Muscular dystrophy by merosin deficiency decreases acetylcholinesterase activity in thymus of *Lama2dy* mice

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Abbreviations used: ACh, acetylcholine; AChE, acetylcholinesterase; anti-mrAChE, antiserum against mouse recombinant AChE; APA, *Abrus precatorius* agglutinin; ATCh, acetylthiocholine iodide; B-96, Brij 96 (polyoxyethylene$_{10}$-oleyl ether); BuChE, butyrylcholinesterase; BuTCh, butyrylthiocholine iodide; BW284c51, 1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide; ChEs, cholinesterases; CMD, congenital muscular dystrophy; Con A, concanavalin A; DT, dystrophic mice thymus; DTNB, 5,5'-dithio-bis-2-nitrobenzoic acid; GPI, glycosylphosphatidylinositol; *iso*-OMPA, tetraisopropyl pyrophosphoramide; LCA, *Lens culinaris* agglutinin; NT, normal mice thymus; PBL, peripheral blood lymphocytes; PIPLC, phosphatidylinositol-specific phospholipase C; RCA, *Ricinus communis* agglutinin (RCA$_{120}$); TEC, thymic epithelial cells; TX-100, Triton X-100; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; WGA, wheat germ (*Triticum vulgaris*) agglutinin.

ABSTRACT

Half of congenital muscular dystrophy (CMD) cases arise from laminin α2 (merosin) deficiency, and merosin-deficient mice (*Lama2dy*) exhibit dystrophic phenotype. The abnormal development of thymus in *Lama2dy* mice, the occurrence of acetylcholinesterase (AChE) in the gland and the impaired distribution of AChE molecules in skeletal muscle of the mouse mutant prompted us to compare the levels of AChE mRNAs and enzyme species in thymus of control and *Lama2dy* mice. AChE activity in normal thymus (NT; 1.42 ± 0.28 μmol of acetylthiocholine.h^{-1}.mg $protein^{-1}$, U/mg) was ~50% decreased in dystrophic thymus (DT; 0.77 ± 0.23 U/mg, p = 0.007), whereas BuChE activity was little affected. RT-PCR assays revealed variable levels of R, H, and T AChE mRNAs in thymus, bone marrow and spinal cord. Control thymus contains amphiphilic AChE dimers (G_2^A, 64%) and monomers (G_1^A, 19%), besides hydrophilic tetramers (G_4^H, 9%) and monomers (G_1^H, 8%). The dimers consist of glycosylphosphatidylinositol (GPI)-anchored H subunits. Western blot assays with anti-AChE antibodies suggest the occurrence of inactive AChE in mouse thymus. Despite the fall of AChE activity in *Lama2dy* thymus, no differences between control and dystrophic thymuses were observed in the distribution of AChE forms, PIPLC sensitivity, binding with lectins and size of AChE subunits.

Keywords: AChE mRNAs; GPI-anchored proteins; laminin; peripheral blood lymphocytes

Running title: Acetylcholinesterase in dystrophic mice thymus

(Introduction)

Congenital muscular dystrophies (CMD) are a genetically and clinically heterogeneous group of autosomal recessive neuromuscular disorders. CMD patients often show joint deformities, inability to stand or walk and variable abnormalities in the CNS. In the 'classical' form of CMD, clinical signs are restricted to skeletal muscle. Evidences of the involvement of the laminin α2 chain in nearly half of the patients with classical CMD have been accumulated by the identification of mutations in its gene (LAMA2) (Tomé, 1999; Miyagoe-Suzuki et al., 2000). The laminin α2 chain (also called merosin) forms the laminin-2 heterotrimer (α2β1γ1), which in muscle connects the extracellular matrix with the membrane cytoskeleton by interacting with α-dystroglycan and integrin α7β1 (Vachon et al., 1997). In humans and mice, the defect of merosin produces muscle degeneration and dysmyelination of peripheral nerves (Miyagoe-Suzuki et al., 2000; Feltri et al., 2002), which makes the merosin-deficient *Lama2dy* mouse a suitable animal model for studying CMD pathology (Xu et al., 1994).

Acetylcholinesterase (AChE, EC 3.1.1.7) hydrolyzes rapidly the neurotransmitter acetylcholine (ACh) at cholinergic synapses (Taylor and Radic, 1994; Massoulié et al., 1999). The action of butyrylcholinesterase (BuChE, EC 3.1.1.8) is unclear, but it possibly collaborates in cholinergic transmission when AChE is diminished or inactive (Li et al., 2000). AChE and BuChE are encoded by separate genes and can be distinguished by their preferred substrate and sensitivity to inhibitors (Taylor and Radic, 1994). In the last few years, the production of ACh in non-neuronal cells, such as epithelial, mesothelial, endothelial and blood cells, has attracted much attention suggesting that the 'neurotransmitter', acting as a local signaling molecule, might regulate cell proliferation, differentiation, apoptosis, and other functions (Wessler et al., 2003). Cholinergic activity in non-excitable tissues would depend on ACh availability, which in turn depends on ChEs.

Apart from their hydrolytic action, AChE and BuChE may play additional functions unrelated to their capacity to degrade ACh, which could explain their appearance in embryonic tissues (Layer and Willbold, 1995), non-cholinergic neurons (Webb et al., 1996), and cells undergoing apoptosis (Zhang et al., 2002). In addition, many observations point to the involvement of ChEs in hematopoiesis, osteogenesis and tumorigenesis. Selected features are: 1) the change on the cellular composition of bone marrow after blocking AChE mRNA with antisense oligonucleotides (Grisaru et al., 1999); 2) the enhanced expression of AChE mRNA with osteoblast differentiation (Grisaru et al., 1999); 3) the blockade of megakaryocytopoiesis with BuChE antisense oligonucleotides (Patinkin et al., 1990); and 4) the impaired expression of AChE and BuChE in tumors of diverse origin (Perry et al., 2002; Ruiz-Espejo et al., 2003).

Three catalytic AChE subunits have been identified in mammals: the T subunit is encoded by exons E2-4 and part of E6 of the AChE-T mRNA; the glycosylphosphatidylinositol (GPI)-linked H subunit by E2-E4 and a piece of E5 of the AChE-H mRNA; and the R subunit by E2-4 and part of intron I4 of the AChE-R mRNA (Massoulié et al., 1999) (see Figure 4). A single BuChE-mRNA has been found so far and its protein product (the T subunit) suffices for making a range of molecules. The association of AChE-T (or BuChE) subunits produces homo or heterooligomers, according to the lack or addition of structural proteins. The globular AChE (and BuChE) forms occur as hydrophilic (G^H) or amphiphilic (G^A) monomers (G_1), dimers (G_2) and tetramers (G_4); G_4^A AChE (and BuChE) predominates in mammalian brain, its amphiphilic domain consisting of a structural subunit referred to as PRiMA (Perrier et al., 2002). The asymmetric ChE forms display hydrophilic properties and consist of one to three tetramers, made of T subunits, linked to a collagenic ColQ tail (Feng et al., 1999).

We have previously reported that the defect of merosin alters the composition of AChE and BuChE forms in skeletal muscle of *Lama2dy* mice (Cabezas-Herrera et al., 1997; Moral-

Naranjo et al., 1999), and decreases the content of G_4^A AChE and BuChE forms in sciatic nerves (Moral-Naranjo et al., 2002). Besides striated muscle and peripheral nerve, merosin mRNA and protein have been identified in mouse placenta, testis, lymph node, thymus, spleen and other organs (Sasaki et al., 2002), but whether the merosin defect alters their functioning remains to be studied.

Experimental evidence indicates that mouse spleen (Gómez et al., 2000) and human lymph nodes (Lev-Lehman et al., 1994) express AChE mRNAs, and rat thymus (Cavallotti et al., 2000a), mouse thymocytes (Rossi et al., 1991) and spleen (Nieto-Cerón et al., 2004) display AChE activity. The synthesis of AChE in lymphoid tissues, the ChE abnormalities in muscle and nerve of merosin-deficient mice (Cabezas-Herrera et al., 1997), the strong decrease in the number of thymocytes in thymus of merosin-deficient *Lama2dy* mice, which leads to an abnormal development of the gland (Magner et al., 2000), and the contribution of immune responses to muscular dystrophy, as it is shown by the reduction of pathological signs in dystrophin-deficient *mdx* mice depleted of $CD4^+$ or $CD8^+$ T cells by antibody immunosuppression (Spencer and Tidball, 2001), led us to study if the deficiency of merosin alters the expression of thymus ChEs.

