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Molecular Cloning of a Novel Transmembrane Protein MOLT Expressed by Mature Oligodendrocytes

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A novel oligodendrocyte (OL)-specific cDNA was isolated from brain capillary endothelial cells and characterized. The cDNA encodes a protein of 1099 amino acids that contains a signal peptide and a transmembrane domain. The protein was expressed in mature OLs *in vivo* and *in vitro* cell cultures and was thus designated as mature OL transmembrane protein (MOLT). RT-PCR analysis showed that MOLT mRNA was expressed in brain, lung, pancreas, and testis. A polyclonal antibody raised against a part of the mouse MOLT reacted specifically with multipolar OLs possessing radially oriented processes that penetrated into the gray matter. More cells were detected in the white matter, and these had longitudinally oriented processes. In a rat OL lineage culture system, oligodendrocyte precursor cells did not initially produce MOLT mRNA and protein, but when they begun to differentiate into mature OLs, they started expressing MOLT. Consequently, MOLT may function as OLs become mature and may serve as a cell-surface marker for OL differentiation.

Key words: differentiation, glial cell, myelination, oligodendrocyte, oligodendrocyte precursor cell.

Abbreviations: AA, amino acid residue; DIV, day *in vitro*; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; E-MEM, Eagle's minimal essential medium; FBS, fetal bovine serum; HBCE, human brain capillary endothelial cell; hMCT, COS-7 cells transfected with h-MOLT cDNA; HSMEC, human skin microvessel endothelial cell; HUVEC, human umbilical vein endothelial cell; MAG, myelin-associated glycoprotein; MBP, myelin basic protein; mMCT, COS-7 cells transfected by m-MOLT cDNA; MOG, myelin/oligodendrocyte glycoprotein; MOLT, mature oligodendrocyte transmembrane protein; O-2A, oligodendrocyte-type 2 astrocyte progenitor cell; OL, oligodendrocyte; OPC, oligodendrocyte precursor cell; D-PBS, Dulbecco's phosphate-buffered saline; PDGF, platelet-derived growth factor; PLP, proteolipid protein; RACE, rapid amplification of cDNA ends; RT, room temperature; SMA, smooth muscle actin; vWF, von Willebrand factor.

The central nervous system comprises the brain and the spinal cord, which contain nerve cells and their processes together with various many specialized support cells, namely astrocytes, oligodendrocytes (OLs), and microglial cells, which are collectively called the glia. OLs, the myelin-producing cells of the central nervous system, are perineuronal and may serve to regulate the microenvironment around neurons. Characterization of specific markers of OLs has increased our knowledge of the stages of OL maturation *in vivo* and *in vitro*. The sequential expression of markers identifies the stages of the lineage of the OL development characterized by proliferative capacities, migratory activities, and drastic morphological changes (*1*).

The presence of oligodendrocyte precursor cells (OPCs) *in vivo* was recognized by their reaction with anti-platelet-derived growth factor (PDGF) receptor antibody and anti-NG2 antibody (*2*). Premature OLs were also characteristically detected by O4 (*3*), and mature OLs were possibly stained by immunological markers that also strongly stain myelin. Therefore, their processes and cytoplasm were mostly masked, so that it was sometimes difficult for OLs to be preferentially recognized. Further, as maturation of OLs proceeds the staining of myelin basic protein (MBP) and proteolipid protein (PLP) decreases, since the concentration of these proteins in the cytoplasm and processes decreases; and, therefore, antibodies against these proteins do not function as good markers (*4*). Thus, it is prerequisite to have specific markers that recognize mature OLs in vivo and thus allow the study of their biological function.

