Age-Related Increase in a Cathepsin D Like Protease That Degrades Brain Microtubule-Associated Proteins

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ABSTRACT: In microtubules isolated from brains of very old rats, two of the major microtubule-associated proteins, MAP1 and MAP2, are found only in degraded form. MAP1 is present as a piece whose molecular weight on sodium dodecyl sulfate–polyacrylamide gel electrophoresis is circa 50 000 smaller than the native protein, and MAP2 is extensively fragmented. The native forms of both proteins are present in tissue homogenates but are rapidly degraded during microtubule isolation. The proteolytic activity responsible for this degradation is cathepsin D like, being more active at acid pH than neutral and being completely blocked by pepstatin at 10^{-7} M. Fractionation of aged brain supernatant by gel permeation chromatography showed that the MAP1 and MAP2 degrading activity elutes with a single peak of cathepsin D like activity. MAP1 and MAP2 are known to promote microtubule assembly, and their degradation by a protease whose levels increase with age could be related to defective microtubule assembly which is known to occur in age-related degenerative conditions such as Alzheimer's disease.

In a variety of animals, including man, old age is frequently accompanied by degenerative changes in axons and dendrites. One factor upon which the structural maintenance of neuronal processes depends is the presence of intact assembled microtubules in the neuronal cytoplasm (Daniels, 1972; Bray et al., 1978; Matus et al., 1986). Consequently, any age-related degradative changes in the component proteins of brain microtubules could contribute to axonal and dendritic degeneration. We have examined the protein composition of microtubules prepared from the brains of very old rats and found that two of the major microtubule-associated proteins, MAP1 and MAP2, are extensively degraded. The responsible proteolytic activity has been traced to a single brain cathepsin D like enzyme.

Materials and Methods

Microtubule Preparation. Albino rats of strain RA22 were used; 27-month-old animals were obtained from the Pharma research group of Ciba-Geigy AG, Basel. Four groups of 27-month-old animals and two groups of 3-month-olds, each group containing five individuals, were separately processed to produce microtubules by three cycles of repolymerization from brain homogenates following the method of Karr et al. (1979).

Electrophoresis and Immunoblotting. Analysis of microtubule proteins was performed on 4–14% gradient sodium dodecyl sulfate (SDS)[1]–polyacrylamide slab gels according to the method of Laemmli (1970). Proteins were transferred onto nitrocellulose and immunoperoxidase stained with monoclonal antibodies as described by Towbin et al. (1979). The monoclonal antibodies used were those described by Huber and Matus (1984).

Degradation of Microtubules by Brain Supernatant. Brain supernatant was prepared by homogenizing rat brain in buffer A (0.1 M MES buffer, pH 6.6, and 10 mM EGTA) at a ratio of 2 g of brain to 5 mL of buffer; 400 5-μg samples of 3-month-old brain microtubule protein, in buffer A at 10 mg/mL, were added to 80 5-μL aliquots of supernatant and incubated at 37 °C for various times as described. The incubation was terminated by adding SDS–PAGE sample buffer (Laemmli, 1970) and heating to 90 °C for 5 min.

Fractionation and Assay of Brain Supernatants. One-milliliter aliquots of brain supernatant were fractionated by gel permeation chromatography using a Superose 6 column and FPLC equipment (Pharmacia). The column was developed with buffer A, and 1-mL fractions were collected and assayed for cathepsin D activity using the oligopeptide substrate Phe-Ala-Ala-Phe(NO_2)-Phe-Val-Leu-4-methoxy-β-naphthylamide as described by Agarwal and Rich (1983). The linearity of response over the range of activity studied was established by using bovine cathepsin D standards (Sigma).

Results

Figure 1 shows the protein content of microtubules prepared from four separate groups of aged (27-month-old) rats (lanes 1–4) and a control group of young adults (3-month-olds) rats (Figure 1, lane 5). Microtubules from 3-month brain presented the conventional pattern of microtubule-associated proteins among which the high molecular weight forms MAP1 and MAP2 were the most prominent species (Figure 1, lane 5). By contrast, 27-month microtubules show only a single high molecular weight component (indicated by arrow with asterisk; Figure 1, left).

