The microtubule-associated protein tau aggregates intracellularly by unknown mechanisms in Alzheimer’s disease and other tauopathies. A contributing factor may be a failure to break down free cytosolic tau, thus creating a surplus for aggregation, although the proteases that degrade tau in brain remain unknown. To address this issue, we prepared cytosolic fractions from five normal human brains and from perfused rat brains and incubated them with or without protease inhibitors. [D-Phenylalanyl-L-prolylarginyl chloromethyl ketone, a thrombin-specific inhibitor, prevented tau breakdown in these fractions, suggesting that thrombin is a brain protease that processes tau. We next exposed human recombinant tau to purified human thrombin and analyzed the fragments by N-terminal sequencing. We found that thrombin proteolyzed tau at multiple arginine and lysine sites. These include Arg155–Gly156, Arg209–Ser210, Arg230–Thr231, Lys257–Ser258, and Lys340–Ser341 (numbering according to the longest human tau isoform). Temporally, the initial cleavage occurred at the Arg155–Gly156 bond. Proteolysis of the resultant C-terminal tau fragment then proceeded bidirectionally. When tau was phosphorylated by glycogen synthase kinase-3β, most of these proteolytic processes were inhibited, except for the first cleavage at the Arg155–Gly156 bond. Furthermore, paired helical filament tau prepared from Alzheimer’s disease brain was more resistant to thrombin proteolysis than following dephosphorylation by alkaline phosphatase. The results suggest a possible role for thrombin in proteolysis of tau under physiological and/or pathological conditions in human brains. They are consistent with the hypothesis that phosphorylation of tau inhibits proteolysis by thrombin or other endogenous proteases, leading to aggregation of tau into insoluble fibrils.

Intracellular aggregates of the microtubule-associated protein tau are one of the pathological hallmarks of Alzheimer’s disease. They are also the hallmark of a number of other neurodegenerative diseases that are now collectively referred to as tauopathies. Thus far, the biochemical features of aggregated tau have been most extensively studied in the paired helical filaments (PHFs) of Alzheimer’s disease brains. They include hyperphosphorylation (1), glycation (2, 3), glycosylation (4), ubiquitination (5), isomerization (6), and nitration (7). Of these, hyperphosphorylation is the most common feature of aggregated tau in tauopathies. The mechanism of tau aggregation still remains unknown, however. One hypothesis is that impaired proteolysis of tau perturbs tau turnover, leading to its availability for aggregation (8). In vitro, tau has been reported to be a substrate for a number of proteases such as trypsin, chymotrypsin, cathepsin D, calpains, caspases, proteasomal proteases, double-stranded DNA-stimulated protease, and thrombin (9–15). However, the proteases that degrade tau or the factors that affect its function in brain are still unclear.

To clarify these issues, we examined tau breakdown in cytosolic fractions extracted from five normal human brains incubated at 37 °C with or without various kinds of protease inhibitors. Here, we show that a thrombin-specific inhibitor, [D-phenylalanyl-L-prolylarginyl chloromethyl ketone (PPACK), completely repressed tau breakdown in this fraction. PPACK also inhibited tau breakdown in the same fraction prepared from perfused rat brains, indicating that thrombin in sufficient quantity for degrading tau is present in brain cells. These results suggest that thrombin is a brain protease that degrades tau. Furthermore, we have studied the in vitro proteolysis by thrombin of human recombinant tau and Sarkosyl-insoluble tau from a PHF preparation (referred to as PHF-tau) (9). We show that thrombin initially cleaved recombinant tau at the Arg155–Gly156 bond. It then cleaved the resultant C-terminal tau fragment bidirectionally at multiple Arg/Lys–Xaa bonds. Moreover, we show that recombinant tau phosphorylated by glycogen synthase kinase-3β (GSK-3β) and PHF-tau were more resistant to thrombin proteolysis than non-phosphorylated recombinant tau and dephosphorylated PHF-tau, respectively. These results suggest a possible role for thrombin in proteolysis of tau under physiological and/or pathological conditions.

