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Association of brain spectrin isoforms with microtubules

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The relationship of rat brain spectrin isoforms to microtubules of newborn and adult animals was studied. Spectrins were minor components in microtubule preparations. The microtubule-associated spectrin is a major calmodulin-binding protein. Radiolabelled brain spectrin(240/235) revealed specific microtubule binding activity in vitro, possibly via a tubulin.

Brain spectrin; Isoform; Microtubule; Calmodulin-binding

1. INTRODUCTION

Brain spectrin, an essential component of the membrane-related cytoskeleton [12,19], links cytoplasmic actin filaments to the plasma membrane [10]. It was also referred to as 'fodrin' [16], and as 'brain-calmodulin-binding protein' [7,11], however, the significance of such calmodulin binding is as yet not clear. Brain spectrin was shown to induce bundling of microtubules in vitro [14]. A major amount of brain spectrin co-isolated with microtubules under non-depolymerizing conditions, but such microtubules lost brain spectrin in subsequent cycles of cold and warm centrifugations [9]. The issue is further complicated by the identification of two brain spectrin isoforms in mammalian brain tissue, named brain spectrin(240/235) and brain spectrin(240/235E) with differential location and development-dependent appearance [17,18]. In contrast to brain spectrin(240/235) which was found in neuroblasts and axons, brain spectrin(240/235E), an immunologically related protein to red blood cell spectrin, appeared late during postnatal brain development in perikarya and dendrites of differentiating neurons, and in some glial cells [18]. The relationship of these two brain spectrin isoforms to microtubules during development has not been investigated and is the issue of this study.

2. MATERIALS AND METHODS

Brain microtubules were isolated from newborn and adult Sprague-Dawley rats by 3 cycles of cold and warm centrifugations [20]. The third microtubule pellet (MT3) was dissolved in 0.1 M MES (pH 7.4), 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM GTP to obtain 9 mg microtubule proteins/ml and were centrifuged at 4°C at $100000 \times g$ for 20 min. The soluble microtubule proteins were used for the microtubule sedimentation assay.

Brain spectrin(250/235) was isolated as described [17]. 100 μg protein was labeled with Na[^{125}I] by the method of Bolton and Hunter [4], and dialysed against blot overlay- or microtubule-sedimentation assay buffer. The specific radioactivity of labeled brain spectrin was 6.5×10^5 cpm/μg brain spectrin(240/235).

Calmodulin was isolated and biotinylated by standard procedures [1].

Protein amounts were determined by the Bio-Rad protein dye assay.

Electrophoresis and Immunoblots: Protein samples were separated on SDS-PAGE [15] and stained with Coomassie blue R250, or transferred to nitrocellulose sheets [21]. Western blots were probed with poly- and monoclonal antibodies and peroxidase-conjugated secondary antibodies and reaction was visualized with 4-chloro-1-naphthol [17].

All *antibodies* have been previously described: Rabbit polyclonal antibodies against spectrin/240/235) and (240/235E) and pre-immune serum [17]; and monoclonal antibodies against MAP2 [13], γ- [2], β-tubulin [3].

Blot overlay: Calmodulin binding to proteins on Western blots was demonstrated by biotinylated calmodulin, avidin-conjugated alkaline phosphatase, and Nitroblue tetrazolium chloride and 5-bromo-4-chloro-3 inoyl phosphate *p*-toluidine salt as chromogens [1]. 1 mM EGTA abolished biotinylated calmodulin-binding to proteins on blots. Brain spectrin(240/235) binding was also determined by a blot overlay method [8]. ^{125}I-labeled brain spectrin(240/235) was used at 2.3×10^5 cpm/ml. Dried blots were exposed to Kodak XAR5 film for 65 h at −20°C. In control experiments a 10-fold excess of unlabeled brain spectrin(240/235) reduced binding of iodinated brain spectrin to proteins on blot.

Microtubule-sedimentation assay: For this assay proteins were used at a final concentration of 1 μg ^{125}I-labeled brain spectrin-(240/235)/ml with, or without 3 mg microtubule proteins/ml, and 10 μg brain spectrin(240/235)/ml. Proteins were incubated for 30 min at 37°C in motility buffer of Vale et al. [22] including protease inhibitors leupeptin and antipain (10 μg/ml). Aliquots of 200 μl were overlayed on top of a 200 μl cushion of 30% glycerol in motility buffer and centrifuged for 30 min at $39000 \times g$ at room temperature. Supernatant and pellet fractions were counted for radioactivity and submitted to electrophoresis and autoradiography. Each measurement was done at least in triplicate.

