The relationship of the rat brain 68kDa microtubule-associated protein with synaptosomal plasma membranes and with the *Drosophila* 70kDa heat-shock protein

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A protein of molecular mass 68kDa and pI5.6 is a major translation product of rat brain mRNA [Hall, Mahadevan, Whatley, Biswas & Lim (1984) *Biochem. J.* **219**, 751–761]. In the rat brain this protein was associated with microtubule preparations and was present together with tubulin as a component of the synaptosomal plasma membranes, synaptic vesicles and post-synaptic structures. The brain mRNA for this protein was found to hybridize specifically to the *Drosophila* gene for the 70kDa heat-shock protein, thus enabling its rapid isolation.

The brain is the most transcriptionally diverse of organs (reviewed by Kaplan, 1982). This is reflected in the composition of its mRNA, which codes for ubiquitous cytoskeletal proteins such as tubulin and actin (Schmitt *et al.*, 1977), as well as for brain-specific proteins including myelin basic protein (Hall *et al.*, 1982), neuron-specific enolase, the brain isoform of creatine kinase and the neuron-specific protein 14-3-3 (Hall *et al.*, 1984). In the rat brain, the mRNA for the cytoskeletal proteins is particularly abundant, as are the proteins themselves (Schmitt *et al.*, 1977). The mRNA for another cytoskeletal protein, the 68kDa microtubule-associated-protein (68K MAP) (Strocchi *et al.*, 1981), is present in lower concentrations (Hall *et al.*, 1984). In human foetal brains we found that the mRNA for the 68K MAP is one of the few species which are specifically increased as a result of trisomy-21, i.e. Down's syndrome; whether this is directly related to a gene-dosage effect is currently unresolved (Whatley *et al.*, 1984).

We have employed two-dimensional-gel analyses as a first step in the identification of brain proteins, followed by peptide analysis to confirm the preliminary identification; this has proved invaluable in characterizing a number of specific proteins and translational products of brain mRNA, including the 68K MAP (Lim *et al.*, 1983; Hall *et al.*, 1984). An examination of the migration

characteristics on two-dimensional gels of a wide range of proteins revealed surprisingly that the 68K MAP shared the same co-ordinates as the 70kDa heat-shock protein (HSP 70) (see, e.g., Bensaude *et al.*, 1983). The heat-shock proteins are a family of proteins whose synthesis is induced on exposure to elevated temperatures (heat shock) as well as to a variety of metabolic stresses (Ashburner & Bonner, 1979; Schlesinger *et al.*, 1982). The heat-shock response was originally observed in *Drosophila* and subsequently in other species, although the exact composition of the induced proteins varies according to the cell type. Some of the proteins are constitutively synthesized in mammalian cells, particularly the highly conserved HSP 70 (Bensaude *et al.*, 1983). Their functions remain obscure, although it has been speculated that they have a cytoskeletal role (Tanguay, 1983).

We now show that the 68K MAP is a structural component of synapses and its mRNA is related to the *Drosophila* HSP 70 gene (Torok & Karch, 1980). Our studies point to the potential value of the readily available *Drosophila* HSP 70 gene in investigations on the genetic expression of the brain 68K MAP.

Materials and methods

Materials

The sources of all the materials used in this paper are given in our previous papers (Hall *et al.*, 1984; Whatley *et al.*, 1984), unless stated other-

Abbreviations used: SPM, synaptosomal plasma membranes; 68K MAP, 68kDa microtubule-associated protein; HSP 70, 70kDa heat-shock protein.

wise. Restriction enzymes were from Amersham International, Amersham, Bucks., U.K.

Preparation of fractions

Forebrains were obtained from 40–50-day-old Wistar rats bred in our laboratory. Synaptosomes and SPM were prepared as previously described (Lim *et al.*, 1983), essentially by the method of Whittaker & Barker (1972), which was also used to derive synaptic vesicles. Post-synaptic densities were prepared from the SPM by using the detergent method of Kelly & Cotman (1978). A preparation of microtubules was obtained by two cycles of polymerization, and the microtubule-associated proteins were isolated by phosphocellulose chromatography as previously reported (Whatley *et al.*, 1984) by the method of Fellous *et al.* (1977).