We report here the levels of AChE activity and AChE mRNAs in normal and dystrophic mouse thymuses. In addition, details concerning the structure, distribution and origin of AChE forms in mouse thymus are provided. Finally, the possible functional significance of AChE in thymus and other lymphoid tissues is discussed.

MATERIALS AND METHODS

Materials

Acetylthiocholine iodide, butyrylthiocholine iodide, DTNB, BW284c51, *iso*-OMPA, HEPES, proteinase inhibitors, Triton X-100 (TX-100), Brij 96 (B-96), Tween 20, markers for

sedimentation analysis (beef liver catalase and beef intestine alkaline phosphatase), human transferrin, Sepharose 4B, and agarose-bound lectins (APA, Con A, LCA, WGA, RCA), dimethyl sulfoxide (DMSO), DNA size markers, agarose and ethidium bromide were all provided by Sigma Chem. Co. (St. Louis, MO, U.S.A.) and Trizol from Life Technologies (GibcoBRL, U.K.). Moloney murine leukaemia virus (MMLV) reverse transcriptase and AChE custom primers were obtained from Invitrogen (U.S.A.). The random decamers and the 'QuantumRNA 18S internal standards' kit were both provided by Ambion (U.S.A.), and ribonuclease inhibitor was from Amersham-Pharmacia (U.K.). Hotmaster *Taq* DNA polymerase and dNTPs were from Eppendorf (Germany). Protein G-agarose was provided by Boehringer Mannheim, (Germany) and peptide-N-glycosidase F (PNGase F) from *Flavobacterium meningosepticum* was purchased from Glyco (Novato, CA, U.S.A.). PIPLC purified from *B. thuringiensis* was kindly donated by Dr. Nigel M. Hooper (University of Leeds, U.K.). A rabbit antiserum against recombinant AChE of mouse (anti-mrAChE) was generously sent by Dr. Palmer Taylor (USCD, U.S.A.). The chemiluminescent ECL Plus reagent was from Amersham-Pharmacia, and the peroxidase-conjugated anti-rabbit IgG antibody from Sigma. The Lymphoprep solution was provided by Nycomed Pharma AS (Norway).

Solubilization of ChEs

Breeding pairs of B6.129P1-Lama2dy/J mice giving phenotypically normal (+/?) and dystrophic (*Lama2dy*) animals (dystrophin-positive, merosin-negative) were purchased from Jackson Memorial Lab. (Bar Harbor, ME, U.S.A.). They were bred and kept in the animal care unit of the University of Murcia under strict ethical rules. Control and dystrophic mice, 3 months old, were anaesthetized with ether, and, after heart perfusion with 5.4 mM EDTA,

154 mM NaCl, pH 7.4, the thymus was excised, washed with HEPES-saline buffer (HSB, 1 M NaCl in 15 mM HEPES buffer, pH 7.4), and weighed.

Thymuses of 16-18 normal mice (normal thymuses, NT) or of dystrophic mice (DT) were homogenized in HSB (5 ml/g of tissue) containing a fresh mixture of antiproteinases (Moral-Naranjo et al., 1996). The homogenate was centrifuged at 100,000 *g*, 1 h at 4ºC, to recover the soluble and loosely-bound ChEs in the S1 supernatant. The pellet was re-extracted with HSB containing 1% w/v TX-100 and antiproteinases and, after centrifuging as above, membrane-bound ChEs were saved in S2. ChE activity and the protein content were measured in the homogenate and supernatants.

For peripheral blood lymphocyte (PBL) isolation, blood was taken from mice by heart puncture using EDTA as anticoagulant. Then, 3 ml of blood was diluted with 3 ml of 0.9% NaCl, the mixture was layered onto 3 ml of Lymphoprep and, after centrifugation 20 min at 800 *g*, the layer rich in PBL was saved. AChE activity was released using the procedure already described for extracting the erythrocyte enzyme (Gómez et al., 2003).

Measurement of ChE activity

AChE and BuChE activities were assayed by the Ellman method; AChE with 1 mM acetylthiocholine (ATCh) and 50 µM *iso*-OMPA, and BuChE with 1 mM butyrylthiocholine (BuTCh) and 10 µM BW284c51 (Cabezas-Herrera et al., 1997; Moral-Naranjo et al., 1999). Inhibition of BuChE activity by TX-100 was relieved by adding 0.5% B-96 to the assay mixture. One unit (U) of AChE or BuChE activity represents the amount of enzyme which hydrolyzes one µmol of the substrate per h at 37°C. Parallel assays were made in the presence of both BW284c51 and *iso*-OMPA for measuring the hydrolysis of ATCh and BuTCh owing to unspecific esterases. The ATCh hydrolysis due to unspecific esterases in the homogenates and S1 or S2 supernatants of NT and DT was negligible (2-5%). The BuTCh hydrolysis

amounted to 10-15% for NT or DT samples. ATCh or BuTCh hydrolysis due to unspecific esterases was subtracted for gaining true AChE or BuChE activities.

In sedimentation analysis experiments, AChE activity was determined by a microtiter assay (Cabezas-Herrera et al., 1997; Moral-Naranjo et al., 1999), in which case the activity is expressed in arbitrary units, one unit referring to an increase of 0.001 absorbance units per microliter of sample, and per minute, but normalized for the volume of sample loaded onto the gradient. Protein content was measured using the BioRad reagent.

Sedimentation analysis

Molecular forms of AChE in the S1 and S2 supernatants from thymus and the TX-100 extract from PBL were resolved by centrifugation analysis and characterized by their sedimentation coefficients. Samples (0.2-0.3 ml) with added sedimentation markers (catalase, 11.4S, and alkaline phosphatase, 6.1S) were centrifuged on 5-20% (w/v) sucrose gradients containing 0.5% B-96 or 0.5% TX-100 (Moral-Naranjo et al., 1996). After centrifugation at 150,000 *g*, 20 h at 4°C, fractions were collected from the tube bottom, assayed for AChE activity and enzyme markers, and the sedimentation coefficients of AChE forms were calculated (Cabezas-Herrera et al., 1994a). After twenty centrifugation runs with thymus extracts and five with PBL samples, mean values and standard deviation for sedimentation values of AChE species were obtained. Sedimentation profiles with overlapping peaks were decomposed in Gaussian curves using the Peak-Fit program from SPSS Inc., version 4, for improving the assignment of the peaks of AChE activity to individual enzyme molecules.

Cleavage of the amphiphilic anchor in AChE

The presence of glycosylphosphatidylinositol (GPI) residues in thymus AChE and the possible differences between AChE of thymus and PBL regarding the sensitivity to

phosphatidylinositol-specific phospholipase C (PIPLC) were tested by sample incubation without (control) and with PIPLC (3 U/ml), 2 h at 37ºC. Removal of the GPI anchor in AChE forms was assessed by sedimentation analysis in gradients with B-96. An increase in the conversion of the G_2^A AChE of PBL into their hydrophilic G_2^H derivatives was attempted by treatment with alkaline hydroxylamine prior to PIPLC incubation (Gómez et al., 2003).

Relative level of AChE transcripts

The relative content of AChE mRNAs in thymus, bone marrow and spinal cord from normal and dystrophic mice was estimated by RT-PCR. Total RNA was isolated from freshly obtained organs using Trizol, according to the manufacturers' instructions.

For obtaining the cDNAs by reverse transcription (RT), 5 μg of RNA were denatured at 70ºC for 10 min, and cooled on ice. A mixture containing buffer, dithiothreitol, dNTPs, random decamers, and ribonuclease inhibitor was added, and samples heated 2 min at 42ºC. Then, 200 U of Moloney murine leukaemia virus (MMLV) reverse transcriptase were added, and synthesis of cDNAs was carried out for 50 min at 42ºC, in a volume of 20 μl. Finally, samples were heated 15 min at 72ºC and kept frozen.

