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# Identification of 3- and 4-repeat tau isoforms within the PHF in Alzheimer's disease

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**The microtubule associated protein tau is incorporated into the pronase resistant core of the paired helical filament (PHF) in such a way that the repeat region is protected from proteases, but can be released as a major 12 kDa species from the PHF core by formic acid treatment and by boiling in SDS. This fragment retains the ability to aggregate in the presence of SDS. Detailed sequence analysis of the 12 kDa species shows that it consists of a mixture of peptides derived from the repeat region of 3- and 4-repeat tau isoforms comigrating as a single electrophoretic band. However, the 4-repeat isoforms released from the core lack either the first or the last repeat. The pronase-protected region of tau within the PHF core is therefore restricted to three repeats, regardless of isoform. The alignment of cleavage sites at homologous positions within tandem repeats after protease treatment indicates that the tau–core association is precisely constrained by the tandem repeat structure of the tau molecule.**

*Key words:* Alzheimer's disease/paired helical filaments/ sequence analysis/tau protein isoforms

## Introduction

Paired helical filaments (PHFs) which accumulate in Alzheimer's disease are the principal fibrous constituent of the neurofibrillary tangle (Kidd, 1983), one of the characteristic neuropathological lesions seen in this disorder. The structurally regular part of the PHF is a double helical stack of C-shaped subunits, which accounts for ~100 kDa per subunit. This core structure survives digestion with the broad spectrum protease, pronase, whereas a fuzzy outer coat, which accounts for a further 20 kDa per subunit, is lost after pronase digestion (Wischik *et al.*, 1988a,b).

Many so-called 'anti-PHF' antibodies cross-react with normal tau proteins (Flament and Delacourte, 1989) and recognize epitopes located outside the repeat region of tau (Ksiezak-Reding *et al.*, 1990; Steiner *et al.*, 1990). PHF immunoreactivity with many anti-tau antibodies is lost after pronase digestion (Wischik *et al.*, 1988b; Brion *et al.*, 1991). Nevertheless, the repeat region of the tau molecule is now known to be embedded within the pronase resistant core structure of the PHF, whereas the N-termina! domain of some 200 amino acids projects into the pronase sensitive fuzzy coat (Wischik *et al.*, 1988a,b; Kondo *et al.*, 1988; Crowther *et al.*, 1989). Thus, the same region of the tau molecule which normally functions as the microtubule binding domain (Aizawa *et al.*, 1988; Ennulat *et al.*, 1989; Joly and Purich, 1990) is integral to the PHF core, and is protected from pronase digestion.

Tau protein has been shown to exist in several alternatively spliced isoforms which have either three or four repeats (Goedert *et al.*, 1989a,b). Four-repeat tau (with repeats denoted R1, R2, R3 and R4) differs from 3-repeat tau (with repeats denoted R1, R3 and R4) by the insertion of the second repeat, R2, encoded by exon 10 in the scheme of Himmler (1989). Although mRNA encoding 3- and 4-repeat isoforms is found in adult brain tissues, only 3-repeat tau mRNA is found in the fetal brain (Goedert *et al.*, 1989a). Based on sequence analysis of HPLC purified tau peptides released by SDS treatment from PHF preparations, Mori *et al.* (1989) have reported finding only 3-repeat tau isoforms. From this they conclude that fetal tau isoforms are selectively incorporated into PHFs.

We have devised a protocol which makes it possible to release the protected repeat region of PHF tau after pronase digestion by formic acid treatment, followed by extraction at pH 5.5 (Wischik *et al.*, 1988a). The major species liberated by this procedure is a 12 kDa band which is recognized both by mAb 7.51, which recognizes an epitope located in R3 and R4 of 3- and 4-repeat tau isoforms, and by mAb 423, which detects a configuration of the repeat region of tau which is unique to PHF tau (Novak *et al.*, 1991). We now report the further biochemical characterisation of the 12 kDa band, showing that it consists of a mixture of peptides derived from the repeat region of 3- and 4-repeat tau isoforms, but encompassing only three repeats.

## Results

### *Immunoblotting of PHF-core derived tau*

An enriched preparation of pronase treated PHFs, called 'if-II', has served as the starting point for isolating PHF-core derived tau fragments (Wischik *et al.*, 1988a). In this protocol, soluble contaminants are first removed by sonication at pH 5.5. The first pH 5.5 extract is termed 'A5.5'. The PHF-core derived fragments can be distinguished by the fact that they can be extracted at pH 5.5 only after a subsequent formic acid sonication step. The resulting post-formic acid pH 5.5 extract, termed 'F5.5', is a substantially pure preparation of PHF-core derived tau, of which the major SDS–PAGE bands are a doublet at 12 and 14 kDa, and variable weaker bands with gel mobilities corresponding to 24, 28 and 32 kDa (Figure 1). These bands are recognized by two monoclonal antibodies, mAbs 7.51 and 423 (Figure 1).