Although this high molecular weight material in the aged rat microtubules migrated in approximately the same position on SDS–PAGE as the MAP2 of the young rat microtubules, immunoblot analysis with monoclonal antibodies revealed that it was not MAP2 but was instead derived from MAP1 (Figure 2). This is shown that the left-hand panel of Figure 2 where aged rat brain microtubules (lanes 1–4) and young rat brain microtubules (lane 5) were run in a single gel, blotted onto nitrocellulose, and stained with monoclonal antibody against

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[1] Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MAP1 and MAP2, microtubule-associated proteins 1 and 2, respectively; MES, 2-(*N*-morpholino)ethanesulfonic acid; FPLC, fast protein liquid chromatography.

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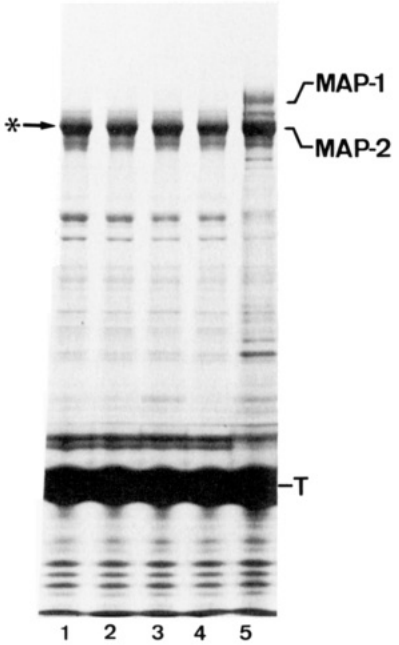

FIGURE 1: Protein content of microtubules isolated from the brains of four separate groups of 27-month-old rats (lanes 1–4) and a control group of 3-month-old rats (lane 5). In this and the following figures, 40 μg of protein was loaded in each lane of SDS–PAGE. The positions of MAP1, MAP2, and tubulin (T) are marked against the young brain microtubules (right). The single high molecular weight component in the aged rat brain preparations is indicated by an asterisk-marked arrow (left).

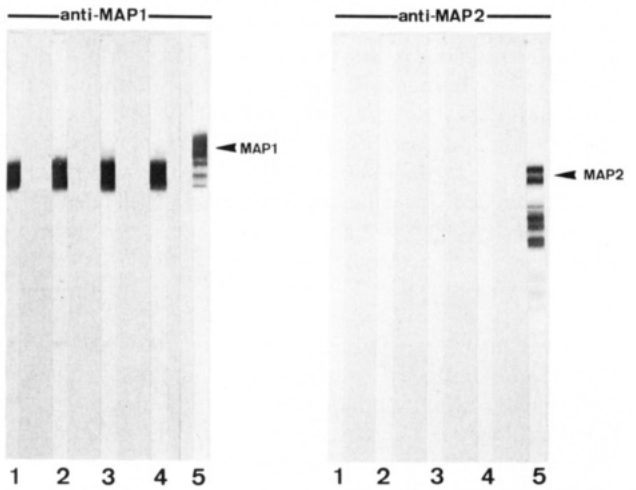

FIGURE 2: Identification of MAP1 and MAP2 in young and old rat brain microtubules by immunoperoxidase-staining strips from nitrocellulose "Western" blots with monoclonal antibodies against MAP1 (left-hand panel) and MAP2 (right-hand panel) using an immunoperoxidase procedure. Lanes 1–4 contain 27-month-old microtubules; lanes 5 contain 3-month-old brain microtubules. The positions of MAPs 1 and 2 are indicated by the numbers to the right of each panel. The smaller anti-MAP1- and anti-MAP2-stained bands in the young brain microtubules are proteolytic degradation products of the intact proteins.

MAP1. In the young brain microtubules (lane 5), native MAP1 is present together with small amounts of three degradation products [see Matus and Riederer (1986)]. In the aged brain microtubules (lanes 1–4), all the anti-MAP1-reactive protein migrates as a single broad band running ahead of the native MAP1 protein position. Regression analysis using myosin (200 000) and native MAP2 (280 000) and native MAP1 (350 000) as molecular weight standards suggests a mean molecular weight for the material in this band of circa 300 000; i.e., it is ~50 000 less than that for native MAP1.

