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Isolation of a fragment of tau derived from the core of the paired helical filament of Alzheimer disease

(molecular pathology/neurodegenerative disease/neurofibrillary tangles)

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ABSTRACT **A substantially enriched preparation of Alzheimer paired helical filaments (PHFs) has been used as a starting point for biochemical studies. Pronase treatment, which strips off adhering proteins, leaves a resistant core that is structurally intact. This has been used to raise a monoclonal antibody that decorates the filament core. The antibody has been used to follow the extraction of two peptide fragments (9.5 and 12 kDa) by immunoblotting. The link between the PHF as a morphological entity and these peptides has been established independently by photoaffinity labeling with a chemical ligand to the PHF core. Sequence analysis of these peptides was used to design oligonucleotide probes for cloning a cognate cDNA, which leads to its identification as human microtubule-associated tau protein. The sequencing of the 9.5- and 12-kDa peptides shows they are derived from a conserved region of tau containing three repeating segments. Since these fragments have been copurified with the Pronase-resistant core and are only released by subsequent steps, the corresponding part of the tau molecule must be tightly bound in the PHF core.**

The paired helical filaments (PHFs) that constitute the bulk of the Alzheimer neurofibrillary tangle (1) consist of repeating three-domain structural subunits (2–4). The subunits that give rise to PHFs clearly do not normally polymerize in this way. Therefore, characterization of the structural protein(s) and of the factors that drive and stabilize their assembly into PHFs is crucial for understanding the origin of the Alzheimer tangle.

It has not proved possible to purify PHFs to homogeneity. Since the PHF is defined by its morphology in the electron microscope, the only way to identify its protein constituents is to establish the link between filament and protein extract by way of a specific label.

Establishing this kind of link is not straightforward. Antibodies selected on the basis of reactivity with tangles in histological sections (5–11) are not reliable, because PHFs accumulate in the cytoplasm as densely packed aggregates that are likely to trap or become associated with many proteins that may have no bearing on the assembly of the PHF. Even antibodies that can be shown to label isolated PHFs (12, 13) do not necessarily establish the identity of the structural subunit of the PHF. We have shown (14) that PHFs are associated with a fuzzy outer coat, which can be stripped off by a proteolytic digestion leaving the three-domain structure morphologically intact. This we define as the PHF core. Anti-tau reactivity of the kind that has been reported (11, 13) is abolished by this procedure. It might therefore be argued that tau is simply attached to the surface of the PHF. We show, however, in this and the companion papers (14, 15) that fragments of tau protein are tightly associated with the Pronase-resistant core of the PHF.

We previously reported a protocol for preparing tangles in nondenaturing conditions (2). To serve as a reliable basis for further studies, a preparation of PHF cores, stripped of coating or adhering proteins but still retaining their characteristic morphology, has been produced. This has been used as an immunogen to raise a monoclonal antibody against the PHF core and is sufficiently rich in free PHFs to permit routine electron microscope screening of antibodies for core reactivity. We report the isolation and characterization of a number of peptides released from the Pronase-resistant core. These fragments have been shown by two independent labeling procedures to originate from the PHF core, and their identity has been established by sequence analysis.

MATERIALS AND METHODS

Further Purification of Tangle Fragments. Tangle-enriched fractions termed ifII were prepared as follows. Fractions (ifI) prepared as described (2) were homogenized in buffer (9 ml) containing 20 mM Tris·HCl, pH 7.0/1 mM $CaCl_2$/3 mM $MgCl_2$. To this was added 100 μl of a solution of micrococcal nuclease (200 units/ml) in 20 mM Tris, pH 7.0/1 mM $CaCl_2$, and the tangle suspension was digested for 1 hr at 35°C. Next, 100 μl of a solution of Pronase (10 mg/ml) in 20 mM Tris, pH 7.0/1 mM $CaCl_2$/3 mM $MgCl_2$ was added, and the protein was digested for 1 hr at 35°C. The composition of the digest was adjusted to 100 mM NaCl/3 mM EGTA/3 mM EDTA/50 mM Tris, pH 8.0, and 100 mg of cholic acid was added. After thorough mixing, the suspension was filtered through Ballotini 8 glass beads in a Pasteur pipette plugged with glass wool.

