ASTROGLIAL FILAMENT AND FIBROBLAST INTERMEDIATE FILAMENT PROTEINS IN CYTOSKELETAL PREPARATIONS FROM SPINAL CORD AND OPTIC NERVE

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1. Introduction

Astroglial filaments isolated from mouse optic nerve are composed of 2 major polypeptides with M_r 55 000 and 45 000 (referred to as P55 and P45 below; [1]). These two polypeptides occur in an approximate molar ratio of 1:1 under a variety of circumstances, i.e., in the normal and degenerated optic nerve in situ, in isolated 10 nm filaments, after being solubilised in a hypotonic medium, and in filaments reassembled in vitro, which suggests that they are closely associated. One-dimensional peptide map analyses have indicated that P55 is distinct from α-tubulin and that P45 is not a degradation product of P55.

Considering that glial fibrillary acidic (GFA) protein has so far been thought to comprise a single protein species (review [2]), and also that astroglial filaments isolated from human gliosed brain were mainly composed of a single species of polypeptide of 49 000 M_r [3], the simultaneous occurrence of two polypeptides in our purified astroglial filaments requires explanation.

An attempt to characterise these 2 polypeptides here indicates that the larger polypeptide is homologous to vimentin, the protein subunit of a type of intermediate filaments in many cells, especially in mesenchymal cells [4–6], and that only P45 cross-reacts with anti-GFA protein antiserum. This finding is in accord with the coexistence of vimentin and GFA protein shown in the cytoskeleton of the rat optic nerve [7]. In contrast to the optic nerve, vimentin in the spinal cord cytoskeleton prevails only in immature animals, and becomes almost undetectable in adults. Interspecies differences in M_r and in one-dimensional peptide maps of GFA protein among rodent species are also shown.

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2. Materials and methods

2.1. *Cytoskeletal preparations from the optic nerve and spinal cord*

Male Wistar rats at various ages and adult male *dd* mice were used. The animals were killed by decapitation, and the spinal cord and optic nerve were quickly dissected out. All the following procedures were carried out at 0–4°C. The tissue was washed in physiological saline, and ground in 5 vol. lysis solution consisting of 1% Triton X-100, 130 mM NaCl and 20 mM Na-phosphate (pH 6.9) in a loosely fitting Teflon–glass homogeniser. The homogenate was centrifuged at 100 000 × *g* for 60 min and the sediment was re-homogenised in the same solution. Extraction with the lysis solution was repeated 2 more times. The final sediment was suspended in a small volume of 100 mM NaCl–1 mM EDTA–10 mM piperazine-*N*,*N*′-bis(2-ethanesulphonic acid) [PIPES] (pH 6.9) and the suspension was centrifuged at 150 000 × *g* for 90 min on 1.5 M sucrose dissolved in the same solution. The sediment at the bottom of the sucrose layer was taken as a cytoskeletal fraction.

2.2. *Preparation of intermediate filament protein from cultured fibroblasts*

Fibroblasts (Don-6 and L · P3) were grown in Dulbecco's modified Eagle's medium (Gibco H21) in plastic flasks, with the addition of 5% calf serum for Don-6, or without serum for L · P3. On reaching confluency, the cells were treated with 1% Triton X-100 and 150 mM NaCl in 10 mM PIPES (pH 6.9) similarly to [8]. The cytoskeletal preparation was electropho-

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rested in SDS–polyacrylamide gel, and the band representing the intermediate filament protein (vimentin [5]) was cut out from the gel and subjected to one-dimensional peptide map analysis.

2.3. *Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis*

The cytoskeletal preparation from the spinal cord, optic nerve, or fibroblasts was homogenised in the medium consisting of 2.3% (w/v) SDS, 5 mM β-mercaptoethanol, 10% (w/v) glycerol and 62.5 mM Tris–HCl (pH 6.8) and heated in a boiling water bath for 5 min. Aliquots were subjected to electrophoresis [9] in 10% SDS–polyacrylamide slab gels, and the gels were stained with 0.25% Coomassie brilliant blue R-250.

