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CELL FREE SYNTHESIS OF RAT BRAIN MYELIN PROTEOLIPIDS AND THEIR IDENTIFICATION BY IMMUNOPRECIPITATION

A. Dautigny[a], P.M. Alliel[a], J.-L. Nussbaum[b] and P. Jollès[a*]

a) Laboratoire des Protéines (E.R. CNRS N°102 and Unité INSERM U-116)
Université de Paris V, 45 rue des Saints-Pères, F 75270 Paris Cedex 06
and b) Centre de Neurochimie du CNRS, 5 rue Blaise Pascal,
F 67084 Strasbourg Cedex (France)

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SUMMARY. Rat-brain poly(A)-containing RNAs were prepared by affinity chromatography on Poly(U)-Sepharose and translated in the rabbit reticulocyte lysate system in the presence of [^{35}S]-methionine. By using anti-myelin proteolipids antibodies, immunoreactive polypeptides of 23.5 K and 19 K were isolated from the translation products : they had electrophoretic mobilities similar to those of the rat brain myelin proteolipids apoproteins.

The protein moiety of the major proteolipid of the central nervous system (CNS) myelin, which is called PLP or P7 (1) or 25000 component (2) accounts for around 50 % of the total protein content of this membrane (3). Minor myelin proteolipid bands have also been described after polyacrylamide gel electrophoresis including the DM 20 band (4) (also referred as P8M (1) or 20000 component (2)) as well as a few other bands corresponding to higher molecular weights forms of the PLP (1, 2, 5). Partial sequence data have shown that the PLP and the DM 20 proteins have similar N- and C-terminal sequences (6). A number of studies by our group and others have been devoted to the determination of the primary sequence of the PLP from different origins (7-14).

The elucidation of the molecular mechanisms of myelin biogenesis and the regulation of myelination awaits further understanding of myelin proteins

*To whom all correspondence should be addressed.

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synthesis. So far, only a few reports have been published on the synthesis of rat myelin basic proteins using a cell-free system programmed with brain messenger RNA (15, 16). In this communication, evidences that the apoproteins corresponding to the PLP and DM 20 bands are translated in a cell-free mRNA dependent system will be presented as well as their characterization by immunoprecipitation with specific anti-myelin proteolipids antibodies.

MATERIALS AND METHODS

All reagents were obtained from usual commercial sources in the highest available degree of purity.

Purification of anti-myelin proteolipids antibodies. The myelin proteolipids antigens (PLP + DM 20) were prepared from a total proteolipid extract of whole rat brain by gel filtration on methylated Sephadex G-100. The purified proteolipid proteins were converted into a water soluble form (17) and then coupled in the presence of 1 % (v/v) Triton X-100 to cyanogen bromide activated Sepharose 4B (Pharmacia). The antibodies were isolated from the immune serum of a rabbit inoculated with purified myelin (18). 1 ml of immune serum was diluted twofold with phosphate buffered saline (PBS) and Triton X-100 was also added to a final concentration of 0.1 % (v/v). This solution was applied to the proteolipid-Sepharose column (0.8 x 10 cm), which was then washed with 100 ml of PBS containing 1 % Triton X-100 (v/v) and with 50 ml of PBS. The bound anti-myelin proteolipids antibodies were eluted with a glycine 0.1 M HCl buffer, pH 2.8, containing 4 M urea ; this solution was rapidly adjusted to pH 7.0 with Tris and dialyzed against PBS for two days with several changes. The solution containing the antibodies was then lyophilized, dialyzed once against PBS and adjusted to a known volume.

Electrophoretic blotting. The purity of the antibodies was tested by immunoblotting experiments using either total brain proteins, pure myelin or a total brain proteolipid extract as antigens. Proteins were separated by SDS-polyacrylamide gel electrophoresis in 2 mm thick slab gels consisting of a linear gradient of acrylamide (10-20 %). The gel preparation as well as all other experimental parameters were previously described (1). After electrophoresis, the proteins were electrophoretically transferred onto a nitrocellulose sheet (0.45 µm pore size Millipore Corp., Bedford, MA) under a 300 mA current for 90 min (19), in the presence of a filter paper soaked in 1 % (w/v) SDS applied on the cathodal side of the gel. The nitrocellulose was then incubated overnight in PBS containing 3 % bovine serum albumin (BSA). The blot was rinsed once and incubated in PBS containing 3 % BSA and 2.5 % normal sheep serum with appropriate dilution of anti-myelin proteolipids antibodies for 2 h. The sheet was then washed with several changes of PBS and incubated thereafter for another 2 h with horseradish peroxidase labeled sheep anti-rabbit IgG antibodies (diluted 1/1000) (Institut Pasteur Production) in PBS containing BSA and normal sheep serum as above. After a thorough washing in PBS, antibody binding was detected by incubation with 4-chloro-1-naphtol (0.018 % w/v) (Merck) in PBS containing 0.02 % (v/v) hydrogen peroxide (30 %, w/w) (20). Total proteolipids from whole adult rat brain myelin were prepared as previously described (1). Proteins were estimated by the method of Lowry et al. (21) with BSA as a standard.

