Volume 248, number 1,2, 87–91 FEB 07099 May 1989

Microtubule-associated protein tau

Identification of a novel peptide from bovine brain

Khalid Iqbal, Alan J. Smith*, Tanweer Zaidi and Inge Grundke-Iqbal

New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York, NY 10314 and **Department of Biological Chemistry, School of Medicine, University of California, Davis, CA 95616, USA*

Received 2 March 1989

The microtubule-associated protein tau isolated from bovine brain was cleaved with CNBr and the 3 largest peptides of approx. 21, 19 and 18 kDa were obtained. Dephosphorylation of the CNBr digest of tau with alkaline phosphatase changed the electrophoretic mobility of these peptides to 19, 18 and 17 kDa. Amino acid sequencing of the total CNBr digest of tau revealed at least 3 sequences, two of which were highly homologous to previously published mouse and human tau sequences derived from cDNAs. A third amino acid sequence of 17 residues with heterogeneity at position 11 showed no homology with the cDNA-derived tau sequences. These studies suggest that the amino acid sequences of mammalian tau predicted from their cDNAs might be incomplete.

Microtubule; Protein, tau; Protein phosphorylation; Amino acid sequencing; Cyanogen bromide cleavage

1. INTRODUCTION

Microtubule-associated protein tau is a family of polypeptides of molecular mass in the range 55–68 kDa on SDS-PAGE [1,2]. These polypeptides also differ in isoelectric points (6.5–8.0) but are closely related to one another in primary structure, since they have similar peptide maps and amino acid compositions [2]. Recent studies [3,4] have shown that, depending on the degree of phosphorylation, tau polypeptides have different electrophoretic mobilities on SDS-PAGE. During preparation of this manuscript, the cDNA-derived sequences of mouse and human tau became available [5,6]. Here, we describe the presence of peptides homologous to these derived sequences in bovine brain tau and a unique peptide not present in either cDNA-derived sequence.

Correspondence address: K. Iqbal, NYS Institute for Basic Research in Developmental Disabilities, Staten Island, NY 10314, USA

Abbreviations: KLH, keyhole limpet hemocyanin; PITC, phenylisothiocyanate; OPA, *o*-phthalaldehyde; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Isolation of tau*

Tau was isolated from bovine brain, obtained from a local slaughterhouse, by the method of Grundke-Iqbal et al. [7]. Microtubule proteins obtained by three cycles of assembly-disassembly were heat-treated at pH 2.7 and tau from the heat-stable MAPS was then extracted in 2.5% perchloric acid [8] and dialyzed vs 2.5 mM Tris (pH 7.6). No protein bands other than tau were detected on Coomassie blue staining in SDS-PAGE. The tau preparation, 0.05 mg protein in 10 ml, was concentrated to 400 μl by evaporation in a Speed Vac (Savant, Hicksville, NY), heated with 2 μl of 10% SDS in a boiling water bath for 5 min, dialyzed vs several changes of 0.02% SDS for 24 h, divided into aliquots of 100 μg protein each in Eppendorf tubes and again dried in a Speed Vac.

2.2. *Generation of tau peptides by chemical cleavage for amino acid sequencing*

Tau in 100-μg aliquots was digested with 40 mM CNBr in 250 μl of 70% formic acid overnight at room temperature in darkness, dried in a Speed Vac, resuspended in 50 μl H_2O and redried twice as above to remove CNBr and formic acid. The digest was taken up in 500 μl derivatizing solution containing alcohol, water, triethylamine and PITC (7:1:1:1, v/v), dried

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50 © 1989 Federation of European Biochemical Societies

and then redried from alcohol, water, triethylamine (2:2:1, v/v) solution in a Speed Vac. In some experiments the CNBr digest without drying was diluted with 250 μl derivatizing solution and dialyzed vs 3 changes (2 h each) of 20 ml per portion of derivatizing solution using 3.5 kDa cut-off dialysis bags.