**EXPERIMENTAL PROCEDURES**

**Effect of Protease Inhibitors on the Breakdown of Endogenous Tau in Soluble Human Brain Extracts**—Five subjects without any neurological symptoms were employed in this study. The age, sex, brain weight, post-mortem interval, brain regions examined, and diagnosis are given in Table I. Frozen brain tissue was homogenized in 5 volumes of ice-cold TS buffer (50 mM Tris-HCl [pH 7.6] and 150 mM NaCl). The homogenate was aliquoted into multiple ultracentrifugation tubes, and a different protease inhibitor was added to each tube. The protease inhibitors used were as follows: protease inhibitor cocktail, Pefabloc SC (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride), aprotinin, chymostatin, trypsin inhibitor from chicken egg white, leupeptin, antipain dihydrochloride, E-64, calpain inhibitors I and II, pepstatin, bestatin, and DTT, dithiothreitol; PIPES, 1,4-piperazinediethanesulfonic acid.

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1 The abbreviations used are: PHF, paired helical filament; PPACK, [D-phenylalanyl-L-prolylarginyl chloromethyl ketone; GSK-3β, glycogen synthase kinase-3β; DTT, dithiothreitol; PIPES, 1,4-piperazinediethanesulfonic acid.
Proteolysis of Unmodified and Phosphorylated Tau by Thrombin

**TABLE I**

| Case no. | Age at death | Sex | Brain wt | PMI\(^a\) | Region | Diagnosis |
|----------|--------------|-----|----------|-----------|--------|-----------|
| 1        | 71           | Female | 1200     | 4         | Frontal | Myocardial infarction |
| 2        | 63           | Female | 1150     | 3.5       | Frontal | Adenoma of adrenal cortex |
| 3        | 75           | Female | 1260     | 10        | Temporal | Lung cancer |
| 4        | 93           | Female | 1180     | 24        | Temporal | Pneumonia |
| 5        | 78           | Male  | NA        | 7         | Frontal | Pulmonary failure |

\(^a\) PMI, post-mortem interval; NA, not available.

**TABLE II**

| Antibody | Type | Specificity | Recognition site | Dilution/conc | Source |
|----------|------|-------------|------------------|---------------|--------|
| AB5868   | Goat pAb* | PI | 1–16 | 1:5000 | Chemicon International, Inc. |
| HT7      | mAb  | PI | 159–163 | 0.01 μg/ml | Innotogenics |
| Anti-phospho-Thr\(^{312}\) tau | Rabbit pAb | P | Thr\(^{312}\) | 0.1 μg/ml | BIOSOURCE |
| Anti-phospho-Thr\(^{311}\) tau | Rabbit pAb | P | Thr\(^{311}\) | 0.1 μg/ml | BIOSOURCE |
| PHP-1    | mAb  | P | Ser\(^{396/404}\) | 1:10,000 | P. Davies |
| A0024    | Rabbit pAb | PI | C terminus | 1:50,000 | Dako Corp. |
| T46      | mAb  | PI | 404–414 | 0.5 μg/ml | Zymed Laboratories Inc. |

\(^*\) pAb, polyclonal antibody; mAb, mouse monoclonal; PI, phosphorylation-independent; P, phosphorylated.

Phosphoramidon (Roche Diagnostics, Basel, Switzerland); PPACK (BIOMOL Research Labs Inc., Hamburg, Germany); and caspase-3 inhibitor III (acetyl-DEVD chloromethyl ketone), potato carboxypeptidase inhibitor, and lactacystin (Calbiochem). The homogenates were centrifuged at 100,000 × g for 20 min at 4 °C, and the supernatants were collected. Part of the supernatant from each tube was mixed with Laemmli SDS sample buffer and boiled for 5 min. The remaining samples were incubated at 37 °C for 1, 2, and 4 h; mixed with Laemmli SDS sample buffer; and boiled. The protein concentrations in the supernatant were measured using the DC protein assay system (Bio-Rad) according to the manufacturer’s instructions.

The effect of each inhibitor on tau breakdown was evaluated by the following procedure. Samples were separated by 12.5% SDS-PAGE. Proteins in the gel were electrotransferred to a polyvinylidene fluoride membrane (Millipore Corp., Bedford, MA). After blocking with 5% skim milk and 0.1% Tween 20 in TS buffer, membranes were incubated with anti-tau antibody T46 or A0024, both of which recognize the C-terminal region of tau (see Table I). Following incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies, immunoreactivity was visualized by the chemiluminescence method using the ECL Western blotting system (Amersham Biosciences AB, Uppsala, Sweden).