In an alternative protocol, the A5.5 pellet is not taken through formic acid, but is instead sonicated at pH 8.5

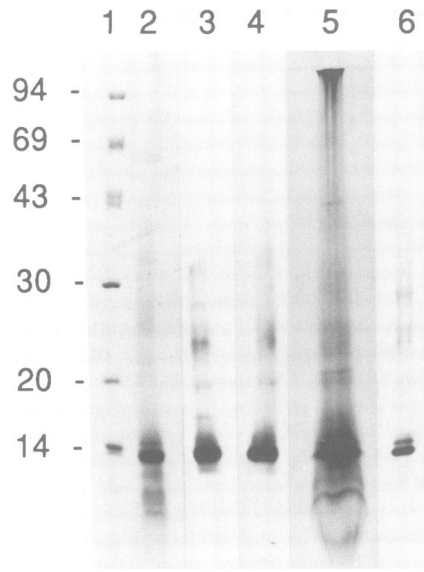


**Fig. 1.** The PHF-core derived tau fragments used in this study were prepared by two different protocols. The F5.5 fraction is released from the pronase resistant core of the PHF by formic acid treatment (Wischik *et al.*, 1988a) and contains a major 12 kDa band species as well as a variable higher molecular weight species (lane 2, Coomassie stained). The 12 kDa band is recognised by two monoclonal antibodies, 423 (lane 3) and 7.51 (lane 4), raised against PHF-core preparations (Wischik *et al.*, 1988a; Novak *et al.*, 1991). Similar bands can be released from an enriched preparation of fragmented, but morphologically recognizable, PHFs (Figure 2B) by boiling in SDS sample buffer (lane 5). The 12 kDa band produced by this method is also recognized by the PHF-tau specific mAb 423 (lane 6) as well as by mAb 7.51 (not shown). Molecular weight standards, lane 1.

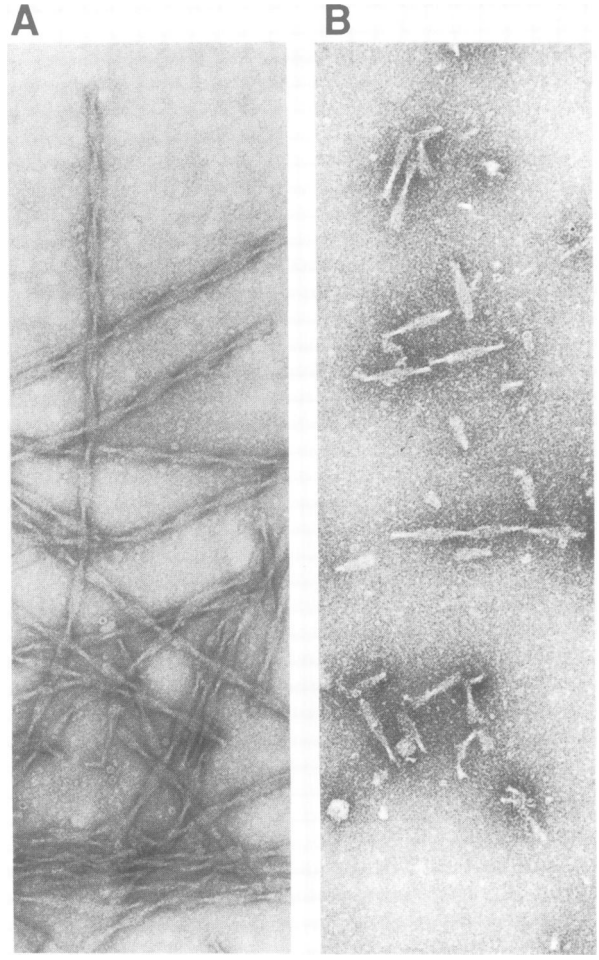


**Fig. 2.** Electron microscopy of morphologically intact pronase-treated PHFs from if-II (**A**) which serves as the starting point for extraction of F5.5. Sonication of the PHF-core preparation in ammonium bicarbonate (50 mM, pH 8.5) produces a suspension of morphologically recognizable PHF fragments termed 'ABCsup' (**B**).