2.3. *Manual Edman degradation and blockage of amino-termini with OPA*

Amino acid sequencing of the CNBr digest of tau after PITC treatment (see above) revealed several peptides, one of which had proline as the second residue. Taking advantage of this observation, a CNBr digest of tau was subjected to one manual cycle of Edman degradation, followed by blockage with OPA of all amino-termini except that starting with proline. The overnight CNBr digest of 100 μg tau (see above) was subjected to a 2nd digestion with CNBr but for only 3 h to achieve increased digestion of the tau polypeptides. The digest was dried twice from 50 μl water as above. The digest was resuspended by mixing with a vortex mixer, in 500 μl derivatizing solution containing alcohol, water, triethylamine and PITC (7:1:1:1, v/v), dried and then redried in 500 μl redrying solution comprising alcohol, water and triethylamine (2:2:1, v/v). Cleavage of PITC-labeled amino-terminal residues was then carried out by heating the peptide mixture with 250 μl of 100% trifluoroacetic acid at 50°C for 5 min. The sample was then dried and redried from 50 μl H_2O. Blockage of amino-termini with OPA (Pierce, Rockford, IL) was then carried out by heating the sample to 65°C for 10 min with 250 μl of a solution prepared by mixing 20 μl of 7% OPA in methanol and mercaptoethanol (5:1, v/v) solution with 1 ml ethanol, H_2O, triethylamine (2:2:1, v/v) solution. The sample was dried in a Speed Vac as above and subjected to automated gas-phase sequencing.

2.4. *Amino acid analysis and sequencing*

Amino acid analysis was performed using either a Waters Pico Tag system (Waters, Millipore Corp., Milford, MA) or a Beckman 6300 ninhydrin-based system (Beckman, Palo Alto, CA). All hydrolyses were carried out in 6 N HCl, at 110°C for 24 h. Sequencing was performed on both a Beckman 890 M sequencer and an Applied Biosystems 470A sequencer in the presence of polybrene.

2.5. *SDS-PAGE*

Evaluation of the CNBr digestion of tau was carried out on 5–22% polyacrylamide gradient (8 × 6 × 0.75 cm) gels using Laemmli's Tris-glycine discontinuous buffer system [9]. Tau before and after CNBr digestion were run on the same gel. Gels were stained with Coomassie blue.

3. RESULTS

3.1. *Structural relationship between bovine and other mammalian tau sequences*

Amino acid analysis of intact bovine tau showed good agreement with published data for whole porcine tau [2] (table 1).

Intact bovine tau was subjected to automated N-terminal sequencing which resulted in no sequence being found. This indicated the presence of a blocked amino-terminus. The protein was then subjected to CNBr cleavage. The 3 larger fragments generated were approx. 21, 19 and 18 kDa in molecular mass (plots not shown) as determined by SDS-PAGE (fig.1, lane 3). After dialysis from 3.5 kDa cut-off dialysis bags, the mixture was sequenced directly and resulted in 3 amino acids at each cycle of Edman degradation. Sequences were recovered at the 700 pmol level from 100 μg tau digest. A proline residue was identified as the second cycle of the mixture. This provided the opportunity for reacting the mixture with OPA and blocking all α-amino groups. Subsequent sequencing should leave only one peptide commencing with proline. To this end, the CNBr mixture was subjected to a single cycle of manual Edman degradation, treated with OPA, and loaded onto an automated protein sequencer. This procedure resulted in a sequence, peptide I, which was 17 residues in length. Microheterogeneity was found at residue 11 where both proline and glycine were identified (fig.2). Subtraction of the peptide I sequence from data obtained by sequencing the CNBr mixture indicated the presence of 2 additional sequences, peptides II and III.

