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Expression of recombinant forms of human 21.5 kDa myelin basic protein and proteolipid protein in CHO cells

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MBP and PLP are major structural protein components of myelin. Both proteins play a functional role in formation of myelin sheath and in maintenance of its compaction. Immune responses to MBP and PLP have been implicated in the pathogenesis of multiple sclerosis (MS), an auto-immune disease of the central nervous system. Recombinant forms of both proteins isolated and purified from bacterial or insect cell systems are commonly used to study the specificity of auto-response in MS. We have prepared recombinant forms of MBP and PLP stably expressed in CHO cells. Several clones with proper cytoplasmic MBP or surface PLP localization were obtained and characterized by flow cytometry and indirect immunostaining. CHO cells expressing the recombinant forms of MBP and PLP can be very useful in studies on the autoimmune mechanism of MS.

Keywords: recombinant MBP and PLP, expression in CHO cells

MBP and PLP are major components of myelin sheaths accounting for about 30 and 50% of total myelin protein, respectively (Schmidt, 1999). Human MBP exists in four main isoforms (17.3, 18.5, 20.2, 21.5 kDa) generated by alternative splicing (Roth *et al.*, 1987). The 21.5 kDa isoform encoded by seven exons occurs early in myelin formation or during remyelination (Nye *et al.*, 1995). The major classic 18.5 kDa form, in which exon 2 is deleted, is most abundant in mature myelin. The polypeptide chain of the human 18.5 kDa MBP consists of 180 amino acid (aa) residues including many basic ones (12 Lys and 19 Arg). Post-translational modification of arginine residues to citrulline can create additional microheterogeneity of MBP molecules, giving rise to charge isomers (C1–C8) (Wood & Moscarello, 1989). Recently, using three-dimensional reconstructions obtained by electron microscopy, it has been proposed that MBP is a flexible, C-shaped molecule with β-sheet core structure (Ridsdale *et al.*, 1997a; 1997b). However, recent molecular dynamics simulations of models of several forms of MBP have revealed that the protein was observed, in fact, extended and flat within the myelin sheath. Considerable formation of short α-helices, which would be stabilized in the presence of lipids and which could serve to anchor the protein in the myelin membrane (Bates & Harauz, 2003). Indeed, it has been shown that human MBP region comprising aa residues 85–96 can form an amphipathic α-helix responsible for lipid bilayer anchoring (Bates *et al.*, 2003; 2004).

PLP is a highly hydrophobic integral membrane protein of oligodendrocytes (Stoffel *et al.*, 1984). Two isoforms encoded by one gene comprising seven exons are generated by alternative splicing: a 26.6 kDa classic form and a 23.5 kDa DM20 protein (Diehl *et al.*, 1986; Kronquist *et al.*, 1987). The polypeptide chain of human PLP consists of 277 aa residues, forming four hydrophobic transmembrane domains and five external hydrophilic regions (Stoffel *et al.*, 1984). The DM20 isoform is shorter due to deletion of 35 aa residues encoded by exon 3 (Nave *et al.*, 1987). PLP and DM20 are posttranslationally acylated by covalent attachment of long fatty acids to cysteine residues *via* thioester linkages. There are six acylation sites in PLP and Cys 108 is the major one (Weimbs & Stoffel, 1992). The PLP/DM20 isoproteins together with MBP are responsible for myelin compaction resulting in an increased electrical conductance of the tightly wrapped axons (Boison & Stoffel, 1994).

Abbreviations: CHO, Chinese hamster ovary; CSF, cerebrospinal fluid; EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; MBP, myelin basic protein; PBS, phosphate-buffered saline; PLP, proteolipid protein.

MBP and PLP are the most extensively studied candidate auto-antigens in MS (Schmidt, 1999; Jaskiewicz, 2004). Both myelin proteins are strong encephalitogens in many species, causing EAE — an animal model of MS. MBP- and PLP-specific T cells can be readily isolated from CSF or peripheral blood of MS patients. Moreover, increased numbers of B cells secreting anti-MBP and anti-PLP antibodies have been demonstrated in CSF of MS patients (O'Conor *et al.*, 2001).

