Immunological characterization of microtubule-associated proteins specific for the immature brain

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Immunoblotting analysis was used to detect the microtubule-associated proteins present at different stages of rat brain development. Polyclonal antibodies were raised against the two main adult brain microtubule-associated proteins: MAP-2 (300 kDa) and TAU (60–70 kDa). Whatever the stage of development, anti-MAP-2 serum detected high molecular mass proteins and at immature stages a protein of 62 kDa. This protein which has previously been referred to as 'young TAU slow' is, therefore, immunologically related to MAP-2. The anti-TAU serum (but not the anti-MAP-2 serum) detected at immature stages of development a 48 kDa protein which also disappears at adulthood. This 48 kDa entity which has been referred to as 'young TAU fast' is progressively replaced by the closely spaced bands (60–70 kDa) of adult TAU proteins. The 62 and 48 kDa proteins appear therefore to be immunologically distinct and represent two microtubule-associated proteins specific to the immature brain.

Microtubule *Microtubule-associated protein* *Brain* *Development*

1. INTRODUCTION

Brain microtubules contain, at an adult stage, several microtubule-associated proteins (MAPs): a group of >200 kDa [1,2] and a group (TAU) of 52–68 kDa [3.] One of the high molecular mass entities, MAP-2 (~300 kDa), has been resolved into 2 discrete peaks [1,4] whereas at early stages of brain development MAP-2 is present as a single peak [4]. Similarly, at an adult stage of development, the TAU fraction is composed of several entities of 58–65 kDa [3] whereas at immature stages only 2 peaks are observed [5] which differ from adult TAU in several respects [5–8]. We show here that the proteins detected at fetal, young post-natal stages of development and at adulthood react differently when tested with polyclonal antibodies raised against MAP-2 and TAU, respectively. At least 2 transitions in MAP composition occur during brain development and some MAPs are highly specific markers of the immature brain.

Abbreviations: MAPs, microtubule-associated proteins; MAP-1(2), microtubule-associated protein 1(2); TAU, another microtubule-associated protein(s); Mes, 2-(*N*-morpholino)ethanesulfonic acid

2. MATERIALS AND METHODS

2.1. *Products*

GTP and Mes were from Boehringer. EDTA was from Prolabo. EGTA, leupeptin, phenylmethylsulfonyl fluoride, trypsin inhibitor, benzamidine, Tween 20 and bovine serum albumin (fraction V) were purchased from Sigma. Acrylamide, bisacrylamide, SDS and nitrocellulose sheets were from Biorad. Anti-rabbit (Ig ^{125}I)-labelled whole antibody from donkey (spec. act. 5–20 Ci/g) was from Amersham.

2.2. *Preparation of brain extracts*

Total and heat-stable supernatants were prepared from rat brain. Brains were homogenized in 1 ml/g tissue of buffer A: 0.1 M Mes (pH 6.4) containing 0.5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM EDTA, 1 mM GTP, 1 mM 2-mercaptoethanol, 10 mM benzamidine, 5 μM leupeptin, 25 mg/l trypsin inhibitor and 1.5 ml/l phenylmethylsulfonyl fluoride (50

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mg/ml DMSO). The homogenate was incubated at 4°C for 30 min and then centrifuged at 100 000 × *g* for 60 min. The supernatant was incubated at 4°C for 60 min in the presence of 0.75 M NaCl and 2 mM dithiothreitol. The preparation was immersed in boiling water for 5 min. After centrifugation (20 000 × *g*, 30 min), the supernatant was dialyzed overnight at 4°C against buffer A.

2.3. *Preparation of the antigens and antisera*

Rat brain microtubules were purified by the temperature-dependent assembly-disassembly procedure [9]. MAPs were prepared as in [10]. TAU was obtained by electrophoresis of the MAPs on SDS-polyacrylamide slab gels. The same technique was applied to obtain MAP-2 prepared from calf brain. The bands containing TAU or MAP-2 were excised from the gel, minced and homogenized with a Potter Dounce in the presence of 2 ml physiological saline.

Antiserum to TAU or MAP-2 was produced by injecting male 'Fauve de Bourgogne' rabbits subcutaneously with 75 μg of the corresponding antigen in complete Freund's adjuvant. The rabbits were boosted 3 and 5 weeks after the first injection. Blood was collected once a week from the third week. Preimmune sera were obtained from the same animals prior to immunization.

2.4. *Qualitative and quantitative gel electrophoresis*

Proteins were analyzed on a linear 4–15% polyacrylamide slab gel gradient in the presence of 0.2% SDS as described in [6] with some modifications. Electrophoresis was carried out overnight at a constant current of 13 mA per gel. A better separation of the high molecular mass proteins was obtained by a modification of the method of Laemmli [11], the separation gel containing 0.2 M Tris-HCl (pH 8.8) and 0.1% SDS.