A variety of centrifugation schemes were used to enrich the tangle fragments further. Equilibrium gradient centrifugations were performed after addition of CsCl, Rb_2SO_4, or KI, at 400,000 × *g*. Preformed gradients of CsCl/sucrose mixtures were centrifuged at 300,000 × *g*. Fractions recovered from these experiments were examined by fluorescence microscopy with thioflavin S, and by electron microscopy using 1% phosphotungstic acid as a negative stain. The standard PHF-rich fraction was prepared in a linear sucrose gradient poured between densities 1.05 and 1.18, and collected on a CsCl cushion at density 1.45. The ifII fractions were washed by overnight centrifugation at 150,000 × *g* with a 10-fold excess of 5 mM citrate/5 mM phosphate buffer, pH 5.5.

Abbreviations: PHF, paired helical filament; mAb, monoclonal antibody.

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Antibodies. Monoclonal antibody (mAb) 423 is an IgG2b produced by the hybridoma clone NOAL 6/66.423.2, which was derived by using the spleen of a C3H mouse hyperimmunized with ifII (i.e., Pronase treated) preparations and NSO myeloma as described (16). The chosen line was selected on the basis of ImmunoGold decoration of the PHF core filament seen in the electron microscope as described (Fig. 2*b*). Details will be described elsewhere (M.N., P.C.E., R. Pannell, C.M.W., and C.M., unpublished data). Anti-tau antisera came from two sources. One is the ICN 65-095 anti-tubulin preparation, which contains substantial anti-tau reactivity, having been raised against a twice-cycled tubulin preparation (17). The second is an antiserum raised against a porcine tau preparation affinity-purified and kindly supplied by G. Perry (Case Western Reserve University, Cleveland), who has shown it to decorate isolated PHFs (13). An anti-biotin mAb prepared in this laboratory was conjugated with colloidal gold (18). Gold-conjugated goat anti-mouse IgG G5 was purchased from Janssen Life Sciences (Wantage, U.K.).

Isolation of a PHF Core Component. Two methods were used in attempts to solubilize the PHF core. The preparation was monitored by using a chemical ligand in the first method (*i*) and mAb 423 in the second method (*ii*).

(*i*) Washed ifII fractions were taken up in 8 M urea/50 mM borate (1 ml, pH 9.0) and sonicated (E/MC microprobe sonicator, setting 8, tuning 3.5, 5 min), 1 M succinic anhydride in acetone was added to a final concentration of 250 mM succinate in 4 ml, and the pH was maintained at 8.5 with sodium hydroxide. The solution was clarified by centrifugation and applied to a Sephacryl S200 column preequilibrated in either 0.1% ammonia or 50 mM ammonium bicarbonate. A 24-kDa component found to run at a K_{av} of 0.21, and identified by chemical labeling, was concentrated by ultrafiltration through an Amicon YM2 membrane (10 ml), digested with chymotrypsin (0.01 mg/ml) in 50 mM ammonium bicarbonate. Chymotryptic fragments for sequence analysis were isolated by reverse-phase HPLC using a C_{18} column, with a 0–100% acetonitrile gradient, with 0.1% trifluoroacetic acid.

(*ii*) Alternatively, washed ifII fractions were extensively sonicated in pH 5.5 buffer (5 mM citrate/5 mM phosphate), and centrifuged for 1 hr at 300,000 × *g*. The supernatant fraction, called A5.5, which contained no mAb 423 activity, was discarded. The resulting pellet was sonicated in formic acid (1 ml). Insoluble material was removed by centrifugation and the supernatant was lyophilized. The freeze-dried material was redissolved in 5 mM ammonium acetate (pH 5.5, 1 ml) and clarified by centrifugation. The supernatant fraction, termed F5.5, was subjected to gel electrophoresis. Two major bands of 9.5 and 12 kDa were identified by mAb 423.

