Differential distribution in white and grey matter of tau phosphoisoforms containing four tubulin-binding motifs

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The microtubule-associated protein tau has been isolated and purified from both white and grey brain matter. Tau isoforms were fractionated, based on their different phosphate contents, by iron-chelating affinity chromatography. Differences were observed in the proportions of phosphorylated isoforms of tau protein (containing four tubulin-binding motifs) present in white

and grey matter. In white matter, isoforms containing four tubulin-binding motifs are mainly present in a phosphorylated form. Thus there appears to be a correlation between the modification, by phosphorylation, of some sites in the tau molecule and the subcellular localization (axonal or somatodendritic compartments) of the modified isoforms.

INTRODUCTION

Microtubule-associated tau is present in the brain in different isoforms (Buttler and Shelanski, 1986). This diversity is the consequence of alternative splicing of a common RNA precursor molecule (Lee et al., 1988; Himmler, 1989; Goedert et al., 1988; Kosik et al., 1989; Kanai et al., 1989) and of differing degrees of phosphorylation at different residues (Lindwall and Cole, 1984; Baudier et al., 1987; Papasozomenos and Binder, 1987; Correias et al., 1992). These isoforms, which can be identified on the basis of their electrophoretic mobility, show different tubulin-binding characteristics and self-aggregate with variable efficiency (García de Ancos et al., 1993). Immunofluorescence studies (Papasozomenos and Binder, 1987) have shown that tau phosphorylated at a serine/proline-rich region (Grundke-Iqbal et al., 1986; Ledesma et al., 1992) is mainly present in the somatodendritic compartment of the neuron, whereas its unphosphorylated counterpart is located at the neural axon (Papasozomenos and Binder, 1987).

For biochemical analyses, it has been proposed (Vallee et al., 1986) that an axonal-rich region should correspond to brain white matter and that somatodendritic regions should correspond to brain grey matter. Thus we have isolated white and grey matter for biochemical analyses of tau protein. Tau isoforms present in both fractions have been analysed, mainly based on their phosphorylation characteristics, and some differences have been found in the phosphorylation level of those tau isoforms which contain four tubulin-binding motifs and are present in white or grey matter.

MATERIALS AND METHODS

Protein preparation

White and grey matter were separated from bovine brain cortex as previously described by Vallee (1982). In some cases, the corpus callosum was used as a source of white matter; the results observed were similar to those obtained using white matter isolated after removal of grey matter cortex. Tau protein was isolated by the procedure of Lindwall and Cole (1984), based on the solubility of the protein in 2.5% perchloric acid (method I). Tau was also isolated from microtubule protein obtained by

polymerization/depolymerization cycles (Shelanski et al., 1973) (method II).

Protein was characterized by PAGE and was stained or blotted on to nitrocellulose to test its interaction with tau antibodies, as previously indicated (García de Ancos et al., 1993).

Densitometric analyses, on photographed gels, were carried out by using a Chromoscan3 apparatus (Joyce Loeb).

Phosphatase treatment of tau

Tau was incubated with alkaline phosphatase (Boehringer Mannheim; cat. no. 1097075; high concentration) at 0.03 i.u./mg of tau in dephosphorylation buffer (Boehringer) containing 1 mM phenylmethanesulphonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 10 µg/ml pepstatin for 16 h at 37 °C. The reaction was stopped by placing the sample on ice and bringing to 2.5% perchloric acid. Samples were then centrifuged at 10000 g for 5 min and the supernatant was dialysed by ultrafiltration (Centricon 30000; Amicon) in buffer A (0.1 M Mes, 2 mM EGTA, 0.5 mM MgCl₂, pH 6.4).

Iron-chelated affinity chromatography (i.c.a.c.)

The procedure for the addition of FeCl₃ to the chelating Sepharose beads, and the fractionation of tau isoforms on the preformed column, have been described previously (García de Ancos et al., 1993). Briefly, columns were prepared using chelating Sepharose Fast Flow (Pharmacia Fine Chemicals, Uppsala, Sweden) as described by Muszynska et al. (1986). The gel bed was washed with 4 vol. of distilled water, loaded with 4 vol. of 50 mM FeCl₃ and finally washed again with distilled water. The column was equilibrated in binding buffer [0.1 M Mes, 1 M NaCl and 1 mM dithiothreitol (DTT), pH 5.7] prior to sample loading.

