Human neurochordins: identification of several P-epitope-bearing polypeptides and a study of their ontogenetic expression

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Received 24 September 1990; revised version received 9 October 1990

Neurochordins are a family of immunologically related high-M_r neural tissue glycoproteins. After SDS-agarose electrophoresis of human neural tissue extracts, two main neurochordins (A1 and B2) as well as several minor ones (O, A2, A3, B1, B3, C1, C2, D) were vizualized on immunoblots stained with monoclonal antibody At5. Neurochordin expression starts in human embryos before 6 weeks of gestation. General antigenic activity of neurochordins increases between 6 and 24 weeks of gestation while its level does not alter from the second half of gestation up to the age of 11–13 years. Neurochordins extracted from large hemispheres of brain, from cerebellum and spinal cord of a 24-week embryo display a similar pattern after electrophoresis. Partially different pattern of neurochordins was observed with a brain tumor.

High-M_r glycoprotein; P-epitope; Nervous tissue; Restricted tissue specificity; Human embryo

1. INTRODUCTION

P-Epitope was found to be the most immunogenic epitope of chordin, a notochord-specific glycoprotein of giant sturgeon [1]. Immunohistochemical studies performed on several vertebrate species showed that MAb At5 directed against chordin P-epitope strongly crossreacted with an antigen or antigens within neural tissue. In non-neural tissues this reaction was negative or weak [2]. Similar results were obtained in immunochemical experiments when different tissue extracts were tested for the ability to prevent immune complex formation between labelled chordin and At5 [2]. In higher vertebrate species notochord disappears within early embryonic stages and the P-epitope is restricted mainly to neural tissue. In the case of human, chicken and newt brains target antigens for anti-P MAbs were preliminarily characterized by gel chromatography of tissue extracts in the presence of SDS [2]. The antigens distributed like polydisperse macromolecules of M_r about several hundred kDa distinguishing clearly from chordin and therefore were termed 'neurochordins'. After prolonged pronase digestion of human or chicken brain extracts or of giant sturgeon chordin, 3 kDa fragments still capable of binding anti-P MAbs (presumably glycopeptides) were revealed in each case [1-3]. In an attempt to further characterize neurochordins, we have found that they did not enter 7.5% gels during SDS-PAGE [3]. In this paper we describe a convenient technique for electrophoretic analysis of high M_r protein molecules, agarose-gel electrophoresis in the presence of SDS, SDS-AGE. The use of this technique allowed us to identify several discrete neurochordin components in human brain extracts and to compare neurochordins of adult and embryonic brain. The P-epitope antigenic activities were compared in the extracts from brain and spinal cord of human embryos at several developmental stages by a liquid-phase competitive-binding assay.

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2. MATERIALS AND METHODS

Embryos at 17–23 Carnegie stages and 9–10 week embryos resulted from interrupted pregnancies. Older embryos resulted from miscarriages. A 24-week embryo was frozen immediately after the operation, and several hours later brain and spinal cord were excized without thawing. In other cases embryonic, postnatal and adult brains were frozen within several hours post-mortem. A malign oligodendroglyoma sample was described in our previous paper [3]. Brain tissue samples were homogenized in a Dounce homogenizer at 0–4°C with 'homogenation buffer': 0.14 M NaCl, 0.01 M Na-phosphate, 0.01 M EDTA, 0.02% NaN_3, 1 mM phenylmethylsulfonyl fluoride, 1 mM *N*-ethylmaleimide, pH 7.4 (1:4, w/v). In some cases homogenization buffer contained 5% of Triton X-100. Homogenates were centrifuged at 15 000 × *g* for 15 min. Whole brains and spinal cords of early embryos were disrupted with homogenization buffer supplied with 5% of SDS, at 37°C (1:10, w/v). Extracts were obtained by centrifugation of homogenates in an Eppendorf microcentrifuge at 15 000 rpm for 15 min. Submarine electrophoresis was performed on gels of 3% agarose (Sigma, type II-A), 12.2 × 8.2 × 0.2 cm, made in covers from microtiter plates. Gel buffer and electrode buffer was the same: 90 mM Tris-HCl, 90 mM borate, 2 mM EDTA, 0.1% SDS, pH 8. Samples were mixed with 5 × Laemmli sample buffer [4] (1:4) and incubated at 100°C for 2–3 min. 15 μl of sample were placed in each well. The separation proceeded at 50 V first for 30 min, and then at 100 V in a cold-room. For vacuum transfer of proteins onto