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3. RESULTS

3.1. *Spectrins in microtubule preparations*

Both brain spectrin isoforms as well as contaminating red blood cell spectrin were present in microtubule preparations. They are minor components making up less than 0.1% of microtubule proteins. On Western blots the antibody against brain spectrin(240/235) reacted with with 240 kDa subunit of brain spectrin present in both newborn and adult microtubules (Fig. 1, lane 3 and 4). Little brain spectrin(240/235E) was detected by an antibody which was raised against rbc spectrin and usually reacts strongly with the 235 kDa brain spectrin subunit. It was seen as faint staining of the 240 kDa and 235 kDa brain spectrin(240/235E) subunits in microtubule preparations of adult animals (Fig. 1, lane 6). This 235 kDa protein staining was lacking in microtubules of newborn rats (lane 5); but the staining of two other proteins of 240 kDa and 220 kDa in the same sample indicated the presence of rbc spectrin subunits possibly due to the presence of contaminating erythrocytes in the microtubule preparation.

3.2. *Brain spectrin(240/235) binding to microtubules*

The ability of brain spectrin(240/235) to bind to microtubules was demonstrated in a microtubule-sedimentation assay. Without the presence of microtubule proteins in the assay $87.0 \pm 8.4\%$ ($n = 4$) of ^{125}I-labeled brain spectrin(240/235) remained in the supernatant. When microtubule proteins were present in the assay, radiolabeled brain spectrin was binding to microtubule proteins, and was found to be $58.7 \pm 16.0\%$ in the pellet fraction. With addition of a ten-fold excess of brain spectrin(240/235) to microtubule proteins and radiolabeled brain spectrin, the radioactive brain spectrin remained in the supernatant fraction (with $72.1\% \pm 5.2\%$). Electrophoresis and autoradiography of corresponding aliquots confirmed these findings (data not shown).

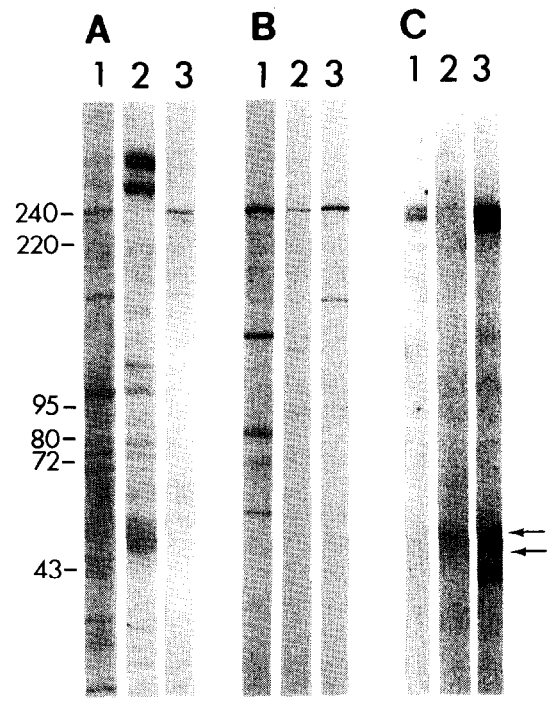

Fig. 2. Calmodulin-binding and ^{125}I-labeled brain spectrin binding by blot overlay. Adult rat brain tissue (lane 1), microtubules of adult rats (lane 2) and rat brain spectrin(240/235) (lane 2) were separated on a 4–12% SDS-PAGE. Western blots were stained with Amido black (A), or used for the calmodulin blot overlay (B), or for the blot overlay with radiolabeled brain spectrin(240/235) (C). The M_r is indicated on the left in kDa. Arrows point to α- and β-tubulins.

3.3. *Calmodulin-[^{125}I]brain spectrin/240/235)-, and immuno-blot overlay*

In Fig. 2B the calmodulin blot overlay showed the 240 kDa spectrin subunit as a major calmodulin binding

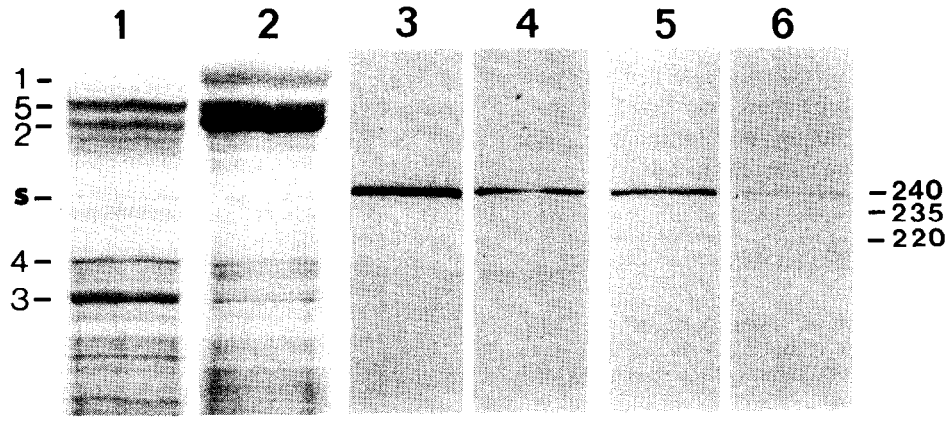

Fig. 1. Brain microtubule preparations of newborn animals (lanes 1, 3 and 5) and adult rats (lanes 2, 4 and 6) were separated on a 4% SDS-PAGE. Gels were stained with Coomassie blue (lanes 1 and 2) and blots were stained for brain spectrin(240/235) (lanes 3 and 4), and for brain spectrin(240/235E) (lanes 5 and 6). The M_r is indicated on the right in kDa. The location of microtubule-associated proteins 1 to 5 and spectrin(s) are indicated on the left. Only the relevant portions of the gels and blots are shown.