Tau protein purified by either method I or method II was loaded on to the i.c.a.c. column after exchanging binding buffer for buffer A in a Sephadex G-50 column previously equilibrated in binding buffer. Once tau protein was loaded on to the i.c.a.c. column, it was eluted with a stepwise pH gradient composed of the following solutions: 0.1 M Mops, 1 M NaCl and 1 mM DTT at pH 7.0, 7.1, 7.2, 7.4 and 7.6; and 0.1 M Tris/HCl, 1 M NaCl

Figure 1 Tau isoforms present in white and grey matter from brain

Tau protein was purified by the perchloric acid method, as indicated in the Materials and methods section, from the grey (G) or white (W) matter from bovine brain, and the different tau isoforms present in each fraction were characterized by gel electrophoresis (a). The bands correspond to: 1, the phosphorylated form containing four tubulin-binding motifs and an extra region at the N-terminal end of the molecule; 2, as for 1, but unphosphorylated; 3, as for 1, but with three tubulin-binding motifs; 4, the unphosphorylated counterpart of 3; 5, as for 1, but without the extra region at the N-terminus; 6, the unphosphorylated counterpart of 5; 7, the phosphorylated form containing three tubulin-binding motifs and without the extra region at the N-terminus site; 8, the unphosphorylated counterpart of 7. The proposed structures of the different isoforms, based on previous analyses (Correas et al., 1992) are also indicated (b). (c) Composition of tau isoforms from white and grey matter in the absence (—) or after (+) phosphatase treatment. Large boxes denote the extra region close to the N-terminal end of tau protein; small boxes denote the tubulin-binding motifs in tau.

and 1 mM DTT at pH 7.8, 8.0 and 8.5. Fractionation was monitored by PAGE.

RESULTS

Tau isoforms from white and grey matter

Tau isolated from bovine brain grey matter contained the four

main tau isoforms previously described for the whole brain (García de Ancos et al., 1993) (Figure 1a). The different phosphorylation states of these tau forms generated eight bands in PAGE, as described in Figure 1.

However, with tau isolated from white matter, only small amounts, if any, of tau isoforms 2 and 6 were observed (Figure 1a). These results have been confirmed by densitometric analysis. Figure 2 shows the presence of tau isoforms 1, 3, 4, 5, 7 and 8 in both white and grey matter in similar proportions, but tau isoforms 2 and 6 were present in greater amounts in grey compared with white matter. These isoforms correspond to the unphosphorylated forms of tau containing four tubulin-binding motifs. Treatment with phosphatase, which converts phosphorylated into unphosphorylated forms, resulted in the conversion of isoforms 1 and 5 into isoforms 2 and 6, as confirmed by gel electrophoresis (Figure 1c).

As previously indicated (García de Ancos et al., 1993), the dephosphorylated isoforms (numbers 2, 4, 6 and 8) bind with lower affinity to microtubules than do the phosphorylated forms (1, 3, 5 and 7). Thus, as shown in Figure 3, no difference in the isoform pattern was observed for microtubule-bound tau between white and grey matter.

Phosphorylation of tau isoforms

Papasozomenos and Binder (1987) observed differences in the degree of phosphorylation, at a single region of the protein, of tau present in axons compared with that present in the somato-dendritic compartment. This phosphorylation can be identified by using monoclonal antibody, Tau-1, which recognizes the unmodified epitope (Papasozomenos and Binder, 1987) located in a proline-rich region present in every tau isoform (Kosik et al., 1988; Ledesma et al., 1992). The reaction of Tau-1 antibody with both microtubule-bound tau and total tau has been studied (Figure 4). Since it has been indicated that microtubule-bound

Figure 2 Densitometric profile of tau from white matter and from grey matter

Tau protein from brain grey (a) or white (b) matter was fractionated by gel electrophoresis and subjected to densitometric analyses. The densitometric profiles are shown. The isoforms with lower electrophoretic mobility are towards the left of the Figure.

Figure 3 Microtubule-associated tau from white and grey matter

Microtubules from white (W) and grey (G) matter from bovine brain were isolated by polymerization/depolymerization cycles and tau protein was isolated from them. The presence of isoforms 1, 3, 5 and 7 was determined by running a sample containing whole brain tau protein in parallel.

Figure 4 Immunoreactivity of Tau-1 antibody with brain tau isoforms

Total or microtubule-bound (cycled) tau was fractionated by gel electrophoresis and stained with Coomassie Blue (CB), or transferred to nitrocellulose sheets to study its interaction with Tau-1 antibody by Western blot analysis (WB). The reaction of the antibody with possible tau dimers (García de Ancos et al., 1993) is indicated. The electrophoretic mobilities of reference markers and that of microtubule-associated protein 2 (MAP-2) protein are also shown.

tau is phosphorylated at some unknown residues, and it is known that Tau-1 antibody recognizes an unphosphorylated epitope, it can be suggested that the phosphorylation of microtubule-bound tau occurs in a region different from that recognized by Tau-1. When the reaction of the Tau-1 antibody was tested by Western blotting using the same amount of tau protein from white or grey matter, a greater reaction (2.8 times) was observed with tau from white matter compared with that from grey matter (Figure 5). This result is compatible with the previous observation (Papazosomenos and Binder, 1987) indicating that somatodendritic (grey) tau isoforms are more highly phosphorylated at the region recognized by Tau-1 antibody than are axonal (white) tau isoforms.