In previous studies, primary cultures of OPCs from newborn rats made an important contribution to the study of OL differentiation (*5–7*). Many OL differentiation markers were first identified in tissue cultures. Raff et al. prepared OPCs from perinatal rat optic nerve and

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cultured them. These were bipolar cells, formerly called oligodendrocyte-type 2 astrocyte progenitor cell (O-2A), and were able to differentiate into OLs and type 2 astrocytes that produced glial fibrillary acidic protein (GFAP) (*8*) under appropriate culture conditions. OPCs were recognized by their reactivity with A2B5 monoclonal antibody (*6*, *9*, *10*), anti-PDGF receptor antibody (*11*), and anti-NG2 antibody (*12*, *13*). These cells were shown to differentiate into pre-mature OLs, which were becoming multipolar and could be marked by a monoclonal antibody, O4, that reacted with sulfatide, seminolipid, and to some extent with cholesterol (*14*), and sequentially into mature OLs, which were also multipolar in shape but also contained long processes forming a myelin sheath. These OLs were recognized by O1 antibody, which reacted with galactocerebroside, monogalactosyl diglyceride and psychosine (*14*, *15*) and with myelin structural proteins such as MBP, PLP, myelin-associated glycoprotein (MAG) and myelin/oligodendrocyte glycoprotein (MOG) (*1*). In primary cell cultures, the discrete stages of OL differentiation from neural stem cells to mature OLs can be reproduced quite faithfully. However, although some markers are very useful in culture to determine the sequence of maturation and ultimately its mechanism, they may be more difficult to use *in situ*, because some of them are present on other cell types *in vivo* (*1*).

The major function of mature OLs is to form a myelin sheath around most of the axons in the central nervous system. Multiple sclerosis is a representative demyelinating disease and is characterized by multifocal loss of myelin, OLs, and axons (*16*). Since OLs form the myelin sheath as their maturation proceeds, the polypeptides that are produced by the mature OLs could function for myelination. The identification of such polypeptides produced by these mature OLs should help to solve the mechanism of myelination and/or pathogenesis of multiple sclerosis.

Our original purpose in this study was to isolate genes closely involved in blood-brain barrier function, for which we adopted the strategy of cloning cDNAs by subtracted hybridization (*17*). Our idea was that such genes might be expressed specifically in brain capillary endothelial cells, and therefore we subtracted cDNAs of human brain capillary endothelial cells from those of endothelial cells outside of the brain. Although the cloning experiment proceeded nicely, we realized later that the original cell line was a mixture of endothelial cells, astrocytes and other cells and unexpectedly but successfully isolated a clone originated from OLs. This present communication describes the isolation of a previously unrecognized gene, MOLT, which is not expressed by cultured OPCs but is active in mature OLs. We also generated a polyclonal antibody against MOLT and found that it did not stain the myelin but stained the entire cytoplasm including the processes of mature OLs in the white matter and the gray matter. This antibody may thus serve as a useful tool to further explore the biological function and differentiation of OLs.

MATERIALS AND METHODS

Animals—All animal experiments were carried out in accordance with the Guidelines for Animal Experiments at Okayama University Medical School, Japanese Government Animal Protection and Management Law (No. 105), and Japanese Government Notification on Feeding and Safekeeping of Animals (No. 6).

Cells—Human brain capillary endothelial cells (HBCEs), human skin microvessel endothelial cells (HSMECs) and human umbilical vein endothelial cells (HUVECs) were obtained by procedures described previously (*18*).

After several passages, we stained HBCEs, HSMECs, and HUVECs with anti-von Willebrand factor (vWF), GFAP, and smooth muscle actin (SMA) antibodies as general markers for endothelial cells, astrocytes, and smooth muscle cells, respectively. HBCEs always showed strong signals for vWF but sometimes faint signals for GFAP and SMA. HSMECs and HUVECs were immunopositive with anti-vWF antibody but not with either anti-GFAP or SMA antibodies.

COS-7 cells were obtained from RIKEN Cell Bank and cultured in Dulbecco's modified Eagle medium (Sigma) supplemented with 10% fetal bovine serum (FBS; JRH Bioscience). These cells were transfected, by the lipofection method using Lipofectamine 2000 reagent (Invitrogen), with pcDNA3.1/V5-His vector (Invitrogen) bearing the inserted genes of interest, according to the manufacturer's instructions. The pcDNA3.1/V5-His construct for transfection contained the inserted cDNA of the full-length coding sequence of human MOLT (h-MOLT) (Met1–Val1099) or mouse MOLT (m-MOLT) (Met1–Val1097) fused to a V5 and His tag at the end of its C-terminus.