**Effect of PPACK on the Breakdown of Endogenous Tau in Soluble Fractions Prepared from Perfused Rat Brain—**Adult Wistar rats were deeply anesthetized with ether and perfused with ice-cold saline containing 50 mM Tris-HCl (pH 6.9), 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride, 10 μM pepstatin. Cells were sonicated 15 times on ice, and the supernatant was collected after removing the cell debris by centrifugation at 10,000 × g for 15 min. NaCl was added to the supernatant to a final concentration of 0.5M, and the supernatant was boiled and then collected by centrifugation at 10,000 × g for 15 min. Finally, the extracted supernatant was dialyzed completely at 4 °C against buffer A (20 mM Pipes (pH 6.9), 50 mM NaCl, 2 mM DTT, 1 mM EGTA, 1 mM MgSO\(_4\), and 0.1 mM phenylmethylsulfonyl fluoride). The dialyzed tau supernatant was further purified on a Mono S HR 5/5 column. Before loading the supernatant, the column was equilibrated with buffer A. After loading, the column was washed with buffer A and eluted with a gradient of NaCl from 0 to 0.5 M in buffer A, and fractions of 1 ml were collected. The pooled fractions containing tau were then dialyzed at 4 °C against 40 mM HEPES, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride. Protein concentrations were determined by the method of Lowry et al. (50). The purified protein was stored at −70 °C until used.

Identification of the Sites of Thrombin Cleavage of Tau—TSDC stock buffer (14) was added to the recombinant tau samples (T39 or T40 at a concentration 0.5 or 1.0 mg/ml) to a final concentration of 17.5 mM PIPES (pH 6.9), 50 mM NaCl, 2 mM DTT, 1 mM EGTA, 1 mM MgSO\(_4\), and 0.1 mM phenylmethylsulfonyl fluoride). The dialyzed tau supernatant was further purified on a Mono S HR 5/5 column. Before loading the supernatant, the column was equilibrated with buffer A. After loading, the column was washed with buffer A and eluted with a gradient of NaCl from 0 to 0.5 M in buffer A, and fractions of 1 ml were collected. The pooled fractions containing tau were then dialyzed at 4 °C against 40 mM HEPES, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride. Protein concentrations were determined by the method of Lowry et al. (50). The purified protein was stored at −70 °C until used.

**Proteolysis of Phosphorylated Recombinant Tau by Thrombin—**In vitro phosphorylation was carried out by incubating T37 or T40 (0.05 mg/ml) at 30 °C in a reaction mixture containing 40 mM HEPES (pH 7.5), 10 mM MgCl\(_2\), 0.5 mM DTT, 0.2 mM ATP (Sigma), 0.05 or 0.5 mg/ml GSK-3β (EMD Biosciences, San Diego, CA), 2 mM EGTA, and protase inhibitor cocktail. After incubation for 3 h, the reaction was stopped by heating at 95 °C for 5 min. The denatured kinase was removed by centrifugation at 10,000 × g for 10 min, and the supernatant was collected. Western blotting was performed as described above to confirm the phosphorylation state of tau. This was done using phosphorylation-dependent anti-tau antibodies, including anti-phospho-Thr\(^{312}\) tau, anti-phospho-Thr\(^{311}\) tau, and PHP-1 (Table II). Antibody T46, which recognizes the C-terminal region of both nonphosphorylated and phosphorylated tau, was also used to detect total tau.

α-Thrombin was then added to the supernatant at a final concentration of 6.0 units/ml. Part of the sample was collected immediately, mixed with Laemmli SDS sample buffer, and boiled for 5 min. The remainder was incubated at 37 °C for 3 h, and the proteolytic reaction was stopped by the addition of Laemmli SDS sample buffer and boiling. For a negative control, T37 or T40 was treated in the same way, except that GSK-3β was eliminated from the first reaction mixture. The sum-

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For additional context, the provided text describes experiments involving the proteolysis of tau protein under various conditions, including the effects of various inhibitors and enzymes. It also mentions the use of antibodies to detect specific sites of tau breakdown and the role of thrombin in phosphorylated tau breakdown. The text references various proteins and conditions used in these experiments, such as PPACK, thermolysin, and phosphorylation-independent tau antibodies. The results are tabulated and graphically represented in tables and figures.
In each lane was 25 cubated at 37 °C for 1, 2, and 4 h.