Polymerase chain reactions require primer pairs which specifically amplify the cDNA for one of the three AChE mRNAs: R, H or T. Therefore, primers were designed to cover exon-exon splicing sites of the ACHE sequence (Rachinsky et al., 1990; Li et al., 1991) (see scheme in Fig. 4). The forward primer for the three mRNAs, named p51, is targeted to the exon 3/exon 4 (E3/E4) junction, in order to amplify only processed transcripts (mature mRNAs). Reverse primers, specific for each mRNA, were: for R mRNA, primer p52 (located in intron 4); for H mRNA, p53 (in E4/E5); and for T mRNA, p55 (in E4/E6). Primer sequences were: p51, ATTTT GCCCG CACAG GGGAC; p52, CCCCA CTCCA TGCGC CTAC; p53, AAGGA GCCTC CGTGG CGGT; p55, CGCCT CGTCC AGAGT ATCGG T.

The expected PCR products for R, H and T mRNAs have 206, 207 and 210 bp, respectively. The 18S rRNA was employed as internal standard, using the primers provided in the 'QuantumRNA 18S internal standards' kit. The amount of 18S PCR product was optimized by means of competitor primers (competimers) as indicated by the manufacturers.

Preliminary PCR assays were performed to determine the cycle number more adequate for comparing the relative AChE mRNA contents. PCR reactions were carried out in a 50 μl buffered medium with 1-2 μl of cDNA, AChE primers (0.3 μM), 18S rRNA primers and competimers, dNTPs, dimethyl sulfoxide (5%) and Hotmaster *Taq* DNA polymerase (50 U/ml). Reactions included an initial denaturing step of 2 min at 94ºC, followed by 29 cycles with 20 s at 94ºC, 20 s at 63ºC, and 40 s at 65ºC.

PCR products were visualized with ethidium bromide in 1.8% agarose gels. Results were recorded with a digital camera, using an orange filter, and relative amounts of DNA in each band were calculated with the GelPro software. The ratio of PCR products for the AChE transcript and for the 18S rRNA in each given sample was calculated and used to estimate the relative content of AChE mRNA in the various samples. Results are expressed as means ± SD of three to four RT-PCR, each performed with RNA obtained from a different animal.

Lectin interaction assays

The binding of several lectins to AChE of NT and DT was studied for exploring whether, as in mouse muscle (Moral-Naranjo et al., 1997) and liver (Gómez et al., 2000), the deficiency of merosin in mice alters the glycosylation of thymus AChE. The lectins used and the terminal sugars preferentially recognized by them are as follows: Con A, α-mannose; LCA, α-mannose with fucose at the 'fucosylation core' (α1-6-linked fucose near the N-glycosidic linkage); WGA, mainly GlcNAc, and to a lesser extent NeuNAc; RCA,

Galβ1-4GlcNAc; and APA, Galβ1-3GalNAc. Lectin interaction studies provide indirect evidence for the transit of ChEs along the secretory pathway (Cabezas-Herrera et al., 1997).

For lectin binding assays, samples of the S1 and S2 extracts (0.2-0.5 ml each) were mixed and incubated with 0.4-1 ml of lectin-free Sepharose 4B or lectin-bound agarose. After overnight incubation at 4ºC, the beads with AChE-lectin complexes were separated by centrifugation, and the unbound AChE forms were quantified by sedimentation analysis. The peak area of each AChE form in the Sepharose 4B supernatant (control) was set as the 100% value, and the extent of lectin binding was calculated from the difference between peak areas of sedimentation profiles of control and lectin assays (Moral-Naranjo et al., 1997). The same procedure was used for evaluating the extent to which lectins bind to AChE of PBL, and this allowed us to explore possible differences between the oligoglycans linked to AChE of thymus and PBL.

Electrophoresis and Western blotting

Prior to electrophoresis, the amount of natural mouse antibodies in samples was reduced by incubating them with protein G-agarose. SDS-PAGE were performed under non-reductive and reductive conditions in 7.5% acrylamide gel slabs. Afterwards, proteins were transferred to nitrocellulose membranes, which were blocked with BSA and Tween 20, and incubated overnight at 4ºC with an antiserum against mouse recombinant AChE (anti-mrAChE; 1:2500), and then with an appropriate peroxidase-conjugated IgG. The immunocomplexes were detected by a chemiluminescent reaction using ECL Plus. Suitable protein markers and the GelPro Analyzer program, version 3.1, were used for calculating the size of AChE protein bands.

In addition, AChE protein in thymus and PBL extracts was immunoprecipitated with the anti-mrAChE and protein G-agarose, and bound proteins were electrophoresed and detected as above.

To determine whether the multiplicity of immunolabeled AChE bands arises from differences in glycosylation, the glycans linked to AChE of thymus and PBL were removed by PNGase F. After immunoprecipitation with anti-mrAChE, the immunocomplexes were detached from the agarose beads and treated or not (control) with PNGase F according to manufacturers' instructions, but doubling the enzyme units. Proteins were resolved by reductive SDS-PAGE and AChE subunits were identified by Western blot with the anti-mrAChE antiserum. Human transferrin was used as a positive control for deglycosylation.

Statistical analyses

AChE and BuChE activities in the homogenates and soluble fractions of NT, DT and PBL, and sedimentation coefficients of AChE forms are given as means ± S.D. The statistical differences in the weight of thymuses excised from normal and dystrophic mice, ChE activity and protein content in extracts were evaluated by the Student's *t* test.

RESULTS

AChE and BuChE activities in control and dystrophic mouse thymuses

AChE activity was nearly one-half lower in the homogenates of dystrophic mice thymus (DT) compared to those of normal mice thymus (NT), while BuChE activity was similar (Fig. 1). Although the higher protein content in DT extracts can justify the small reduction in BuChE activity, it cannot explain the drop in AChE activity.

Most of the AChE (90 ± 9%) and BuChE activities (95 ± 11%) were detached from NT and DT by using a double extraction protocol, first with saline buffer alone to save soluble and loosely bound ChEs in the S1 supernatant, and later with saline buffer plus TX-100 to recover the tightly bound ChEs in the S2 supernatant. One half of the releasable AChE activity of NT or DT was measured in S1 (NT, 0.97 ± 0.14 U/mg; DT, 0.48 ± 0.15 U/mg,

$p = 0.004$), and the rest in S2 (NT, 1.49 ± 0.40 U/mg; DT, 0.69 ± 0.15 U/mg, $p = 0.007$). Almost two-thirds (65%) of the BuChE activity of NT or DT was saved in S1 (NT, 0.17 ± 0.04 U/mg; DT, 0.11 ± 0.02 U/mg, $p = 0.084$), and the remaining activity (30%) in S2 (NT, 0.13 ± 0.05 U/mg; DT, 0.07 ± 0.03, $p = 0.095$ U/mg). Thus, apart from the reduction of AChE activity in DT, no statistically significant differences were observed between NT and DT on extraction yields of AChE and BuChE activities, indicating that the merosin deficiency does not affect the interaction of the enzymes with the thymus membranes. AChE activity in the TX-100 extract of PBL was 10.9 ± 1.2 U/mg, and BuChE activity about 3,000-fold less.

Separation of AChE components

Molecular forms of AChE and BuChE in the S1 and S2 supernatants were identified by sedimentation analysis. In order to prevent excess of information, we report here the results on AChE mRNAs and enzyme components in thymuses of normal and dystrophic mice; the results concerning the BuChE molecules will be given in a forthcoming paper.

Sedimentation profiles with AChE forms in the salt-soluble (S1) and detergent-soluble extracts (S2) are shown in Fig. 2. While dimers and monomers are better resolved in gradients with TX-100, those with B-96 give information on their amphiphilic behavior. In gradients with TX-100, AChE activity in S1 peaked at 4.0 ± 0.2S (32%), 5.4 ± 0.2S (58%) and 10.7 ± 0.2S (10%). According to their sedimentation values (Moral-Naranjo et al., 1996; 2002; Nieto-Cerón et al., 2004), the peaks were assigned to AChE monomers (G_1), dimers (G_2) and tetramers (G_4). In gradients with B-96, the activity peaks migrated at 2.9 ± 0.2S (15%), 3.9 ± 0.2S (75%) and 10.7 ± 0.3S (10%). A comparison of the profiles with TX-100 or with B-96 showed the amphiphilic properties of the light forms (G_1^A and G_2^A) and the hydrophilic behavior of the 10.7S components (G_4^H). However, since G_1^H AChE overlaps with G_1^A in TX-100 gradients, but with G_2^A in those with B-96, we calculated that the S1

extract contains 10% of G_4^H (10.7S), 58% of G_2^A (5.4S or 3.9S), 15% of G_1^A (4.0S or 2.9S) and 17% of G_1^H forms.