2.4. *One-dimensional peptide mapping*

The protein bands to be examined were cut out from the stained gel. The gel slices were placed in sample wells of a second SDS–polyacrylamide slab gel (15%), and overlayered with *Staphylococcus aureus* V8 protease (0.5 ng). Limited proteolysis was carried out directly in the stacking gel during re-electrophoresis [10].

2.5. *Direct detection of GFA protein by antibody binding in SDS–polyacrylamide gel*

Cytoskeletal proteins were electrophoresed in a small-sized gel (4 cm × 8 cm), and the gel was fixed in water:methanol:acetic acid (5:5:1, by vol.), and washed with 150 mM NaCl, 0.1% NaN_3 in 50 mM Tris–HCl (pH 7.5). The gel was then treated with anti-GFA protein antiserum raised in a rabbit against the degraded antigen from the human spinal cord [2,11] followed by fluorescein isothiocyanate-conjugated IgG fraction of goat antiserum directed against rabbit IgG (Miles-Yeda, Israel) [12]. Photographs were taken under ultraviolet light.

3. Results

3.1. *Cytoskeletal proteins from the optic nerve and spinal cord – Interspecies variations*

On electron microscopy, cytoskeletal fractions from optic nerves and spinal cords of the adult rats consist mainly of 10 nm filaments of smooth contour, either loosely reticulated or tightly packed (fig.1). On SDS-gel electrophoresis, the optic nerve cytoskeleton contains, besides the neurofilament triplet (P200, P160, P68), two major polypeptides, i.e., P56 and

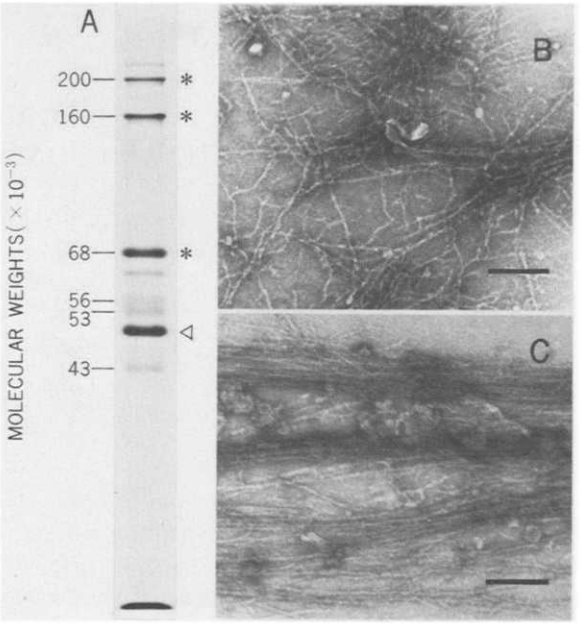

Fig.1. Cytoskeletal preparation isolated from the rat spinal cord: (A) electrophoretic banding pattern; (*) neurofilament polypeptide triplet; (◁) P51; (B,C) negatively stained electron micrographs. Samples in 140 mM NaCl–10 mM PIPES (pH 6.9) were fixed on collodion–carbon-coated grids with 5% formaldehyde and 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.3) and were stained with saturated uranyl acetate. Loosely reticulated (B) and tightly packed (C) filaments are shown. Bar, 0.2 μm.

P51 in rats, and P56 and P45 in mice (fig.2A). P56 and P45 in the mouse optic nerve were shown to be copurified with the astroglial 10 nm filaments ([1]; we amend the M_r of the larger component from 55 000 in [1] to 56 000). In contrast to optic nerve cytoskeletons, those prepared from spinal cords of adult animals lack P56; P51 or P45 thus represents the major non-neuronal cytoskeletal protein in the spinal cord of the adult rat or mouse.

