine-coated glass-bottom dish (MatTek) at a density of 10⁵ cells/plate. 24 h after plating, cells were transfected by Jet-PEI (Polyplus) either with one plasmid or for co-localization studies with two plasmids. 24 h after transfection cells were analyzed in a Leica TCS SL (SP5) laser confocal microscope, and Leica Confocal Software was used for imaging. Images were taken using the HCX PL APO ×63 oil objective lens.

**Quantitative RT-PCR (qRT-PCR)**

qRT-PCR was carried out as described (42) using the following primers: ratHtrA1 forward, CCTTTTTGTAGACATCATC and ratHtrA1 reverse, GATGTAATCTCGGAG-CATATATC; rat β-actin forward, GATTACTGCTCTGGCTCCTAG and rat β-actin reverse, ACTCATGTCACTCTGGC- TTGC; human tau forward, CCATGCCAGACTCGAAGAT and tau reverse, TGCTCAGGTCAACTGGTTTG. The housekeeping gene β-actin was used to normalize the results.

**Human Brain Tissue**

Brain tissue was obtained from the German Brain Bank “Brain-Net” and collected at the Institute of Neuropathology, University Hospital Muenster. Prior to autopsies, consent from patients’ families was obtained to use samples for research. In all cases (AD brain and controls) staging of AD-related neurofibrillary pathology according to Braak was performed (43, 44). All control cases have Braak stages I or II (absence of neurofibrillary tangles in the frontal cortex); all AD cases have Braak stages V or VI (abundant neurofibrillary tangles in the frontal cortex). 100 mg of tissue was taken from frozen postmortem samples of the frontal gray matter of 29 AD cases (mean age 76.2 years; range 63–88; mean postmortem time 23.8 h, range 5–48 h) and 24 sex-matched controls (mean age 71.3 years; range 59–92; mean postmortem time 19.3 h, range 5–43 h).
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Human Brain Homogenates

Tissue was homogenized in 250 mM sucrose, 20 mM Tris, 1 mM EDTA, and 0.1 mM EGTA by sonication. Protein extraction was performed by formic acid treatment as described (45).

Quantitative Analysis of Neuritic Plaques and Neurofibrillary Tangles

Quantitative analysis of neuritic plaques and neurofibrillary tangles was performed using sections from the medial frontal gyrus (46). Briefly, immunophenotypes of AD brains were analyzed quantitatively by determining the number of Aβ/τ-positive neuritic plaques and the number of τ-positive neurofibrillary tangles (anti-Aβ, 6F/3D, 1:1,000; anti-τ, AT8, 1:2,000). Eight consecutive representative fields from severely affected tissue areas were evaluated by computer-based image analysis.

HTRA1, Tau, and TauP396 ELISA

HTRA1 in human brain samples was quantified by HTRA1-specific ELISA as described (14). Tau and tauP396 levels in human brain samples were determined by tau and tauP396-specific ELISAs (Invitrogen).

Statistical Analyses

HTRA1 mRNA levels in PC12 cell lines were analyzed by ANOVA, data are presented as means. The HTRA1 and tau protein levels as well as numbers of neuritic plaques and tangles in patient brains were analyzed by two-tailed p values with correlation coefficient using Prism (GraphPad) and SPSS version 16.0 (SPSS) statistical analysis software. A Pearson r value within 0 to −1 indicates that one variable increases as the other decreases.

Antibodies

Rabbit polyclonal antibodies against HTRA1 were generated using the purified recombinant PDZ domain of HTRA1 (residues 377–480). Production of mouse monoclonal antibody is described elsewhere (24). Antibodies against tubulin, WT-tau, phosphorylated tau396, tau repeat, β-actin, Aβ were from Invitrogen, Innogenetics, MP Biomedicals, IBL, and DAKO, respectively.