The percentages of the G_4^H, G_2^A and G_1^A AChE in the S2 extract were 7, 72 and 21% in gradients with TX-100, or 7, 68 and 26% in those with B-96. Thus, S2 contains about 7% of G_4^H, 70% of G_2^A and 23% of G_1^A forms. Since AChE activity is almost fully extracted from thymus and evenly divided among S1 and S2, the composition of AChE forms in thymus is: 9% G_4^H, 64% G_2^A, 19% G_1^A and 8% G_1^H. Apart from the 50% drop of AChE activity in DT, no significant differences were noticed between the patterns of AChE forms in NT and DT (profiles not shown).

As regards mouse PBL, sedimentation analyses of the TX-100 extract in gradients with B-96 (Fig. 3) revealed G_2^A (4.0 ± 0.2S, 80%) and G_1^A species (2.8 ± 0.1S, 20%). Thus, although it has been stated that PBL only possess G_2 AChE (Bartha et al., 1987), they also contain monomers.

Detachment of the amphiphilic domain in AChE dimers

The abundance of G_2^A and G_1^A AChE forms in mouse thymus led us to explore whether their amphiphilic properties are due to the presence of GPI residues. The lipid moiety of the GPI anchor can often be removed by exposure to bacterial PIPLC (Toutant et al., 1990; Hooper, 2001), which provides a simple method for converting the GPI-anchored AChE forms into their hydrophilic variants, and, therefore, for distinguishing AChE-H from AChE-T subunits. However, modifications of the GPI residue can make it resistant to PIPLC (Toutant et al., 1990).

More than 85% of the G_2^A forms of mouse thymus were converted into their hydrophilic variants by PIPLC (Fig. 3, left panel), demonstrating that they consist of subunits encoded by the AChE-H mRNA. No significant differences in the phospholipase C sensitivity were

observed when comparing the AChE dimers of NT and DT (profiles not shown). In contrast to the dimers of thymus, which were mostly PIPLC-sensitive, a great fraction (60%) of the PBL dimers were PIPLC-resistant (Fig. 3, right panel). Nevertheless, the fact that more than 80% of PBL dimers were converted into the G_2^H variants by treatment with alkaline hydroxylamine and PIPLC (profile not shown) demonstrated that most of the PBL dimers consist of AChE-H subunits (Gomez et al., 2003). The different sensitivity to PIPLC alone revealed differences in the structure of the GPI residues in G_2^A AChE of thymus and PBL. The majority of the PBL G_1^A species were converted into their G_1^H variants with PIPLC alone (Fig. 3, right panel).

Levels of AChE transcripts in thymus, bone marrow and spinal cord of control and dystrophic mice

The identification of AChE mRNAs in lymph nodes (Lev-Lehman et al., 1994) and blood lymphocytes (Ando et al., 1999) prompted us to compare the relative abundance of AChE transcripts in thymus with respect to bone marrow and spinal cord, and to investigate the effect of the merosin deficiency on the content of messengers. In tissues of healthy mice, the relative content of the AChE-H mRNA was higher in thymus than in bone marrow, and in both tissues much greater than in spinal cord (relative abundance 100: 68±21: 10±4) (Fig. 4). The data confirmed the capacity of hematopoietic and lymphoid tissues, or blood cells nested in them, for producing AChE-H subunits.

The content of the AChE-R messenger was similar in thymus and bone marrow, and it was about ten times higher than in spinal cord (proportions 100: 116±19: 12±5). Finally, the relative amount of the AChE-T transcript was lower in thymus or bone marrow than in spinal cord (100: 55±18: 184±52). The abundance of the AChE-T mRNA in spinal cord agrees with the necessity of T subunits in neural tissues for building PRiMA-anchored AChE tetramers.

As the result of the merosin deficiency, the level of the AChE-T mRNA remained unchanged in thymus, bone marrow and spinal cord; the relative content of the AChE-H transcript was 35% lower in spinal cord, and that of the AChE-R mRNA 40% lower in bone marrow.

Binding of AChE components to immobilized lectins

The presence of GPI-linked AChE dimers in mouse thymus, erythrocytes (Gómez et al., 2003) and lymphocytes led us to test the possible blood origin of the enzyme activity measured in thymus. Lectin interaction is useful for studying the origin of ChEs in tissues. In addition, since the deficiency of merosin alters the glycosylation of AChE forms in mouse muscle (Cabezas-Herrera et al., 1994b) and liver (Gómez et al., 2000), the level of lectin binding with AChE species of NT and DT was measured.

Nearly the totality (~90%) of AChE activity in NT was bound by Con A and LCA, slightly less by WGA (~85%), and much less by either RCA or APA (~65%). The lectin binding percentages of separate AChE forms were calculated from sedimentation analysis of the unbound AChE activity (Fig. 5, left panel). For the G_4^H, G_2^A (+ G_1^H), and G_1^A molecules, these percentages were: 90, 85, and 70% with WGA; and 60, 70, and 55% with RCA or APA. The lack of significant differences regarding the extent of lectin binding with AChE forms of NT and DT revealed that, in contrast to mouse skeletal muscle (Cabezas-Herrera et al., 1997) and liver (Gómez et al., 2000), the defect of merosin does not alter the glycosylation of thymus AChE molecules.

On the other hand, the greater binding of WGA and RCA with AChE dimers of thymus (85% and 70%) than of PBL (32% and 14%; right panel of Fig. 5) means that AChE of the two sources differ in their sugar composition.

Subunit size of AChE in thymus and PBL

Possible differences between the mass of the AChE subunit in NT and DT, or between NT and PBL, were explored. Non-reductive SDS-PAGE of NT samples followed by Western blotting with the anti-mrAChE antiserum revealed principal bands of ~150 kDa and fainter bands of 95, 65 and 61 kDa (Fig. 6A). Since the mass of mouse erythrocyte AChE (without the GPI anchor) is 66 kDa (Gómez et al., 2003), and each GPI residue adds ~3 kDa, the ~150 kDa protein band probably corresponds to the amphiphilic AChE dimers. A larger mass for the PBL dimers (~165 kDa) than for the thymus variants (~150 kDa) was observed. The similar protein profile in NT and DT extracts allowed us to discard that the merosin deficiency causes any change in the size of AChE dimers, but the deeper staining of the 150 kDa band in thymus than the 165 kDa band in PBL, despite the greater AChE activity in the latter samples, pointed to an increased ratio AChE protein/AChE activity in thymus compared with PBL or, alternatively, to a lower affinity of the antibodies for the PBL enzyme.

Reductive SDS-PAGE and Western blot of NT, DT and PBL samples with anti-mrAChE provided protein bands ranging 96-45 kDa in NT or DT, and 74-61 kDa in PBL (Fig. 6B). Again, the labeling of AChE protein in PBL required samples containing 5 times more AChE activity than the thymus ones.

For testing if the set of labeled proteins could be assigned to AChE subunits, AChE protein was first adsorbed to immobilized anti-mrAChE antibodies and the immunocomplexes bound to the agarose beads were subjected to Western blot, loading ten times more AChE activity in PBL than thymus samples (Fig. 6C). Proteins of 64-61 kDa with thymus and of 74-61 kDa with PBL samples were immunoprecipitated and labeled. It is noteworthy the weaker labeling of the DT than NT lanes, despite the equal units of activity added to them, which raised the possibility that the ratio of AChE protein/AChE activity was higher in NT than DT. Other protein bands were not immunoprecipitated by the anti-mrAChE antibody, which may be due

to the fact that they are recognized by the antibody only in their denatured state (Western blots) but not in their native form (immunoprecipitation). Nevertheless, the possibility remains that some of the stained bands of figure 6B correspond to cross-reacting proteins. As a whole, the above results show that AChE of thymus is principally made of 64-61 kDa subunits and AChE of PBL consists of 74-61 kDa proteins.

The different sizes of AChE bands could arise from their linked oligoglycans and/or from the variety of AChE subunits (R, H or T). For studying this issue, AChE was stripped of oligoglycans by PNGase F. After deglycosylation, the immunolabeled proteins from thymus migrated slightly faster, whereas those of PBL changed from 74-61 kDa to 63-59 kDa (Fig. 6D), which revealed that the higher size of PBL AChE subunits is due to the oligoglycan residues. Nevertheless, the maintenance of several bands after deglycosylation suggested that part of the mass heterogeneity may arise from the variety of AChE subunits.