Oligodendrocyte Lineage Cell Culture—OL lineage cell cultures were prepared as described previously (*19*). Briefly, OPC and OL cultures were prepared from an E16 rat cerebrum, which was mechanically dissociated through 140-μm-pore stainless mesh. The cells were further dissociated by pipetting the tissue suspension in and out of a fire-polished Pasteur pipette, suspended, centrifuged, and resuspended in 10% FBS/Eagle's minimal essential medium (E-MEM, Invitrogen) and finally sieved twice through 70-μm-pore nylon mesh. Dispersed cells were seeded on poly L-lysine (100 μg/ml)–coated 90-mm-diameter culture dishes at a density of 1×10^7 cells/dish. Cells were incubated in an atmosphere of 90% air and 10% CO_2 and approximately 98% humidity at 37°C. After 7 d of culture, the cells were passaged by digestion with 0.05% trypsin in Dulbecco's phosphate-buffered saline (D-PBS, Invitrogen) (1st passage). They were then resuspended in 10% FBS/E-MEM, sieved through a 10-μm-pore nylon mesh, and cultured for 7 d at a density of 8×10^6 cells per non-coated culture dish. After 7 d of culture, the cells were passaged with 0.05% trypsin in D-PBS, then cultured for 2 d in 10% FBS/E-MEM at a density of 3×10^6 cells per non-coated Petri dish (2nd passage). Thereafter, the medium was changed to serum-free chemically defined Dulbecco's modified Eagle medium supplemented with 10 μg/ml insulin, 0.5 μg/ml transferrin, 100 μg/ml bovine serum albumin, 60 ng/ml progesterone, 16 μg/ml putrescine, 40 ng/ml sodium selenite, 60 ng/ml *N*-acetyl-L-cysteine, 5 μM forskolin, and 10 ng/ml PDGF-AA (*6*), and the OPCs were cultured for another 2 d. To induce differentiation, OPCs were cultured continuously with 30 ng/ml triiodothyronine, 40 ng/ml thyroxine, and 10 ng/ml neurotrophin-3, but without PDGF, for 3–5

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d. All of these procedures were necessary to eliminate neurons and astrocytes.

cDNA Cloning—The PCR select cDNA subtraction method (Clontech) was employed for cDNA cloning. Poly (A^+) RNAs were isolated from HBCEs, HSMECs and HUVECs according to the standard protocol (*20*). Tester cDNAs were obtained from 2 μg of Poly (A^+) RNA from HBCEs, and driver cDNAs were obtained from 1 μg each of Poly (A^+) RNA from HSMECs and HUVECs. The subtraction hybridization was performed by the method recommended by Clontech. Finally, the subtracted PCR products were subcloned into the pAMP10 vector by the method used by the pAMP10 system (GIBCOBRL).

Since we obtained the interesting subtracted clone 120, 5′ and 3′ RACE were performed based on the sequence of 120 by using a Human Fetal Brain Marathon-Ready cDNA library (Clontech) according to the manufacturer's protocol. Primers for the first PCR of 5′ RACE were 5′-CCATCCTAATACGACTCACTATAGGGC-3′ (AP1 primer) and 5′-AACTCCATCGTGATGAGTAGCGAGG-3′. The nested PCR primers of 5′ RACE were 5′-ACTCACTATAGGGCTCGAGCGGC-3′ (AP2 primer) and 5′-TAAGTGGGTCTGCCAGGATCTGG-3′. For 3′ RACE, the first primers were AP1 and 5′-CCATACAGCCTCAGCCAATGACCCC-3′, and the nested primers were AP2 and 5′-CCAGGATGTGTAGCAACGGTGCC-3′. The PCR was performed with LA Taq (Takara).

A mouse brain cDNA library (Clontech) was repeatedly used to screen for cDNAs covering the entire coding region of the m-MOLT. 5′ RACE was employed by using a Mouse Brain Marathon-Ready cDNA library (Clontech).

For cloning rat MOLT (r-MOLT) cDNAs, RT-PCR was performed with degenerated PCR primers according to the sequences of h- and m-MOLT. 5′ RACE was performed with a Rat Brain Marathon-Ready cDNA library (Clontech).