As indicated in Figure 1, other modes of tau phosphorylation have been described, and it would be of interest to determine the

Figure 5 Immunoreactivity of Tau-1 antibody with tau protein from white and grey matter

Tau protein from brain white (W) or grey (G) matter was fractionated by gel electrophoresis and preprocessed as indicated in the legend to Figure 4. CB, Coomassie Blue staining; WB, Western blotting.

Figure 6 Fractionation of tau protein by iron-chelating Sepharose

Purified tau from whole (total) bovine brain (a), microtubule-bound tau (b), and tau from grey (c) and from white (d) matter was chromatographed as described in the Materials and methods section on an iron-chelating Sepharose column and eluted with a stepwise pH gradient. The proportion of protein eluted at each step with respect to total protein is shown.

different phosphorylated forms of tau isolated from white or grey matter.

Fractionation of tau protein by I.c.a.c.

Tau was fractionated on iron-chelating Sepharose in order to analyse the pattern of phosphorylated isoforms. Tau was isolated from four different sources: total brain, microtubules isolated from total brain, white matter and grey matter. The chromatography pattern of the first two samples has been previously described (García de Ancos et al., 1993), but it is included again for comparison with the remaining samples.

Figure 6 shows the elution profiles of the samples. Tau isolated from grey matter behaves essentially like tau from total brain. The pattern for tau from white matter shows some similarities to that of microtubule-bound tau, except that the peak at pH 8.5 is greater for tau from white matter than in microtubule-bound tau.

Figure 7 Characterization of the protein eluted from the iron-chelating Sepharose column

Tau protein from grey (a) or white (b) matter, chromatographed as indicated in Figure 6, was characterized by gel electrophoresis. The pH used to elute the protein and the electrophoretic mobility for forms 2, 4, 6 and 8 are indicated.

Electrophoretic analysis of the fractions obtained in the i.c.a.c. separations of grey and white matter samples is shown in Figure 7. With regard to the grey matter sample, the electrophoretic pattern of the i.c.a.c. fractions shows a very similar pattern to that obtained with total brain tau (García de Ancos et al., 1993). Isoforms 2, 4, 6 and 8, showing a faster electrophoretic mobility, elute first at low pH; these are the unphosphorylated tau isoforms. Increasing the pH induces the elution of isoforms 1, 3, 5 and 7 which have a slower electrophoretic mobility and a higher degree of phosphorylation.

With tau from the white matter sample, a very small amount of isoforms 2 and 6 was found (pH 7.0 and 7.1). By densitometric analysis it was found that 78% of total tau isoforms in white matter correspond to isoforms 4 and 8, whereas for grey matter the proportions of isoforms 2 and 6 were 54% and 46% respectively. This agrees with the results shown in Figure 1, and indicates that tau isoforms, containing four tubulin-binding motifs, are mainly present in white matter in a phosphorylated form.

DISCUSSION

Tau is a phosphoprotein that contains an average of 2–3 mol of phosphate per tau molecule (Khatoon et al., 1992). In a previous study it was indicated that phosphorylation of tau protein at a site in the middle portion of the molecule, in a proline-rich region, correlates with the location of these phosphorylated forms in the somatodendritic compartment (Papazomenos and Binder, 1987). By using an antibody, Tau-1, which specifically binds to the unphosphorylated proline region, Papazomenos and Binder (1987) showed that a more intense reaction occurred when the antibody was mixed with axonal tau than when it was mixed with somatodendritic tau.

This result has been confirmed in the present study, in which brain tissue has been fractionated into white and grey matter by using the approach of Vallee (1982), in order to determine the similarity or difference in the tau isoforms present in these fractions.

We have shown that phosphorylation at other sites of tau molecules correlates with their specific localization in the axon (white matter) or the somatodendritic compartment (grey matter). The occurrence of phosphorylated tau isoforms, identified by their electrophoretic mobility (García de Ancos et al., 1993), is different (for those isoforms containing four tubulin-binding motifs) in the axonal fraction compared with in the whole neuron.

In white matter a low content, if any, of tau isoforms 2 and 6 is present. The region that is not phosphorylated in these forms is not known, but the fact that only those tau isoforms containing four tubulin-binding motifs showed differences in their level of phosphorylation suggests that the modified region is specifically present in those isoforms. Analysis of the primary sequence of tau isoforms containing three or four motifs shows that a region that is specifically present in these isoforms is that containing the sequence INKKLDLSN. This sequence is only present in tau and it is not found in other cytoskeletal proteins such as neurofilaments, or microtubule-associated protein families 1 and 2 (see Garner et al., 1988a,b). On the other hand the sequence IINKK is similar to an actin-binding motif AIKKK (Kabsch and Vandekerckhove, 1992). By taking into account that tau is an actin-binding protein (Correas et al., 1990), a hypothesis could thus be presented in which tau containing four tubulin-binding motifs may bind to actin to a higher degree than do the other isoforms. It should also be noted that the tau isoforms that have the greatest capacity for self-aggregation are those which are preferentially transported through the axon. This may also suggest a possible correlation between the presence of tau aggregates and their axonal transport. Further work needs to be done to analyse this possible correlation.

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