MBP and PLP purified from human myelin or obtained as recombinant proteins from bacterial or insect cells (Nye *et al.*, 1995; Fukuzono *et al.*, 1998; Bates *et al.*, 2000) are used in numerous studies to characterize the specificity of anti-MBP and anti-PLP auto-response in MS (Jaskiewicz, 2004). Here we describe isolation and characterization of CHO cell clones with stable expression and proper cellular localization of recombinant MBP or PLP. The rationale for using a mammalian system is the posttranslational modification of the recombinant proteins expressed in CHO cells. The obtained clones could be a very useful tool for characterization of immuno-response in MS.

MATERIALS AND METHODS

Construction of MBP/pcDNA3 and PLP/pcDNA3 expression vectors. cDNAs encoding the 21.5 kDa isoform of human MBP in Bluescribe cloning vector and the 26.6 kDa classic form of human PLP in Bluescript vector were kindly provided by Dr. C. Campagnoni (University of California, Los Angeles, CA, USA). Both cDNAs were excised with *Eco*RI restriction enzyme from the cloning vectors provided and inserted into *Eco*RI cloning site of pcDNA3 eukaryotic expression vector (Invitrogen), giving the MBP/pcDNA3 and PLP/pcDNA3 vectors.

Cell culture and transfection. Wild type CHO cells were cultured in OPTI-Mem (Gibco BRL) containing 10% fetal calf serum (Gibco BRL) and 2 mM glutamine (Sigma Chemical Co.). Stable transfection of CHO cells with MBP/pcDNA3 or PLP/pcDNA3 was performed in serum-free medium using FuGENE 6 reagent (Roche Diagnostic) according to the manufacturer's protocol. Transfected CHO cells were selected in complete OPTI-Mem medium containing 0.4 mg/ml active geneticin (G418, Gibco BRL) and analysed for MBP or PLP expression by flow cytometry. Clonal cell lines expressing MBP or PLP recombinant proteins were isolated by repetitive cloning by limiting dilution.

Flow cytometry analysis. The transfected CHO cells detached with 0.2% EDTA in Hank's balanced salt solution, pH 7.4, were washed with cold phosphate-buffered saline, pH 7.4, and then fixed and permeabilized with 0.1% saponin and 2% *p*-formaldehyde solution in PBS for 15 min at 4°C. After washing with PBS the cells were incubated for 30 min at 4°C with mouse monoclonal anti-MBP (MAB387, Chemicon International) or anti-PLP (clone plpc 1, Serotec Ltd) antibodies. Incubation with fluorescein-conjugated rabbit anti-mouse immunoglobulins antibody (Dakopatts) was performed for 30 min at 4°C with intervening washing with PBS. Directly after labeling the cells were analyzed for fluorescence intensity using a flow cytometer (FACSCalibur, Becton Dickinson).

Indirect immunofluorescence. Cells were grown on ethanol-sterilized cover slips for 24 h, rinsed three times with PBS at 4°C and then fixed and permeabilized for 15 min at room temp. in 3% *p*-formaldehyde in PBS and for 20 min in 0.1% Triton X-100 in PBS. After washing with PBS the cells were incubated with a primary antibody (either anti-MBP or anti-PLP) and a secondary antibody (Cy^{TM} 3-conjugated goat anti-mouse IgG, Jackson ImmunoResearch Lab. Inc.) for 45 min, with intervening washing with PBS. Cover slips were mounted in complete OPTI-Mem medium, sealed with nail polish and examined using a confocal scanning microscope (BioRad MRC 1024).

Polymerase chain reaction analysis (RT-PCR). The presence of MBP/pcDNA3- or PLP/pcDNA3-specific message was evaluated by PCR of cDNA generated by reverse transcription of RNA prepared from chosen clones of CHO cells. The following primers were used: sense 5'CGCTCTGGATCACCCATGGC3' within exon 7 of MBP cDNA and 5'CATGGGCCGAGGCACCAAGT3' within exon 7 of PLP cDNA; antisense 5'TAGATGCATGTCCGAGCGGC3' within the multiple cloning site of pcDNA3 vector. PCR conditions were 94°C for 1 min, 56°C for 1 min and 72°C for 1 min for 35 cycles.