Antibody T46.

Shown on the right in kilodaltons. Molecular mass markers are increased over time. With PPACK, a thrombin-specific inhibitor, the quantity of full-length tau did not change during incubation (Fig. 1) (22).

Identification of Thrombin as a Protease That Processes Tau in Soluble Human Brain Extracts—Fig. 1 shows a representative Western blot of human brain supernatant incubated at 37 °C and stained with antibody T46. Without any protease inhibitors, the quantity of the full-length tau isoforms decreased, and several C-terminal tau fragments from 18.5 to 27 kDa appeared, increasing with time. A thrombin-specific inhibitor, PPACK, completely inhibited these tau breakdowns. Table III summarizes the effects of protease inhibitors on tau breakdown in human brain supernatant. In addition to PPACK, protease inhibitor cocktail, Pefabloc SC (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride), leupeptin, and antipain dihydrochloride inhibited tau breakdown. Inhibitors that had no effect included aprotinin, chymostatin, trypsin inhibitor, E-64, calpain inhibitors, caspase-3 inhibitor III, pepstatin, bestatin, phosphoramidon, potato carboxypeptidase inhibitor, and lactacystin. Western blotting with anti-tau antibody A0024 showed the same results as obtained with antibody T46 (data not shown). These findings were consistent among all cases examined, indicating that thrombin is a major protease that degrades tau in human brain supernatant. Western blotting of human brain supernatant using anti-prothrombin (1:20,000; Dako Corp., Glostrup, Denmark) and anti-human thrombin (0.5 µg/ml; American Diagnostica Inc., Stamford, CT) antibodies showed bands corresponding to prothrombin, prothrombin-1, prothrombin-2, the B chain of α-thrombin, and the B2 chain of β-thrombin (Fig. 2) (22).

Tau Breakdown by Thrombin Takes Place in Soluble Fractions Prepared from Perfused Rat Brain—Fig. 3 shows the Western blots of a perfused rat brain supernatant incubated at 37 °C with and without PPACK and immunostained with antibody T46. In the sample without PPACK, the quantity of endogenous full-length tau decreased over time, with most of it disappearing after 24 h. On the other hand, in the sample with PPACK, the quantity of full-length tau was unchanged at 24 h. These results suggest that tau breakdown by thrombin can occur in brain that has been cleared of residual plasma.

Proteolysis of Non-phosphorylated Recombinant Tau by Thrombin—Fig. 4 illustrates the time course of proteolysis of T39 (A and B) and T40 (C and D) by thrombin, which was analyzed by 12.5% (A and C) or 16.5% (B and D) SDS-PAGE. The incubation time ranged from 0 to 180 min in A and C and was 24 h in B and D. The latter experiment was performed to detect fragments below 10 kDa (fragments j, k, s, and t) in sufficient quantity for sequencing, even though trace amounts of them were observed after 1 h of incubation (data not shown). The fragments indicated by arrows (fragments a–t) were subjected to N-terminal sequence analysis. The results are given in Table IV.

Sequence analyses of the fragments revealed five corresponding to tau residues. Four of them are common to T39 and T40. They are GAAPPG (fragments b, c, i, and l), SRTPSL (fragments d, f, and o), TPPKSP (fragments e, h, and p), and...
Proteolysis of Unmodified and Phosphorylated Tau by Thrombin

The amount of total GSK-3β was estimated to be 0.5 μg/ml compared with 0.05 μg/ml in the non-phosphorylated tau samples. The phosphorylation-dependent antibody (anti-phospho-Thr212 tau) and a phosphorylation-independent antibody (T46) indicated that a 10-fold higher concentration of GSK-3β (0.5 mg/ml) is appropriate for phosphorylating tau under the conditions used (Fig. 9A).

We then compared thrombin proteolysis of non-phosphorylated and phosphorylated tau by Western blot analysis using antibody T46. In unmodified tau, a 31-kDa band (corresponding to tau fragment b in Fig. 4A) and a full-length tau band were observed at 0 min of incubation with thrombin (Fig. 9B, lane 1). After 3 h of incubation, the full-length tau band disappeared, and three bands of 25, 22.5, and 17.5 kDa appeared (corresponding to tau fragments d, e, and h, respectively, in Fig. 4A) (Fig. 9B, lane 2). In phosphorylated tau, only a full-length tau band was observed at 0 min of incubation (Fig. 9B, lane 3). After 3 h of incubation, a 37-kDa band appeared (possibly corresponding to the phosphorylated tau fragment b in Fig. 4A) while part of the full-length tau band remained (Fig. 9B, lane 4). These results suggest that phosphorylation of tau by GSK-3β inhibits tau proteolysis by thrombin and causes delayed cleavage at the first cleavage site. Phosphorylation of T40 showed the same effect on its proteolysis by thrombin (data not shown).