Northern Blot Analysis—Total RNA was isolated from cultured cells by the standard protocol (*20*), electrophoresed on 1% agarose gel under denaturing conditions, blotted onto a Hybond nylon filter (Amersham Pharmacia Biotech), and hybridized with the probes specific to h-MOLT labeled with [^{32}P]dCTP by random priming (Amersham Pharmacia Biotech). All images were processed for publication using Adobe® Photoshop™ v5.0.

PCR Analysis of cDNA Library and RT-PCR of Oligodendrocyte mRNA—cDNAs from multiple human tissue cDNA panels (Clontech) were analyzed by PCR using the primers 5′-CCACCAGGAACTCCATCGTG-3′ and 5′-CGACGCACACATTCCAGAGT-3′, which were designed from the h-MOLT sequence. Reaction mixtures were amplified for 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 60 s, and polymerization at 72°C for 50 s. PCR products (5 μl) were analyzed on 1% agarose gel with ethidium bromide and visualized by UV.

Total RNA extracted from equal amounts of RNA (25 ng) from cultured OLs was reverse transcribed by using Superscript RNaseH-reverse transcriptase II (GIBCO-BRL) and random primers for 60 m at 42°C and heated for 10 m at 95°C to stop the reaction. The synthesized cDNA (1 μl) was amplified in a total volume of 15 μl with 0.5 U AmpliTaqGold DNA polymerase (Perkin-Elmer) in the presence of 0.25 mM dNTP, 1 mM $MgCl_2$ and 25 pmol each of two primers for the r-MOLT fragment (633 bp): 5′-primer TGTCTTCGTCAATGGGAAGG and 3′-primer CTCATCCAACACTGTGATGG. The PCR product of 18S-rRNA (488 bp) was used as an internal control. Reaction mixtures were amplified for 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and polymerization at 72°C for 50 s for r-MOLT and 18S-rRNA. PCR products (10 μl) were analyzed on 3% NeuSieve 3:1 agarose gel (FMC) and visualized by UV after ethidium bromide staining. These signals were semiquantified by IPLab Gel.

Antibodies—Rabbit polyclonal antibody (Ab238) was raised against the peptide 238CAKEDSRKSGGTPAGHNDVDE258 (prepared by Sawady Technology) selected from the m-MOLT amino acid sequence and affinity-purified by using the peptide. The antibodies used for Western blotting and immunohistochemistry were the following: mouse monoclonal antibody (mAb) anti-GFAP (GA5) and mouse mAb anti-β actin from Sigma-Aldrich; mouse mAb anti-V5, from Invitrogen Corp, polyclonal anti-NG2 antibody from Dr. Stallcup, and O1 from American Type Culture Collection. As secondary antibodies, FITC-conjugated, affinity-purified, goat anti-rabbit IgG with minimal cross-reaction to IgGs of other species (Jackson Immunoresearch Laboratories) and HRP-conjugated goat anti-rabbit and anti-mouse IgG (Cappel) were used.

Western Blot Analysis—To obtain cell extracts, we washed cultured cells with PBS briefly, added 2% Triton X-100, 1% DTT, and 9 M urea in PBS to the dish, and scraped the cells off the dish surface. The scraped cells and extraction fluid were centrifuged at 12,000 rpm for 5 m at 4°C, and the supernatant was used as the sample for SDS-PAGE. The samples were electrophoresed in 10% SDS-PAGE gels, transferred onto nitrocellulose membranes (Advantech), and sequentially incubated with primary antibody and secondary antibody conjugated to HRP (Cappel), with a washing step between each incubation step. The peroxidase was visualized by use of ECL reagents (Amersham Pharmacia Biotech). All images were processed for publication by using Adobe® Photoshop™ v5.0.

Immunohistochemical Analysis—Immunohisochemistry was performed as described previously (*21*, *22*) with slight modifications. In brief, cryostat sections (10 μm) were prepared from adult BALB/c murine brain embedded in OCT medium (Sakura) after the brain had been snap-frozen in hexane cooled by liquid nitrogen. The sections were fixed with acetone for 10 m at 4°C, blocked with 1% bovine serum albumin for 1 h, and sequentially incubated with primary antibody and secondary antibody diluted with blocking solution. The samples were examined under a BX50 microscope (Olympus) equipped with an AxioVision system (Carl Zeiss). A 10× or 20× UplanFl objective lens was used. All images were processed for publication by using Adobe® Photoshop™ v5.0.