RESULTS AND DISCUSSION

Transfection of CHO cells and isolation of stable clones

The MBP/pcDNA3 or PLP/pcDNA3 plasmid was stably transfected into CHO cells. After fixation and permeabilization transfected cells were analyzed for MBP or PLP expression by flow cytometry. Following transfection approx. 87% and 89% of geneticin-resistant CHO cells were positive for MBP and PLP expression, respectively. From both transfected CHO cell populations several stable MBP or PLP expressing cell clones were isolated by limiting dilution and characterized in flow cytometric analysis using anti-MBP or anti-PLP monoclonal antibodies (Fig. 1).

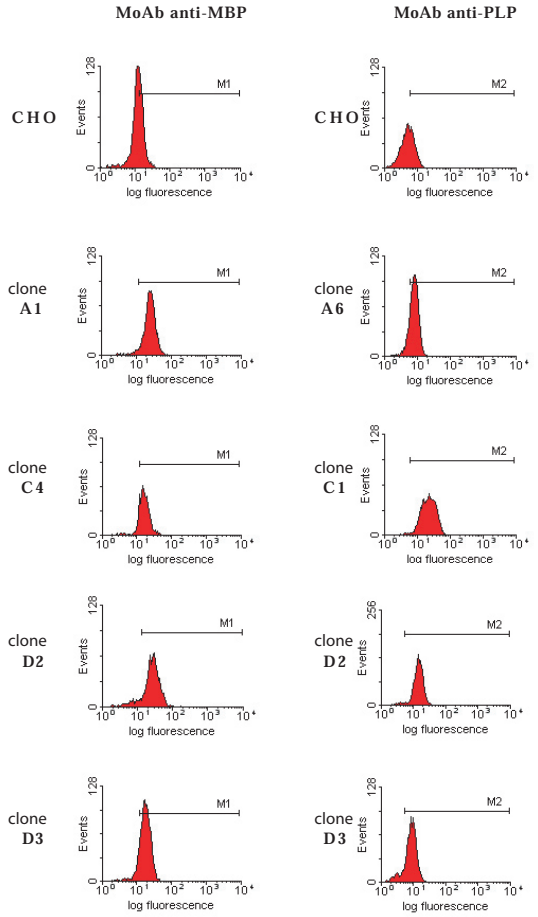

Figure 1. Stable CHO clones expressing recombinant forms of MBP or PLP characterized by flow cytometry using monoclonal anti-MBP or anti-PLP antibodies.
Untransfected CHO cells served as control.