Proteolysis of PHF-tau by Thrombin—To further investigate the effect of phosphorylation on tau proteolysis by thrombin, we analyzed thrombin degradation of PHF-tau extracted from Alzheimer’s disease brain with or without alkaline phosphatase treatment (Fig. 10). The results of Western blotting with antibody HT7, which recognizes the middle region of tau, showed that bands corresponding to full-length tau disappeared after 1 h of incubation with thrombin in both the non-dephosphorylated and phosphorylated samples. However, the degradation rates of the resultant tau fragments differed. In the non-dephosphorylated sample, two major fragments of 42.5 kDa (fragment a) and 37 kDa (fragment b) were observed at 0 min decreased after 1 h of incubation, and significant amounts remained even after 3 h of incubation. On the other hand, in the dephosphorylated sample, six fragments of 41 kDa (fragment c), 38 kDa...

Fig. 2. Western blot analysis of human brain supernatant and human plasma using anti-prothrombin (A) and anti-thrombin (B) antibodies. Lanes 1, human brain supernatant containing 25 μg of total protein; lanes 2, human plasma diluted at 1:10,000 in TSB buffer. Fragment a corresponds to prothrombin (85 kDa), fragments b and c to prethrombin-1 (50 kDa), fragment d to prethrombin-2 (36 kDa), fragment e to the B chain of α-thrombin (50 kDa), and fragment f to the B2 chain of β-thrombin (25 kDa). Molecular mass markers are shown on the right in kilodaltons.

Fig. 3. Western blot analysis of perfused rat brain supernatant incubated at 37 °C for 1, 3, 6, 9, 12, and 24 h. The amount of total protein loaded in each lane was 25 μg. The quantity of full-length tau decreased over time in the sample without PPACK (lanes 1–7), but did not change in that with PPACK (lanes 8–14). Staining was performed with antibody T46. Molecular mass markers are shown on the right in kilodaltons.

SEKLF (fragments j, s, and t). Sequence SKGIS (fragment t) was identified only in T40. These results suggest that thrombin can cleave tau at five different sites, which are Arg155–Gly156, Arg209–Ser210, Arg230–Thr231, Lys340–Ser341, and Lys257–Ser258 (numbering according to the longest human tau isoform). (23) These findings are consistent with a previous report that thrombin preferentially cleaves polypeptide substrates at Arg/Lys–Xaa bonds (24).

Other sequences identified were those of thrombin. They are IVEGSD (fragments a, g, and n), YERNIE (fragments e and q), GQPSVL (fragment i), and SPFNNR (fragment t), which correspond to the B chain of α-thrombin, the B2 chain of β-thrombin, the B4 chain of γ-thrombin, and C-terminal fragment of the B4 chain of γ-thrombin, respectively (Fig. 5) (22, 25). These results suggest that autolytic degradation of α-thrombin takes place during incubation as reported previously (26). The remaining three fragments (fragments k, m, and r) revealed no decipherable sequences. The reason for fragments k and r not revealing definite sequences might be an insufficient amount of protein. The reason for the failure of fragment m to yield a definite sequence is uncertain.