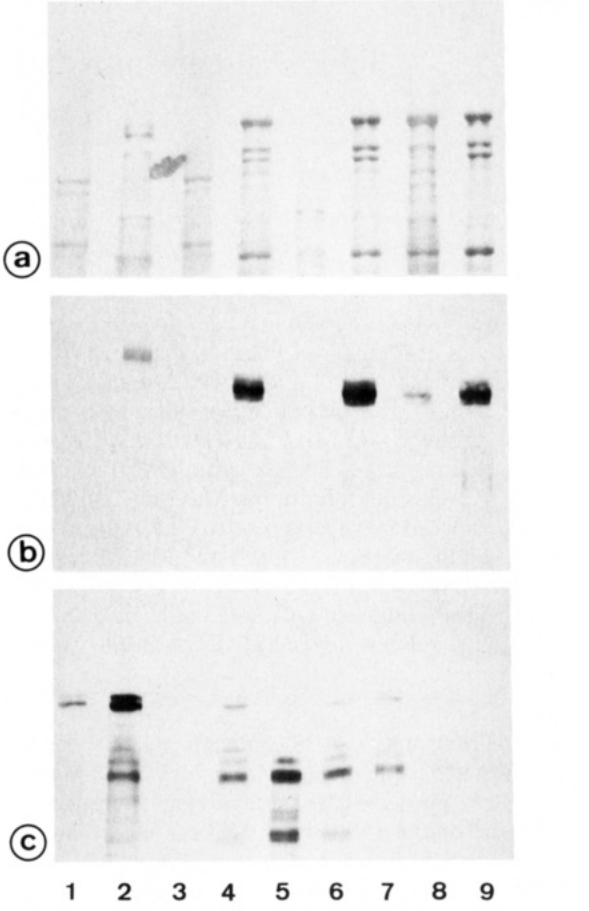

FIGURE 3: Degradation of MAPs 1 and 2 during the isolation of microtubles from aged rat brain. Nitrocellulose blots of three identical SDS–polyacrylamide gels are shown differing only in that they were stained with amido black for protein (panel a), anti-MAP1 (panel b), or anti-MAP2 (panel c). The lanes contain protein from (1) brain homogenate, (2 and 3, respectively) supernatant and pellet from the first centrifugation, (4) first-cycle microtubules, (5) supernatant to microtubules, (6 and 7, respectively) supernatant and pellets obtained by centrifuging cold-depolymerized first-cycle microtubules, and (8 and 9, respectively) second- and third-cycle microtubules.

The change in the pattern of MAP2 between young and aged brain was very striking (Figure 2, right-hand panel). Young brain microtubules showed the usual native MAP2 doublet with the characteristic pattern of breakdown products (lane 5). However, among the proteins of the 27-month-old brain microtubules, no trace of MAP2 antigen was detectable (lanes 1–4). As we show below, this is because the MAP2 has been extensively degraded, presumably into fragments too small to appear on SDS–PAGE.

The monoclonal antibodies were used to follow the fate of MAP1 and MAP2 during the isolation of microtubules from 27-month-old brain (Figure 3). This established that in brain homogenates the undenatured native forms of both MAP1 and MAP2 are present but that they are rapidly degraded during the 37 °C incubation steps of the microtubule isolation procedure. Staining for protein (Figure 3, panel a) showed that the initial soluble, supernatant of aged brain contains a high molecular weight doublet (panel a, lane 2) but that subsequent microtubule fractions (lanes 4, 8, and 9) contained only the single MAP1-derived high molecular component.

The course of events with respect to MAP1 was revealed by staining Western blots with monoclonal antibody (panel b, Figure 3). The presence of native MAP1 in the initial brain supernatant was confirmed (lane 2). The subsequent degradation is very rapid; after the 30-min incubation at 37 °C to