The cDNA-derived sequences of both mouse

Table 1

Amino acid composition of tau (mol%)

Amino acid	Bovine tau (this study)	Porcine tau [2]
Asp	9.4	9.7
Thr	7.0	7.6
Ser	9.7	10.1
Glu	12.1	10.8
Pro	9.7	10.4
Gly	11.6	10.9
Ala	8.8	7.9
Val	5.0	5.1
Met	0.2	1.2
Ile	3.0	2.6
Leu	5.4	5.5
Tyr	1.2	1.0
Phe	1.4	0.9
Lys	9.6	9.7
His	2.2	2.6
Arg	3.9	3.6

Bovine tau: data obtained from 24 h hydrolysis in 6 N HCl at 110°C; mean of 4 experiments. Porcine tau: data obtained from Cleveland et al. [2]

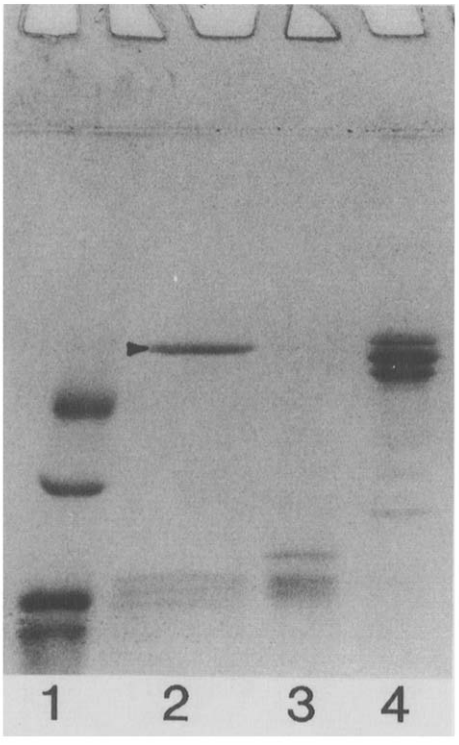


Fig.1. Electrophoretic patterns of bovine tau, its peptides generated by CNBr cleavage and effect of dephosphorylation on migration of these peptides. Lanes: 4, tau isolated from thrice-cycled calf brain microtubules; 3, same amount of tau as lane 4 (10 μg) digested with 40 mM CNBr in 70% HCOOH overnight; 2, CNBr digest of tau (10 μg) dephosphorylated with alkaline phosphatase (1 μg) (upper darkly stained band, marked by an arrowhead) overnight at 37°C; 1, molecular mass (in kDa) standards (from top to bottom): ovalbumin (43), α-chymotrypsinogen (25.7), β-lactoglobulin (18.4), cytochrome *c* (12.3). Amounts of tau employed for lanes 2–4 were identical (10 μg). Coomassie blue staining of CNBr peptides of tau is much weaker than the same amount of the native protein.

and human tau have been published [5,6]. They show strong homology to each other especially in the C-terminal region. Comparison of the two bovine sequences II and III with these published sequences showed two regions of striking homology, both of which coincided with the highly conserved C-terminal region. The derived sequence of peptide II could be shown to be identical in 17 of 23 positions when compared to regions 181–203 of mouse and 192–214 of human tau sequences. The derived sequence of peptide III could be shown to be identical in 17 of 22 positions to regions 319–340 and 330–351 of the published mouse and human tau sequences, respectively. The sequence of peptide I showed no region of homology with either predicted tau sequences nor homology to other published sequences in the available databanks. A comparison of the predicted sequences with those derived from this study is shown in fig.3. Subtracting these two sequences from the mixture shows that alanine or valine must precede proline in peptide I. Since valine is present at this position in peptide III, it was decided that alanine would be designated as preceding proline. In the case of peptides II and III, this type of analysis generates ambiguities at positions 2, 4 and 11 where specific assignments cannot be made to either polypeptide.

3.2. *Dephosphorylation of bovine tau CNBr digest*

Dephosphorylation of the CNBr digest of bovine tau changed the electrophoretic mobility of the three large peptides on SDS-PAGE (fig.1). The apparent molecular masses of these peptides changed from 21, 19 and 18 kDa to 19, 18 and 17

																Pro
(Met)	(Ala)	Pro	Gly	Leu	Lys	Glu	Ser	Pro	Leu	Gln	Ile	Gly
		1									10	

Ala	Ala	Pro	Gly	Leu	Lys
					17

Fig.2. Amino acid sequence of bovine tau peptide I. Sequence 1–17 obtained by automated Edman sequencing of a CNBr digest after one cycle of manual Edman degradation and subsequent blocking with OPA. CNBr digestion was performed on 100 μg purified tau and the sequence was recovered at the 700 pmol level. Residues in parentheses were deduced from mixed sequence information of the total tau CNBr digest (see fig.3).