Gel Electrophoresis and Immunoblots. After succinylation, protein fractions prepared by method *i* could not be visualized on gels with Coomassie blue or silver stain or by immunoblot. They were detected by autoradiography after specific chemical labeling of the protein with tritiated ligand (see text), or by reaction with Bolton–Hunter reagent (Amersham). Fractions isolated by method *ii* were visualized by Coomassie stain or, after electrophoretic transfer to polyvinylidene difluoride membranes, by peroxidase-conjugated second antibody with chloronaphthol color development after incubation with mAb 423.

Protein Sequence Analysis. For sequence analysis, the 9.5- and 12-kDa bands were transferred to polyvinylidene difluoride membrane (19) and stained with Coomassie blue. Then they were excised, eluted, and digested with trypsin, and the resulting peptides were fractionated by microbore HPLC. The methods used in these experiments will be described fully elsewhere (J.E.W., M.J.R., and I. M. Fearnley, unpublished data). The sequences of tryptic peptides from the 9.5- and 12-kDa fragments and of chymotryptic peptides from method *i* were determined with an Applied Biosystems gas-phase sequencer with "on-line" HPLC detection of phenylthiohydantoin-derivatized amino acids. The sensitivity of this instrument has been increased by a modification to permit 90% of the phenylthiohydantoin-derivatized amino acid released at each cycle to be analyzed (J.E.W., F. D. Northrop, and R. A. Blows, unpublished results).

RESULTS

An Enriched Tangle Fraction: ifII. The majority of PHFs prepared by the method reported here remain in the form of tangle fragments that can be seen in the fluorescence microscope. Pronase was found to reduce amorphous contaminants substantially without affecting tangle yields. Enzymes such as proteinase K, crude collagenase, or V8 protease were found to reduce yields, whereas enzymes such as trypsin, chymotrypsin, subtilisin, mixed-function glycosidase, and neuraminidase were not efficient in reducing amorphous contaminants. In addition to preserving the morphology of isolated PHFs, Pronase abolished the axonal fragments seen by fluorescent labeling with RT97 (a mAb directed against neurofilaments, kindly supplied by B. Anderton, St. George's Hospital Medical School, London).

The problem of further separating tangle fragments from Pronase- and micrococcal nuclease-resistant contaminants was solved by a combination of velocity and density centrifugation. After equilibrium centrifugation in CsCl, tangle fragments banded at density 1.30. Since this is the average density of proteins, equilibrium centrifugation was only partially successful in reducing the amorphous background. By preforming a low-density sucrose gradient, and by relying on velocity separation, relative retardation of the amorphous contaminants contributed to a dramatic enrichment of tangle fragments collected at the CsCl interface. The final difference between ifI and ifII is shown in Fig. 1.

Identification and Isolation of PHF Components. To identify components originating from the Pronase-resistant core of the PHF, we used two independent methods, chemical and immunological.