DISCUSSION

Acetylcholinesterase activity in thymus

The presence of merosin in thymus (Sasaki et al., 2002), the anomalous development of thymocytes in merosin-deficient *Lama2dy* mice (Magner et al., 2000), the ChE abnormalities in muscle and nerve of merosin-deficient mice (Cabezas-Herrera et al., 1997; Moral-Naranjo et al., 1999; 2002), and the contribution of immune responses to muscular dystrophy pathology (Spencer and Tidball, 2001) prompted us to study the possible effects of the merosin deficiency on mouse thymus ChEs. To the best of our knowledge, this is the first time that the content of the three AChE messengers in mammalian thymus and the effects of the merosin defect on their levels, and on AChE activity and molecular components in this organ have been investigated.

Our results show that mouse thymus expresses the R, H and T AChE mRNAs, as well as enzyme activity, which confirms previous observations showing the presence of AChE activity in the gland (Cavallotti et al., 2000a; 2000b). We find a much higher AChE activity in mouse thymus (1.42 U/mg) than that reported in the rat (0.03 U/mg; Cavallotti et al., 2000a) or human organ (0.06-0.08 U/mg, Cavallotti et al., 2000b; Mihovilovic and Butterworth-Robinette, 2001). Since these authors extract thymus with a detergent-free buffer, when the release of AChE needs detergent, the reported activity values are probably undervalued.

Molecular forms of AChE in mouse thymus and peripheral blood lymphocytes

Sedimentation analyses and PIPLC exposure allowed us to identify in thymus abundant GPI-anchored AChE dimers (G_2^A, 64%), less monomers (G_1^A, 19%), and a few hydrophilic tetramers (G_4^H, 9%) and monomers (G_1^H, 8%). The results extend previous data concerning the molecular composition of AChE in mouse thymus and clarify the controversy on the presence or not of G_4^H species in it (Bulloch and Lucito, 1988; Rossi et al., 1991). Our data disagree with the exclusive presence of G_2^A AChE in the gland (Rossi et al., 1991), the discrepancy being explained by the improved resolution of the G_2^A and G_1^A forms in sucrose gradients with 0.5-1% TX-100 (this work) than with 0.1% TX-100 (Rossi et al., 1991). The finding in PBL of a few (20%) AChE monomers (Fig. 3), which had remained so far undetected (Bartha et al., 1987; Bulloch and Bossone, 1987), besides the abundant dimers, highlights the usefulness of B-96 for resolving G_2^A and G_1^A AChE molecules.

The finding of AChE-H mRNA in mouse thymus (Fig. 4), lymph nodes (Lev-Lehman et al., 1994) and spleen (Gómez et al., 2000) agrees with the abundance of GPI-anchored AChE in lymphoid tissues (Nieto-Cerón et al., 2004). According to Dr. Taylor results, bone marrow displays a high level of AChE-H transcript (Li et al., 1991). Therefore, the elevated content of the AChE-H mRNA in thymus may reflect the bone marrow origin of thymus cell precursors,

which after development, differentiation and maturation produce a range of thymic cells, some of the T-cells finally leading to PBL (Pear et al., 2004). In addition, the finding of the AChE-T transcript explains the presence of G_4^H species in thymus and asymmetric A_4 forms in lymph nodes (Ruiz-Espejo et al., 2003). Whether the G_1^H AChE in thymus and spleen (Nieto-Cerón et al., 2004) comes from H or T mRNAs requires a further investigation. Considering the secretory nature of G_4^H AChE (Flores-Flores et al., 1996), the synthesis in thymus of GPI-linked G_2^A AChE for membrane anchorage and G_4^H for secretion agrees with the histochemical observation of AChE in intracellular structures of T lymphocytes, epithelial cells of thymus and their surrounding space (Topilko and Kaillou, 1985).

Identification of inactive AChE in thymus

The finding of the AChE-H, AChE-T and AChE-R mRNAs in thymus besides the resistance of the G_1^A forms to PIPLC (Fig. 3) prevented us from establishing whether they consist of H, T or R subunits. Because of the similar mass of the three AChE subunits (all with 60-70 kDa in mouse and man; Randall, 1994; Uccelletti et al., 2002; Darreh-Shori et al., 2004), the results of Western blotting alone (Fig. 6) failed to clarify this issue. Nevertheless, the Western blot assays allowed us to detect size differences between AChE dimers of thymus and PBL and to observe a heavier immunolabeling of AChE proteins in thymus than PBL, in spite of loading more AChE units of the latter (Fig. 6). The last finding can be explained considering that the ratio of AChE protein/AChE activity is higher in thymus than PBL, or that the antibody affinity is lower for AChE of PBL than of thymus. Nevertheless, the deeper labeling of the 61-64 kDa AChE bands in NT than DT samples containing the same units of AChE activity (Fig. 6B and C) adds support to the possible presence of inactive AChE (non-catalytic variants) in mouse thymus. The occurrence of inactive AChE in mouse tissues, its aggregation state and functional significance are currently being investigated.

Origin of thymus AChE activity

The abundance of G_2^A AChE in both thymus and erythrocytes (Gómez et al., 2003) could lead to the belief that thymus AChE arises from the red cells. The different extent to which lectins bind to G_2^A AChE of thymus (WGA, 85%; RCA, 70%) and erythrocyte (WGA, 70%; RCA, 25%; Gómez et al., 2003) discards the erythrocytic origin of thymus AChE.

If thymus AChE arises from the organ itself, in which kind of cells is it produced? This is a difficult issue due to the tremendous complexity of the thymus cytoarchitecture. The gland is formed by many cell elements, which arise from the ectoderm, endoderm, mesenchyme and neural crest. It also contains a network of diverse epithelial cells, which besides immune cells create the microenvironment required for thymocyte development and immune homeostasis (Botham et al., 2001). The widespread histochemical staining of AChE both in thymic cells (reticular epithelial cells, lymphocytes and Hassall's corpuscles) and thymic structures (blood and lymphatic vessels along with nerve fibers-like elements) (Cavallotti et al., 2000b) illustrates how difficult it is to attribute the synthesis of AChE to particular thymus cells.

Current information on the distribution of AChE activity in the true cellular elements of thymus is incomplete: while some authors say that the activity is similar in T cells and stromal cells (which include thymus epithelial cells, TEC) (Rossi et al., 1991), others report that most activity resides in the thymocytes (Bulloch and Lucito, 1988; Mihovilovic and Butterworth-Robinette, 2001). If the preferential localization of AChE in thymocytes is admitted, the higher PIPLC resistance of AChE dimers from PBL than from thymus (Fig. 3) and their distinct patterns of lectin binding (Fig. 5) may reflect developmental changes of AChE along T cell maturation. The presence of G_4^H AChE in thymus and its absence from PBL (see for instance Fig. 5) may reflect changes in the biosynthetic program as the T cells mature.

Functional aspects of AChE in thymus

Before discussing the possible function of AChE in thymus, it should be reminded: 1) the expression of cholinergic receptors, AChE and ChAT in thymocytes (Rinner et al., 1994; Kawashima and Fujii, 2000) and TEC (Rossi et al., 1991; Wakkach et al., 1996; Mihovilovic and Butterworth-Robinette, 2001); 2) the proliferative response of mature thymocytes to nicotinic agonists (Rossi et al., 1989); and 3) the role of muscarinic receptors in thymocyte maturation (Maslinski et al., 1987). Thymocyte-secreted ACh has been involved in the control of T-cell proliferation and blockade of T-cell apoptosis (Rinner et al., 1998). In the context of cholinergic stimulation can be explained: 1) the anti-apoptotic action of carbachol, a nicotinic agonist, on thymocytes (Rinner et al., 1994); 2) the need of nicotinic activation for thymocyte and TEC surviving (Mihovilovic and Butterworth-Robinette, 2001); 3) the proliferative response of Con A-stimulated rat spleen T cells to ACh (Qiu et al., 1996); and 4) the anti-proliferative action of atropine, a muscarinic antagonist, on spleen T cells (Rinner et al., 1998). These observations highlight the importance of cholinergic signaling for T cell surviving and proliferation, and lend support to the possible participation of AChE in them.