RESULTS

HTRA1 Digests Tau in Vitro—First, we examined whether soluble tau is a substrate of HTRA1 in vitro. We used purified normal human tau (WT), PHP human tau in which the phosphorylated Ser/Thr residues were mutated to Glu residues, and a corresponding Ala mutant (Ala-tau) in which Ser/Thr residues were mutated to Ala residues (36). Tau substrates were incubated with purified HTRA1, and the proteolytic products were identified by mass spectrometry. HTRA1 cleaved each tau substrate into 45 fragments. 22 cleavage sites that are located in the microtubule binding and self-assembly regions were identical in WT-tau, PHP-tau, and Ala-tau (Fig. 1A). The produced fragments varied in length between 8 and 38 residues with the majority of products ranging from 9 to 22 residues (supplemental Fig. S1). Sequence analysis of the products revealed a preference for Leu, Val, and lle at the P1 position but little further specificity (supplemental Fig. S2). These results are in agreement with recent data of complete digests of citrate synthase and malate dehydrogenase (10). For convenient determination of enzymatic parameters of tau processing, we synthesized peptide substrates derived from the produced tau fragments by introducing a C-terminal pNA group, i.e. KHQPGGGKVK-pNA, VYKPVDLSKV-pNA, and PGGGNKKIETHKL-pNA. Whereas KHQPGGGKVK-pNA was not processed by HTRA1, the specific activities for VYKPVDLSKV-pNA and PGGGNKKIETHKL-pNA were 0.9 and 2.2 nmol × mg⁻¹ × min⁻¹, respectively. We therefore chose PGGGNKKIETHKL-pNA for further experiments. PGGGNKKIETHKL corresponds to residues 364–376 of tau and contains the C terminus of the fourth repeat region (Fig. 1A, UniProt accession no. P10636). Processing of PGGGNKKIETHKL-pNA followed Michaelis-Menten kinetics with a $K_m$ of 1.9 ms and $V_{max}$ of 64 nmol/mg of protease per min. The fact that a 13-mer peptide is a substrate suggests that HTRA1 in addition to full-length tau could also degrade tau fragments that are produced for example by other proteases.

We subsequently tested whether HTRA1 digests tau aggregates by incubating two purified tau samples. One, termed 3RS, was the supernatant fraction following ultracentrifugation, and the insoluble form, termed 3RP, corresponded to the pellet fraction following ultracentrifugation. The two fractions were subsequently characterized further with respect to their β-sheet content and the size of the tau particles present in both samples. ThT, a common amyloid-specific fluorescent dye (47), showed characteristic fluorescence emission at $\lambda = 490$ nm, suggesting a high β-sheet content (supplemental Fig. S3A). The ThT signal of soluble tau is assumed to be caused by soluble oligomers rich in β-sheet structures. Dynamic light scattering suggested the enrichment of larger particles, probably aggregated tau protein, in the insoluble fraction as indicated by increased hydrodynamic radii and total scattering intensities (supplemental Fig. S3, B and C, and supplemental Experimental Procedures). AFM was employed to assess further the tau species found in the soluble and insoluble fractions. Whereas soluble tau is present as small particles, such as monomeric and oligomeric tau species, the sizes of the insoluble material obtained from ultracentrifugation showed a much broader distribution with a major fraction representing large aggregated material (supplemental Fig. S3, D–F), which was of amorphous structure. At a molar substrate:protease ratio of 10:1, HTRA1 digested soluble and insoluble tau within 30 and 120 min, respectively (Fig. 1, B and C). We concluded that in addition to soluble proteins, HTRA1 is able to digest insoluble aggregated tau protein. To compare the activity of HTRA1 with two other proteases known to cleave tau we incubated both tau samples with human calpain 1 and recombinant human caspase 3 (48, 49). Calpain digested soluble and insoluble tau about twice and 10 times as fast as HTRA1, respectively (Fig. 1, D and E). Furthermore, and in contrast to HTRA1 and calpain 1, caspase 3 digested tau not completely, but generated large tau fragments (Fig. 1, F and G), which can be explained by the pronounced substrate specificity of caspases (50). It should be noted that in the insoluble tau fractions, an additional band of unknown
identity migrates slightly above the tau monomer. This protein is not digested by either protease.

