Proteolytic processing of tau

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Abstract

Tau aggregation is a hallmark of several neurodegenerative diseases, including AD (Alzheimer’s disease), tauopathies [1,2]. In AD, tau is modified in several ways, e.g. phosphorylation, ubiquitination, truncation, prolyl isomerization, glycation, glycosylation, amino acid modifications, nitrosylation and change in isoform distribution (splicing) (reviewed in [3]). In addition, there are mutations in FTDP-17 (frontotemporal dementia and parkinsonism linked to chromosome 17) [4,5]. The relationship of these modifications to the diseases process is poorly understood. Phosphorylation is typically regarded as one of the most important changes of tau; however, a number of studies show that proteolysis of tau may play an important role as an early step in neuronal degeneration [6].

In our studies on inducible cell models, we found that, regardless of the phosphorylation status of tau, small amyloidogenic tau fragments generated by sequential cleavage of the repeat domain of tau harbouring ΔK280 mutation (deletion of Lys280) (tauRDΔK), induce the aggregation of tauRDΔK or full-length tau, which leads to cytotoxicity [7,8]. The sequence of these cleavages of tauRDΔK is: first, at the N-terminus after Lys257 to generate a fragment termed F1; then at the C-terminus after Val163 to generate fragment F2, and finally after Ile165 to generate fragment F3 (Figure 1). The first cleavage (Lys257→Ser258) is exerted by an intracellular protease with thrombin-like properties since it can be prevented by the thrombin inhibitor PPACK (D-Phe-Pro-Arg chloromethane). Interestingly, the second and third cleavages (near the C-terminal, generating F2 and F3) occur only after the first one, suggesting that the repeat domain must have a particular conformation, such that the N-terminal part protects the C-terminal part against proteolytic attack (Figure 1). Among these fragments, F1 aggregates rather slowly, but fragments F2 and especially F3 are much more amyloidogenic and rapidly lead to aggregates. Blocking the generation of those amyloidogenic fragments (F2 and F3) by mutating the residues at the cleavage site (Lys257 to alanine) disrupts the aggregation of tauRDΔK. Thus the limited proteolysis is necessary for tau aggregation in this cell model.

Since observations have been made by several authors on proteases affecting tau and implications for tau toxicity or aggregation, we take this opportunity to summarize the major results reported on tau cleavage and the proteases and mechanisms involved.

Introduction

Tau aggregation is a hallmark of a series of neurodegenerative diseases, including AD (Alzheimer’s disease), termed tauopathies [1,2]. In AD, tau is modified in several ways, e.g. phosphorylation, ubiquitination, truncation, prolyl isomerization, glycation, glycosylation, amino acid modifications, nitrosylation and change in isoform distribution (splicing) (reviewed in [3]). In addition, there are mutations in FTDP-17 (frontotemporal dementia and parkinsonism linked to chromosome 17) [4,5]. The relationship of these modifications to the diseases process is poorly understood. Phosphorylation is typically regarded as one of the most important changes of tau; however, a number of studies show that proteolysis of tau may play an important role as an early step in neuronal degeneration [6].

In our studies on inducible cell models, we found that, regardless of the phosphorylation status of tau, small amyloidogenic tau fragments generated by sequential cleavage of the repeat domain of tau harbouring ΔK280 mutation (deletion of Lys280) (tauRDΔK), induce the aggregation of tauRDΔK or full-length tau, which leads to cytotoxicity [7,8]. The sequence of these cleavages of tauRDΔK is: first, at the N-terminus after Lys257 to generate a fragment termed F1; then at the C-terminus after Val163 to generate fragment F2, and finally after Ile165 to generate fragment F3 (Figure 1). The first cleavage (Lys257→Ser258) is exerted by an intracellular protease with thrombin-like properties since it can be prevented by the thrombin inhibitor PPACK (D-Phe-Pro-Arg chloromethane). Interestingly, the second and third cleavages (near the C-terminal, generating F2 and F3) occur only after the first one, suggesting that the repeat domain must have a particular conformation, such that the N-terminal part protects the C-terminal part against proteolytic attack (Figure 1). Among these fragments, F1 aggregates rather slowly, but fragments F2 and especially F3 are much more amyloidogenic and rapidly lead to aggregates. Blocking the generation of those amyloidogenic fragments (F2 and F3) by mutating the residues at the cleavage site (Lys257 to alanine) disrupts the aggregation of tauRDΔK. Thus the limited proteolysis is necessary for tau aggregation in this cell model.