Hybrid selection of rat brain 68 K MAP mRNA by *Drosophila* HSP 70 gene

The pML plasmid (a pBR322 derivative) containing the *Drosophila* HSP 70 gene (sequence 56H8; see Torok & Karch, 1980) in *Escherichia coli* HB 101 (kindly provided by Dr. Hugh Pelham, M.R.C., Cambridge; see Pelham, 1982) was amplified in the presence of chloramphenicol. Plasmid from a cleared lysate (Maniatis *et al.*, 1982) was extracted with phenol, and precipitated with ethanol and also poly(ethylene glycol) by the method of Lis (1980). RNA was removed by consecutive treatment with 2.5 M-LiCl and ribonuclease. The purified DNA was cut with the restriction endonuclease *Xba*I, which excised a DNA fragment containing the coding sequence of the HSP 70 gene (Torok & Karch, 1980). Denatured DNA was bound to nitrocellulose filters as described by Maniatis *et al.* (1982). For control experiments purified pBR322 DNA was cut with endonuclease *Eco*RI, denatured and bound to nitrocellulose.

The brain mRNA was polyadenylated RNA (Lim & Canellakis, 1970) isolated from free polyribosomes of rat brain as previously described (Hall *et al.*, 1984). This mRNA was subjected to hybrid-selection procedures with the DNA-cellulose filters essentially by the procedure of Ricciardi *et al.* (1979), except that hybridization was for 16 h at 45°C and filters were washed extensively with $0.1 \times$ SSC buffer [0.015 M-sodium citrate (pH 7)/ 0.15 M-NaCl] at 60°C. Hybridized and non-hybridized mRNA were re-purified by phenol extraction.

The mRNA was translated in a reticulocyte-lysate system with [35 S]methionine as a radioactive precursor, as previously described (Hall *et al.*, 1984). The translation products were then

subjected to two-dimensional-gel analysis and fluorography.

Analytical procedures

Proteins and translation products were subjected to two-dimensional gel analysis as previously described (Lim *et al.*, 1983; Hall *et al.*, 1984; Whatley *et al.*, 1984), as was fluorography of separated translated proteins on the two-dimensional gels.

Results

The two-dimensional-gel analysis of rat brain preparations showed the 68 K MAP to be a constituent of microtubule-associated proteins (Fig. 1a), isolated by phosphocellulose chromatography of microtubules (Fellous *et al.*, 1977), as reported by Strocchi *et al.* (1981) [for a two-dimensional-gel analysis of unextracted microtubules and its various constituents, including tubulin and actin, see our previous paper (Mahadevan *et al.*, 1984)]. The 68 K MAP was found to be present, together with tubulin, in synaptosomes where these proteins occurred as major constituents of the SPM (Fig. 1b) (Strocchi *et al.*, 1981; Lim *et al.*, 1983), synaptic vesicles (Fig. 1c) and post-synaptic densities (Fig. 1d). Actin was another common component. In the SPM, in addition to the 68 K MAP, the enzymes neuron-specific enolase and the brain form of creatine kinase, which are constituents of a membrane ATP-generating system (Lim *et al.*, 1983), were also present. When the SPM was subjected to treatment with 0.4% Triton X-100 followed by extraction with 0.5 M-NaCl, the cytoskeletal extract contained most of the SPM 68 K MAP (result not shown).

The two-dimensional-gel migration characteristics of the 68 K MAP appear identical with those of the 70 kDa heat-shock protein (HSP 70) (result not shown; Bensaude *et al.*, 1983). To determine the relationship of these two proteins, we used a DNA fragment containing the *Drosophila* HSP 70 gene in hybrid-selection experiments. Rat brain mRNA was hybridized to the *Drosophila* DNA and both bound and unbound mRNA were translated in the reticulocyte-lysate system. The hybrid-selected mRNA was found to direct the exclusive synthesis of the brain 68 K MAP (cf. Figs. 2a and 2b). The protein was absent from translation products of the unbound mRNA (Fig. 2c), and MAP mRNA was not bound to control DNA, i.e. plasmid pBR322 lacking the *Drosophila* gene (Fig. 2d). The *Drosophila* HSP 70 gene thus specifically and exclusively hybridizes to the 68 K MAP mRNA under highly stringent conditions. This close homology of the coding sequences is strongly supportive of homology of the proteins, originally indicated by their