(ammonium bicarbonate, 100 mM). This produces in the supernatant a suspension of short fragments which are recognizable as PHFs (Figure 2). As judged by electron microscopy, this fraction, termed 'ABCsup', appears to be a purer source of PHFs. Electrophoresis of ABCsup after boiling in SDS sample buffer releases the same tau fragments seen in F5.5. These fragments also immunoblot with mAbs 423 (Figure 1) and 7.51 (not shown). This demonstrates that the core-binding fragment of tau can be released from the PHF by boiling in SDS or formic acid extraction.

When gel slices of single Coomassie stained electrophoretic bands from F5.5 were subjected to re-electrophoresis, dimeric and higher molecular weight aggregates were shown to form from the 12 kDa and 14 kDa species (Figure 3). This indicates that these fragments retain the potential for aggregation in the presence of SDS.

### *Sequence analysis of the 12 kDa band*

The 12 kDa band has yielded N-terminal sequence which has been reported previously (Wischik *et al.*, 1988a), but analysis was complicated by staggered end cleavage and background contamination. By excising and re-running the SDS−PAGE gel slice of this 12 kDa band (Plaxton and Moorhead, 1989), we have been able to enhance the signal and reduce the background contamination substantially. We have analysed this fragment at the 10 pmol level from a PVDF blot on the gas phase sequencer.

Residues identified at each cycle are shown in Figure 4A. It was possible to interpret this data in the light of known cDNA sequences of 3- and 4-repeat tau isoforms (Goedert *et al.*, 1988, 1989). The presence of I, L and H at position 1 demonstrates the existence of cleavage sites two residues apart within each isoform in the band. The presence of V at position 2 is unique to R2 from 4-repeat tau, and cannot be explained by alternative alignment of 3-repeat tau. Likewise, the presence of V at position 4 is unique to R2 from 4-repeat tau. G alone at position 6 confirms the alignment of the cleavage positions for the first residue. Finally Y and N at position 12 can only be explained by the presence of R3 from either 3- or 4-repeat tau, and R2 from 4-repeat tau respectively. Quantitatively, the predominant species are the 3-repeat isoforms, which constitute 70%, estimated from PTH-amino acid recoveries (V at positions 1, 4, 8, 11 and 15; I at positions 1, 10, 12; L at positions 1, 15 and 17).

These peptides are shown in Figure 5A and B assuming an approximate length of 100 amino acids. The C-terminus of the fragments has not been determined; however, the presence of a chymotryptic fragment (FRENAKAKT) in F5.5 digests defines the minimum length as 90 residues (Wischik *et al.*, 1988a). No sequences beyound this residue (marked by '*' in Figure 6) have been found so far by direct sequencing in F5.5 preparations.

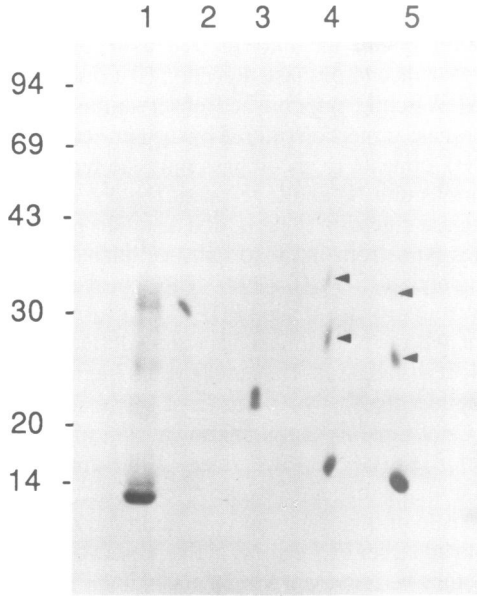


**Fig. 3.** Electrophoretic gel bands from the F5.5 fraction were excised and re-electrophoresed on a 10–20% acrylamide gradient SDS (0.1%) minigel without further boiling in sample buffer and were visualized by immunoblotting using mAb 423. F5.5 (lane 1); 32 kDa gel slice (lane 2); 22/24 kDa gel slice (lane 3); 14 kDa gel slice (lane 4); 12 kDa gel slice (lane 5). Arrow heads indicate the presence of higher molecular weight aggregates which have formed from material originally running at 12 and 14 kDa.