Since observations have been made by several authors on proteases affecting tau and implications for tau toxicity or aggregation, we take this opportunity to summarize the major results reported on tau cleavage and the proteases and mechanisms involved.

Analytical proteolysis of tau

Tau is a natively unfolded protein and therefore very sensitive to protease digestion. A number of studies have shown that tau is a substrate for various proteases. Limited proteolysis, as a useful tool to assess the surface exposure of polypeptide chains and to dissect the substructure of proteins and protein assemblies, has been widely used to assess the structure of tau or tau aggregates [9]. Tau bound to taxol-stabilized microtubules can be cleaved by chymotrypsin at Tyr197 (numbering according to the longest human brain tau), resulting in the separation of the projection domain (residues Met1–Ytr197) from the microtubule-binding domain [10]. Digestion of PHFs (paired helical filaments) isolated from AD-affected brain with pronase showed that this protease clipped off the fuzzy coat of PHFs, leaving a pronase-resistant core [11]. This core consisted of sequences derived from the repeat region and the C-terminal flanking region [12]. Limited digestion of PHFs by trypsin in vitro revealed that the PHF core comprises roughly the second half of R1 (first repeat), R2, R3 and the first half of R4, as the other

Key words: aggregation, Alzheimer’s disease, fragmentation, proteolysis, tau, tauopathy.

Abbreviations used: Aβ, amyloid β-peptide; AD, Alzheimer’s disease; FTDP-17, frontotemporal dementia and parkinsonism linked to chromosome 17; GSK3β, glycogen synthase kinase 3β; NFT, neurofibrillary tangle; PHF, paired helical filament; PSA, puromycin-sensitive aminopeptidase.

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Figure 1 | Sequential cleavage and aggregation of tauRDΔK in a cell model of tau aggregation
The N-terminal region of tauRDΔK protects the C-terminal part (state 1). Cleavage near the N-terminus (state 2) removes the protection and allows two cleavages near the C-terminus (states 3 and 4). The fragment F3 thus generated can aggregate by itself and nucleate paired helical filaments (state 5) and can then also co-aggregate with tauRDΔK or full-length tau (state 6). Modified from [76] with permission of S. Karger AG, Basel.

regions of tau can be quickly proteolysed, in good agreement with the above results on PHFs from AD-affected brain [9].

Proteolysis of tau by endogenous proteases
Besides the digestion of tau by trypsin, chymotrypsin and other proteases which are commonly used for in vitro analytical proteolysis, tau is also a substrate of several endogenous proteases. Among them, caspases and calpain have been investigated most intensively and are described first. In addition, we consider several other proteases reported to play a role in tau proteolysis.

Caspases
Caspases belong to the family of cysteine proteases, which play essential roles in apoptosis. All caspases are activated through proteolysis of procaspase zymogens [13]. Caspases recognize at least four contiguous amino acids on the substrates upstream of the cleavage site, named P4-P3-P2-P1, and absolutely require an aspartate residue in the P1 position before the scissile bond. However, most of them are promiscuous at P2 and P3 [14,15], thus the value of prediction of caspase cleavage sites is quite limited.

In vitro experiments showed that tau (2N4R variant) can be cleaved by caspases 1, 3, 6, 7 and 8 at Asp421, resulting in the generation of a fragment ~5 kDa smaller than intact tau [16,17]. In addition, tau is also cleaved in vitro behind Asp13 by caspase 6, even more efficiently than behind Asp421. This N-terminal domain of tau interacts with the C-terminal domain to keep tau in its soluble state [15a]. Both the cleavage at Asp421 by caspase 3 and at Asp13 by caspase 6 were validated by N-terminal protein sequencing or/and MS [16–18]. The truncation of tau behind Asp421 can be found in AD-affected brain and in mouse models [16,17,18a]. Whether the proteolysis at Asp13 occurs in AD is not clear, although activated caspase 6 was found to co-localize with tau aggregates in AD-affected brain [19]. Through the use of site-directed antibodies, truncation of tau at Asp25 (putative caspase 3 cleavage site) and Asp402 (putative caspase 6 cleavage site) were observed in AD-affected brain or transgenic animals [19,20], but these sites cannot be cleaved by known caspases in vitro [16]. Thus, so far, only the truncation of tau at Asp421 is validated both in vitro and in vivo. Also, a caspase-cleaved N-terminal tau fragment (residues 26–230) elicits NMDA (N-methyl-D-aspartate) receptor-mediated neurotoxic effects in primary neuronal cultures and is generated during apoptosis [20a].