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00145793/90/$3.50

nitrocellulose sheets PBS was used (0.14 M NaCl, 0.01 M Na-phosphate, 0.02% NaN_3, pH 7.4). Then nitrocellulose was incubated with PBS supplied with 5% of human serum albumin and normal rabbit globulin at a concentration of 0.5 mg/ml, for 17–20 h at 4°C. MAb At5 [1] was diluted to 10 μg/ml in PBS (without NaN_3) supplied with 0.1% of bovine serum albumin and 0.05% of Tween 20. The same buffer was used for dilution of peroxidase conjugate (goat anti-mouse IgG, Sigma). Peroxidase reaction was carried out with the use of a CND mixture of substrates [5]. In controls, a DEAE-absorbed fraction of normal mouse IgG was substituted for MAb At5. All control blots were blank. Liquid phase competition-binding assay was performed as described elsewhere [3,6].

3. RESULTS AND DISCUSSION

Our first attempts to fractionate human brain extracts on 1.5% agarose SDS-gels resulted in vizualization of slowly migrating diffuse material after staining with anti-P-epitope MAb At5. Discrete bands appeared when 3% gels had been used (Fig. 1). Two main bands were seen in the cases of embryonic and adult brain. These bands were primarily designated as A and B (Fig. 1A). In the next experiments better resolution was achieved by distancing the wells from the edge of gel-supporting covers for not less than 3 cm. As a result two main and usually several minor bands were seen on immunoblots, the last ones were not equally well reproduced in different experiments. In Fig. 1B seven of eight characteristic neurochordin bands are seen (in this case brain extract was obtained without proteinase inhibitors). The following nomenclature is proposed for the revealed neurochordin components: the main, well-