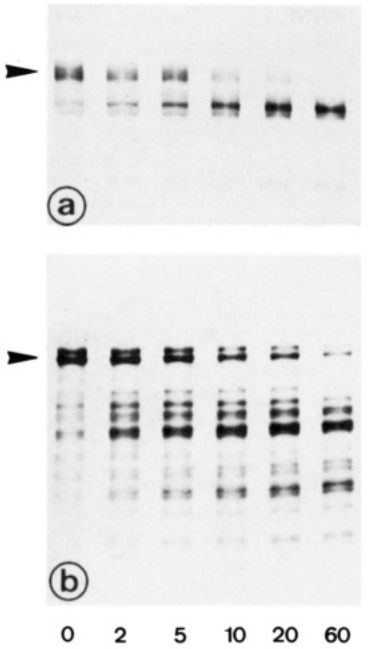

FIGURE 4: Western blots of 3-month-old rat brain microtubule proteins stained with either anti-MAP1 (panel a) or anti-MAP2 (panel b) after exposure to 27-month-old rat brain supernatant at 37 °C for the times indicated below (in minutes). The positions of intact MAP1 and MAP2 are indicated by the arrowheads to the left of panels a and b, respectively.

produce first-cycle microtubules, all the MAP1 has been clipped to produce the circa 50 000-dalton smaller fragment (lane 4). This fragment binds efficiently to tubulin in subsequent cycles of microtubule assembly (lanes 5–9) and is not further degraded.

The degradation of MAP2 was equally rapid but much more extensive. The native MAP2 doublet was present in the 27-month-old brain homogenate and supernatant (panel c, lanes 1 and 2), but degradation products are rapidly generated even during the 30-min spin at 2 °C used to produce the initial supernatant (lane 2). After the 30-min incubation to polymerize the first-cycle microtubules, almost all the native doublet protein had been degraded (lane 4). The intermediate-sized fragments initially generated were further degraded, and by the time second-cycle microtubles were collected, no anti-MAP2-reactive material remained (Figure 3, lane 8; see also Figure 2, right-hand panel).

The proteolytic activity that causes this degradation was present in 100000*g* brain supernatants (Figure 4). To demonstrate this, we incubated young adult brain microtubules (which contain intact MAP1 and MAP2) with aged brain supernatant for various lengths of time and prepared SDS–PAGE Western blots stained with monoclonal antibodies to assess the extent of degradation (Figure 4). After 20 min, more than 90% of the MAP1 had been degraded, and after 1 h, almost all the native MAP2 was degraded. The degradation patterns of young adult MAP1 and MAP2 produced by the aged brain supernatant was clearly the same as those seen in aged brain microtubules. This indicates that the cleavage sites of MAP1 and MAP2 are the same whether they are derived from young or adult brain. Thus, the difference in MAP1 and MAP2 breakdown between young and aged tissue lies not in the MAP proteins but in the protease that degrades them.

The limited breakdown of MAP1 and the observation that tubulin and some other components are not degraded at all (Figure 1) suggested that the cleavage of MAPs 1 and 2 is specific and might be the result of a single proteolytic activity.

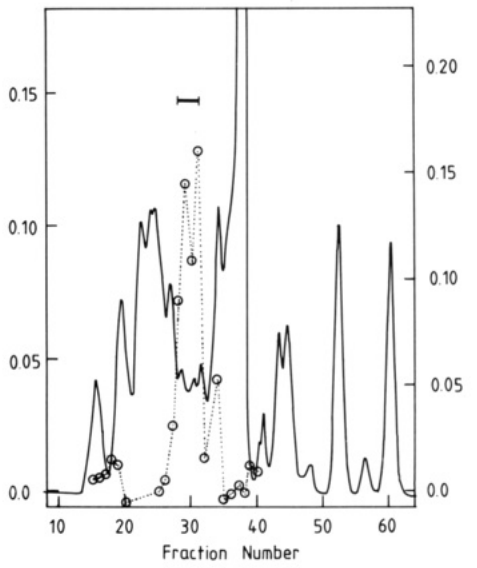

FIGURE 5: Fractionation of 27-month-old rat brain supernatant on Superose 6 gel filtration. The solid line shows the protein elution profile as measured by the absorbance at 280 nm (left scale); the circled points joined by a dotted line indicate the cathepsin D activity measured by using oligopeptide substrate expressed as arbitrary fluorescence units (right scale). The elution positions of MAP1 and MAP2 degrading activity are shown by the bar centered on fraction 30.

