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Direct measurement of inositol in bovine myelin basic protein

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Myelin basic protein has been isolated from bovine central-nervous-system myelin by four methods, none of which exposes the protein to acid. After purification the inositol content of both hydrolysed and unhydrolysed protein was quantified by g.c.-m.s. Basic protein prepared by all methods contained less than 4 mol % of inositol. It is concluded, contrary to a previous proposal, that covalent binding to phosphoinositides does not represent a general mechanism for attachment of this cytoplasmically-oriented protein to its membrane.

INTRODUCTION

Several cell surface proteins, including Thy-1 antigen, alkaline phosphatase, acetylcholinesterase, and variant-surface glycoproteins of *Trypanosoma brucei* are linked covalently to phosphatidylinositol, which has been shown to anchor these proteins to their membranes [1,2]. It has been reported [3,4] that a cytoplasmically-oriented protein, bovine myelin basic protein (MBP), is covalently bound through a phosphodiester linkage with serine-54 to phosphatidylinositol bisphosphate. Yang and coworkers arrived at their conclusion from the observation that, following addition of radioactive ATP to myelin, a ^{32}P-labelled peptide from MBP could be isolated which had hydrophobic characteristics until treated with phospholipases C or D, or with strong acid or alkali. It has been suggested [3] that the method routinely used to prepare MBP, which includes extraction of a chloroform/methanol pellet of brain at pH 1.6–1.7, would cleave the lipid–protein bond leaving only the phosphate attached to the protein, though this would imply an unusually acid-labile phosphodiester linkage.

Yang *et al.* [3,4] provided considerable evidence in favour of their hypothesis, but since phosphoinositides were only identified by radioactivity they were unable to assess the proportion of MBP molecules bearing phosphatidylinositol. Clearly, any interpretation of the functional role of covalently-attached lipid is dependent on this stoichiometry. The phosphatidylinositol could be linked to most MBP molecules, or to a small subset of MBP molecules: if the latter were the case it would argue againt a general anchoring role for this lipid.

Here we report the isolation of MBP from myelin by four methods, none of which employs acid or alkaline conditions. After purification the MBP was analysed for free and covalently-bound inositol by a sensitive g.c.–m.s. method.

EXPERIMENTAL

Preparation of MBP

Method A. MBP was extracted from the acetone powder of brain using a variation of a method developed for the purification of myelin 2′,3′-cyclic nucleotide 3′-phosphodiesterase [5]. Bovine brain acetone powder (20 g) was washed with buffer and then extracted with 1 M-guanidinium hydrochloride in 0.2 M-Tris/HCl, pH 7.5. The guanidinium hydrochloride concentration was reduced by dialysis against phosphate buffer as described by Drummond *et al.* [5], then the protein, in 50 mM-sodium phosphate/1 mM-EDTA/1 mM-dithiothreitol/5% glycerol, pH 6.8, was concentrated by ultrafiltration through an Amicon YM-30 membrane (Amicon, Lexington, U.S.A.), rather than by precipitation with $(NH_4)_2SO_4$. The concentrated protein was applied to a 3 cm × 35 cm column of carboxymethyl Sepharose CL-6B (Pharmacia, Uppsala, Sweden) in the above buffer and eluted with a 0–1.0 M-NH_4Cl gradient. The basic protein eluted last as a prominent, well-separated peak as shown in Fig. 1 of Drummond *et al.* [5] (though with better resolution in our experiments). After reconcentration by ultrafiltration the protein was finally purified by gel permeation chromatography on a 1.5 cm × 40 cm column of Sephadex G-100–40 equilibrated with 20 mM-Hepes/0.15 M-NaCl, pH 7.4. Fractions from this column were analysed by SDS/polyacrylamide-gel electrophoresis, [6]; those containing only basic protein were dialysed at 4 °C against several changes of water, and then freeze-dried.

A portion (2 mg) of the freeze-dried protein was extracted with 3 × 5 ml of chloroform/methanol (1:1, v/v) for 24 h at room temperature and the insoluble residue was dried under vacuum.

Method B. The second method took advantage of the

Abbreviations used: MBP, myelin basic protein; TMS, trimethylsilyl.

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observation (R. Smith & P. E. Braun, unpublished work) that incubation of myelin with the phospholipase C (EC 3.1.4.3.) from *Bacillus cereus* releases a significant proportion of the basic protein. Bovine central-nervous-system myelin was prepared essentially by the method of Autilio *et al.* [7]. Myelin suspension containing 30 mg of protein was incubated with 500 units of phospholipase C in 4.0 ml of 20 mM-Hepes buffer, pH 7.4. After incubation at 37 °C for 1 h the myelin was sedimented at 16000 *g* in a microfuge for 7 min, and the supernatant was drawn off. The pellet was incubated with a further 200 units of enzyme for another 10 h. The combined supernatants containing 4.5 mg of protein (including about 0.3 mg of enzyme) were concentrated by ultrafiltration (see above) and applied to the Sephadex G-100 column described above. Fractions containing only basic protein were dialysed against water and freeze-dried.