Immunocytochemical Analysis—For cell-surface staining with O1 and Ab238 antibodies, OLs growing on coverglasses were fixed with 4% paraformaldehyde solution for 15 m at RT. After washing, the cells were incubated with the primary antibody Ab238 over night at 4°C, washed again, and incubated with Alexa Fluor 488-conjugated anti-rabbit IgG (1:3000, Molecular Probes) for 1 h at RT. After incubation with the secondary antibody, the cells were incubated with the primary O1 antibody (1:50)

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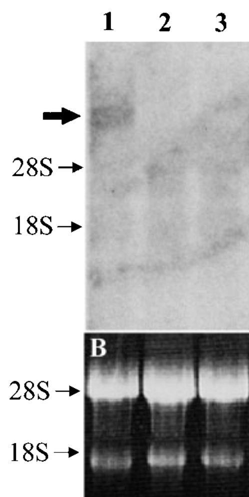

Fig. 1. **Northern blot analysis for the h-MOLT mRNA.** (A) Total RNA (15 μg) samples isolated from HBCE (lane 1), HSMEC (lane 2), and HUVEC (lane 3) were electrophoresed on 1% agarose gels, blotted onto nylon filters, and hybridized with the h-MOLT-specific probe. A thick arrow indicates the location of h-MOLT mRNA. Locations where the 28S and 18S rRNAs migrated are indicated at the left side. (B) The ethidium bromide staining pattern following electrophoresis but before blotting is shown in the bottom panel to indicate the location of the 28S and 18S rRNAs.

for 1 h at RT, washed, and incubated with Alexa Fluor 594-conjugated anti-mouse IgM (1:1,500, Molecular Probes) for 1 h at RT.

Stained cells were mounted on slide-glasses by using Vectashield® mounting medium (Vector Lab.). Fluorescence microscopy was performed with a Zeiss confocal laser scanning LSM510 system equipped with an inverted microscope (Axiophot; Carl Zeiss).

RESULTS

Molecular Cloning of MOLT—Our original purpose was to clone cDNAs specific for brain endothelial cells; and, therefore, we subtracted cDNAs of HSMECs and HUVECs from those of HBCEs. By the subtracted hybridization, we characterized 189 clones, from which we identified 13 independent clones by nucleotide sequence and Northern blot analyses. They were all relatively highly expressed in HBCE. One of these clones, called 120, displayed a weak expression of mRNA of approximately 5.5 kb in size in HBCEs, but none in HSMECs and HUVECs, by Northern blot analysis (Fig. 1). This is the clone we characterized further and designated MOLT as its coding protein in this report. To extend the clone in both 5′ and 3′ directions, we conducted 5′ and 3′ RACE and isolated two clones, both of which overlapped with the original 120 clone at its 5′ and 3′ ends. Nucleotide sequence analysis revealed that the cDNA had a total size of 5,790 bp, of which the central 3,297 bp represented a coding sequence of 1,099 amino acid residues (AAs; Accession number: AB061814), as shown in Fig. 2. A part of the coding sequence had already been deposited in the DNA database as KIAA1944 protein (Accession number: BAB85530).

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Human  MCPSEMGTLWHHWSPVLISLAALFSKVTEGRGILESIQRFSLLPTYLPVTYHINNADVSF 60
Rat    MCPSEMGTLWYLWSPVLISLAALFSKVTEGRGILESIQRFSLLPTYLPVTYHINNADVSF
Mouse  MCPSEMGTLWYLWSPVLISLAALFSKVTEGRGILESIQRFSLLPTYLPVTYHINNADVSF 60

Human  FLKEANQDIMRNSSLQSRVESFLIYKSRRLPVLNASYGPFSIEQVVPQDLMLPSNPFGFT 120
Rat    FLKEANQDIMRNSSLQSRVESFLIYKSRRLPVLNASYGPFSLEQVVPQDLMLPSNPFGFT
Mouse  FLKEANQDIMRNSSLQSRVESFLIYKSRRLPVLNASYGPFSLEQVVPQDLMLPSNPFGFT 120