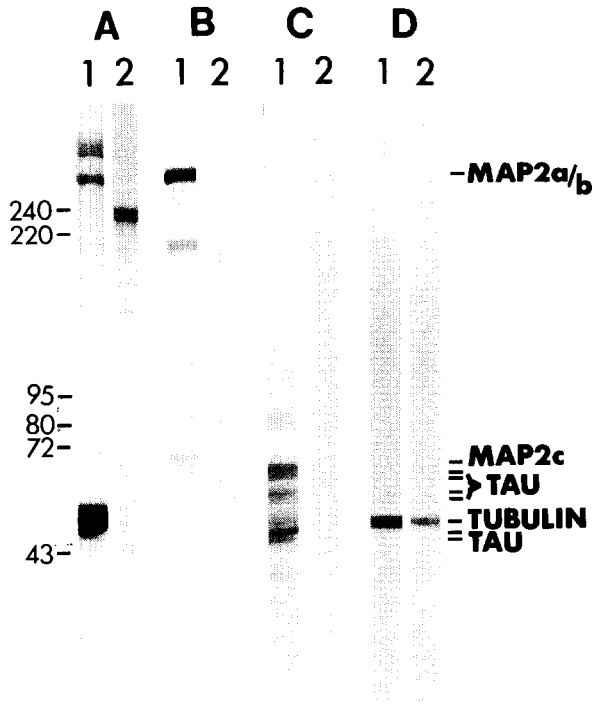

Fig. 3. SDS-electrophoresis and Western blots of microtubules (lanes 1) and brain spectrin (lanes 2), isolated from adult rat brain. 4–15% SDS-PAGE were stained with Coomassie blue (A), or blots were probed with anti-MAP2 (B), anti-τ- (C) or anti-β-tubulin (D). M_r values are indicated on the left in kDa.

protein in adult rat brain tissue (lane 1), in a microtubule preparation (lane 2), and in purified brain spectrin (lane 3). The autoradiography of a blot overlay with radiolabeled brain spectrin(240/235) in Fig. 2C indicated that brain spectrin(240/235) had the tendency to self-association (lanes 1 and 3). In a microtubule fraction (lane 2) the radioactively labeled brain spectrin exhibited binding to proteins in the M_r range of tubulins (indicated by arrows).

In Fig. 3, Western blots of microtubules (lane 1) and of purified brain spectrin(240/235) (lane 2) were probed with monoclonal antibodies against MAP2, τ or β-tubulin and showed that all 3 antibodies reacted specifically with MAP2, τ or β-tubulin in the microtubule preparation (Fig. 3B–D, lane 1). However, only a trace of β-tubulin was found present in purified brain spectrin (Fig. 3D, lane 2).

4. CONCLUSIONS

The results presented suggest that all spectrin isoforms have limited ability to co-isolate with microtubules through several temperature-dependent centrifugation cycles. The difference, that only brain spectrin(240/235) but not (240/235E) binds to microtubules of newborn animals, could be explained by that brain spectrin(240/235) is present in fetal and newborn tissue while brain spectrin(240/235E) appears at later stages during development and is not present at birth [18]. Our results on brain spectrin-microtubule binding are in agreement with previous studies [5,9,14] and furthermore suggest that brain spectrin may bind to microtubules of juvenile and adult animals. The binding site for brain spectrin is possibly a tubulin form with a high brain spectrin binding affinity but which is present at low abundancy in microtubules – what could explain the low tubulin labeling efficiency by radioactive brain spectrin (Fig. 2C, lane 2). For erythrocyte spectrin the binding site in microtubules was shown to be a τ-protein [5], but it remains to be seen if the red blood cell spectrin-related brain spectrin(240/235E) also binds to τ-proteins once its isolation procedure has been established.

Brain spectrin was shown to be a major calmodulin-binding protein in postsynaptic densities [6]. In addition, our data suggest that brain spectrin may be a major calmodulin-binding protein in association with microtubules. It remains to be determined if both brain spectrin isoforms bind calmodulin equally well. An immuno-electron microscopic study has shown that both brain spectrin isoforms occur along microtubules [23], and therefore such a brain spectrin coat along microtubules, with the ability to bind calmodulin could be of significance for the stabilization of microtubule and for the regulation of calmodulin-dependent enzymatic processes.

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