2.5. *Electrophoretic transfer and immunological detection of proteins*

The procedure used for transfer of proteins from polyacrylamide gels was that of Towbin et al. [12] with several modifications. The proteins were transferred to nitrocellulose sheets at room temperature in the Biorad Trans Blot Cell at a constant current of 0.25 A for 3 h. The nitrocellulose blots were cut into several vertical strips and incubated for 2 h at 50°C in 10 mM Tris-HCl (pH 7.4), 0.9% NaCl containing 3% bovine serum albumin (buffer A). After washing with 10 mM Tris-HCl (pH 7.4), 0.9% NaCl and 0.05% Tween 20 (buffer B), the blot was incubated at room temperature for 60 min with the respective antiserum diluted as required in buffer A. The nitrocellulose blot was then washed with agitation in buffer B for 90 min, changing the buffer (20 ml/lane) every 15 min. The blot was incubated at room temperature overnight with ^{125}I-labelled donkey anti-rabbit Ig diluted to 10^6 cpm/ml buffer A containing 10% new born calf serum and 0.01% NaN_3. The blot was washed in buffer B as described above and dried between 2 sheets of Whatman 3 mM paper. The wet blot was then autoradiographed on Kodak X-AR5 X-ray films. In all cases no significant band was detected when the nitrocellulose blot was incubated with the preimmune serum. Following electrophoretic transfer the gels were stained with Coomassie blue to test the efficiency of the transfer.

3. RESULTS AND DISCUSSION

Polyclonal antibodies were raised against adult MAP-2 and adult TAU. The antisera obtained after 2–3 weeks immunization reacted essentially with the parent antigen (fig.1). In contrast, the antisera obtained after longer periods of immunization against TAU protein revealed not only the injected antigen but also other MAPs (see figs.2a and 3a). This suggests that several MAPs share antigenic determinants, probably those related to their common property of co-assembling with microtubules, to induce tubulin assembly (see [13]) and/or to interact with the calmodulin-Ca^{2+} complex [14,15]. For instance, structural homology of MAP-1 and MAP-2 has been demonstrated by peptide mapping analysis and immunoreactivity [16]. Fig.1 also shows that the anti-TAU serum revealed 3 bands whereas 4 were seen after Coomassie blue staining. The reasons for this difference are unknown but it is possible that some diffusion of the 2 very closely spaced heaviest TAU bands occurs during the electrophoretic transfer.

These antisera were used to characterize, by polyacrylamide gel electrophoresis and immunoblotting, the MAPs present at various stages

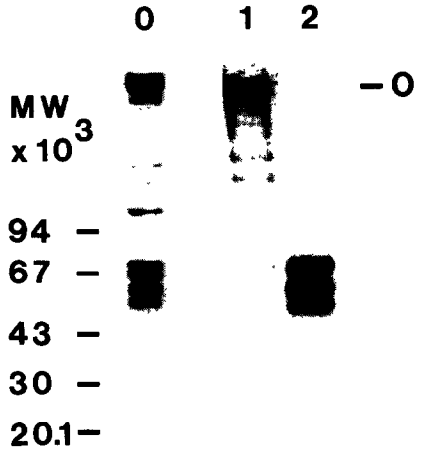

Fig.1. Immunoblotting of adult rat brain MAPs. Heat-treated adult brain supernatants were analyzed by polyacrylamide gel electrophoresis and immunoblotting. Immunoblotting was performed with the anti-MAP-2 (lane 1) and anti-TAU (lane 2) sera. Lane 0: Coomassie blue staining of total adult brain heat-stable MAPs. The positions of the molecular mass markers are those of the immunoblotting experiments.

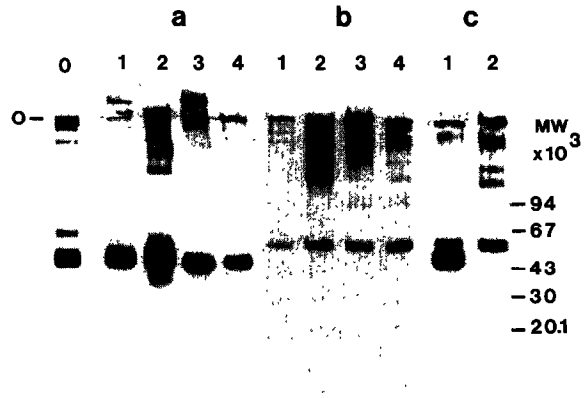

Fig.2. Immunoblotting of rat brain fetal and 3-day old MAPs. (a,b) Immunoblotting results obtained with different 3-day-old brain preparations. Lanes 1, total $10^5 \times g$ supernatant; lanes 2, heat-treated $10^5 \times g$ supernatant; lanes 3, microtubules; lanes 4, heat-stable MAPs. Immunoblotting was performed with the anti-TAU (a, lanes 1–4) and anti-MAP-2 (b, lanes 1–4) sera. Heat-stable supernatants prepared from fetal brain (17 days gestation) were also analyzed: immunoblotting was performed with the anti-TAU (c, lane 1) and anti-MAP-2 (c, lane 2) sera. The same amount of thermostable proteins (5 μg) was analyzed in (a) lane 2, (b) lane 2 and (c) lanes 1 and 2. Lane 0: Coomassie blue staining of total 3-day-old brain heat-stable MAPs. The positions of the molecular mass markers are those of the immunoblotting experiments.