Fig. 1. Two-dimensional gel analysis of the distribution of the rat brain 68 K MAP

Microtubule-associated-proteins (a), SPM (b), synaptic vesicles (c) and post-synaptic densities (d) were subjected to two-dimensional-gel analysis (see the Materials and methods section): SDS, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis; IEF, isoelectric focusing. In Figs. 1 and 2, only the relevant portion of gels containing the 68 K MAP, tubulin and actin is shown. Note presence of the 68 K MAP (arrowed) and tubulin in all fractions except in (a), where the MAP was separated from tubulin by phosphocellulose chromatography. Abbreviations: A, actin; CK, brain isoform of creatine kinase; NSE, neuron-specific enolase; MAP, 68 K MAP; α T and β T, α - and β -tubulin respectively. The migration characteristics and identification of all these proteins have been previously described in detail (Lim *et al.*, 1983; Hall *et al.*, 1984; Whatley *et al.*, 1984; Mahadevan *et al.*, 1984).

Fig. 2. Hybrid-selection of rat brain 68 K MAP mRNA by *Drosophila* HSP 70 gene

The Figure shows translation products of (a) brain mRNA before hybrid-selection with the *Drosophila* HSP 70 gene and of brain mRNA either hybridizing (b) or not hybridizing (c) to the HSP 70 gene; (d) shows that the brain MAP mRNA does not bind to plasmid DNA lacking the HSP 70 gene. Full experimental details are provided in the Materials and methods section. B represents radioactivity binding to endogenous lysate proteins; identification of all the translation products designated has been previously described (Hall *et al.*, 1984); for abbreviations see Fig. 1 legend. Note in (b) the presence of the 68 K MAP as the sole product of HSP 70-DNA-selected mRNA and in (c) the absence of MAP after the brain mRNA has undergone hybrid-selection. The arrow points to the position of the 68 K MAP, which is also that of HSP 70.

identical molecular mass and pI, as analysed on two-dimensional gels.

Discussion

Our results show that the 68 K MAP, the mRNA of which is present in significant amounts in rat and human brain (Hall *et al.*, 1984; Whatley *et al.*, 1984), occurred together with the other cytoskeletal proteins tubulin and actin in various structures of the synaptosome. In microtubules separation of MAP from tubulin is achieved by phosphocellulose chromatography (Fig. 1a). With the SPM, which also contains calmodulin (Hall *et al.*, 1984; Grab *et al.*, 1979), we have since found that agents such as trifluoperazine (Cheung, 1980) and melittin (Comte *et al.*, 1983), which disrupt calmodulin interactions with other proteins, are also effective in separating MAP from tubulin (T. Leung & L. Lim, unpublished work). Strocchi *et al.* (1981) have proposed that the 68 K MAP which they also found to be present in the SPM could act as an anchoring point between microtubules and membranous components. Our results are compatible with their proposal.

The 68 K MAP appears to be similar to the 68 kDa cytoskeletal protein associated with a number of intermediate filaments, including neurofilaments and microtubules (Wang *et al.*, 1980; Granger & Lazarides, 1983), particularly with respect to their migration on two-dimensional gels, although their co-identity has yet to be established. Wang *et al.* (1981) have commented on an apparent homology of the 68 kDa protein with the constitutive highly conserved HSP 70. Our hybrid-selection experiments concur with this view.

The specific hybridization of the *Drosophila* HSP 70 gene with rat brain mRNA for the 68 K MAP (Fig. 2) is not surprising, considering that the HSP 70 gene is known to be highly conserved (Bensaude *et al.*, 1983). If the 68 K MAP and HSP 70 are indeed identical (definitive evidence of this has to await sequence analysis of the MAP gene, which could be isolated by using the HSP gene as a probe), this would lend credence to the hypothesis that heat-shock proteins have a cytoskeletal role.

Nevertheless, regardless of whether these two proteins are identical or not, the *Drosophila* HSP 70 gene, because of its exclusive hybridization with the rat brain 68 K MAP mRNA, will prove invaluable in investigations on the regulation of the expression of the gene for the brain MAP.

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