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Neuraxin corresponds to a C-terminal fragment of microtubule-associated protein 5 (MAP5)

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From cloned DNA, neuraxin has been identified as a tubulin binding protein of predicted molecular weight of 94 kDa. The deduced sequence of the rat protein exhibits high homology to the C-terminal region of mouse microtubule-associated protein 5 (MAP5). Here, we show that different neuraxin antibodies recognize MAP5, but fail to detect a protein of 94 kDa, in subcellular and microtubular fractions of the rat central nervous system. Furthermore, tubulin binding by neuraxin was found to be dependent on taxol. These data are consistent with neuraxin corresponding to a C-terminal fragment of MAP5 that contains a low-affinity tubulin binding site.

Neuraxin; Microtubule-associated protein 5; Tubulin; (Rat brain)

1. INTRODUCTION

Neuronal shape and cell surface differentiation depend on elaborate interactions of cytoskeletal and plasma membrane components. The elements mediating these interactions in various compartments of the nerve cell have not been identified. Biochemical and immunological data, however, indicate that specialized membrane-associated proteins are implicated in the topological organization of adhesion proteins, ion channels and receptors at synapses, the nodes of Ranvier, and along axons and dendrites (for examples see [1-3]). Upon screening for a cytoplasmic peripheral membrane protein associated with the inhibitory glycine receptor, we have isolated a cDNA which encodes a novel putative structural protein, neuraxin [4]. Neuraxin has a predicted molecular weight of 94 kDa, contains a characteristic heptadecarepeat domain and is expressed in all regions of the central nervous system (CNS). Bacterial neuraxin fusion protein binds tubulin in the presence of taxol and cross-react with antibodies to microtubule-associated protein 5 (MAP5). Conversely, neuraxin antibodies recognize MAP5 on Western blots. Neuraxin therefore was suggested to represent a related or alternatively spliced variant of the MAP5 protein family that may be implicated in membrane-cytoskeleton interactions [4].

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Abbreviations: CNS, central nervous system; mAb, monoclonal antibody; MAP, microtubule-associated protein; PBS, phosphate-buffered saline

Previous work has established MAP5 (also termed MAP1B, MAP1.2, or MAP1-X) as a prominent large MAP (M_r about 250-300 kDa) that promotes tubulin polymerization in vitro and is highly expressed during neurite outgrowth (reviewed in [5]). Recently, the primary structure of MAP5 has been unraveled by DNA sequencing [6]. Comparison of mouse MAP5 and rat neuraxin sequences now reveals that the open reading frame of the latter is highly homologous to the 3′ coding region of the MAP5 cDNA (not shown). Throughout the coding sequences, 93% nucleotide identity are found. This raises the question whether MAP5 gene products corresponding to neuraxin indeed exist in the rodent CNS. Here, we present immunoblotting and microtubule copolymerization studies which indicate that the predicted neuraxin protein corresponds to a C-terminal fragment of MAP5 harbouring a tubulin binding domain of low affinity.

2. MATERIALS AND METHODS

2.1. *Antibodies*

The glycine receptor monoclonal antibody mAb 9b has been characterized previously [7]. Antisera raised against different domains of neuraxin were as described by Rienitz et al. [4]. MAb aM5 directed against MAP5 was obtained from H. Langbeheim, Sigma Immunochemicals. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG (goat) were purchased from Jackson Immunoresearch Laboratories.

2.2. *Microtubule purification*

Microtubules were purified from rat brain at different developmental stages (embryonic day 17 (E17), newborn (P0), 12 days after birth (P12), and adult) using the taxol method [8]. Synaptosomes and fractions thereof were prepared from brain and spinal cord according to

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Gray and Whittaker [9], as modified by Wolf and Kapatos [10]. A protease inhibitor cocktail [11] was included in all preparations.

2.3. *Gel electrophoresis and Western blot analysis*

Electrophoresis on 5–10% gradient SDS-polyacrylamide gels was performed according to Laemmli [12]. Protein bands were visualized using Coomassie brilliant blue R250 (Sigma). Transfer of proteins to nitrocellulose (Schleicher & Schuell, 0.45 μm pore size) was performed according to Towbin et al. [13]. After transfer, blots were blocked for 30 min in PBS supplemented with 5% (w/v) non-fat milk powder. Incubation with the first antibody (diluted 1:500 in PBS) was for 1.5 h at 35°C. After three washes in PBS, the blots were incubated with peroxidase-conjugated second antibody (diluted 1:2.000 in PBS) for 1 h at 35°C. Antigen-antibody complexes were visualized by 4-chloro-1-naphtol (Sigma).

2.4. *Copolymerization assay*

Tubulin was purified from rat brain microtubules using a phosphocellulose column [14] and stored at −70°C. Increasing amounts (2–10 μg) of tubulin in MEM buffer (0.1 M MES-NaOH, 1 mM EGTA, 1 mM $MgSO_4$, 1 mM dithiotreitol, pH 6.6) were mixed with 5 μg neuraxin-MS2 polymerase fusion protein, and GTP and taxol were added to final concentrations of 1 mM and 20 μM, respectively. After incubation for 30 min at 35°C, the microtubules were centrifuged through a cushion of MEM buffer containing 10% (w/v) sucrose in a Beckmann airfuge (approx. 83 000 rpm for 10 min). Both supernatant and pellet were analyzed by SDS-polyacrylamide gel electrophoresis. Protein concentrations were determined according to Bradford [15].