Fig. 6 illustrates the Western blot results of proteolysis of T40 by thrombin, detected by antibody AB5868, which recognizes the N-terminal portion of tau. A major fragment of 29 kDa initially appeared and increased after incubation. Based on the molecular mass, this fragment could correspond to the N-terminal fragment after cleavage at the Arg155–Gly156 bond. Thereafter, proteolysis of the resultant C-terminal fragment progressed bidirectionally. Based on the molecular masses of the fragments, it can be estimated that both T39 and T40 have at least three other cleavage sites, which are located from the microtubule-binding domain to the C terminus, respectively. The smallest fragments identified in both T39 and T40 consist of part of the microtubule-binding domain.
(fragment d), 32.5 kDa (fragment e), 32 kDa (fragment f), 28 kDa (fragment g), and 20 kDa (fragment h) observed at 0 min were sequentially degraded during incubation, even though a small amount of fragment f and a significant amount of fragment h remained after 3 h of incubation. These results again suggest that phosphorylation represses tau proteolysis by thrombin. Upon Western blotting with antibody A0024, which recognizes the C-terminal part of tau containing the microtubule-binding domain, fragments a, b, and f–h could be recognized as positive despite diffuse smear-like background staining (data not shown) as reported previously (27). These results suggest that they are C-terminal tau fragments that appear after thrombin cleavage at the N-terminal site of the HT7 epitope (positions 159–164). The results are again consistent with an initial cleavage at the Arg155–Gly156 bond.

**DISCUSSION**

In this study, we have shown that tau in cytosolic fractions prepared from normal human brain tissues was degraded during incubation at 37 °C and that this degradation was inhibited by a thrombin-specific inhibitor, PPACK. Other protease inhibitors that repressed tau breakdown included protease inhibitor cocktail, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, leupeptin, and antipain dihydrochloride, all of which inhibit thrombin activity (28). These results suggest that thrombin is a brain protease that processes tau. The results of Western blot analysis showing the presence of prothrombin, prethrombin-1, and prethrombin-2, the B chain of /H9251-thrombin, and the B2 chain of /H9252-thrombin in the same fraction further support these findings.

At present, the source of thrombin in human brain supernatant is uncertain. Thrombin is a trypsin-like serine protease that is an important component of the blood coagulation system. It is generated by proteolytic cleavage of its precursor, prothrombin, which is made primarily in the liver and circulates in plasma (29). Recent studies indicate that prothrombin mRNA is expressed in rat brain neurons as well as in neural cell lines (30, 31), and both the mRNA and protein appear to be synthesized by cultured human astroglial cells (32). Factor X,

![Fig. 4. Proteolysis of non-phosphorylated recombinant tau by thrombin. T39 (A and B) and T40 (C and D) were incubated with thrombin and analyzed on 12.5% (A and C) and 16.5% (B and D) SDS-polyacrylamide gels. Proteins were transferred to polyvinylidene fluoride membranes and stained with Coomassie Brilliant Blue R-250. See "Experimental Procedures" for details. A and C: lanes 1, tau alone; lanes 2–6, tau incubated with thrombin for 0, 2, 15, 60, and 180 min, respectively. B: lane 1, thrombin alone; lane 2, T39 incubated with thrombin for 24 h. D: T40 incubated with thrombin for 24 h. Fragments a–t (indicated by arrows) were subjected to N-terminal sequence analysis, and the results are listed in Table IV. Molecular mass markers are shown on the left in kilodaltons.

| Table IV | Summary of amino acid sequencing of each fragment after incubation of human recombinant tau with thrombin |
|----------|----------------------------------------------------------------------------------------------------------|
| **Fragment** | **Molecular mass** | **Sequence** | **Residues** |
| T39 + thrombin | | | |
| a | 32 | IVEGSD | B chain of α-thrombin |
| b | 31 | GAAPPG | tau 156–161 |
| c | 28 | GAAPPG | tau 156–161 |
| d | 25 | SRTPSL | tau 210–215 |
| e | 22.5 | YERNIE | B2 chain of β-thrombin |
| e | 22.5 | TPPKSP | tau 231–236 |
| f | 20.5 | SRTPSL | tau 210–215 |
| g | 19 | IVEGSD | B chain of α-thrombin (after γ-cleavage) |
| h | 17.5 | TPPKSP | tau 231–236 |
| i | 12 | GQPSVL | B4 chain of γ-thrombin |
| j | 8 | GAAPPG | tau 156–161 |
| k | 5 | SEKLDF | tau 310–315 |
| T40 + thrombin | | | |
| l | 33 | GAAPPG | tau 156–161 |
| m | 32 | ns | |
| n | 30 | IVEGSD | B chain of α-thrombin |
| o | 27 | SRTPSL | tau 210–215 |
| p | 25 | TPPKSP | tau 231–236 |
| q | 22 | YERNIE | B2 chain of β-thrombin |
| r | 21 | ns | |
| s | 8 | SEKLDF | tau 341–346 |
| t | 5 | SKIGST | tau 258–263 |
| t | 5 | SEKLDF | tau 341–346 |
| t | 5 | SPFNNR | C-terminal fragment of B4 chain of γ-thrombin |