The rise or fall of AChE activity in thymus, and the corresponding change in ACh bioavailability, may have physiological and pathological consequences. Thus, a high level of AChE activity (with a decreased content of ACh) can direct T cells to apoptosis (or to cease proliferation), and a low AChE activity (with a rise in ACh content) to T cell proliferation (or protection against apoptosis). Evidences in support of the proposal are: 1) the rise of AChE activity as thymus shrinks in puberty, aging and stress conditions (Micic et al., 1994; Cavallotti et al., 2000b); 2) the robust increase of thymus AChE activity after cortisone administration to mice, specially in thymus areas where lymphocyte death is prevalent (Bulloch and Lucito, 1988); 3) the protective effect of the anti-AChE drug physostigmine against thymocyte apoptosis induced by atropine (Rinner et al., 1999); and 4) the increased

response of splenocytes to mitogens after *in vivo* inhibition of AChE with malathion (Rodgers and Ellefson, 1990).

Concerning AChE in dystrophic mouse thymus, our results show that the levels of AChE mRNAs are little affected, the enzyme activity is one-half decreased, and the distribution of enzyme forms and the extent of lectin binding are not modified by dystrophy. Assuming that AChE resides mostly in thymocytes, the fall of AChE activity in *Lama2dy* mice thymus can reflect the reported loss of $CD4^+8^+$ (immature) T cells (Iwao et al., 2000; Magner et al., 2000). Alternatively, it may reveal thymus efforts to overcome the fall in thymocyte number by increasing the ACh level and thereby cholinergic stimulation.

In summary, mouse thymus contains AChE activity and expresses the R, H, and T AChE mRNAs. The activity is distributed between GPI-bearing AChE dimers, made of AChE-H subunits, hydrophilic tetramers, made of T subunits, and amphiphilic and hydrophilic monomers. AChE probably arises from the organ itself and, considering the differences in PIPLC sensitivity and lectin binding between AChE dimers of thymocytes and blood lymphocytes it is probable that their processing change with thymocyte maturation. The fall of AChE activity in dystrophic thymus may reflect the loss of T cells; the resulting rise in the ACh content may be functionally important for enhancing cell division as a means to overcome the severe atrophy caused by the deficiency of merosin in the gland.

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FIGURE LEGENDS

Figure 1. Variation of cholinesterase activity in dystrophic mouse thymus. Histograms compare thymus weight, the protein content as well as AChE and BuChE activities in thymus homogenates obtained from healthy (empty bars) and dystrophic (black bars) mice. Mean values plus standard deviation (SD) are given.

Figure 2. Molecular forms of AChE in mouse thymus. Healthy organs were extracted with a HEPES-saline buffer (HSB), and after centrifugation, the soluble/loosely bound AChE was recovered in the S1 supernatant. After a second extraction, with HSB plus 1% TX-100, the tightly bound AChE was saved in S2. AChE forms were resolved by centrifuging S1 and S2 on sucrose gradients containing 0.5% TX-100 (TX-100) or 0.5% B-96. AChE activity is expressed in arbitrary units (A.U.). Catalase (C, 11.4S) and alkaline phosphatase (P, 6.1S) were used as sedimentation markers. AChE forms were characterized by their sedimentation coefficients, and the amphiphilic (G^A) or hydrophilic (G^H) behavior was assessed by their distinct or equal migration in gradients with TX-100 or B-96. Overlapped sedimentation peaks were decomposed in various curves using the Peak-Fit program, which allowed a correct quantification of each AChE form. Note the similar distribution of AChE activity in S1 and S2, the amphiphilic properties of the dimers and most monomers, and the hydrophilic behavior of the tetramers. Almost the same profiles of AChE forms were obtained with S1 and S2 supernatants of *Lama2dy* mice thymuses (not shown).

Figure 3. Sedimentation profiles showing that AChE of thymus and PBL differ in the sensitivity to PIPLC. To study the presence of GPI residues in AChE, and possible changes in the composition of the GPI anchor in AChE of thymus and PBL, a mixture of the S1 and S2 supernatants of thymus and the TX-100 extract of PBL were exposed to PIPLC from

B. thuringiensis. Control (o) and PIPLC-treated samples (●) were centrifuged on gradients with B-96, which allowed the separation of G_2^A and G_2^H forms. PIPLC exposure of normal thymus (NT) samples converts most G_2^A forms (4.0S) into the G_2^H species (6.1S) (left panel), which demonstrates the existence of GPI residues in G_2^A AChE of thymus, and, therefore, that they consist of H subunits. The lack of any difference between AChE of NT and DT samples (profiles not shown) means that the composition of the GPI anchor in thymus AChE is unaffected by the defect of merosin. The low conversion of G_2^A AChE of PBL into the G_2^H variant with PIPLC alone denotes differences in the phospholipase sensitivity between AChE of thymus and peripheral blood lymphocytes (compare left and right panels).

Figure 4. RT-PCR of R, H and T AChE mRNAs in thymus of control and dystrophic mice. The relative content of the AChE mRNAs in normal and dystrophic thymus, bone marrow and spinal cord was estimated by RT-PCR. A scheme of the splicing pattern of the AChE gene is shown, along with diagrams of the 3' ends of the three mRNAs indicating the position of the primers pairs used for PCR, which amplify exclusively the cDNA of the desired AChE mRNA variant: R, H or T. PCR products (206-210bp for AChE and 488bp for 18S rRNA, used as standard) were observed in agarose gels, and their relative amounts estimated with the GelPro software. Fluorescence values for AChE PCR products were normalized with respect to the 18S rRNA signals, and normalized values for the different samples were compared. Note the intense signal for the three AChE mRNAs in thymus and bone marrow, whereas spinal cord had the greatest signal for the T mRNA, but very low ones for R and H mRNAs. The amount of none of the AChE mRNAs is greatly affected by the merosin deficiency in thymus of dystrophic mice.

Figure 5. Lectin binding of AChE from thymus and peripheral blood lymphocytes. Insights into the possible lymphocytic origin of thymus AChE were gained by lectin binding assays with the enzyme of thymus and PBL. A mixture of S1 and S2 extracts of thymus and the TX-100 extract of PBL were incubated with the agarose-bound lectins indicated in the sedimentation profiles, as well as with lectin-free Sepharose B (control, Ct). After separating the lectin-bound AChE activity by centrifugation, the unbound AChE forms were quantified by sedimentation analysis. The percentages of binding between lectins and AChE molecules were calculated from AChE activity in the peaks of the profiles obtained for lectin-incubated and lectin-free supernatants. Note the lower WGA and RCA interaction of PBL AChE compared to that of the thymus enzyme. The profile displaying the interaction of AChE with the lectin APA (not showed) was similar to that for RCA.

Figure 6. Western blots showing immunoreactive AChE protein in thymus and PBL. Proteins were electrophoresed, transferred to nitrocellulose membranes, and detected with anti-mrAChE antibodies. A) Western blotting of NT, DT, and PBL after non-reductive SDS-PAGE. AChE activity in lanes is given in milliunits (mU). Note the stronger labeling of AChE protein in thymus than PBL samples despite the greater activity loaded in the latter. B) A similar experiment, but under reductive conditions. C) Reductive SDS-PAGE and Western blot of AChE immunoprecipitated with anti-mrAChE. D) As C, but with samples treated (DG) or not (Cont) with PNGase F. In the immunoprecipitation assays, the added anti-mrAChE antibodies are observed as bands with ~45 kDa.