3. RESULTS

3.1. *Western blot analysis of subcellular fractions from adult rat CNS*

Western blot analysis was performed to detect the possible presence of neuraxin in various subcellular fractions prepared from rat brain and spinal cord. The following antibodies were employed in these experiments: mAb 9b which recognizes the 93 kDa polypeptide associated with the postsynaptic glycine receptor [7], mAb aM5 directed against MAP5, and polyclonal antibodies raised against the head region (G15) or a repeat peptide (HPA 1) of the predicted neuraxin sequence [4]. These antibodies are known to react with a bacterially expressed neuraxin fusion protein [4]. Fig. 1 shows a Western blot of subcellular fractions from spinal cord using the polyclonal antibody HPA1.

All antibodies stained the neuraxin-MS2 polymerase fusion protein and a high-molecular weight component, which has previously been identified as MAP5 [4]. MAP5 was the only protein that was consistently labelled on Western blots of subcellular fractions by all antibodies employed in this study. The HPA1 antiserum in addition cross-reacted with polypeptides of about 75 and 30 kDa that were enriched in synaptosomes (fig.1). However, a protein of relative electrophoretic mobility close to that of the predicted neuraxin protein was not found. We therefore conclude that neuraxin is not present in detectable amounts in the adult rat CNS.

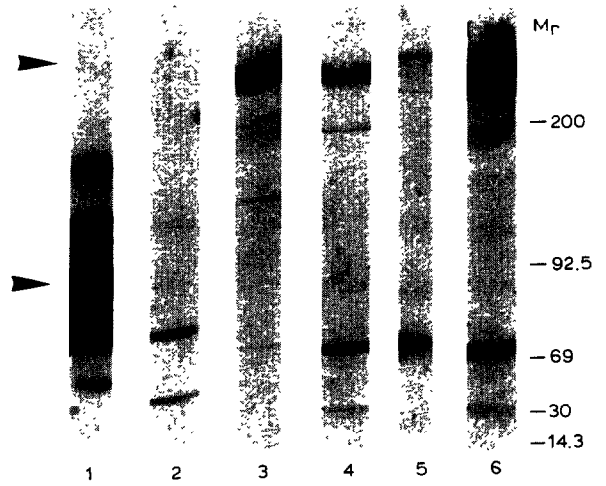

Fig.1. Anti-neuraxin antibodies detect MAP5, but not a 94 kDa protein, in various fractions of rat spinal cord. Subcellular fractions were separated by SDS-polyacrylamide gel electrophoresis and subjected to Western blotting using the polyclonal antibody HPA1. Lane 1, neuraxin-MS2 polymerase fusion protein; 2, total homogenate; 3, 100 000 × *g* supernatant; 4, synaptosomal fraction. Microtubules were precipitated from the synaptosomal fraction using taxol, and supernatant; (5) and microtubular pellet (6) were analyzed as described above. Lanes 2–6 contained 100 μg protein each, except lane 3 where 500 μg were applied. Note that in addition to MAP5 and the neuraxin-MS2 polymerase fusion protein (arrows) the antibody decorates bands of about 75 kDa and 30 kDa that are enriched in synaptosomes. Similar data were obtained with subcellular fractions from rat brain (not shown).

3.2. *Analysis of microtubular fractions isolated at different stages of development*

To exclude the possibility that a protein corresponding to the open reading frame of the neuraxin cDNA is selectively expressed at specific developmental stages, as reported for the MAP2 protein family [16,17], additional data were collected. Taxol-purified brain

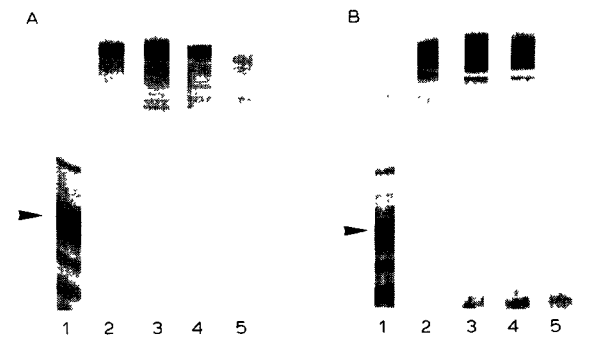

Fig.2. Western blot analysis of rat brain microtubules isolated at different developmental stages. Neuraxin-MS2 polymerase fusion protein (lane 1) and taxol-purified microtubules isolated at E17 (2), PO (3), P12 (4) and adulthood (5) were analyzed by Western blotting using antibodies G15 (A) and HPA1 (B). Approximately equal amounts of tubulin were loaded per slot. Note the decrease of MPA5 immunoactivity with rat brain development (see [20]). Arrows indicate the positions of the neuraxin-MS2 polymerase fusion construct.