In AD-affected brain, tau truncated at Asp421 co-localizes with NFTs (neurofibrillary tangles), and Aβ (amyloid β-peptide) treatment can induce this cleavage in cultured neurons [16,17]. Experiments in vitro show that this truncated tau (tau4–421) can facilitate the aggregation of intact tau in the presence of polyanion. These findings lead to a proposal linking extracellular amyloid plaques and intracellular NFTs: Aβ exposure induces caspase activation and subsequent generation of tau truncated at Asp421, which then leads to filament formation [16,17]. However, whether the truncated tau can indeed induce intact tau aggregation...
in cells remains unclear. Subsequent studies showed that overexpression of this truncated fragment did not result in aggregation in cultured cells, except when it was co-expressed with GSK3β (glycogen synthase kinase 3β) [21]. In addition, pseudophosphorylation at Ser422 can inhibit truncation of tau at Asp421 by caspase 3 in vitro, and phosphorylation at Ser422 in AD precedes truncation at Asp423 during NFT maturation. Thus tau phosphorylation at Ser422 may be a protective mechanism that inhibits cleavage in vitro [22].

Another issue still under debate is whether the generation of caspase-3-cleaved tau represents a beneficial or toxic process. Some authors reported that this tau truncation induces apoptosis or mitochondrial dysfunction in cultured hippocampal neurons or cell lines [23,24]. On the other hand, using a novel method to observe NFTs and caspase activation directly in a living brain of a transgenic mouse model of tauopathy, Hyman and colleagues found most cells with activated caspases contain NFTs and such neurons do not undergo apoptotic degeneration [25,26]. They thereby proposed that caspase activation in tauopathy may be more important in cleaving tau, thus causing tau fibrillogenicity rather than in promoting apoptosis [26]. Using similar methods, they showed that caspase activation preceded and led to NFTs in the same transgenic mouse model. Most NFTs were formed after caspase activation; however, the majority of NFT-bearing neurons were not caspase-positive, indicating that such neurons survived the initial caspase attack and became caspase-negative [26,27]. In line with this proposal, other authors found that human neurons do not undergo the rapid apoptosis observed in many cell lines [28].

**Calpains**

Calpains are cytosolic Ca²⁺-activated cysteine proteases. Calpain 1 and calpain 2, also known as μ-calpain and m-calpain, are the two major forms. Besides Ca²⁺, calpain activity is regulated by a Ca²⁺-dependent heat-stable inhibitor, calpastatin [29]. Early investigations proposed that cleavage by calpains follows a P2-P1 rule, which states that the preferred residues on the substrates for calpains are leucine or valine at P2, and arginine or lysine at P1, just before the scissile bond [30]. However, it is now well established that the cleavage by calpains is only poorly related to amino acid sequence, but rather to the conformation of the polypeptide chain [31,32].

In AD-affected brain, calpastatin is significantly decreased and calpain 1 and calpain 2 are abnormally activated [33,34]. Several studies showed that tau can be degraded by calpains and phosphorylation can retard this process [35–37]. On the basis of the P2-P1 rule, nine potential calpain cleavage sites have been suggested for tau [38]. A 17 kDa tau fragment was thought to induce apoptosis in cerebellar granule cells [38]. This fragment was ascribed to cleavage by calpain, as treatment with calpain inhibitor could block its generation, and in vitro digestion of recombinant tau by calpain resulted in its appearance. The fragment was located within residues 73–315 of tau, on the basis of antibody-reaction assays [38], but the exact N- and C-terminal ends were not clearly demonstrated. A 17 kDa fragment of tau was also found in transgenic AD animal models which showed neurodegeneration [39]. Park and Ferreira [40] reported that pre-aggregated Aβ treatment of cultured neurons led to activation of calpain and production of a 17 kDa fragment. On the basis of the potential calpain cleavage sites on tau and the apparent molecular mass, the 17 kDa fragment was assumed to be tau45−230. Overexpression of tau45−230 induced apoptosis in CHO (Chinese-hamster ovary) cells or cultured neurons. Thus tau45−230 was proposed to mediate Aβ-induced neurodegeneration [40], but the link to apoptosis remains ambiguous. The role of the N-terminal part of tau in neurodegeneration is also highlighted by the finding that this region of tau is essential for prefibrillar Aβ-induced microtubule disassembly in cultured cells [41]. However, until now, the presence of the 17 kDa calpain-induced fragment has not been detected in AD-affected brains, so that the relationship to neurodegeneration in AD remains to be determined.