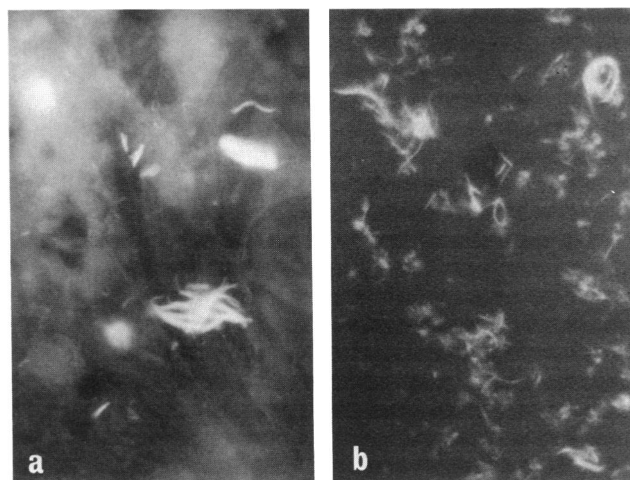

FIG. 1. Fluorescence microscopy of isolated tangle preparations. (*a*) ifI. Smear of ifI preparation prepared as described (2) and stained with thioflavin S prior to centrifugation in 1.5 M sucrose. Tangle fragments are seen in the fluorescence microscope as bright yellow against a dense amorphous background that fluoresces with a weak blue color. (×860.) (*b*) ifII. Suspension of ifII stained with thioflavin S. Tangle fragments are more dispersed and stand out against a clear background. Nothing else is visible by phase-contrast microscopy, but electron microscopy shows some contamination by collagen, lipofucsin, and amyloid aggregates. (×390.)

A chemical ligand, whose synthesis is discussed elsewhere (H.C.T. and C.M.W., unpublished observations), was found to bind with high affinity to PHF cores. A biotinylated derivative of the ligand produces strong ImmunoGold labeling, by gold-conjugated antibiotin, of Pronase-digested PHFs (Fig. 2*a*). A ^{125}I-labeled photolabile derivative was used as a photoaffinity ligand to identify peptides released after partial solubilization of PHF cores by succinylation. The labeled peptides released by succinylation ran at 24, 36, and 56 kDa (Fig. 3*a*) with various quantities of a 12-kDa component running in later column fractions (data not shown). The 24-kDa component, being the most abundant, was isolated by gel-filtration chromatography and digested with chymotrypsin for sequence analysis (Fig. 4).

Immunological identification relied on mAb 423, which labeled PHF cores strongly, and fuzzy PHFs only at high antibody concentration. Thus, mAb 423 identifies an epitope that is intimately associated with the Pronase-resistant core of the PHF and is not directed against epitopes present in the fuzzy coat of the PHF (14) (Fig. 2*b*).

The second method was introduced because the chemically labeled peptides solubilized initially by succinylation were negative when tested in immunoblots with mAb 423. The supernatant after the initial sonication of ifII at pH 5.5 (A5.5) contained contaminants that were loosely associated with PHFs, including a doublet in which the prominent band at ≈20 kDa (Fig. 3*b*) was found to contain a ubiquitin sequence (data not shown). The same doublet was found in an ifII fraction prepared from juvenile brain (data not shown). All the mAb 423 reactivity remained in the pellet at this stage, despite repeated sonication at pH 5.5. The mAb 423 reactivity was released by sonication in formic acid. Lyophilization and subsequent sonication at pH 5.5 of the material extracted with formic acid gave a supernatant, termed F5.5 (Fig. 3*b*). This contained several peptides, all recognized by mAb 423 (Fig. 5*a*), the 9.5- and 12-kDa components being the most abundant. Amino acid sequences (Fig. 4) derived from both bands were found to overlap with each other and with sequences derived from peptides prepared by method *i*.

The protein sequence Gln-Ile-Val-Tyr-Lys-Pro was used to design a mixture of oligonucleotides that served as a hybridization probe to isolate a cDNA clone that led to its identification as human tau protein (15). The amino acid sequences determined by peptide sequencing correspond to residues 211–297 of the protein sequence deduced from the human cDNA. Therefore, these residues account for most or all of the 9.5-kDa component. However, some microheterogeneity at the N terminus of the 9.5-kDa fragment (Fig. 4) could be interpreted in terms of the presence of two types of tau sequence identified from cDNA clones (15), implying that both types of tau are present in PHF core preparations.