of brain development. Preimmune sera were also used as controls and gave negative results. Several brain preparations were analyzed: (i) total $10^5 \times g$ supernatants from rat brain homogenates; (ii) heat-treated $10^5 \times g$ supernatants prepared as described to obtain heat-stable MAPs [10] (heat denaturation results in the elimination of 90% of the brain soluble proteins with only some of the MAPs being lost during this treatment); (iii) microtubule obtained as in [9]; (iv) total heat-stable MAPs prepared as described [10].

Whatever the 3-day-old preparation used the anti-TAU serum revealed a major component of 48 kDa (fig.2a, lanes 1–4). When using the anti-MAP-2 serum very little immunoreactivity was present at the level of this 48 kDa entity; in contrast, the anti-MAP-2 serum revealed a narrow band of 62 kDa (fig.2b, lanes 1–4), which was only slightly detected by the anti-TAU serum (fig.2a, lanes 1–4). Thus, whatever the brain preparation used the 48 and 62 kDa components, which have been referred to as young TAU fast and young TAU slow [6] on the basis of their detection by Coomassie blue staining (fig.2, lane 0), appear to be immunologically distinct since they are preferentially detected by only 1 of the 2 antisera. The 62 kDa entity is antigenically more closely related to MAP-2 than to TAU although it has an apparent molecular mass similar to that of one of the slower bands of the adult TAU complex. In contrast, the 2 antisera both detected high molecular mass components (fig.2a,b). However, depending on the type of preparation and on the antiserum used, the profiles of these high molecular mass components markedly differ both qualitatively and quantitatively. Some bands are absent in the heat-treated preparations (fig.2a,b, lanes 2,4) suggesting that they correspond to heat-labile MAPs. For instance, with the $10^5 \times g$ supernatant (fig.2a, lane 1) and microtubule preparation (fig.2a, lane 3), a very heavy component remaining in the stacking gel was detected by the anti-TAU serum. This unidentified entity is thermolabile

since it is absent in the heat-treated preparations (fig.2a, lanes 2,4).

Two bands of 62 and 48 kDa were also found at a fetal stage (17 days gestation). However, the 62 kDa band reacted at this stage with both the anti-MAP-2 and anti-TAU sera (fig.2c) suggesting that the fetal MAP which migrates in this position is different from that present at day 3 after birth. In contrast, the 48 kDa component reacted only with the anti-TAU serum both at fetal and early post-natal stages. Fig.2 also shows that the concentration of the 62 kDa component is higher at the fetal stage than 3 days after birth.

Previous results [6] showed that the 2 entities detected in the TAU region at early stages of brain development progressively disappear from birth to adulthood. Fig.3 shows that the immunoblotting technique also revealed marked changes in the composition of MAPs during brain development. Heat-stable supernatants prepared at day 3,6,15 and 35 after birth were analyzed by polyacrylamide gel electrophoresis using slightly modified conditions which allowed a better resolution of the high molecular mass components. Under these conditions anti-TAU and anti-MAP-2 sera revealed at least 8 components of >200 kDa. In the 50–65 kDa region the band revealed by the anti-TAU serum at young stages of development was resolved into at least 2 peaks with none of them being stained by the anti-MAP-2 serum (fig.3a). This heterogeneous peak is still present at day 6 and 15 but clearly in lower proportion that at day 3. At day 35, 4–5 closely spaced bands characteristic of adult TAU were clearly revealed by the anti-TAU serum but not by the anti-MAP-2 one. In contrast, the anti-MAP-2 serum revealed the 62 kDa entity in the immature brain preparation but only small amounts of this component at adulthood (fig.3b).