**Thrombin**

Thrombin is a serine protease generated by proteolytic cleavage of its precursor, prothrombin, which is made primarily in the liver and circulates in plasma [42]. However, thrombin was also reported to be present in NFTs in AD [43,44], implying that it may be related to tau aggregation. In vitro, tau can be proteolysed by thrombin at multiple arginine and lysine sites including Arg155-Gly156, Arg229-Ser230, Arg235-Thr236, Lys257-Ser258 and Lys345-Ser346, which can be suppressed by phosphorylation at Thr212, Thr231 and Ser396/Ser404 induced by GSK3β [45]. The presence of prothrombin mRNA in neurons has been demonstrated [46]. It was also claimed that both prothrombin and thrombin exist in neurons, judging by immunohistochemistry [44], and it was therefore proposed that thrombin is a protease that proteolyses endogenous tau in the brain. However, this proposal is still under debate, as the mechanism underlying the proteolysis of prothrombin to thrombin in neurons and the existence of active thrombin within the cytoplasm is not clear.

**Cathepsins**

Cathepsins are a group of proteases which normally locate in lysosomes. Most cathepsins are cysteines proteases, except for cathepsins A and G, which are serine proteases, and cathepsins D and E, which are aspartyl proteases. In pathological conditions, certain cathepsins can be released from lysosomes and circulate in plasma [42]. However, thrombin was also reported to be present in NFTs in AD [43,44], implying that it may be related to tau aggregation. In vitro, tau can be proteolysed by thrombin at multiple arginine and lysine sites including Arg155-Gly156, Arg229-Ser230, Arg235-Thr236, Lys257-Ser258 and Lys345-Ser346, which can be suppressed by phosphorylation at Thr212, Thr231 and Ser396/Ser404 induced by GSK3β [45]. The presence of prothrombin mRNA in neurons has been demonstrated [46]. It was also claimed that both prothrombin and thrombin exist in neurons, judging by immunohistochemistry [44], and it was therefore proposed that thrombin is a protease that proteolyses endogenous tau in the brain. However, this proposal is still under debate, as the mechanism underlying the proteolysis of prothrombin to thrombin in neurons and the existence of active thrombin within the cytoplasm is not clear.
Figure 2 | Diagram of proteolytic cleavage sites on tau

Tau is divided into an N-terminal projection domain and a C-terminal assembly domain containing the microtubule-binding elements, based on the cleavage by chymotrypsin after Tyr197 [10]. The repeat domain of tau (R1-R4) represents the core of PHFs and is highly protease-resistant in PHFs [9,11]. The microtubule (MT)-binding region of tau contains the repeat domains plus the flanking proline-rich domains P2 and P3. Cleavage sites after Asp13 by caspase (Csp) 6 [18], Asp421 by caspase 3 [16–17], Tyr197 by chymotrypsin [10] and Lys257 by an unknown thrombin-like cytosolic protease [7–8] were validated by N-terminal protein sequencing or MS. Cleavage after Asp25 (putative caspase 3 cleavage site) [20], Asp402 (putative caspase 6 cleavage site) [19] and Glu391 was determined with site-directed antibodies. Glu391 is cleaved by an unknown protease [73–74]. Cleavage after Lys44 and Arg230 was proposed from putative calpain cleavage sites found on tau according to the P2-P1 rule [40]. PSA removes residues stepwise from the N-terminus of tau [56].

of hyperphosphorylated tau. However, since cathepsins are lysosomal proteases, the question remains of how they gain access to tau and affect its turnover. One possible mechanism is by incomplete chaperone-mediated autophagy, as suggested by our results [51]. In AD and other conditions of cellular stress, cathepsin D and others might contribute to tau proteolysis once the lysosomal system is disturbed [52].