Analysis of peptides from a tryptic digest of the 9.5-kDa fragment yielded in two cases sequences differing at one amino acid position. In both instances (Fig. 4, *) the alternative assignments were lysine and isoleucine, whereas the cDNA sequence codes for lysine. One explanation for this observation might be that in tau derived from PHF cores,

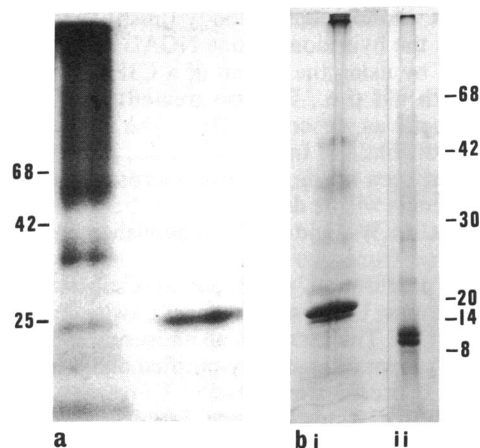

FIG. 3. Gels of ifII subfractions prepared by methods *i* and *ii*. (*a*) An ifII preparation was labeled with a tritiated photolabile derivative of the ligand shown to decorate PHFs in Fig. 2*a*. The fraction solubilized by succinylation (method *i*) was run on a Sephacryl S200 column in 50 mM ammonium bicarbonate. The void volume and a fraction running with a K_{av} of 0.21 were pooled separately, concentrated, electrophoresed on $NaDodSO_4$ gels, and visualized autoradiographically. The major labeled bands shown here appeared at 24, 36, and 56 kDa. (*b*) Lane i, A5.5. $NaDodSO_4$ gel of the first soluble fraction produced by sonicating ifII at pH 5.5. A number of components are seen: the most prominent is a doublet running at 20–22 kDa, the major band of which was found to contain a ubiquitin sequence. The pellet after this sonication contains all the mAb 423 reactivity and abundant morphologically recognizable PHF fragments. Lane ii, F5.5. $NaDodSO_4$ gel of peptides released from Pronase-resistant PHF cores by method *ii* stained with Coomassie blue. The major constituents run at 9.5 and 12 kDa, although 24- and 28-kDa and larger components occur variably in these preparations.

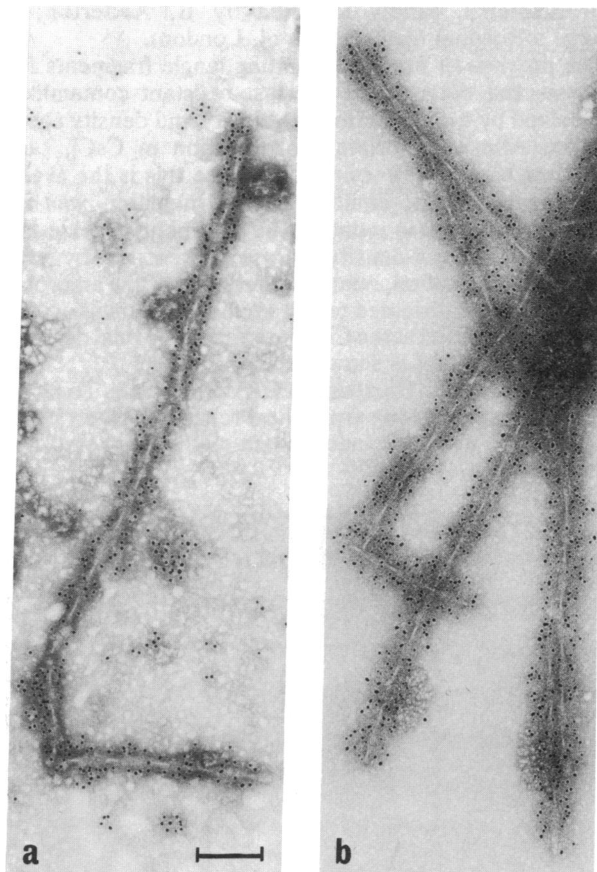

FIG. 2. Electron microscopy of PHFs labeled after Pronase digestion. (*a*) Chemical labeling. PHFs were deposited on a carbon-coated grid after Pronase digestion of an ifI preparation, incubated briefly with a preparation of biotinylated ligand (see text), and then incubated with an antibiotin antibody preparation that had been conjugated with colloidal gold but had not been washed free of colloid aggregates. Decoration of isolated PHFs establishes ligand binding to the PHF core structure. Negative stain, 1% phosphotungstic acid. (Bar = 100 nm.) (*b*) mAb labeling. An ifII preparation was incubated on the grid with mAb 423, followed by incubation with a standard gold-conjugated goat anti-mouse second antibody (14). Isolation of the corresponding hybridoma clone NOAL 6/66.423.2 was based on the criterion of unequivocal decoration of the Pronase-resistant PHF core structure as shown in this figure.