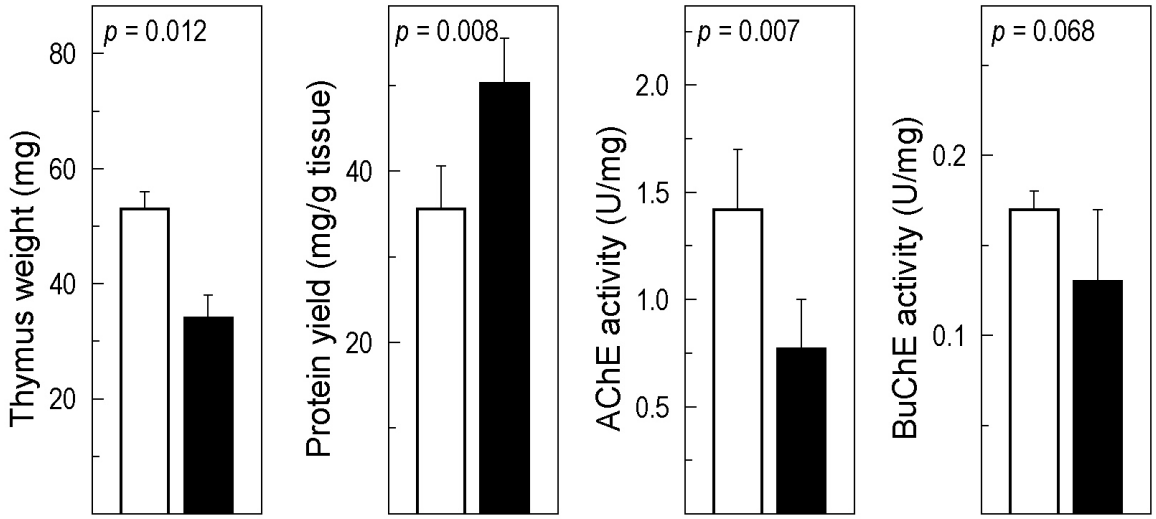

Figure 1
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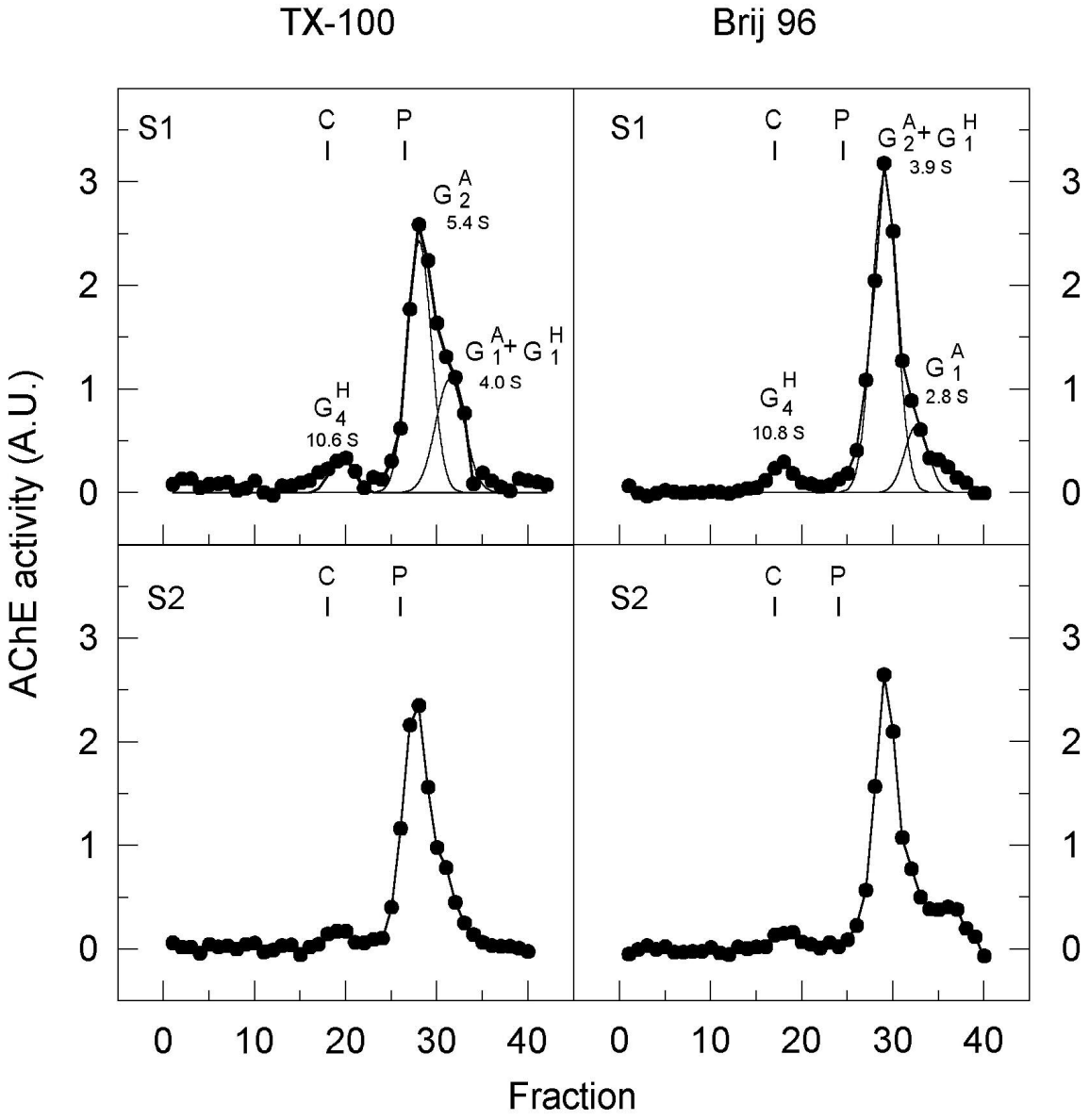

Figure 2
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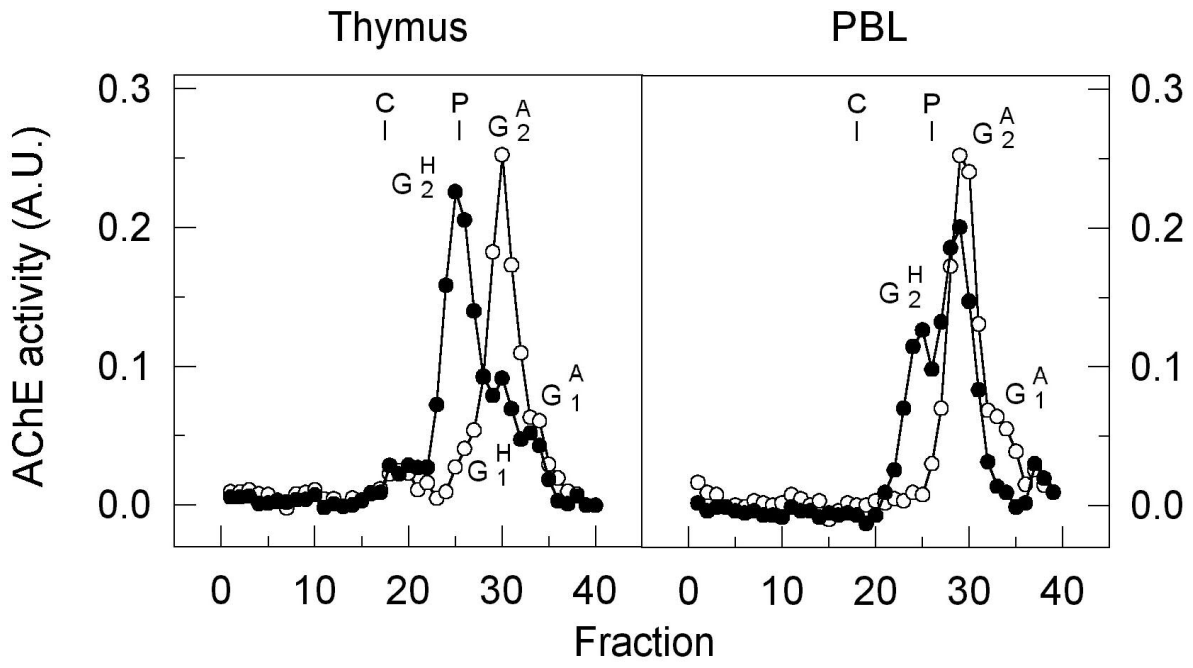