**A**

```
          5         10        15
L K H Q P G G G K V Q I I N K K L D
H Q P G G G K V Q I I N K K L D L S
L K H Q P G G G K V Q I V Y K P V D
H Q P G G G K V Q I V Y K P V D L S
I K H V P G G G S V Q I V Y K P V D
H V P G G G S V Q I V Y K P V D L S
```

**B**

```
          5         10        15
S K V T S K C G S L G N I H H K P G
K D R V Q S K I G S L D N I T H V P
```

**Fig. 4.** When the 12 kDa band from F5.5 (**A**) and the 8 kDa band released from the performic acid oxidized ABCsup fraction after pepsin digestion (**B**) were re-electrophoresed and sequenced, multiple residues but with much reduced background were found at each Edman degradation cycle. It was possible to interpret this data in the light of known sequences of 3- and 4-repeat tau isoforms (Goedert *et al.*, 1988, 1989a). Underlined residues in (A) are discussed in the text.

**A**

```
      1       10        20        30        40        50        60
a     HQPGGGKVQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQVEVKSEKLDFKDRVQSKIGSLDNITHV
b     LKHQPGGGKVQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQVEVKSEKLDFKDRVQSKIGSLDNIT
c     HVPGGGSVQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQVEVKSEKLDFKDRVQSKIGSLDNITHV
d     IKHVPGGGSVQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQVEVKSEKLDFKDRVQSKIGSLDNIT
e     HQPGGGKVQIINKKLDLSNVQSKCGSKDNIKHVPGGGSVQIVYKPVDLSKVTSKCGSLGNIHHKP
f     LKHQPGGGKVQIINKKLDLSNVQSKCGSKDNIKHVPGGGSVQIVYKPVDLSKVTSKCGSLGNIHH

         70        80        90
a     PGGGNKKIETHKLTFRENAKAKTDHGAEIVYK          (97)
b     HVPGGGNKKIETHKLTFRENAKAKTDHGAEIVYK        (99)
c     PGGGNKKIETHKLTFRENAKAKTDHGAEIVYK          (97)
d     HVPGGGNKKIETHKLTFRENAKAKTDHGAEIVYK        (99)
e     GGGQVEVKSEKLDFKDRVQSKIGSLDNITHVPGGGN     (101)
f     KPGGGQVEVKSEKLDFKDRVQSKIGSLDNITHVPGGGN   (103)
```

**B**

```
   266          280       290       300       310       320       330
a    --HQPGGGK-------------------------------VQIVYKPVDLSKVTSKCGSLGNIHH
b    LKHQPGGGK-------------------------------VQIVYKPVDLSKVTSKCGSLGNIHH
c    ---------------------------------HVPGGGSVQIVYKPVDLSKVTSKCGSLGNIHH
d    --------------------------------IKHVPGGGSVQIVYKPVDLSKVTSKCGSLGNIHH
e    --HQPGGGKVQIINKKLDLSNVQSKCGSKDNIKHVPGGGSVQIVYKPVDLSKVTSKCGSLGNIHH
f    LKHQPGGGKVQIINKKLDLSNVQSKCGSKDNIKHVPGGGSVQIVYKPVDLSKVTSKCGSLGNIHH

            340       350       360       370       380       390
a    KPGGGQVEVKSEKLDFKDRVQSKIGSLDNITHVPGGGNKKIETHKLTFRENAKAKTDHGAEIVYK
b    KPGGGQVEVKSEKLDFKDRVQSKIGSLDNITHVPGGGNKKIETHKLTFRENAKAKTDHGAEIVYK
c    KPGGGQVEVKSEKLDFKDRVQSKIGSLDNITHVPGGGNKKIETHKLTFRENAKAKTDHGAEIVYK
d    KPGGGQVEVKSEKLDFKDRVQSKIGSLDNITHVPGGGNKKIETHKLTFRENAKAKTDHGAEIVYK
e    KPGGGQVEVKSEKLDFKDRVQSKIGSLDNITHVPGGGN
f    KPGGGQVEVKSEKLDFKDRVQSKIGSLDNITHVPGGGN
```

**Fig. 5.** The peptides identified from N-terminal sequence analysis of the 12 kDa band are shown in (**A**) extending ~100 amino acid residues. The actual C-terminal extent of the 12 kDa band is unknown. The same peptides are shown in (**B**) aligned according to the known cDNA sequences of 3- and 4-repeat tau isoforms. a and b begin in R1 of 3-repeat tau; c and d begin in R2 of 4-repeat tau; e and f begin in R1 of 4-repeat tau. Numbering of residues correspond to the longest tau isoform (441 residues, Goedert *et al.*, 1989a).