To determine whether this was so, we examined the influence on MAP1 and MAP2 degradation of the following class-specific protease inhibitors: diisopropyl fluorophosphate, E64, *o*-phenanthroline, pepstatin, ethylenediaminetetraacetic acid, *p*-(hydroxymercuri)benzoate, and phenylmethanesulfonyl fluoride. Of these, only pepstatin was effective, indicating that the enzyme involved is an aspartyl acidic protease and most probably cathepsin D, for which pepstatin is an extremely potent inhibitor (Barrett & Dingle, 1972). With the use of a quantifiable oligopeptide substrate assay (Agarwal & Rich, 1983), the sensitivity of the aged brain protease to pepstatin was confirmed, and the enzyme was shown to be more active at pH 5 than at Ph 7 and inactive at pH 8.

When 27-month-old brain supernatant was fractionated by gel permeation chromatography, both measurable cathepsin D activity and the MAP-degrading activity eluted in the same position (Figure 5). In the peak fractions, both activities were completely blocked by 10^{-7} M pepstatin, and some inhibition was detectable at 10^{-10} M, further suggesting that the MAP-degrading activity is cathepsin D.

Measurement of the comparative levels of cathepsin D in young and aged rat brain using the oligopeptide assay showed an approximately 2-fold increase, from 0.0065 ± 0.00014 to 0.0120 ± 0.0029 (means and standard deviations of five determinations expressed as A_{310} units per 10 min per milligram of protein).

Discussion

Our results identify two specific substrates, the microtubule-associated proteins MAP1 and MAP2, whose susceptibility to proteolysis increases with age. These substrates are known to be important for the stability of microtubules, which is itself critical for the maintenance of axons and dendrites.

The protease that degrades these proteins is cathepsin D like, and in aged brain, it is sufficiently active to rapidly degrade MAPs 1 and 2 at near-neutral pH when the cells are broken. Although brain cathepsin D is packaged in lysosomes (Snyder et al., 1985), the rapidity with which it degrades MAP1 and MAP2 when brain is homogenized suggests that once released from lysosomes the elevated levels of this protease in the aged

tissue would produce a rapid degradation of these proteins in the neuronal cytopolasm. This action would be favored by the fact that cathepsin D, unlike other lysosomal proteases such as cathepsins B, H, and L, does not have cytosolic inhibitors to limit its extralysosomal activity.

Fractionation of aged rat brain supernatant showed that all the MAP-degrading activity eluted with the peak of proteolytic activity which is blocked by 10^{-7} M pepstatin, at which level cathepsin D is completely inhibited but other aspartyl proteases are not. Thus, it appears that all the MAP1 and MAP2 degradation we observe can be accounted for by cathepsin D like activity. However, we suspect that this brain enzyme is a specific form of cathepsin D because while total cathepsin D activity measured by the oligopeptide-based assay shows a 2-fold increase between young and aged brain, the apparent increase in MAP-degrading activity, as shown in Figures 1 and 2, is much greater. The possibility that various types of cathepsin D exist in brain is supported by the observation that the human brain enzyme contains multiple forms separable by isoelectric focusing (Azaryan et al., 1983).

While the increased cathepsin D levels do not apparently have a large influence on MAPs in normal aged brain, they suggest interesting pathological consequences in aged tissue compromised by minor damage of whatever source. The structural maintenance of axons and dendrites is absolutely dependent on there being intact microtubules in the neuronal cytoplasm (Daniels, 1972; Bray et al., 1978; Matus et al., 1986). Since MAP1 and MAP2 contribute significantly to the polymerization of brain tubulin (Sloboda et al., 1976; Kuznetsov et al., 1981), their degradation would be expected to have an adverse effect on the assembly of brain microtubules. Recently, it has been shown that microtubules isolated from Alzheimer's diseased brain are defective in assembly (Iqbal et al., 1986) and in addition immunocytochemical staining has shown that in Alzheimer's diseased brain MAP1 and MAP2 antigens accumulate in cell bodies and proximal dendrites (Nukina & Ihara, 1983; Kosik et al., 1984), suggesting that their transport along the neuronal processes may be impaired. Thus, while the results presented here are based on in vitro measurements, taken together they suggest a possible involvement of cathepsin D like activity in age-related neuronal degeneration. An investigation of a possible role for cathepsin D induced degradation of neuronal cytoskeleton in pathological conditions of the human brain, such as Alzheimer's disease, would now seem to be worth investigating.

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