Method C. The third method was an outcome of our recent observations (R. Smith & P. E. Braun, unpublished work) that washing myelin with $CaCl_2$ solutions also liberates much of the basic protein. The purification followed in essence that described in B, except that the extraction was performed with 200 mM-$CaCl_2$ in 20 mM-Hepes buffer, pH 7.4, at 37 °C for 1 h. The myelin residue was twice re-extracted under the same conditions and the supernatants pooled before gel permeation chromatography on Sephadex G-100.

Method D. Finally, lipid was extracted from purified myelin twice using ether/ethanol, ether and then acetone, following the method of Yang *et al.* [3]. The residue was dissolved in buffer containing SDS and then electrophoresed on an SDS/polyacrylamide (12%) gel. Part of the gel was stained to locate the MBP band and the corresponding region of the unstained gel cut out. Slices from two gels were pulverized in a warm 1% SDS solution; this solution was freeze-dried and the residue taken up in a small volume of water before precipitation of the protein with acetone. The precipitate was washed three times with ice-cold acetone to remove remaining SDS.

The purity and identity of the basic protein from each of these isolation procedures, except method D, was verified by gel electrophoresis and by electroblotting, using a well-characterized rabbit antibody against bovine MBP [8]. A sample of the protein prepared by method D was re-electrophoresed to ensure that only MBP had been excised from the original gel.

Inositol analyses

Inositol analyses were performed according to the method of Sherman *et al.* [9]. Samples were freeze-dried and then hydrolysed in 6 M-HCl at 110 °C for 24 h after addition of an internal standard (deuterated *myo*-inositol C-d6 from MSD Isotopes, Montreal, Canada, for samples A and B, or *scyllo*-inositol from Calbiochem, San Diego, CA, U.S.A., for samples C and D). The hydrolysates were dried repeatedly from water to remove HCl and reacted with 5% trimethylsilyl chloride, 45% *NO*-bis(trimethylsilyl)trifluoroacetamide in anhydrous pyridine for at least 24 h at room temperature. Portions were analysed by g.c.–m.s. using electron impact and selected-ion monitoring for the characteristic ions $[TMSOCH{=}C(OTMS){-}CH{=}OTMS]^+$, m/z 305 and $[TMSO{=}CHCH{=}C(OTMS)CHOTMS]^+$, m/z 318. Where deuterated *myo*-inositol was used as an internal standard the ion $[TMSOCD{=}C(OTMS){-}CD{=}OTMS]^+$, m/z 307 was also monitored [9,10]. Preliminary analyses without internal standards of preparations C and D showed an absence of *scyllo*-inositol.

RESULTS AND DISCUSSIONS

The homogeneity of the basic protein prepared by method B is demonstrated in Fig. 1; the other three methods gave MBP of equivalent purity. Electroblots probed with anti-MBP revealed one intense band, corresponding in position to the major dye-stained band, in addition to the usual, weaker MBP dimer band.

The proportion of protein extracted by method A is small: approx. 300 mg of protein was extracted from 20 g of acetone powder which initially contained 7 g of protein, giving an overall MBP yield of 6%. The recovery of MBP from the other methods was considerably higher. Phospholipase C released approx. 50% of the MBP, as assessed by scanning stained gels on which samples of myelin before and after extraction had been electrophoresed. Repetitive washing with 200 mM-$CaCl_2$ removes more than 90% of the MBP from myelin. Comparable washing with buffer alone removes negligible amounts of MBP. Based on the protein content of the myelin that was extracted, the MBP recovered using method D was 8% and > 50% in the two preparations.

The results of analyses for *myo*-inositol are given in Table 1. It is evident that the inositol contents are very small. The inositol detected in unhydrolysed samples represents a background of contaminating free inositol,

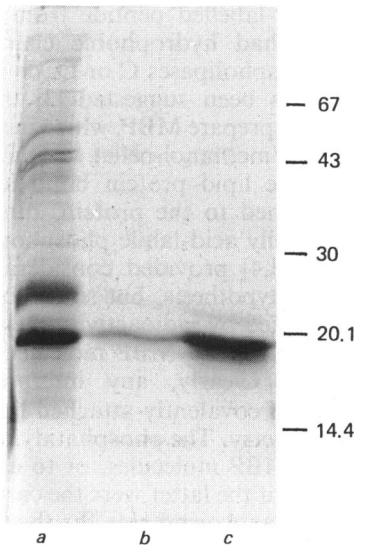

Fig. 1. Purity of basic protein used for inositol analyses

SDS/polyacrylamide (12%) gel stained with Coomassie Brilliant Blue R250. *a*, Whole myelin (approx. 30 μg of protein); *b* and *c*, successive fractions from a gel permeation column (Sephadex G-100) to which MBP prepared by method B (see Experimental section) had been applied. The positions and molecular masses, in kDa, of standard proteins are indicated on the right. The protein in lane *c* was used for inositol analyses. MBP prepared by the other methods was of equivalent purity.