As AFM images of the tau aggregates described above did not show the characteristic, well ordered fibrillar structure of tau aggregates typically found in tauopathies, we produced tau fibrils by incubation of the 441 amino acid isoform of tau (4R) with heparin for 7 days (37). The formation of amyloid aggregates was detected by an increase in ThT fluorescence over time (Fig. 2A). The resulting aggregates detected by AFM revealed fibrillar structures of varying lengths (100 nm up to >2 μm) and a characteristic width of approximately 20–25 nm (Fig. 2B) (51). These highly ordered tau filaments were also subjected to proteolytic cleavage and were digested by both HTRA1 and calpain 1 (Fig. 2, C and D), albeit with lower efficiency compared with the soluble and insoluble material. Only after overnight incubation was almost all of the tau protein proteolyzed, which can be explained by the tight packing of tau monomers within amyloid-like fibers rendering them more protease-resistant. These results suggest the ability of HTRA1 to degrade not only amorphous tau aggregates, but also PHF-like tau filaments characterized by tight intermolecular interactions giving rise to the highly structured, cross-β-sheet-rich assembly typical for amyloid (52, 53).

HTRA1 Is Activated by Tau—Because HTRA1 function can be activated by its substrates (10), we tested whether tau was able to act as an inducer of the proteolytic activity of HTRA1, using calpain 1 and caspase 3 as a control. To quantify HTRA1 activity we used two synthetic substrates PGGGNKKIETHKL-pNA and VFNTLPMMGKASPV-pNA. The addition of tau and PHP-tau caused a 4–6-fold activation of HTRA1 activity, whereas calpain 1 and caspase 3 were only slightly or not at all activated under these conditions (Table 1). To confirm that in contrast to other HtrA proteases, HTRA1 does not employ an allosteric mechanism involving its PDZ domain, we repeated

FIGURE 2. Proteolytic degradation of tau fibrils by HTRA1 and calpain 1. Fibrillization of 4R-tau (20 μM) was induced by incubation the anionic co-factor heparin (50 μM) for 7 days. A, the extent of fibril formation was monitored by ThT fluorescence at an excitation and emission wavelength of 440 and 485 nm, respectively. 10 μl of the aggregation samples was incubated with 12 μM ThT in duplicate at the indicated time points. B, AFM analysis revealed the typical fibrillar morphology of in vitro PHF-like tau aggregates, whereas the control (small image) does not show any fibrillar structures when heparin is not used. C and D, 4R-tau fibrils were subjected to proteolytic cleavage by HTRA1 (C) and calpain 1 (D), performed as described in Fig. 1.
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| Protease       | Substrate        | 0°  | 0.5° | 1°  | 2°  | 0.5° | 1°  | 2°  |
|----------------|------------------|-----|------|-----|-----|------|-----|-----|
| HTRA1          | PGGGNKKEITHKL-pNA| 1°  | 5.6  | 5.7 | 5.5 | 5.9  | 5.0 | 5.0 |
| HTRA1          | VFNTLPMMGKASPV-pNA| 1°  | 4.5  | 4.4 | 3.9 | 4.3  | 4.2 | 3.9 |
| HtrA1 APDZ     | PGGGNKKEITHKL-pNA| 1°  | 22.4 | 230 | 21.7| 21.2 | 20.5| 21.1|
| HtrA1 APDZ     | VFNTLPMMGKASPV-pNA| 1°  | 10.2 | 9.6 | 8.8 | 10.6 | 10.1| 9.2 |
| Calpain 1      | Suc-LLVY-AMC     | 1°  | 1.2  | 1.3 | 1.3 | 1.2  | 1.3 | 1.4 |
| Caspase 3      | Ac-DEVD-pNA      | 1°  | 1.0  | 1.0 | 1.0 | 1.0  | 1.0 | 1.0 |

* Molar ratio tau: protease. Specific activities of the proteases indicated in the absence of added tau proteins. These specific activities were set to 1 for the calculation of the activation factors.