Human  NKFSLNWKLKAHILRDKVYLSRPKVQVLFHIMGRDWDDRSAGEKLPCLRVFAFRETREVR 180
Rat    NTFSLNWRLKAYILQEKVYLSHPKVQVLFHIVGRDWDD-HRDENLPCLRVFAFRETQEVR 179
Mouse  NTFSLNWRLKAYILQEKVYLSHPKVQVLFHIVGRDWDD-HRDENLPCLRVFAFRETREVR 179

Human  GSCRLQGDLGLCVAELELLSSWFSPPTVVAGRRKSVDQPEGTPVELYYTVHPGGERGDCV 240
Rat    GSCRLGGALGLCVAQLEMLPGWFSPPSVVSGRRRPTEQPEGSPVELYYAVQPGDERGDCA 239
Mouse  GSCRLGGALGLCVAQLEMLPGWFSPPSVVSGRRRPTEQPEGNPVELYYAVQPGDERGDCA 239

Human  REDARRSNGIRTGHSDIDESGPPLQRIGSIFLYQTHRKPSLRELRLDNSVAIHYIPKTVR 300
Rat    KEDSRKSRGPPAGHNDVDESSPPLHRIGSVFLRETPSSPPLRELRLDSNVAVHYIPKTVR 299
Mouse  KEDSRKSGGTPAGHNDVDESSPPLHRIGSVFLRETPSSPPLKELRLDSNVAVHYVPKTVR 299

Human  KGDVLTFPVSISRNSTEDRFTLRAKVKKGVNIIGVRASSPSIWDVKERTDYTGKYAPAVI 360
Rat    QGDVLTFPISVSRNCTEDRFTLRAKVKKGVSIVGVRASTSSLWDVKQSTEYTGKYAPAVI 359
Mouse  QGDVLTFPISVSRNCTEDRFTLRAKVKKGVSIVGVRASSSSIWDVRQSTEYTGKYAPAVI 359

Human  VCQKKAAGSENSADGASYEVMQIDVEVEEPGDLPATQLVTWQVEYPGEITSDLGVSKIYV 420
Rat    ICQKKSAGSGKSVDDASYEVMKIDIEVEAPSDPPTTQLVTWQVEYPGEITSDLGVSKIYV 419
Mouse  VCQKKSAGSEKSVADASYEVMKIDIEVEAPSDPPTTQLVTWQVEYPGEITSDLGVSKIYV 419

Human  SPKDLIGVVPLAMEAEILNTAILTGKTVAVPVKVVSVEDDGTVTELLESVECRSSDEDVI 480
Rat    SQKDLIGVIPLAMEAEILNTAILTGKTVAVPVKVISVEEDGTVQGLSDSVECRSSDEDVV 479
Mouse  SQKDLIGVIPLAMEAEILNTAILTGKTVAVPVKVISVEEDGTVQGLLDSVECRSSDEDVV 479

Human  KVSDRCDYVFVNGKEMKGKVNVVVNFTYQHLSSPLEMTVWVPRLPLQIEVSDTELNQIKG 540
Rat    KVSDRCDYVFVNGKEMKGKVNVVVTITYQHLSSPLEMTVWVPRLPLQIEVSDMELNQIKG 539
Mouse  KVSDRCDYVFVNGKEMKGKVNVVVSFTYQHLSSPLEMTVWVPRLPLQIEVSDMELNQIKG 539

Human  WRVPIVSSRRPAGDSEEEEDDERRGRGCTLQYQHAMVRVLTQFVAEAAGPGGHLAHLLGS 600
Rat    WRVPVVSNKRPARDSEEEDDDEKRGRGCTLQYQHAMVRVLTQFVAEAPDPGGHLAHLLGS 599
Mouse  WRVPIVSNKRPARDSEEEDDDEKKGRGCTLQYQHAMVRVLTQFVAEAPDPGGHLAYLLGS 599