Isolation of brain mRNA. Total cytoplasmic brain RNA of 15 day-old rats (Sprague Dawley) was extracted by the guanidine-thiocyanate

procedure described by Kaplan et al. (22). Poly(A)-RNA was prepared according to Shapiro et al. (23) using poly(U)-Sepharose (Pharmacia). Routinely 1 g of tissue yielded 20-30 µg of poly(A)-RNA.

Translation of Poly(A)-RNA in a cell-free translation system and analysis of the translation products. Poly(A)-RNA (0 to 2 µg) was incubated for 60 min at 37°C. Translation of mRNA in a rabbit reticulocyte lysate was performed as described by Pelham and Jackson (24) using a New England translation kit. [^{35}S]-methionine was used at 50 µCi per 25 µl total incubation mixture. The assay of amino acid incorporation is described in the instruction brochure accompanying the translation kit. After translation of 2 µl poly(A)-RNA (0.8 µg/µl) in 23 µl of supplemented lysate, an aliquot was removed for polyacrylamide gel electrophoresis. To the remainder was added 1 volume of 0.15 M NaCl, 1 mM EDTA, 0.1 % NaN_3, 1 % Nonidet P-40, 0.5 % Na deoxycholate, 50 mM Tris-HCl buffer, pH 8.3, containing BSA at 1 mg/ml. Specific antigen-antibody complexes were precipitated with *Staphylococcus aureus* Cowan I (Pansorbin-Calbiochem) as described by Kessler (25). Pre-clearing was performed with 20 µl of a 10 % suspension of formalin-fixed *Staphylococcus aureus* ; after incubation for 30 min at 4°C under stirring, the solution was centrifuged (12,000 g for 5 min). 10 µl anti-myelin proteolipids purified antibodies were added to the supernatant and incubated overnight at 4°C. After addition of 20 µl of a 10 % suspension of formalin-fixed *Staphylococcus aureus* and incubation for 20 min at 4°C, the mixture was layered over a 1 ml cushion of sucrose (30 % w/v sucrose in the buffer saline solution : 500 mM NaCl, 2 mM EDTA, 1 % Nonidet P-40, 0.5 % Na deoxycholate, 0.1 % NaN_3, 50 mM Tris-HCl, pH 8.3) and centrifuged for 5 min (12,000 g). This procedure was repeated three times. The bacteria antigen-antibody complexes were resuspended in 0.3 ml of a 0.1 % SDS, 50 mM NaCl, 2 % Nonidet P-40, 0.125 M Tris-HCl buffer, pH 6.8 and centrifuged again. The resulting pellets were suspended in 40 µl of a sample buffer (2 % SDS, 50 % v/v glycerol, 0.125 M Tris-HCl, pH 6.8), treated at 90°C for 10 min and centrifuged as above. The supernatants were subjected to electrophoresis in 15 % polyacrylamide slab gels as described by Laemmli (26). For quantification, trichloroacetic acid (TCA) precipitable radioactivity was measured with 3 µl aliquots. Fluorography was performed as described by Bonner and Laskey (27). Gels were processed for fluorography and exposed for 2-10 days on a Kodak X-Omat R film.

RESULTS AND DISCUSSION

The specificity of the anti-myelin proteolipids antibodies purified by affinity chromatography from whole anti-myelin serum was assessed by the sensitive method of immuno-electroblotting. Figure 1 shows that after incubation of a gel electroblot of rat myelin proteins with the anti-myelin proteolipids antibodies, only the PLP and the DM 20 proteins bands were detected ; occasionally an additional immunoreactive band, which probably corresponds to a higher molecular weight form of PLP, was also observed. More immunoreactive bands in the high molecular weight region of the gel electroblot were detected when total proteolipids were investigated under similar conditions. This may be due to the formation of multiple stable aggregates of PLP as a result of the va-