** TABLE 1 Proteolytic activities of HTRA1, calpain 1 and caspase 3 in the presence and absence of tau and PHP-tau**

PGGGNKKEITHKL-pNA is derived from tau, VFNTLPMMGKASPV-pNA is a previously introduced synthetic HTRA1 substrate (10). The values shown represent means of four to nine independent experiments for each protease with an S.E. of < 10%.

these assays with an HTRA1 construct lacking its PDZ domain. Here, the addition of tau and PHP-tau caused an activation of the proteolytic activity by a factor of about 10 and 20 for VFNTLPMMGKASPV-pNA and PGGGNKKEITHKL-pNA, respectively. The increase in activation compared with HTRA1 and the differences between the two substrates used were in part due to the differences in basal activities, i.e. the activities without tau.

** Tau Levels and Regulation of HTRA1 Expression in Cell Culture**—Having established tau and tau aggregates as HTRA1 substrates, we wished to address the question of whether tau might be a physiological substrate of HTRA1. Initially, we asked whether a modulation of HTRA1 levels affects tau levels in cells. However, this model is unlikely because RNA levels of WT-tau were similar in patient and control brains whereas the levels of phosphorylated tau were significantly increased in AD versus control brains. Interestingly, total tau and phosphorylated tau levels correlate inversely with HTRA1 levels in AD but not in control brains in statistically relevant manner (p = 0.018–0.026). To confirm our findings, we used contralateral frontal cortex to determine the content of neurofibrillary tangles and neuritic plaques as determined by immunohistochemistry of AD brains (24).

**Correlation of HTRA1 and Tau Levels in Patient Samples**—To obtain further evidence for the correlation of HTRA1 and tau levels, 29 AD patient and 24 control brains were analyzed by ELISAs measuring HTRA1, total tau, and phosphorylated tau-p396 levels (Fig. 4, A–D). As expected, the total tau levels were similar in patient and control brains whereas the levels of phosphorylated tau were significantly increased in AD versus control brains. Interestingly, total tau and phosphorylated tau levels correlate inversely with HTRA1 levels in AD but not in control brains in statistically relevant manner (p = 0.018–0.026). To confirm our findings, we used contralateral frontal cortex to determine the content of neurofibrillary tangles and neuritic plaques as determined by immunohistochemistry of AD brains (24).

** DISCUSSION**

Whereas the involvements of bacterial HtrA proteases in all aspects of protein quality control have been widely studied and convincingly demonstrated (2), there is limited experimental evidence that human HTRAs are involved in similar processes. A recent study showed the association of HTRA1 with amyloid deposits in the human cornea (54). Accordingly, the observations that HTRA1 degrades aggregated tau as well as Aβ pep-
tides (24), its up-regulation on the transcriptional level and the activation of its proteolytic activity in response to the presence of elevated tau concentrations, respectively, suggest that the protein quality control function of HtrA proteases is conserved. The results presented in this work provide direct biochemical evidence for the association of HTRA1 with amyloid aggregates. Even though HTRA1 performs its tasks in an ATP-independent manner, aggregated and fibrillar forms of tau are degraded. The products of the proteolytic reaction are typically between 8 and 22 residues long, and sequence specificity analysis suggests a preference for Leu, Val, and Ile at the P1 position but little further specificities. This feature is similar to the bacterial HtrA family member DegP, which degrades unfolded substrates with little specificity into fragments of a mean length of 13–15 residues (28). However, DegP does not degrade aggregates.