3.2. *Occurrence of the protein subunit of fibroblast intermediate filaments in the optic nerve cytoskeleton*

Because of the virtual absence of P56 in the spinal cord cytoskeleton, we suspected that the polypeptide may represent the subunit of intermediate filaments other than astroglial 10 nm filaments, despite its occurrence in astroglial filaments purified from the optic nerve [1]. This possibility was explored by comparing the one-dimensional peptide maps of optic nerve P56 and of the filament protein that was elec-

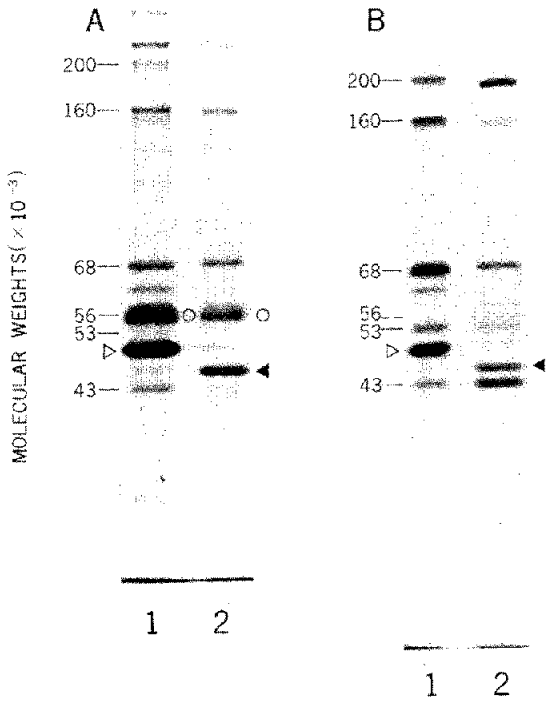

Fig.2. Comparison of cytoskeletal proteins between the optic nerve and spinal cord, and between rats and mice: (A) optic nerve; (B) spinal cord; (1) rats; (2) mice. In both species P56 (○) is seen in the optic nerve, but not in the spinal cord. In both the optic nerve and spinal cord, P51 (▷) is present in rats; this is replaced by P45 (◀) in mice.

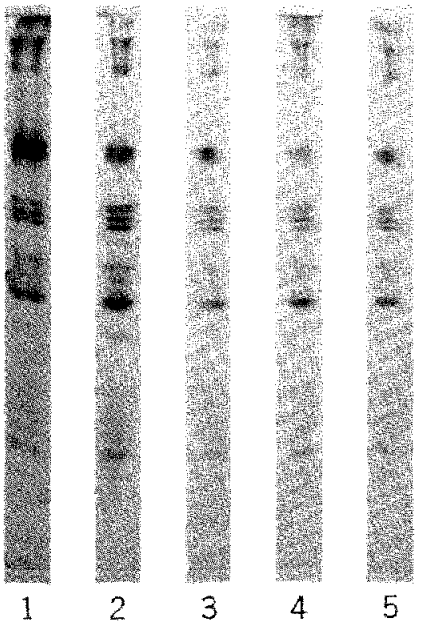

Fig.3. One-dimensional peptide maps of the spinal and optic P56 and of the fibroblast intermediate filament protein (vimentin): (1) P56 from the newborn rat spinal cord; (2) P56 from the adult rat optic nerve; (3) P56 from the adult mouse optic nerve; (4) vimentin from Don-6 cells; (5) vimentin from L - P3 cells. Each lane contained ≃20 μg of the indicated protein, which was subjected to a limited proteolysis with 0.5 ng *Staphylococcus aureus* V8 protease.

trophoretically purified from cytoskeletons of cultured fibroblasts; the fibroblast filaments were reported to consist of the major polypeptide with M_r 57 000 [5] or 58 000 [4,6]. Peptide maps of the optic nerve P56 from rats and mice are very similar to one another (fig.3, lanes 2,3), and the homology was ascertained also between the rodent P56 and filament protein from two different lines of fibroblasts (fig.3, lanes 2–5).

3.3. *Identification of GFA protein in cytoskeletons isolated from the optic nerve and spinal cord*

Attempts have been made to characterise the smaller component of astroglial filament protein that shows the interspecies variation in its molecular weight (fig.2; [1]). One-dimensional peptide mapping indicates the homology between the optic nerve polypeptide and spinal cord polypeptide in each species, i.e., P51 in rats (fig.4, lanes 1,2), and P45 in mice (fig.4, lanes 3,4). However, the peptide maps exhibit many dissimilarities between the rat and mouse.