PSA (puromycin-sensitive aminopeptidase)
PSA belongs to the M1 class of metallopeptidases [53], which is found in neurons, but not in surrounding glial cells or in blood vessels [54]. As an aminopeptidase, PSA comprises over 90% of the aminopeptidase activity in the brain [55]. Recently, in a Drosophila model of AD, it was found that PSA expression reduced tau levels and protected against tau-induced neurodegeneration, whereas PSA loss-of-function exacerbated neurodegeneration [56]. In vitro experiments showed that tau could be degraded by PSA [56,57]. Thus PSA may suppress tau pathology through modulating tau levels. Interestingly, in human brain, the expression of PSA is elevated 5-fold in the cerebellum compared with frontal cortex [56]. Cerebellum is less affected than cerebral cortex in AD and FTDP-17, therefore the expression of PSA is inversely correlated with vulnerability to tau pathology in these diseases.

Proteasome
The proteasome system degrades misfolded proteins which otherwise would form potentially toxic aggregates [58–63]. Lactacystin, a specific inhibitor of the 20S proteasome catalytic core, was shown to inhibit the degradation of tau in transfected SH-SY5Y cells [64]. Furthermore, the direct degradation of unfolded recombinant tau by the 20S proteasome in vitro in an ubiquitin-independent and bi-directional manner led to the formation of stable intermediates during this degradation process (~27 and 17 kDa fragments). In AD-affected brain, proteasome activity is decreased, which could contribute to the accumulation of aggregates including tau filaments [65,66]. This corresponds to a reported inhibition of the endogenous proteasome system by the binding of PHF-tau to the 20S core proteasome in human brain tissue [67].

Non-enzymatic cleavage of tau
Aging of proteins affects their integrity in various ways, including deamidation and isomerization in vitro, which also occurs in vivo with long-lived proteins [68]. Watanabe et al. [69] showed that prolonged incubation of recombinant tau led to non-enzymatic cleavage at the carboxy side of asparagine residues that are followed by bulky hydrophobic residues (e.g. Asn-Val, Asn-Leu or Asn-Ile motifs), a phenomenon also described by others [70]. In the case of tau, most of the asparagine residues are clustered within the microtubule-binding domain. Watanabe et al. [69] also confirmed similar types of degradation products starting at bulky residues preceded by asparagine by sequencing of smeared tau in vivo obtained from AD-affected brain (V256KSKI, L266KHQP, I297KHVP and I328HHKP). This suggests that deamidation/isomerization and non-enzymatic cleavage at asparagine residues through a succinimide intermediate may contribute to the formation of amyloidogenic fragments of tau and their unusual stability in vivo.
Unknown proteases
Some tau fragments found in AD are not well-characterized and the proteases responsible for their generation are not identified. For instance, a 25–35 kDa tau fragment in the CSF (cerebrospinal fluid) has been used as an early marker of AD [71,72], but the proteases responsible are not known. Using a monoclonal antibody (mAb 423) against the PHF core, Wischik et al. [73] found two fragments with apparent molecular masses of 9.5 and 12 kDa in the PHF core after removing the fuzzy coat with pronase. This antibody (mAb 423) specifically recognizes tau terminated at Glu391 [74,75], suggesting that the truncation at Glu391 is produced by an endogenous protease that has not yet been identified.

Summary
Tau can be cleaved by various proteases, resulting in the generation of many different tau fragments both in vitro and in vivo (Figure 2). Although most of these tau fragments are not well characterized and some proteases remain to be identified, the studies performed so far show that the proteolysis of tau plays an important role in both tau aggregation and neurodegeneration. On one hand, truncation of tau may generate amyloidogenic tau fragments that initiate the aggregation of tau, which in turn can cause toxicity, as illustrated by cell models of tau pathology. On the other hand, truncation of tau may result in tau fragments which induce neurodegeneration through an unknown mechanism, regardless of tau aggregation, perhaps by interacting with other cellular components. Blocking the truncation of tau thus may represent a promising therapeutic approach for AD or other tauopathies. In addition, certain proteases such as PSA that can reduce overall tau levels may also be potential targets for the development of therapeutics.

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References

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