Figure 3
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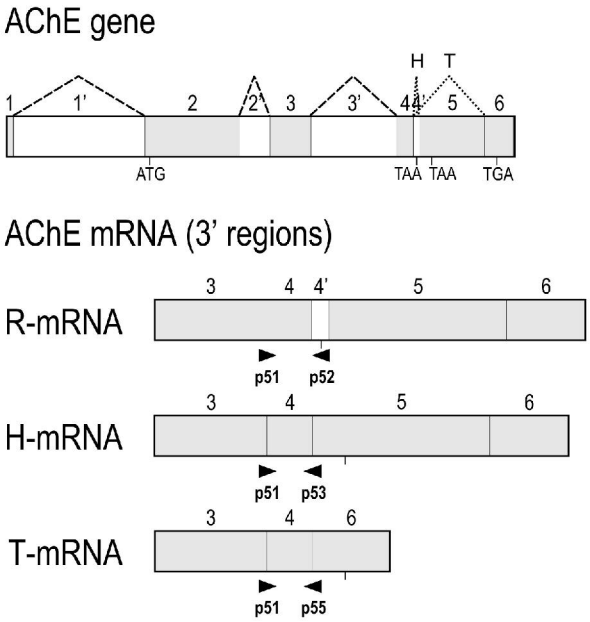

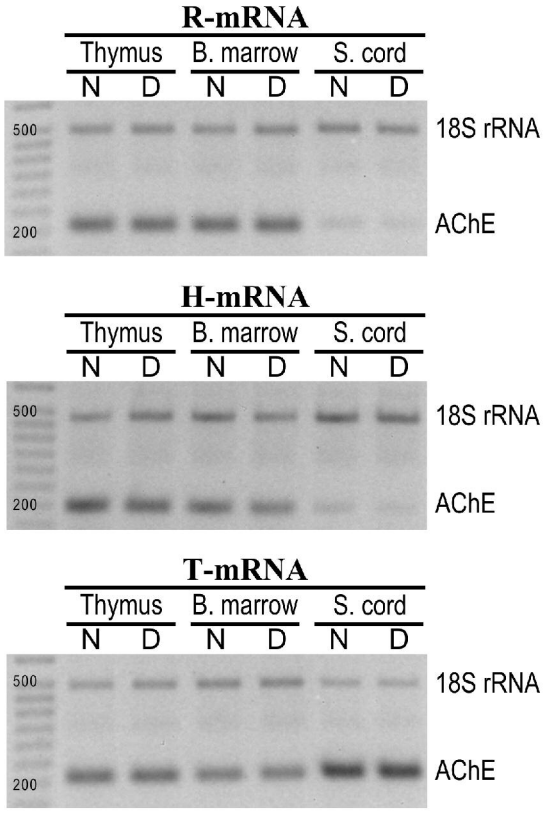

Figure 4
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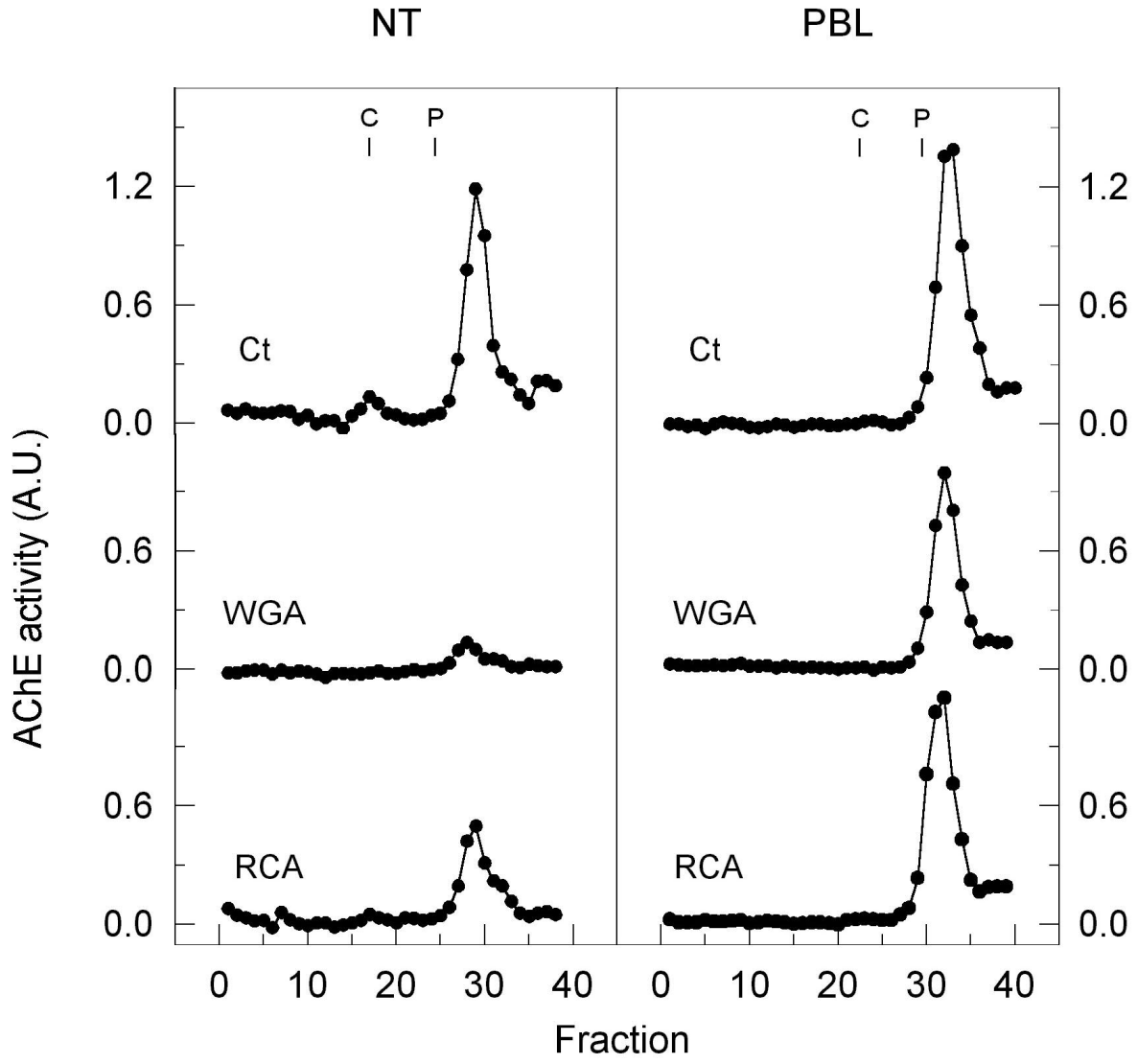

Figure 5
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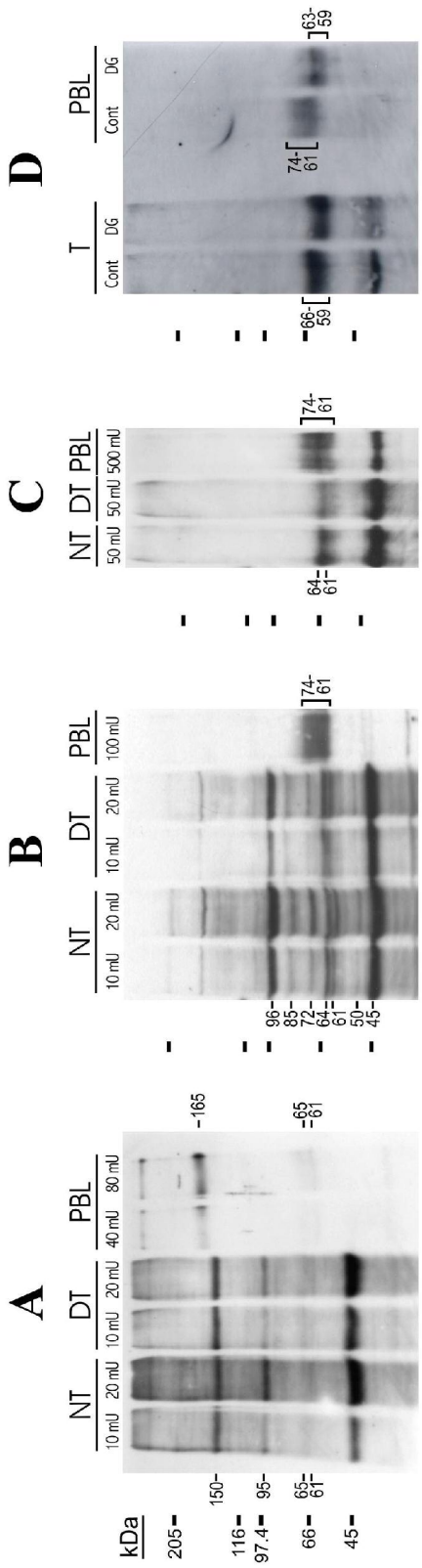

Figure 6
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