In the next step, the immunoreactivity of P51 and P45 with anti-GFA protein antiserum was examined. After electrophoresis of the spinal cytoskeletal fraction, the gel was treated with rabbit anti-GFA protein antiserum, and then with fluorescein isothiocyanate-conjugated goat IgG directed against rabbit IgG. Significant fluorescence was produced by P51 in rats and by P45 in mice (fig.4B), indicating that these are the GFA protein despite their interspecies variations in M_r and in one-dimensional peptide maps. In mice, a weaker fluorescence was seen also with P43 (fig.4B, lane 2), which seems to be related to the presence of this polypeptide in the cytoskeletal preparation from the mouse spinal cord (fig.2B, lane 2). The one-dimensional peptide map of this polypeptide is homologous to that of actin purified from the mouse brain (not shown).

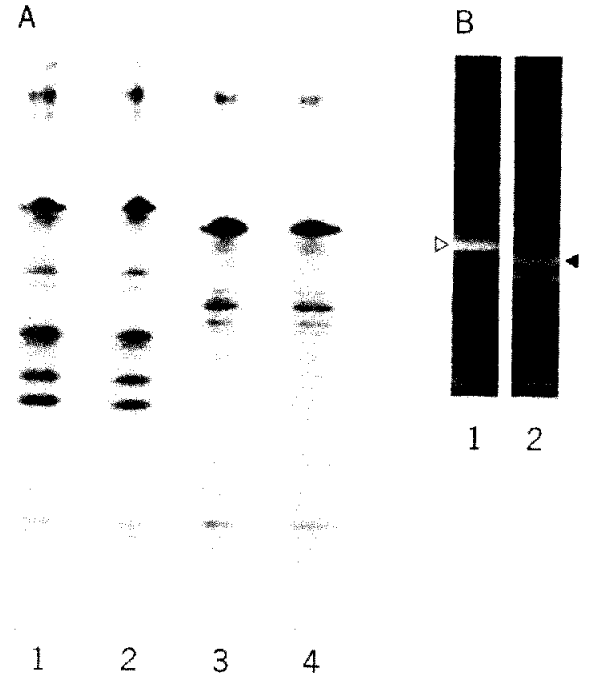

Fig.4. Species difference in GFA protein between rats and mice: (A) one-dimensional peptide maps of rat P51 (spinal (1); optic (2)) and mouse P45 (spinal (3); optic (4)); (B) identification of the rat P51 and mouse P45 as GFA protein. The spinal cytoskeletal preparation from the rat (1) and mouse (2) was electrophoresed in a small gel, and the gel was treated with rabbit anti-GFA protein antiserum, and further with fluorescein isothiocyanate-conjugated goat IgG directed against rabbit IgG. Electrophoretic mobility of P51 in rats (▷) and P45 in mice (◀) was determined by comparison with the equivalent lanes electrophoresed in the same gel and stained with Coomassie blue.

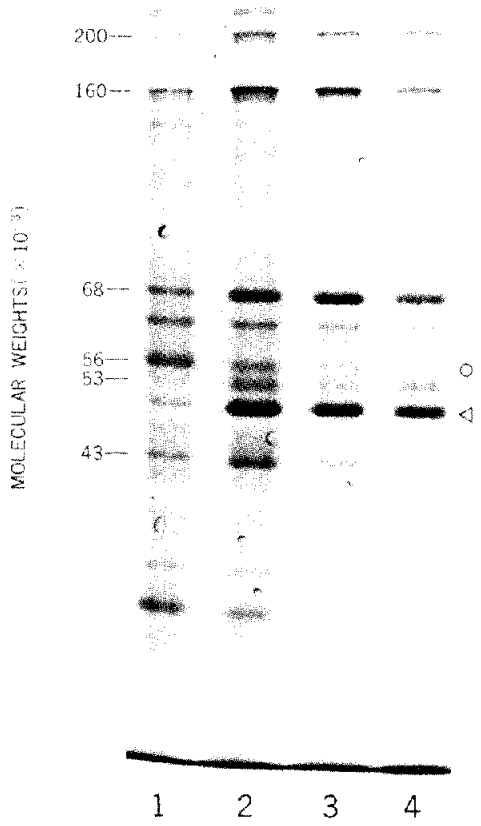

Fig.5. Maturational changes in the composition of cytoskeletal proteins in the rat spinal cord: (○) P56 (vimentin); (◁) P51 (GFA protein); (1–4) correspond to 2 day-, 2 week-, 4 week- and 10 week-old rats, respectively.