				Ser		Gly						Thr
Bovine II	(Met)	Glu	Asp	Leu	Lys	Asn	Val	Gly	Gly	-	Ile	Gly
Mouse	Met	Pro	Asp	Leu	Lys	Asn	Val	Arg	Ser	Lys	Ile	Gly
	181									190		
Human	Met	Pro	Asp	Leu	Lys	Asn	Val	Lys	Ser	Lys	Ile	Gly
	192									201		
Bovine II	Ser	Thr	Glu	Asn	Leu	-	-	Gln	Pro	Gly	Gly	
Mouse	Ser	Thr	Glu	Asn	Leu	Lys	His	Gln	Pro	Gly	Gly	
								200			203	
Human	Ser	Thr	Glu	Asn	Leu	Lys	His	Gln	Pro	Gly	Gly	
								211			214	
				Ser		Gly						Thr
Bovine III	(Met)	Val	Asp	Lys	Lys	Gln	Leu	Ala	Thr	Leu	Ala	Asp
Mouse	Met	Val	Asp	Ser	Pro	Gln	Leu	Ala	Thr	Leu	Ala	Asp
	319											330
Human	Met	Val	Asp	Ser	Pro	Gln	Leu	Ala	Thr	Leu	Ala	Asp
	330											341
Bovine III	Glu	Val	-	-	Ser	Leu	Ala	-	Gln	Gly		
Mouse	Glu	Val	Ser	Ala	Ser	Leu	Ala	Lys	Gln	Gly		
										340		
Human	Glu	Val	Ser	Ala	Ser	Leu	Ala	Lys	Gln	Gly		
										351		

Fig.3. Alignment of bovine tau sequences II and III with the predicted mouse [5] and human [6] tau sequences. Sequences II and III were derived by subtracting peptide I from the mixed sequences obtained after CNBr digestion and generating a 'best-fit' alignment with the predicted mouse tau sequence. At positions 2, 4 and 11 of peptides II and III specific assignments could not be made due to lack of homology.

(plots not shown). However, the number of peptides did not change suggesting that bovine tau contained 3 large CNBr peptides at least one of which could be dephosphorylated. The murine tau sequence predicted from its cDNAs has only two large peptides of approx. 17 and 15 kDa [5].

4. DISCUSSION

The present study demonstrates (i) the structural similarities between two bovine tau peptides and the highly conserved C-terminal regions of both murine and human tau and (ii) identifies an additional peptide not present in either of the cDNA-predicted sequences of tau.

The amino acid composition of bovine tau obtained here is in close agreement with that of porcine brain tau reported in [2]. The failure of total bovine tau to reveal any amino acid sequence information by automated Edman degradation in this study suggests that tau is amino-terminally blocked. Successful isolation of large peptides from bovine tau by CNBr digestion in the present study revealed multiple sequences which could not be ordered with certainty because of the presence of approximately equimolar recoveries of the amino acids after each cleavage cycle. However, the identification of proline at the 2nd cleavage cycle provided an opportunity to block all amino-termini with OPA except that of the sequence beginning with proline. A similar approach has been used previously using OPA to block the non-proline amino-termini of a mixture of peptides from β-adrenergic receptor [10]. The subsequent tau peptide beginning with proline provided a clear 17-amino-acid sequence (peptide I). At position 11, two amino acids, proline and glycine, were observed suggesting that microheterogeneity exists at this position. Subtraction of the peptide I sequence from the mixed sequences obtained from the large CNBr fragments of tau provided a mixture of 2 sequences. A comparison of these remain-

ing mixed sequences with the published tau sequences showed that 2 bovine sequences (II and III) could be generated which possessed a high degree of homology to the murine and human sequences. Peptide I shows no homology with either predicted sequences.