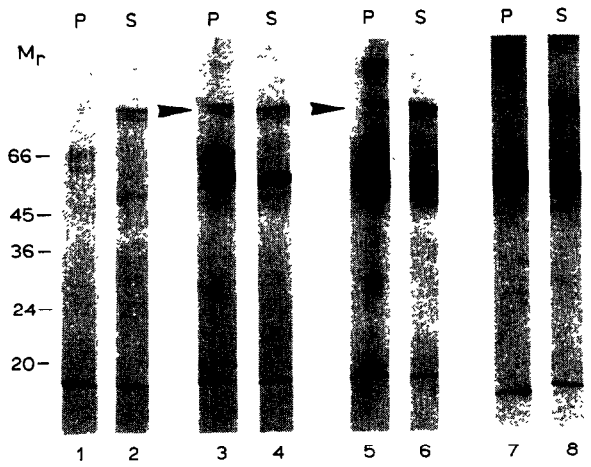

Fig.3. Copolymerization of the neuraxin-MS2 polymerase fusion protein and tubulin requires taxol. The fusion protein was incubated in the absence (lanes 1, 2) or presence (3–8) of tubulin with (1–6) or without (7, 8) taxol. After sedimentation, pellets (P) and supernatants (S) were separated on a 10% SDS-polyacrylamide gel followed by Coomassie blue staining. Tubulin concentrations per assay were 5 μg for 3 and 4, and 20 μg for 5–8, respectively. Note that only in the presence of taxol the neuraxin fusion construct was found in the pellet fractions (arrows).

microtubules were isolated from rats of various ages and subjected to Western blot analysis using all antibodies mentioned above. Fig.2 illustrates the staining seen with the polyclonal antibodies G15 and HPA1. Only a single prominent high molecular weight band of electrophoretic mobility identical to that of MAP5 was consistently detected under these conditions. Similar data were obtained with the other antibodies (not shown).

3.3. *Tubulin binding of the neuraxin fusion protein requires taxol*

The tubulin binding capacity of neuraxin has been established previously in overlay experiments where tubulin was shown to bind a neuraxin-MS2 polymerase fusion protein in the presence of taxol [4]. Attempts to copolymerize purified tubulin with the neuraxin-MS2 polymerase fusion protein in solution revealed that taxol was indeed required for incorporation of the fusion construct into microtubules (fig.3, lanes 3, 5). Even at tubulin concentrations high enough to allow self-polymerization [18], neuraxin was not incorporated into tubulin polymers without the alkaloid (fig.3, lane 7). Thus, tubulin binding by the neuraxin fusion protein appears to be taxol-dependent, suggesting a low affinity of the binding site(s) involved.

4. DISCUSSION

In this study, an attempt was made to identify the predicted neuraxin protein by Western blot analysis. Although four different antibodies reacting with a bacterially expressed neuraxin fusion protein were employed, a polypeptide of about 94 kDa was not found in various subcellular fractions of rat brain and spinal cord. Also, we failed to detect a polypeptide of this size in microtubules isolated at different stages of brain development. Only one of the antibodies, HPA1, which originally was generated by immunization with a peptide derived from the repeat domain of neuraxin, stained polypeptides of about 70 kDa and 30 kDa that were enriched in synaptosomes. The relation of these bands to neuraxin is presently unknown. All antibodies, however, visualized MAP5 in soluble and microtubular fractions. These data are consistent with neuraxin either representing a fragment of MAP5 or being an extremely rare microtubule-associated protein. In favour of the former possibility, an open reading frame extends in the neuraxin cDNA upstream of the predicted translation start site which matches that of mouse MAP5 up to position 12 of the neuraxin nucleotide sequence [4].

Co-assembly of neuraxin with tubulin polymers was found to require taxol. Only in the presence of the drug, incorporation of the neuraxin-MS2 polymerase fusion protein into microtubules was detected. This suggests a low affinity of the observed association. Interestingly, transfection and copolymerization experiments using various deletion constructs have located the microtubule binding domain of MAP5 to a N-terminal basic repetitive region characterized by irregular KKEE(V/I) repeats [6]. This N-terminal domain is not present in the neuraxin sequence [4]. However, an accumulation of basic residues is found in the tail region close to the C-terminus of neuraxin/MAP5. Here, amphipathic α-helical structures characterized by clusters of positive charges and hydrophobic amino acids have been predicted to form potential protein-protein interaction sites [4]. In vivo, this basic tail domain may constitute an additional low-affinity site involved in tubulin binding and may be of potential importance for the cross-linking of filaments. Furthermore, the orientation of tyrosines in the repeat structure of neuraxin/MAP5 suggests that these side chains are involved in hydrophobic interactions [4] similar to those suggested for another microtubule-associated protein, MAP2 [19]. Here, deletion mutants have allowed mapping of the microtubule bundling activity to the hydrophobic C-terminal region of MAP2 [19]. Future experiments of this type should show whether MAPs of unrelated sequence, like MAP2 and MAP5, indeed share a similar topological organization of functional domains.

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