*ns, no significant sequence.*
Proteolysis of Unmodified and Phosphorylated Tau by Thrombin

**Fig. 5. Autolytic cleavage sites of thrombin.** Shown is the sequence of α-thrombin. The *vertical lines* indicate the cleavage sites identified in this study. The arginine and lysine residues at the cleavage sites are in **boldface** and underscored with their respective position number. The A chain of α-thrombin is located at residues 1–36, the B chain of α-thrombin at residues 37–295; the B2 chain of β-thrombin at residues 107–295, and the B4 chain of γ-thrombin at residues 191–295.

which is an activator of prothrombin to thrombin, is also expressed in rat brain as well as in neural cell lines (33). Thus, thrombin can be released into supernatants by these cells and/or residual plasma in brain.

To examine whether degradation of endogenous tau by thrombin takes place in the absence of residual plasma, we incubated the supernatant prepared from a perfused rat brain with or without PPACK. The results of Western blot analyses showed that significant tau breakdown took place in the supernatant without PPACK, but not in that with PPACK, indicating that thrombin is present in brain cells in sufficient quantity to degrade tau. These findings support the notion that thrombin can be involved in the proteolysis of tau in brain. Furthermore, the presence of thrombin in neurofibrillary tangles of Alzheimer’s disease brains (34) suggests that thrombin may be involved in neurofibrillary tangle formation. It is therefore important to investigate the sites of thrombin cleavage of tau and the effect of phosphorylation on thrombin proteolysis of tau.

We have shown that purified human α-thrombin hydrolyzed human recombinant tau at multiple sites. Two of the sites (Arg^{155}–Gly^{156} and Arg^{230}–Thr^{231}) are consistent with a previous report (14). Based on the molecular masses of the fragments of T39 and T40, tau should have at least four other sites that are cleaved by thrombin (see Figs. 7 and 8).

Thrombin exhibits a more restricted specificity toward protein substrates compared with trypsin. The specificity is considered to originate from within the catalytic groove, which contains multiple sites of the protease (designated as S sites) to accommodate multiple sites of the target substrate (designated as P sites) (35, 36). Arginine or lysine in the P1 position is required for thrombin cleavage, with arginine being preferred (37). Furthermore, there is general agreement that proline in the P2 position (37–39) and serine, glycine, threonine, and alanine in the P1’ position (36, 38, 40, 41) are optimal for thrombin catalysis. Based on these findings, among the five cleavage sites identified in this study, Arg^{155}–Gly^{156} seems to be the most optimal one because of the presence of proline in the P1’ position. Eventually, fragment h is N-terminally cleaved at the Lys^{309}–Ser^{310} bond, leaving fragment j. **Fig. 7. Schematic diagram of the temporal sequence of T39 proteolysis and the definite and possible thrombin cleavage sites.** A, T39 is initially hydrolyzed at the Arg^{355}–Gly^{356} bond. Thereafter, the resultant C-terminal fragment (fragment b) is digested bidirectionally. N-terminal cleavage occurs at the Arg^{109}–Ser^{110} (fragment d) and Arg^{230}–Thr^{231} (fragment e) bonds consecutively. By estimations based on the molecular masses of fragments, the C-terminal cleavage of fragment b at the Arg^{355}–His^{356} bond can generate fragment c by losing 35 amino acids from its C terminus. Fragment c could be further digested at the Lys^{357}–Ser^{358} bond, which is the definite cleavage site identified in T40, producing fragment i. The C-terminal cleavages of fragments d and e at the Lys^{364}–Ser^{365} bond can produce fragments f and h, respectively, by losing 46 amino acids from their C termini. Eventually, fragment h is N-terminally cleaved at the Lys^{309}–Ser^{310} bond, leaving fragment j. **B**, shown is the sequence of T39. The **vertical solid lines** indicate the definite cleavage sites identified in this study. The **vertical dashed lines** indicate possible cleavage sites based on the molecular masses of the fragments. The arginine and lysine residues at the cleavage sites are in **boldface** and underscored with their respective position number.
substrates (36, 38). These findings suggest the possibility that the presence of a negative charge in these positions could affect the interaction between thrombin and substrates. Phosphorylation of the residues in these positions of tau can therefore repress thrombin cleavage. Fig. 11 summarizes the sites of GSK-3β phosphorylation of tau (42) and the sites of thrombin cleavage of tau. Regarding the definite cleavage sites, Thr212 and Thr231 are located in the P3 position of the thrombin cleavage of tau. Regarding the definite cleavages sites based on the molecular masses of the fragments. Thereafter, two kinds of bidirectional cleavage of fragment p take place. One is the definite N-terminal cleavage at the Arg155–Gly156 bond and the other is the definite cleavage at the Arg379–Ser380 bond. The resultant C-terminal fragment (fragment o) is then sequentially cleaved at the Arg209–Ser210 (fragment o) and Arg230–Thr231 (fragment p) bonds. Thereafter, two kinds of bidirectional cleavage of fragment p take place. One is the definite N-terminal cleavage at the Arg155–Ser156 bond and the other is the definite cleavage at the Arg379–Ser380 bond and the possible one at the Arg389–His390 bond, generating fragment s. The other is the definite digestion at the Lys298–His299 bond, leaving the 5-kDa fragment (fragment t). The C-terminal fragment of fragment s generates another 5-kDa fragment (fragment t). The candidate sites of this cleavage could be the Arg209–Thr212 site, respectively. Indeed, in this study, phosphorylation of these threonine residues by GSK-3β was confirmed by Western blot analyses. Similarly, Ser210 and Ser214, which were also phosphorylated by GSK-3β, are located in the P1’ position of the possible cleavage sites of the Lys395–Ser396 bond and in the P3 position of the possible cleavage sites of the Arg406–His407 bond. On the other hand, there is no residue to be phosphorylated by GSK-3β in the P3–P3’ positions of the first cleavage site of the Arg155–Gly156 bond. These findings support the notion that phosphorylation inhibits tau proteolysis by thrombin, except at the initial cleavage at the Arg155–Gly156 bond. Further support comes from the finding that, following the initial cleavage, the C-terminal fragments of non-dephosphorylated PHF-tau
were more resistant to thrombin proteolysis than those of dephosphorylated PHF-tau.