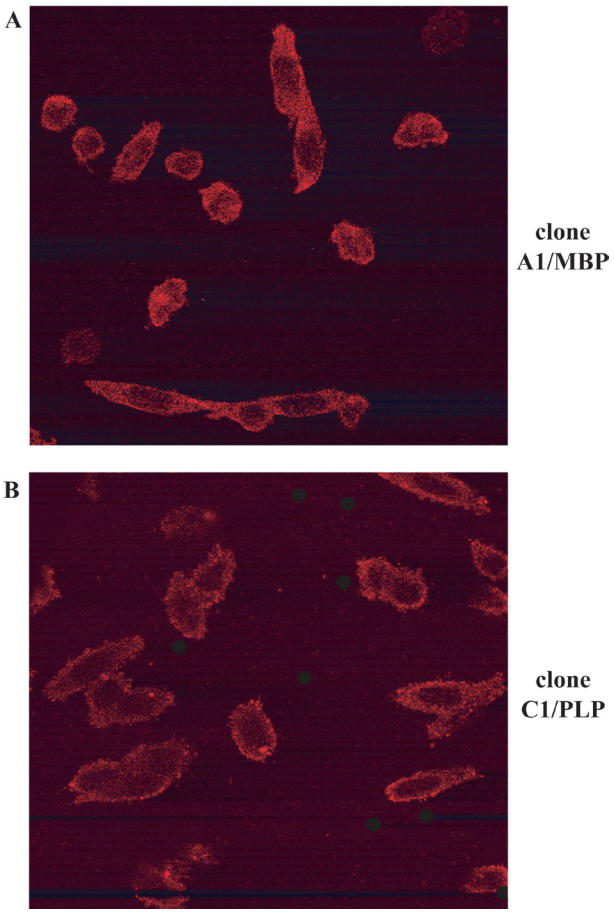

Figure 2. Cellular localization of recombinant forms of MBP and PLP in CHO cell clones analyzed in confocal scanning microscopy (magnification 40×).
Cells cultured for 48 h were fixed and permeabilized and then stained with antibodies: A, anti-MBP, clone A1/MBP; B, anti-PLP, clone C1/PLP and then with Cy-3-conjugated goat anti-mouse Ig. There was no detectable staining of untransfected CHO cells (not shown).

Immunolocalization of recombinant forms of MBP and PLP in CHO cells

Two CHO cell clones A1 and C1 expressing recombinant MBP and PLP, respectively, were subjected to further analysis. After fixation and permeabilization allowing intracellular antigen staining the cells were observed for immunofluorescence by confocal scanning microscopy. Anti-MBP staining of the A1 clone produced extra-nuclear granules uniformly distributed in the whole cell body, thus indicating the cytoplasmic localization of the recombinant MBP (Fig. 2A). Anti-PLP staining of the C1 clone revealed punctate circles reflecting surface localization of the recombinant PLP (Fig. 2B). Both obtained staining patterns of CHO cells were consistent with proper cellular localization of MBP and PLP observed in myelin sheaths (Sedzik *et al.*, 1984; Stoffel *et al.*, 1984; Ridsdale *et al.*, 1997a; 1997b). It is noteworthy that the expression of MBP and PLP was stable (for at least 3 months in selective medium containing 200 μg geneticin/ml) and not lethal for CHO cells, in contrast to stably PLP-transfected oligodendroglial cell lines N19 and N20.1 (Bongarzone *et al.*, 2001).

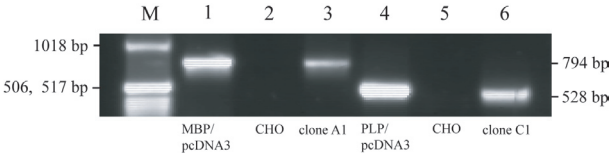

Figure 3. PCR analysis to demonstrate the expression of transfected MBP and PLP plasmid cDNAs in CHO cell clones.
mRNA from untransfected CHO cells and clones A1/MBP or C1/PLP were reverse transcribed and used as template for PCR. Lanes: M, marker (1 kb ladder); 1, MBP/pcDNA3 plasmid; 3, clone A1/MBP; 4, PLP/pcDNA3 plasmid; 6, clone C1/PLP; 2, 5, untransfected CHO cells.

Presence of the transfected genes for MBP and PLP in selected CHO cell clones

To verify that MBP and PLP were expressed in the A1 and C1 clones from the transfected constructs, PCR analysis was performed using as tem-

plate cDNA reverse transcribed from mRNA expressed in either clone. To guarantee that only products of the transfected genes would be amplified, 5′-primer sequence from within the MBP or PLP inserts and 3′-primer sequence from within the pcDNA3 vector were used. Bands at the predicted sizes, 794 bp for MBP and 528 bp for PLP were present in A1 and C1 clones, respectively, but were absent from nontransfected CHO cells (Fig. 3). This was an additional piece of evidence that the A1 and C1 CHO clones expressed the recombinant forms of MBP and PLP.

The recombinant forms of MBP and PLP expressed in CHO cells, most likely with a native secondary and tertiary structure may have an advantage, in immunological studies, over purified and denatured proteins. Both recombinant proteins, posttranslationally modified in the mammalian system similarly as the native proteins expressed in oligodendrocytes in physiological conditions, can be used for characterization of immuno-response in MS. CHO clones expressing MBP or PLP can serve to simplify identification of auto-antibodies with anti-MBP and anti-PLP specificity present in body fluids of MS patients or in further studies for screening of immunoglobulin libraries obtained from lymphocytes of MS patients.

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