### *Pepsin fragments of tau from the PHF core*

The F5.5 tau species are protected from pronase digestion only by their tight association with the core of the PHF, since they are readily digested once released (Wischik *et al.*, 1988a). It is nevertheless possible to achieve further pepsin digestion of tau that remains partially bound in the ABCsup fraction after performic acid oxidation. Pepsin treatment of oxidized ABCsup produces a major 8 kDa species in place of the usual 12 kDa band when the preparation is boiled in SDS and examined by SDS−PAGE. N-terminal sequence analysis of an HPLC fraction of this material revealed the presence of two species derived from R3 and R4 respectively (Figure 4B).

### *Alignment of cleavage sites within the repeat region of 3- and 4-repeat tau*

Although each of the 32 amino acid repeats of tau is slightly different, they can be aligned so as to demonstrate the considerable degree of homology that exists between successive repeats (Figure 6). The pronase cleavage site shown above align precisely according to the tandem repeat structure of the tau molecule (see arrows marked '*' in Figure 6). The cleavage sites in R1 are identical for 3- and 4-repeat tau. The cleavage sites in R2 of 4-repeat tau are at positions which are homologous to those in R1. The pepsin cleavage sites (arrows marked $P_1$ and $P_2$ in Figure 6) are also located at homologous positions in R3 and R4, which are common to 3- and 4-repeat tau isoforms.

## Discussion

The repeat region of tau, which consists of three or four tandem repeats of 32 amino acids each, is known to function as the microtubule binding domain (Aizawa *et al.*, 1988; Ennulat *et al.*, 1989; Joly and Purich, 1990). The C-terminal tail of $\beta$ tubulin is thought to represent the tau binding domain of tubulin (Littauer *et al.*, 1990). The precise nature of the association is unknown. Although it is striking that the same region of the tau molecule also appears to function as the PHF-core binding domain (Wischik *et al.*, 1988a; Crowther *et al.*, 1989), there is no evidence that the inner core of the PHF contains tubulin. The tau−core association in the PHF is quite different from the tau−tubulin association in the microtubule. Tau is not released from the PHFs under conditions that release tau from tubulin, such as high salt concentration, and the tau−tubulin association does not confer resistance to pronase digestion (P.Edwards, R.Jakes and C.M.Wischik, unpublished observations).

The pronase protection offered by the tau−core association in the PHF appears to encompass only the repeat region of the tau molecule. N- and C-terminal regions of tau are lost after pronase digestion *in vitro*, and a similar process occurs as tangles pass from intra- to extra-cellular compartments *in vivo* (Bondareff *et al.*, 1990). The repeat region can be released from pronase-treated PHFs in the form of a 12 kDa fragment whose N-terminus has been defined previously (Wischik *et al.*, 1988a). The length of the fragment is known from peptide analysis to be at least 90 residues, but the precise C-terminal extent is unknown, and the unusual gel mobility of tau (Goedert and Jakes, 1990) makes accurate molecular sizing difficult. We now report that an additional characteristic of this fragment is its ability to dimerize (and to form even larger aggregates) in the presence of SDS. This property may be important for understanding PHF assembly.

The present data demonstrate that several distinct tau peptides derived from 3- and 4-repeat tau isoforms comigrate as the 12 kDa band in the extract released from the pronase resistant core of the PHF. Since all of the peptides are of a similar gel mobility, the peptides derived from 4-repeat tau species must be shortened by the equivalent of one repeat in order to have the same gel mobility as the 3-repeat tau species. A difference of one repeat, i.e. 32 amino acids, would be readily detectable on SDS−PAGE. There is direct evidence in the N-terminal sequence data of the absence of the first repeat of 4-repeat tau in two of the peptides (shown as c and d in Figure 5). The absence of R4 in those 4-repeat tau peptides starting at R1 can be deduced from comigration at 12 kDa with the 3-repeat tau peptides. Thus the species present in the 12 kDa band must be derived from R1-R3-R4 of 3-repeat tau, from R1-R2-R3 of 4-repeat tau and from R2-R3-R4 of 4-repeat tau. The protected region of tau in PHFs is therefore restricted to three repeats.

Although pronase has wide proteolytic specificity, and entirely digests the 12 kDa band once released from the core of the PHF (P.Edwards, R.Jakes and C.M.Wischik, unpublished observation), the cleavage sites in tau molecules which remain bound to the core are at positions which align precisely according to the tandem repeat structure of tau.