3.4. *Postnatal changes in the composition of cytoskeletal proteins*

P56 is present in the optic nerve cytoskeleton of the adult animal, but is almost undetectable in the spinal cord cytoskeleton of the adult animals (fig.2). However, P56 does predominate in the spinal cord cytoskeleton isolated from 2 day-old rats, where the level of GFA protein is still very low (fig.5, lane 1). The homology between the spinal P56 and optic P56 is established by one-dimensional peptide mapping (fig.3, lanes 1,2). The spinal P56 rapidly declines during the subsequent maturational stage, and becomes almost undetectable in the adult spinal cord, while the adult level of GFA protein seems to be attained within 2 weeks at latest (fig.5).

4. Discussion

We have shown that the astroglial 10 nm filaments isolated from degenerated optic nerves of adult mice are composed of two polypeptides [1]. Here, the larger component is shown to be homologous to the subunit of the intermediate filament protein of fibroblast origin (vimentin), and the smaller component to GFA protein. The finding is in accord with the recent immunocytochemical demonstration of the coexistence of vimentin and GFA protein in astrocytes in situ [13,14], and in glioma cells in culture [15]. In [7] vimentin and GFA protein were reported to coexist in the rat optic nerves undergoing Wallerian degeneration. It is thus clear that astrocytes

in situ or in culture are one of the members of a wide variety of cell lines where vimentin occurs simultaneously with other kinds of intermediate filament proteins, i.e., desmin and keratins (review [16]).

Detailed immunocytochemical studies [13] have demonstrated that vimentin in astrocytes and glial precursors is expressed well before the onset of GFA protein and might therefore serve as an early marker of glial differentiation. Here, the relative dominance of vimentin *vs* GFA protein in the rat spinal cord seems to alter some time during 2 days and 2 weeks after birth (fig.5). In [7] vimentin was reported to be the major protein in the newborn and GFA the major protein in the adult cytoskeleton from rats. However, the question remains whether the earlier expression and later decline of vimentin represent events occurring inside the astrocytes in the spinal cord, because vimentin is immunocytochemically demonstrated not only in astrocytes, but also in fibroblasts, cells of larger vessels, and in ependymal cells [13]. In the optic nerve, the coexistence of GFA protein and vimentin in astrocytes seems certain, because these polypeptides are copurified with astroglial 10 nm filaments [1]. Early expression of vimentin and its decline at later stages have been reported in cultured muscle cells [17], though other workers failed to observe the latter decline [18].

Neurofilaments isolated from the rat sciatic nerve are composed exclusively of 3 polypeptides, P200, P160 and P68 [19,20], present in relative molar proportion comparable with that of nerve in situ [21]. Neurofilament preparations isolated from the central nervous system contain, besides the triplet, several additional polypeptides among which the major is a polypeptide with $M_r \sim 50\,000$ [22–24]. Although this polypeptide has generally been suspected to be of glial origin, direct evidence for this has been lacking, except for a recent demonstration that the antiserum raised against 49 000 M_r polypeptide recovered in a filament preparation from the human brain strongly stained Bergmann glial fibres and astroglial cells in white matter fibre tract in the granular layer of the cerebellum [14]. This demonstration of GFA protein and the neurofilament triplet in the spinal cord cytoskeleton, together with the demonstration of GFA protein in the purified astroglial filaments [1], directly indicates that astroglial filaments are inevitably copurified with neurofilaments when the central nervous system is used as starting material.

Interspecies variations of GFA protein do not seem to have been properly documented, though the M_r reported for GFA protein by different workers varies to a considerable extent (e.g. [3,11,25,26]). This study clearly indicates the occurrence of interspecies variation of the GFA protein in its M_r and in its peptide maps between 2 rodent species (fig.2,4); in guinea pigs, the M_r of GFA protein was estimated to be 52 000 (not shown).

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