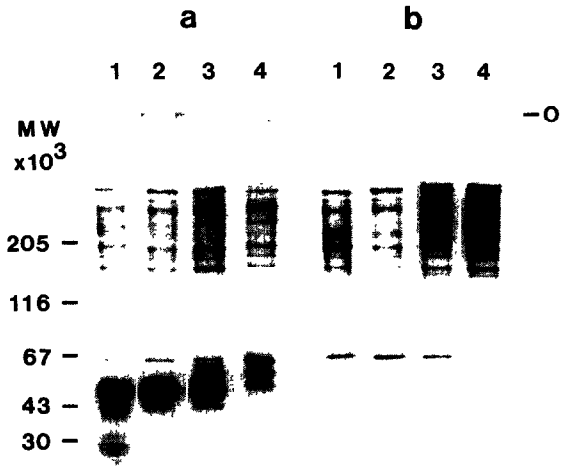

Fig.3. Changes in MAP composition during rat brain development analyzed by immunoblotting. Heat-treated supernatants were prepared from 3,6,15 and 35-day-old brains and analyzed by polyacrylamide gel electrophoresis according to Laemmli [11] except that the separation buffer contained 0.2 M Tris-HCl (pH 8.8) and 0.1% SDS. Immunoblotting was performed with the anti-TAU (fig.3a) and anti-MAP-2 (b) sera. Lane 1, 3 days; lane 2, 6 days; lane 3, 15 days; lane 4, 35 days. The same amount of thermostable proteins (10 μg) was analyzed in each lane.

In conclusion: (i) at the fetal stage 2 MAPs are detected by Western blotting. One of them of 62 kDa is revealed by both the anti-MAP-2 and anti-TAU sera whereas the other, of 48 kDa, is only detected by the anti-TAU serum. At day 3 and 6 after birth the same bands are present but a marked change in immunoreactivity occurs since the 62 kDa band is poorly detected by the anti-TAU serum. This suggests that the 62 kDa bands present at the fetal stage and soon after birth are immunologically different proteins. Two transitions in the composition of the 50–65 kDa MAPs therefore seem to occur during brain development i.e. (a) from the fetal to the new born stage (b) before adulthood. (ii) Both the 62 and 48 kDa MAPs present in the immature brain are progressively replaced by the adult TAU entities; in total brain extracts very small amounts of the 62 kDa component are present at adulthood. (iii) At day 15 after birth the brain contains a mixture of the 'young' and 'adult' MAPs of 50–70 kDa. This timing in the post-natal transition in MAP composition correlates well with the so called 'critical period' of brain development, i.e. with the period at which intensive neurite outgrowth occurs. The functional significance of the heterogeneity of MAPs seen at each stage of brain development and of the changes in MAP composition occurring during the construction of the neuronal network remains to be determined. Recent unpublished results obtained with primary cultures of astrocytes and neurons suggest that such a heterogeneity is related, at least partially, to the presence of different cell types, neuronal or glial, and to the

changes in cell populations occurring during brain maturation. Whatever the validity of these assumptions, the minimal conclusion of this work is that some immunologically different MAPs are specific to the immature brain.

REFERENCES

[1] Murphy, D.B., Vallee, R.B. and Borisy, G.G. (1977) Biochemistry 16, 2598-2605.
[2] Sloboda, R.D., Rudolph, S.A., Rosenbaum, J.L. and Greengard, P. (1975) Proc. Natl. Acad. Sci. USA 72, 177-181.
[3] Cleveland, S.W., Hwo, H.Y. and Hirschner, M.W. (1977) J. Mol. Biol. 116, 207-225.
[4] Binder, L.I., Frankfurter, A., Kim, H., Caceres, A., Payne, M.R. and Rebhun, L.I. (1984) Proc. Natl. Acad. Sci. USA 81, 5613-5617.
[5] Mareck, A., Fellous, A., Francon, J. and Nunez, J. (1980) Nature 284, 353-355.
[6] Francon, J., Lennon, A.M., Fellous, A., Mareck, A., Pierre, M. and Nunez, J. (1982) Eur. J. Biochem. 129, 465-471.
[7] Ginzburg, I., Scherson, T., Giveon, D., Behar, L. and Littauer, U.Z. (1982) Proc. Natl. Acad. Sci. USA 79, 4892-4896.
[8] Drubin, D.G., Caput, D. and Kirschner, M.W. (1984) J. Cell Biol. 98, 1090-1097.
[9] Shelanski, M.L., Gaskin, F. and Cantor, R.C. (1973) Proc. Natl. Acad. Sci. USA 70, 765-768.
[10] Fellous, A., Francon, J., Lennon, A.M. and Nunez, J. (1977) J. Biochem. 78, 167-174.
[11] Laemmli, U.K. (1970) Nature 227, 680-685.
[12] Towbin, H., Staemelin, T. and Gordon, J.C. (1979) Proc. Natl. Acad. Sci. USA 76, 4350-4354.
[13] Dustin, P. (1984) in: Microtubules 2nd edn, Springer, Berlin.
[14] Lee, Y.C. and Wolff, J. (1984) J. Biol. Chem. 259, 1226-1230.
[15] Erneux, C., Passareiro, H. and Nunez, J. (1984) FEBS Lett. 172, 315-320.
[16] Herrmann, H., Pytela, R., Dalton, J.M. and Wiche, G. (1984) J. Biol. Chem. 259, 612-617.