There are at least two possibilities why HTRA1 is able to degrade tau aggregates. First, the active site of HTRA1 is surface-accessible (10); and second, the PDZ domain might be instrumental in this process because it might be involved in binding and thus extracting tau monomers from aggregates. In addition, it is unlikely that HTRA2 and HTRA3 degrade tau
in cells because they are localized to mitochondria (55, 56), and there are still insufficient data on HTRA4 available to speculate about a possible functional redundancy with HTRA1 (22).

Digests of two synthetic substrates revealed that HTRA1 is activated by its native substrate tau. That both HTRA1 and HTRA1\(\Delta\)PDZ domain are activated by substrate indicates that HTRA1 employs an induced-fit mechanism where substrate bound to the active site directly induces a rearrangement of regulatory elements within the protease domain (10) rather than an allosteric mechanism that was previously identified for all other HtrA proteases studied so far (2). In contrast, the mechanism of transcriptional up-regulation of HTRA1 in neuronal cells upon overexpression of tau and PHP-tau remains elusive. Even though there are numerous reports in the literature describing the up-regulation of HTRA1 expression, for example during endometriosis (57), age-related macular degeneration (58), neuronal development (59), following chemotherapy (60, 61), and during arthritis (15, 62), very little is known about the regulation of the HTRA1 gene on the transcriptional level. Therefore, the future identification of the signaling cascades and relevant transcription factors mediating the up-regulation of the HTRA1 promoter in response to the presence of increased levels of intracellular tau protein might be of general importance as these pathways might also play a role in the pathologies mentioned above.

Both the transcriptional and posttranscriptional regulatory mechanisms that contribute to elevated HTRA1 activity are expected to contribute to the marked reduction of tau levels.

FIGURE 4. HTRA1 levels correlate with tau levels and neuritic AD pathology in patient brains. A–D, protein was extracted from homogenized cortex of 29 AD patients (A and C) and 24 normal brain samples (B and D). Subsequently, WT-tau (A and B), tauP396 (C and D), and HTRA1 levels were measured by ELISA. E and F, neurofibrillary tangles (NFT) (E) and neuritic plaques (F) determined by immunohistochemistry (number/mm²) correlated with HTRA1 protein levels in frontal cortex sections of 25 AD brains.
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upon overexpression of HTRA1 in neuronal PC12 cells. Furthermore, a significant inverse correlation between HTRA1 and tau levels is observed in AD patient brains. These data suggest that HTRA1 might function as a tau protease in vivo.

Unfolded protein response systems may represent one strategy of how organisms prevent early onset of protein folding diseases. Because these diseases are more prevalent in aged individuals it is conceivable that the various stress response systems are overwhelmed by additional tasks. In case of AD, additional substrates produced by aged tissues could compete with tau or Aβ for clearance by stress response factors leading to the accumulation and ultimately aggregation of toxic protein fragments. The serine protease HTRA1 represents a viable candidate for a disease-modifying factor that might contribute, perhaps in concert with other proteases such as calpain, to maintaining Aβ and tau levels low. This notion is supported by previous work suggesting that HTRA1 is involved in the β-amyloid pathway by performing alternative processing of various amyloid precursor protein fragments, i.e. Cys99, Aβ_{1-2}, and Aβ_{30}. In line with this hypothesis, accumulation of Aβ was observed in astrocytoma cell culture supernatants following chemical inhibition of HTRA1 and by co-localization of HTRA1 with β-amyloid deposits in human brain samples (24). The recently reported association of HTRA1 with conneal amyloid deposits of TGFB1 (transforming growth factor β-induced gene), however, raises both the possibility of the generation of amyloidogenic fragments and the clearance of amyloid aggregates through proteolysis by HTRA1 (54). Moreover, recent evidence from studying adult macular degeneration suggests that it is the increased activity of HTRA1, resulting from its overexpression that causes disease symptoms (12, 13). These data illustrate conflicting findings with regard to the toxicity of protein fragments, their aggregation, as well as the functional role of proteolytic processing by protein quality control factors. Addressing these contrasting models in future studies is likely an important step for our general understanding of the underlying mechanisms of protein folding diseases.

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