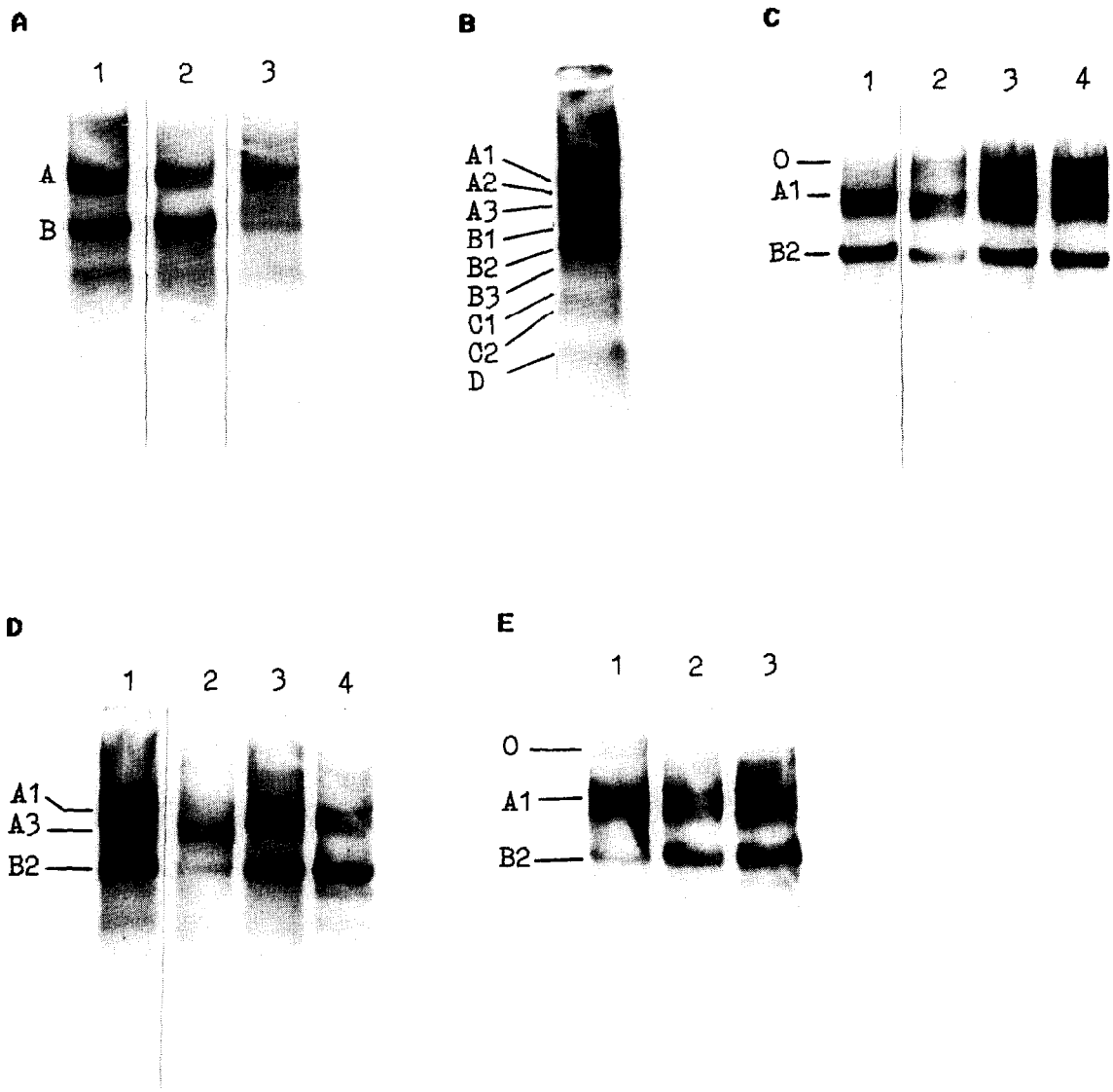

Fig. 1. SDS-AGE and immunoblotting. A_1, embryonic brain, 24 weeks of gestation; A_2, postnatal brain, 4 days; A_3, adult brain, 11 years. (B) Brain at the age of 11 years. (C) A_1, embryonic brain, 37–38 weeks of gestation; B_2, 40 weeks; B_3, postnatal brain, 4 days; postnatal brain, 45 days (Down's syndrome). (D) D_1, embryonic brain, 24 weeks of gestation; D_2, malign oligodendroglyoma; D_3, embryonic brain, 37–38 weeks, homogenization in the buffer supplied with Triton X-100 (10 μl of the sample analysed); D_4, the same but without Triton X-100. (E) 24-week embryo. E_1, brain, lobe part of a large hemisphere; E_2, cerebellum; E_3, spinal cord.

reproducible bands are designated as A1 and B2 and the others as A2, A3, B1, B3, C1, C2, and D. The band nearest to the origin is designated as O (Fig. 1C,E). In Fig. 1C the results of fractionation of embryonic and postnatal brain extracts are shown. Immunostaining patterns of the four blots are almost identical, with characteristic predominance of A1 and B2. Extraction of the brain of a 37–38-week embryo with a buffer supplied with 5% of Triton X-100 and with usual extraction buffer resulted in similar patterns (Fig. 1D, lanes 3 and 4), but in the presence of the detergent weak diffuse staining between and above the main bands was observed. Similar results were obtained with brains of a 24-week embryo and a 45-day newborn (data not shown). In Fig. 1E electrophoretic patterns of extracts from large hemispheres, cerebellum and spinal cord of a 24-week embryo are shown. Characteristic neurochordin components A1, B2 and O are seen. The neurochordin pattern of a malign oligodendroglyoma is distinct from the pattern of normal brain by the predominance of A3 component (Fig. 1D, lane 3). B2 is also present, and A1 is a minor component in this case. Thus, the use of SDS-AGE (3% gels) made it possible to resolve previously polydispersed neurochordin zone into several discrete components. The main components, A1 and B2, are characteristic for brain, cerebellum and spinal cord of 24-week embryos, brain of older embryos, newborns and adults. The detection of A1 and B2 components in different neural tissue samples independent of time duration before freezing, favours the suggestion that these polypeptides are not post-mortem degradation products. As was mentioned above, neurochordins are sensitive to pronase, but P-epitope is not inactivated completely after pronase digestion and can be found in association with relatively low-M_r fragments [2]. In the case of chordin these P-epitope-bearing fragments proved to be glycopeptides with oligosacharides bound to protein through *O*-glycosidic bonds between serine or threonine and *N*-acetylgalactosamine (Likhosherstov and Preobrazhensky, unpublished data). These Ser(Thr)-GalNAc bonds are critical for P-epitope antigenic activity [7]. Consequently, we suggest that neurochordins are also glycoproteins with *O*-bound oligosacharides.