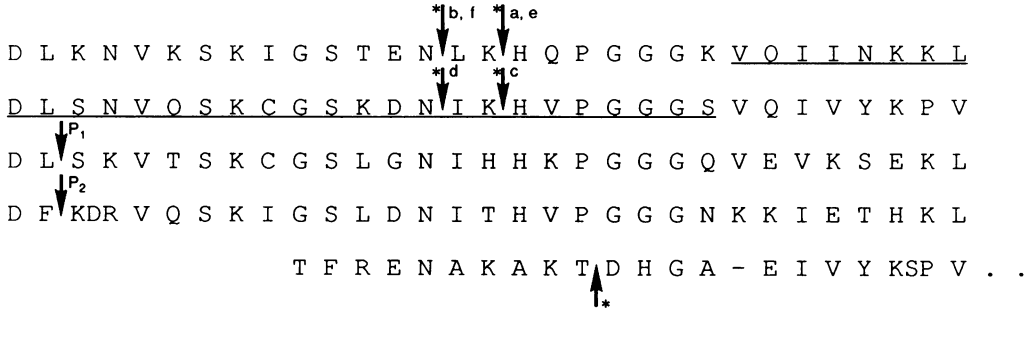


**Fig. 6.** Alignment of tandem repeat region of 3- and 4-repeat tau isoforms showing the extensive homology that exists between successive repeats (Goedert *et al.*, 1988). As reported by Goedert *et al.* (1989a) the 4-repeat isoform differs from the 3-repeat by the presence of the insert amounting to one complete repeat underlined (R2). The pronase cleavage sites deduced from N-terminal sequence analysis of the 12 kDa species are shown by vertical arrows marked '*'. The peptides denoted a−f in Figure 3 (A and B) are shown to begin at precisely homologous positions in R1 and R2 of 3- and 4-repeat tau isoforms. The vertical arrows marked '$P_1$' and '$P_2$' show that the pepsin cleavage sites in performic acid oxidized PHF fragments also align at homologous positions in the last two repeats. The lower arrow marked '*' shows the end position of the most C-terminal peptide observed from direct sequence analysis of tryptic and chymotryptic fragments derived from the 12 kDa band (Wischik *et al.*, 1988a).

The cleavage site in R1 of 3- and 4-repeat tau align to homologous positions in R2 of 4-repeat tau. Likewise the pepsin cleavage sites after oxidation of PHFs are exactly one repeat apart in R3 and R4. In this case, the C-terminal half of the repeat region presumably remains firmly bound after oxidation, limiting pepsin access. Since R3 and R4 are common to 3- and 4-repeat tau, it is not possible to deduce from which isoforms the pepsin peptides originate.

The alignment of pronase cleavage sites within 4-repeat tau provides very strong evidence that 4-repeat tau is indeed contained within the pronase resistant core of the PHF. The absence of fragments derived from 4-repeat tau in the material prepared by reverse phase HPLC after SDS extraction by Mori *et al.* (1989) is presumably due to a difference in preparative procedure. The precise alignment of cleavage sites in 3- and 4-repeat isoforms demonstrates that the tau–core association is definitely constrained by the repeat structure of the tau molecule. However, the protease-protected domain of tau within the pHF core is restricted to three tandem repeats, regardless of isoform. This therefore defines the extent of the binding site for releasable tau within the core of the PHF.

## Materials and methods

The preparation of the pronase treated PHF-core preparation (Wischik *et al.*, 1988a), the F5.5 fraction containing PHF-core derived tau fragments (Wischik *et al.*, 1988a), and the enriched preparation of PHF-core fragments termed 'ABCsup' (Harrington *et al.*, 1990) have been described previously. Protein fractions were separated using 10–20% gradient SDS–PAGE (Matsudaira and Burgess, 1978) and electrophoretically transferred to PVDF (polyvinylidine difluoride) for immunoblotting using peroxidase-labelled rabbit anti-mouse second antibody. Monoclonal antibodies 423 and 7.51, used as first antibodies, were prepared as described (Wischik *et al.*, 1988a; Novak *et al.*, 1989, 1991). Excision and re-running of gel slices was carried out as described (Plaxton and Moorhead, 1989). Amino acid sequencing using an ABI gas-phase sequencer was carried out from bands electrophoretically transferred to PVDF (Matsudaira, 1987) and from fractions obtained by standard reverse phase HPLC. Proteolytic subdigestion of fractions was carried out as described (Novak *et al.*, 1991). Electron microscopy were carried out as previously (Wischik *et al.*, 1988b).

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