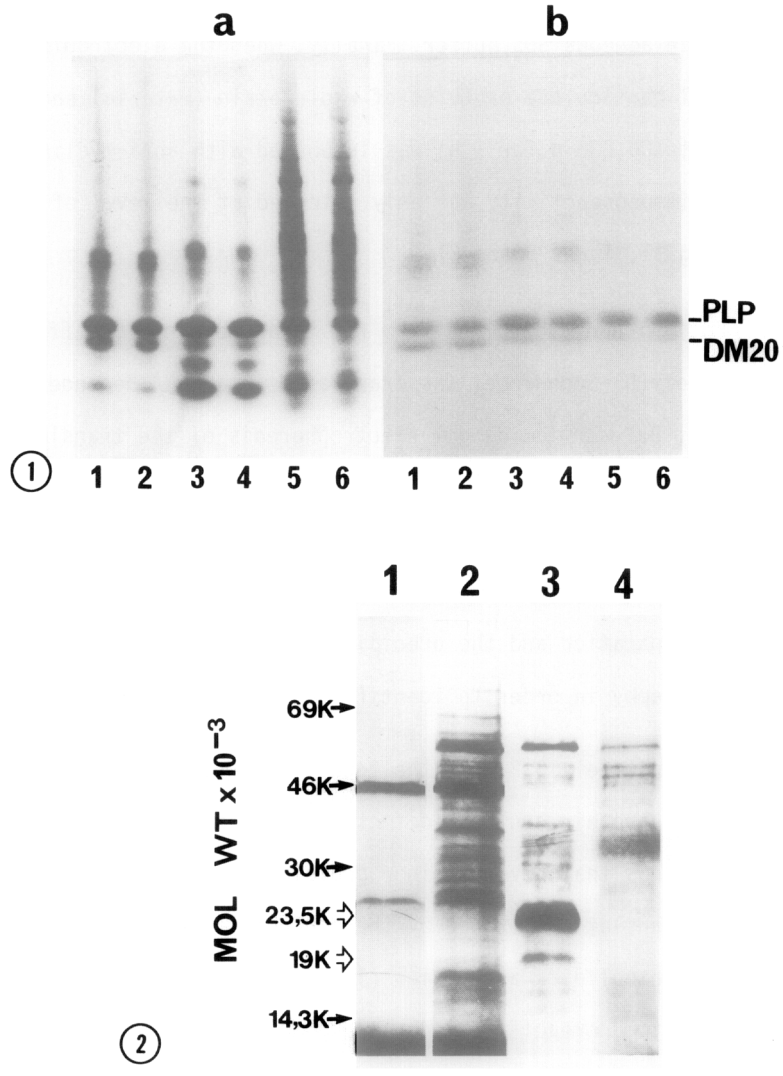

Figure 1 :Binding of anti-myelin proteolipids antibodies, purified by affinity chromatography, to gel electroblots of proteolipids from whole adult rat brain, myelin proteins and total particulate brain proteins. (a) Strip of stained gel (10-20 % acrylamide) : lanes 1 and 2 contain 60 µg and 48 µg of proteolipids of whole rat brain, respectively ; lanes 3 and 4 contain 40 µg and 25 µg of myelin proteins, respectively ; lanes 5 and 6 contain 76 µg and 57 µg of total particulate brain proteins, respectively. (b) Nitrocellulose electroblot incubated with anti-myelin proteolipids antibodies (dilution 1/500).

Figure 2 : *In vitro* translation of total poly(A)-RNA from 15 day-old rat brain and identification of the myelin proteolipids by immunoprecipitation. Fluorograms of the reticulocyte lysate cell-free reaction products after electrophoresis on 15 % polyacrylamide gels. Lane 1 : fluorogram of the reaction products obtained in the absence of exogenous poly(A)-RNA ; lane 2 : fluorogram of the reaction products directed with 1.6 µg rat brain poly(A)-RNA ; lane 3 : fluorogram of the immunoprecipitate by anti-myelin proteolipids antibodies from the cell-free translation products programmed with 1.6 µg rat brain poly (A)-RNA ; lane 4 : same as lane 3, except that non-immune antibodies were used.

rious treatments of the proteolipids, namely their extraction, purification and solubilization in aqueous SDS buffer. Finally, when the electroblot corresponding to the total particulate proteins of whole brain (material sedimented in 0.32 M sucrose at 100,000 g for 1 h) was incubated with anti-myelin proteolipids antibodies, immunoreactivity was only detected at the level of the PLP and DM 20 bands (Fig. 1, lanes 5 and 6).

Poly(A)-containing RNA from rat brain obtained by affinity chromatography on Poly(U)-Sepharose, was translated in a mRNA-dependent rabbit reticulocyte lysate. Polyacrylamide gel electrophoresis of the translation products revealed, after fluorography, the presence of several labeled proteins with molecular weights ranging from 15 K to 100 K (Fig. 2, lane 2). The translation products were purified by immunoprecipitation with an anti-myelin proteolipids antibodies preparation and the precipitate was subjected to gel electrophoresis and fluorography in order to identify the immunoreactive substances.

Approximately 0.2 % of the total [^{35}S]-methionine incorporated into TCA-precipitable protein could be detected in the immune precipitable material. Figure 2 shows that several minor proteins were non specifically precipitated from the translation mixture by the control serum (lane 4) as well as by the anti-myelin proteolipids antibodies (lane 3). However, the sample in lane 3 shows a major immunoreactive band corresponding to a molecular weight of 23.5 K and a faint band corresponding to a molecular weight of 19 K. In contrast, control serum failed to bind any of these two protein bands. These values are in agreement with the previously reported molecular weights of ca. 24 K for PLP and 20 K for DM 20 (1, 2, 4). The present data represent the first evidence for a *de novo* cell-free synthesis of these proteins.

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