Human  DWQVDITELINDFMQVEEPRIAKLQGGQILMGQELGMTTIQILSPLSDTILAEKTITVLD 660
Rat    DWQVDITELITDFMQVEEPRIAKLQGGQILTGQELGMTTIQILSPLSDAILAEKTITVLD 659
Mouse  DWQVDITELITDFMQVEEPRIAKLQGGQILTGQELGMTTIQILSPLSDAILAEKTITVLD 659

Human  EKVTITDLGVQLVTGLSLSLQLSPGSNRAIFATAVAQELLQRPKQEAAISCWVQFSDGSV 720
Rat    EKVTITDLGVQLVTGLSLSLQLSPGSNRAIFATAVAQELLQRPKQEAAISCWVQFSDGSV 719
Mouse  EKVTITDLGVQLVTGLSLSLQLSPGSNRAIFATAMAQELLQRPKQEAAISCWVQFSDGSV 719

Human  TPLDIYDGKDFSLMATSLDEKVVSIHQDPKFKWPIIAAETEGQGTLVKVEMVISESCQKS 780
Rat    TPLDIYDEKDFSLMATSLDEKVVSILQDPKFKWPIIAAENEGQGALVKVEMLISESCQKS 779
Mouse  TPLDIYDEKDFSLMATSLDEKVVSILQDPKFKWPIIAAENEGQGALVKVEMLISESCQKS 779

Human  KRKSVLAVGTANIKVKFGQNDANPNTSDSRHTGAGVHMENNVSDRRPKKPSQEWGSQEGQ 840
Rat    KRKSVLAVGTASIKVKFGQNDANPNGSESGHLGAGLHVEN-INDRRSKKPFQEWGSPEGP 838
Mouse  KRKSVLAVGTASIKVKFGQNDANPNSSESGHLGAGLHVEN-INDRRSKKPFQEWGSPEGP 838

Human  YYGSSSMGLMEGRGTTTDRSILQKKKGQESLLDDNSHLQTIPSDLTSFPAQVDLPRSNGE 900
Rat    YYSSSSMGLMEGWGSTTKRPTFPKKEGQENLLDDIILSQTMATDLTSFPDQVDLPGSNVG 898
Mouse  FYSSSSMGLMEGWGSTTKRPTFQKKEGQENLLDDIILSQTMATDLTSFPDQMDLPGSNVG 898

Human  MDGNDLMQASKGLSDLEIGMYALLGVFCLAILVFLINCVTFALKYRHKQVPFEEQEGMSH 960
Rat    TEEHDPDQAAKGLSDLEIGMYALLGVFCLAILVFLINCVTFALKYRHKQVPFEEQEGLSH 958
Mouse  TEEHDLDQAAKGLSDLEIGMYALLGVFCLAILVFLINCVTFALKYRHKQVPFEEQEGLSH 958

Human  SHDWVGLSNRTELLENHINFASSQDEQITAIDRGMDFEESKYLLSTNSQKSINGQLFKPL 1020
Rat    SHDWVGLSNRTELLGNHMNFASSQEEQITAIDRGLDFEESKLLLSSNSQNSINGQLFRST 1018
Mouse  SHDWVGLSNRTELLGNHMNFASSQEEQITAIDRGLDFEESKLLLSSNSQNSINGQMFRST 1018

Human  GPIIIDGKDQKSEPPTSPTSKRKRVKFTTFTAVSSDDEYPTRNSIVMSSEDDIKWVCQDL 1080
Rat    GAMLTDDKEQKSEPPTSPTSKRKRVTFSTFSAISSDDGCPSGNTMVLSNEDDIKWVCQAL 1078
Mouse  GAMLTDDQEQKSEPPTSPTSKRKRVTFSTFSAISSDDGCPSGNTMVLSNEDDIKWVCQGL 1078

Human  DPGDCKELHNYMERLHENV                                          1099
Rat    DPGECPEPHSCMERLHEHV                                          1097
Mouse  DPGECTEPHSCMERLHEHV                                          1097
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Fig. 2. **Deduced amino acid sequences of the human (upper line), rat (middle line) and mouse (lower line) MOLT.** Putative hydrophobic signal peptide (1–26, underlined), transmembrane sequence (921–943 for human and 919–941 for rat and mouse, dotted underline), cysteinyl residues (squares), and the peptide sequence against which Ab238 antibody was raised (doubled underlines) are marked. Identical amino acid residues among human, rat, and mouse MOLTs are colored with a gray background. Accession numbers for h-MOLT, r-MOLT, and m-MOLT are AB061814, AB100356, and AB100355, respectively.