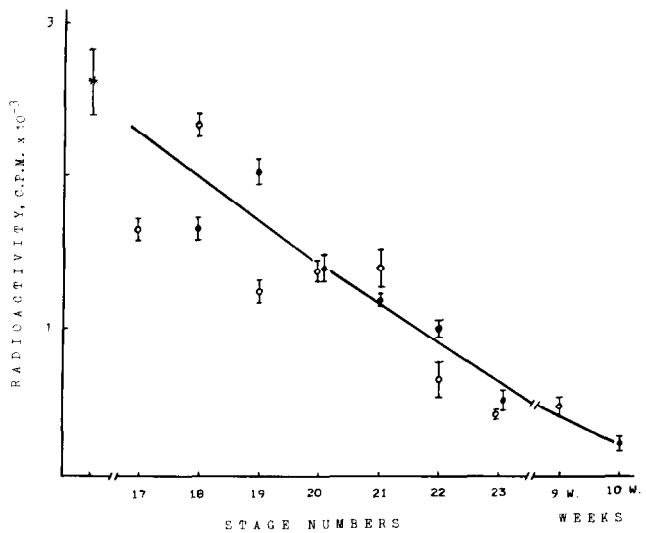

Fig. 2. Inhibition of [^{125}I]chordin-At5 immune complex formation by whole brain and spinal cord SDS-extracts from human embryos. Each point results from analysis of an extract obtained from several brains and spinal cords (except of a 10 week embryo). Asterisk, buffer substituted for extract; open circles, brain; closed circles, spinal cord. The true significance of the determined values lies within the limits shown in the figures with a probability of 95%.

The comparison of the total staining intensity of neurochordins from adult and embryonic brains (Fig. 1A) shows no increase in general neurochordin content from the second half of pregnancy until the age of 11 years. These data were confirmed by liquid-phase competitive-binding assay: extracts from embryonic brains (27–38 weeks of gestation) and from an adult brain (13 years old) caused the same degree of inhibition of immune complex formation between At5 and labelled chordin (data not shown). The same competitive-binding test has been employed to compare the antigenic activity of P-epitope bearing antigens in the brains and spinal cords of human embryos at early stages of development (Fig. 2). At the stages 17–18 (about 6 weeks of gestation) antigenic activity in the extracts is comparatively weak. On the other hand, in 6 week embryos brain and spinal cord are stained well for the P-epitope in immunohistochemical experiments [2]. At later stages the inhibition gradually increases. The data of Fig. 2 as well as the results of blot immunostaining (Fig. 1) show that the bulk of the P-epitope-bearing antigens appears in human embryos within a period between 6 and 24 weeks of gestation.

4. CONCLUSIONS

(i) Human neurochordins are high-M_r P-epitope bearing glycoproteins. Brain and spinal cord contain two main neurochordins designated as A1 and B2, as well as several minor ones.

(ii) While P-epitope expression starts in the neural tissue of human embryos before 6 weeks of gestation, the increase of antigenic activity of P-epitope-bearing antigens in brain and spinal cord of human embryos takes place between 6 and 24 weeks; in brain the content of neurochordins remains approximately at the same level from the second half of gestation until the age of 11–13 years.

Acknowledgement: The authors express their gratitude to Professor A.S. Spirin for constant interest in their work.

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