The amino acid sequence data show differences between bovine and other mammalian tau polypeptides. These differences probably reflect both small species differences, i.e. of peptides II and III with mouse and human tau, and the presence of an additional part of the tau molecule not predicted in both cDNA-derived sequences. It is of particular interest to note that a portion of peptide II shows high homology with residues 187–203 of the mouse and 198–214 of the human sequences. This region is a component of a distinctive repeating sequence [5] which has been identified as the microtubule-binding domain of the tau proteins [11].

Tau is known to be a phosphoprotein, different degrees of phosphorylation being partly responsible for the heterogeneity on SDS-PAGE [3,4]. Dephosphorylation of the CNBr digest of bovine tau with alkaline phosphatase changed the electrophoretic mobility of at least one of three large peptides observed on SDS-PAGE, but the number of the peptide bands was unchanged. The presence of these three large CNBr peptides suggests that the molecular mass of bovine tau must be considerably greater than the predicted 35.7 kDa for mouse and human tau which should contain only two large methionine peptides from the cDNA sequences. The 3 peptides could readily be accommodated by the observed 55–68 kDa size of intact, denatured tau. This also suggests that the bovine tau protein sequences found in this study represent a different form of tau to that proposed previously [5,6]. On Western blots, antisera to peptide I label all bovine tau polypeptides recognized by other antibodies to bovine tau but only two of the four mouse tau molecular species (Iqbal et al., in preparation). The possibility of differential splicing of the mRNA might account for the differences in the amino acid sequences of tau polypeptides.

Acknowledgements: This work was supported in part by New York State Office of Mental Retardation and Developmental Disabilities National Institutes of Health Grants AG 05892, NS 18105 and AG/NS 04220, and a grant from the Alzheimer's Disease Research Program of the Amercian Health Assistance Foundation, Rockville, MD. We thank Dr L.I. Binder, University of Alabama, Birmingham, AL, for critical reading of the manuscript, Biomedical Photography Unit for the preparation of the figures, and Concetta Veneziano for typing the manuscript.

REFERENCES

[1] Cleveland, D.W., Hwo, S.-Y. and Kirschner, M.W. (1977) J. Mol. Biol. 116, 207–225.
[2] Cleveland, D.W., Hwo, S.-Y. and Kirschner, M.W. (1977) J. Mol. Biol. 116, 227–247.
[3] Baudier, J. and Cole, D.R. (1987) J. Biol. Chem. 262, 1757.
[4] Baudier, J., Lee, S.-H. and Cole, D.R. (1987) J. Biol. Chem. 262, 17584–17590.
[5] Lee, G., Cowan, N. and Kirschner, M. (1988) Science 239, 285–288.
[6] Goedert, M., Wischik, C.M., Crowther, R.A., Walker, J.E. and Klug, A. (1988) Proc. Natl. Acad. Sci. USA 85, 4051–4055.
[7] Grundke-Iqbal, I., Iqbal, K., Quinlan, M., Tung, Y.-C., Zaidi, M.S. and Wisniewski, H.M. (1986) J. Biol. Chem. 261, 6084–6089.
[8] Grundke-Iqbal, I., Vorbrodt, A.W., Iqbal, K., Tung, Y.-C., Wang, G.P. and Wisniewski, H.M. (1988) J. Mol. Brain Res. 4, 43–52.
[9] Laemmli, U.K. (1970) Nature 227, 680–685.
[10] Ramachandran, J., Rodriquez, H., Henzel, W. and Trubokawa, M. (1986) in: Methods of Protein Sequence Analysis (Walsh, K.A. ed.) pp.157–169, Humana Press, Clifton, NJ.
[11] Hiroyuki, A., Kawasaki, H., Murofushi, H., Kotani, S., Suzuki, K. and Sakai, H. (1988) J. Biol. Chem. 263, 7703–7707.