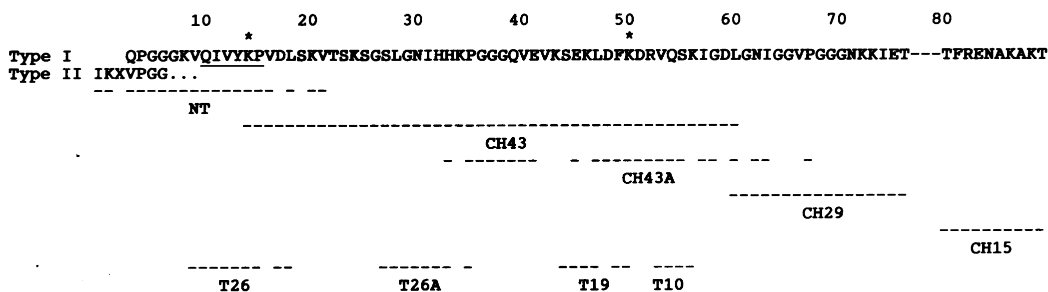

FIG. 4. Amino acid sequence (single-letter code) of peptides prepared from ifII by methods *i* and *ii*. In this tabulation, residues identified in sequencing the peptides are marked by dashes. N-terminal sequence (NT) was derived directly from polyvinylidene difluoride electrotransfers of the 9.5-kDa band prepared by method *ii*. The N terminus of the 12-kDa fragment is blocked. CH43, CH43a, CH29, and CH15 are chymotryptic peptides prepared by method *i*. T26, T26a, T19, and T10 are tryptic peptides from 9.5 kDa prepared by method *ii*. Except for the C-terminal fragment and the first few residues at the N terminus (see text), these sequences have been aligned independently of the cDNA sequences. Types I and II indicate two types of human tau detected in cDNA clones (15). The sequence Q I V Y K P was used to design the oligonucleotide probes used to isolate a human tau cDNA clone. *, Residues were assigned as lysine or isoleucine, whereas the cDNA sequence predicted lysine. This could be due to N-methylation (see text).

lysine residues are partially modified, possibly by N-methylation, and that the phenylthiohydantoin derivative of N^{ϵ}-methyllysine coelutes with phenylthiohydantoin-derivatized isoleucine in the HPLC chromatographic analysis.

Distinction Between mAb 423 and Anti-tau Antisera. Although the sequence of the fragment shown here to originate from the PHF core is essentially identical to human (15) and mouse tau (24), antisera raised in rabbits against porcine and bovine tau preparations failed to give immunoblots against any of the constituents of F5.5 (Fig. 5*a*). Both these antisera recognized epitopes present in the fuzzy coat of the PHF but not the Pronase-resistant core (14). Their failure to label F5.5 was therefore not due to a failure to recognize human tau. Conversely, mAb 423 failed to recognize tau isolated from mammalian sources in immunoblots (Fig. 5*b*). The 423 tau epitope exposed on the surface of Pronase-digested PHFs is therefore distinct from the epitopes normally exposed in soluble preparations of tau protein, and present in the fuzzy outer part of the PHF.