Table 1. Analyses of MBP for inositol

Samples of myelin basic protein, prepared by methods described in the Experimental section, were analysed for inositol by g.c.-m.s. After addition of the standard inositol each sample was divided into two equal parts, one of which was hydrolysed before derivatization. Protein concentrations were measured colorimetrically [15]. The concentration of the standard MBP solution was calculated assuming an $A_{280,1\,cm}$ of 5.44 for a 1% (w/v) solution. Molar ratios were calculated after averaging the figures and subtracting the inositol content of unhydrolysed samples: a molecular mass of 18400 was assumed for MBP [16]. The results for methods A1, A2 and B are from replicate analyses on single preparations. For methods B and C results of analyses on two preparations are given separately.

Protein purification method	Protein content of sample (μg)	*myo*-Inositol content (ng)		*myo*-Inositol /MBP molar ratio
		Hydrolysed sample	Unhydrolysed sample	
A1	320	3.7, 3.7, 3.3	2.8, 3.5	1.3×10^{-4}
A2	320	1.7, 2.1, 1.6	1.3, 1.2	1.6×10^{-4}
B	104	14.8, 14.9	8.9	5.8×10^{-3}
C	1500	13.9, 9.5	4.7, 4.7	9.5×10^{-4}
	3000	91.0	79.2	4.0×10^{-4}
D	80	41.4, 42.3	16.2, 16.9	3.2×10^{-2}
	840	131	0	1.6×10^{-2}

which may be subtracted from the values obtained with hydrolysed inositol samples, to obtain the amount of inositol released by hydrolysis and presumably attached to the protein or some component co-purifying with the MBP. Assuming a M_r for MBP of 18400 it can be calculated that there is less than 1.6×10^{-4} mol of inositol/mol of protein prepared by method A. Extraction of this protein with chloroform/methanol (method A2) diminished the inositol content but had little effect on the amount of inositol released by hydrolysis, suggesting that this treatment removed free inositol. MBP obtained by the other methods also released little inositol (Table 1). The highest level was found in MBP extracted from the SDS gel, but the 0.016–0.03 mol of inositol/mol of MBP may include inositol associated with molecules that co-migrate with this protein during electrophoresis: retention of lipids by MBP through a variety of purification methods has previously been reported [11]. Thus, only a very small proportion of the MBP molecules, if any, could contain phosphatidylinositides.

The MBP prepared using a non-specific phospholipase C (method B) did contain some *chiro*-inositol (data not shown); the unhydrolysed protein had 3.8 ng and the hydrolysed protein 34 ± 1 ng/104 μg of protein. This corresponds to 0.01 mol of *chiro*-inositol/mol of MBP, after allowing for free inositol. *chiro*-Inositol has also been observed as being associated with other proteins but it is not known whether it is covalently linked [10,12].

The mechanism by which the non-specific phospholipase C solubilizes the MBP is unknown. However, if MBP is attached to the membrane via ionic interactions with phospholipid polar head groups then solubilization may simply be a result of their removal by the phospholipase C. Although phosphatidylinositol-specific phospholipase C has been reported to contaminate commercial phospholipase C preparations and is capable of releasing many proteins from membranes by hydrolysing the phosphatidylinositol anchor [1], we have no evidence that such an activity is responsible for the release of MBP.

These results indicate that the majority of the extracted myelin basic molecules do not contain phosphatidylinositides and they are therefore inconsistent with a phosphatidylinositol moiety acting as a membrane anchor for MBP. The possibility does remain that MBP is linked to phosphatidylinositol but that scission of this bond occurs during the extraction procedures used in the current experiments despite the fact that none of the methods uses acid or alkaline conditions. Rapid phospholipase C-mediated hydrolysis of protein–phosphatidylinositol bonds has been observed in trypanosomes [2], but if such hydrolysis did occur a phospholipase C would leave the inositol attached to the MBP, and it would therefore be detected in our analyses. Other explanations, such as a rapid post-mortem hydrolysis by a phosphodiesterase acting between the inositol ring and the protein cannot at present be excluded, but to our knowledge such activities have not yet been described. It is relevant to note that investigations of the incorporation of radiolabelled fatty acids into myelin proteins demonstrated attachment of fatty acids to the proteolipid protein, but not to the basic protein [13,14], supporting the present conclusions.

The results of Yang *et al.* [3,4] could arise from a small minority of phosphoinositide-containing MBP molecules which are rapidly labelled by phosphorylation of an exposed hydroxy group on the inositol ring. Such phosphorylation may itself have significance for the expression of MBP function; our results do not exclude this possibility.

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