The effect of phosphorylation of tau on its proteolysis is still not well understood. PHF-tau is more resistant to proteolysis by calpain than normal tau and fetal tau, indicating that phosphorylation decreases the sensitivity of tau to calpain (11, 43). On the other hand, fetal tau is more vulnerable to proteolysis by cathepsin D than unmodified recombinant tau, suggesting that phosphorylation increases susceptibility of tau to cathepsin D (10). With thrombin, phosphorylation of recombinant tau by the cAMP-dependent protein kinase catalytic subunit delays proteolysis of tau, but phosphorylation by a double-stranded DNA-dependent protein kinase accelerates it (15).

GSK-3β, which was used to phosphorylate recombinant tau in this study, is considered to be one of the major kinases to phosphorylate tau in human brains (44, 45). GSK-3β phosphorylation occurs at Thr212 and Thr231 and at Ser396 and Ser404. Phosphorylation at these sites has been identified in aggregated tau of patients with Alzheimer’s disease and other tauopathies (1, 21, 27). The results of the present study are therefore consistent with the hypothesis that phosphorylation of tau inhibits proteolysis by thrombin or other endogenous proteases, thus promoting aggregation of tau into insoluble fibrils.

Furthermore, it is important that thrombin can cleave tau at the Arg155–Gly156 bond even after phosphorylation because the core portion of PHF in Alzheimer’s disease consists of C-terminal fragments of tau containing the microtubule-binding regions (46, 47), whereas N-terminal fragments are a major component of tau in cerebrospinal fluid (48, 49). The molecular mass of the N-terminal tau fragment after the first cleavage by thrombin upon SDS-PAGE in this study is similar to that of cerebrospinal fluid tau in Alzheimer’s disease patients (48, 49). Thus, thrombin may be involved in the release of the N-terminal tau fragment to cerebrospinal fluid and intracellular aggregates of C-terminal tau fragments in Alzheimer’s disease. In conclusion, the results of this study suggest a possible role for thrombin in tau proteolysis under physiological and/or pathological conditions.

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Proteolysis of Non-phosphorylated and Phosphorylated Tau by Thrombin
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