DISCUSSION

It has not previously been possible to identify any protein as being a constituent of the core structure of the Alzheimer PHF. Although the amorphous material on the outside of PHFs contains tau epitopes, reactivity with antisera raised against standard microtubule-associated tau preparations can be abolished by Pronase digestion (14). Since the Pronase digestion we have used in the course of purification leaves PHFs and tangle fragments morphologically intact, while removing the fuzzy outer coat of the PHF, it is arguable that tau is simply a PHF-associated protein and not an integral part of the Pronase-resistant core. Likewise, previous reports showing the presence of gel bands reacting with anti-tau antibodies in extracts of tangle-enriched fractions (20–22), although consistent with the present results, do not establish whether tau simply adheres to the surface of the PHF or whether it is intimately associated with the core structure as we have defined it. Simple extraction of proteins or peptides from tangle preparations (e.g., see ref. 23) is not sufficient proof in view of the complexity of such preparations.

On the other hand, the protein fragments we have isolated and characterized have been copurified with the Pronase-resistant core of the PHF and are tightly associated with it. The strong association with the core is borne out by two observations. First, mAb 423 reactivity cannot be released into the supernatant merely by extensive sonication at pH 5.5 in the absence of the formic acid step. Second, once separated from the core, the tau fragments released into the pH 5.5 supernatant after formic acid sonication are entirely digested by Pronase at much lower enzyme/substrate ratios than used in the preparation of ifII.

mAb 423, which we have used to follow the peptide isolations and which decorates the cores, does not cross-react with the fuzzy outer coat of the PHF (14). Therefore, the peptides we have characterized do not originate from the fuzzy outer material but are intimately associated with the protease-resistant core of the PHF.

The near identity of the amino acid sequence between the major tau fragment we have characterized and mouse and human cDNA sequences makes the reciprocal failure of mAb 423 and the anti-tau antisera to cross-react with their respective substrates (Fig. 5) somewhat puzzling. A possible explanation is as follows. mAb 423 reacts with epitopes present in the 9.5- and 12-kDa fragments, which originate from the three repeating segments present in the C-terminal portion of tau. If these were to constitute not only its PHF core-binding domain, but also the microtubule-binding domain of tau (24), the microtubule-associated preparations of tau commonly used to immunize animals would not give rise to antibodies recognizing the part of tau that is tightly bound to the core. It thus appears that the reactivity of tau antisera against fuzzy PHFs is directed toward epitopes lost after Pronase digestion, and probably present in the first 200 or last 50 residues of tau. This would agree roughly with the mass difference of 20 kDa per subunit observed after Pronase digestion, which is reported in the third paper of this series (14). As for the

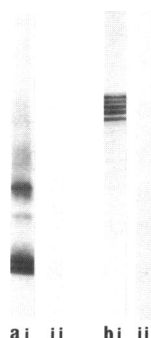

FIG. 5. Immunoblots of F5.5 and tau preparations with mAb 423 and an anti-tau antiserum. (*a*) F5.5. Lane i, immunoblot of F5.5 shows that mAb 423 labels the 9.5- and 12-kDa bands strongly in addition to weaker labeling of the 24- and 28-kDa components. Lane ii, F5.5 is not labeled by anti-tau antisera in immunoblots, although these antisera decorate non-Pronase-treated PHFs (14). (*b*) Tau. Lane i, immunoblot of a purified tau preparation labeled with an affinity-purified anti-tau antiserum. Lane ii, the same preparation fails to be labeled by the anti-PHF core mAb 423.

failure of mAb 423 to recognize tau, this could be due to a difference in epitope. Species-specific residues cannot be excluded at this stage.

The presence of tau in the Pronase-resistant core of the Alzheimer PHF does not necessarily imply that tau polymerizes to form PHFs as has been claimed (25). Although tau could conceivably play a role in the *de novo* polymerization of the PHF, the identity of the major repeating subunit of the PHF, defined structurally in our earlier work (2–4), remains unknown. Tau remains associated with PHFs even after the death of tangle-bearing neurons (C.M.W., P. Barber, M.N., and R. Hills, unpublished data). It is likely therefore that tau is bound permanently once PHFs form. Although PHFs accumulate initially in nerve terminals as neuritic plaques (26), their eventual appearance in cell bodies as neurofibrillary tangles may signal a failure of the cytoskeletal transport system that is due to the sequestration of a large portion of the tau pool of the cell.

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