To isolate mouse cDNAs, we screened a mouse brain cDNA library (Clontech) by use of the human cDNA as probe and obtained two cDNAs that were separated from each other. To cover these 2 cDNAs, we performed RT-PCR and extended the 5′ end by 5′ RACE. The size of the open reading frame was 1097 AAs (Accession number: AB100355), 2 AAs shorter than the human counterpart. The nucleotide sequence of m-MOLT has been made public as an unnamed protein product (Accession number: BAC38810).

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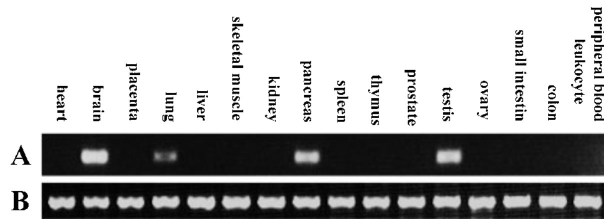

Fig. 3. **Tissue distribution of h-MOLT mRNA.** PCR products were amplified from multiple human tissue cDNA panels (Clontech) by using primers designed from the h-MOLT sequence. (A) h-MOLT mRNA was detected in the brain, lung, pancreas, and testis. (B) GAPDH mRNA was amplified as an internal control.

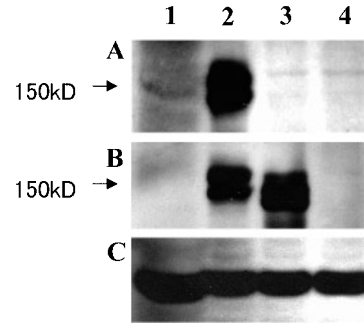

Fig. 4. **Western blot analysis with Ab238 antibody.** Extracts from adult BALB/c mouse brain (lane 1), and cell lysates of m-MOLT transfectants, mMCT (lane 2), h-MOLT transfectants, hMCT (lane 3) and MOCK transfectants (lane 4) were subjected to SDS-PAGE, followed by Western blot analysis using Ab238 antibody (A), anti-V5 mAb (B), and anti-β-actin mAb (C). Molecular mass is indicated at the left side of the panels (A–C). MOLT proteins were detected in mouse brain and mMCT extracts as a 150–160 kDa product but not in hMCT.

In the cloning of the rat counterpart, we designed primers for the regions where the mouse and human sequences were highly homologous, performed the degenerate PCR method, and isolated two cDNAs. The sequence between these two cDNAs was covered by a long PCR, and the uncovered 5′ end region was reached by 5′ RACE. Thus, the entire cDNA was completed and the deduced amino acid sequence was found to be 1097 AAs (Accession number: AB100356), the same as that of the mouse sequence, and again two AAs shorter than the human one. No rat sequence has been reported in the database yet.

MOLT was highly conserved between human and rat with an identity of 83.4% and between human and mouse with one of 83.2% (Fig. 2). From its amino acid sequence, h-MOLT was predicted to include an N-terminal hydrophobic signal peptide (Met1–Lys26) and a transmembrane region (Tyr921–Leu943) by using a simple modular architecture research tool (http://smart.embl-heidelberg.de/). Seven predicted *N*-glycosylation sites (Asp72, Asp94, Asp314, Asp505, Asp805, Asp821, and Asp969) were obtained with the ScanProsite program (http://kr.expasy.org/tools/scanprosite/); two predicted *O*-glycosylation sites (Ser1032 and Thr1036) were found by using the NetOGlyc 2.0 Prediction Server (http://www.cbs.dtu.dk/services/NetOGlyc/); and several phosphorylation sites for different kinases were revealed by the NetPhos 2.0 program (http://www.cbs.dtu.dk/services/NetPhos/).

Tissue Distribution of h-MOLT mRNA—The expression of h-MOLT mRNA in various tissues was examined by RT-PCR using a multiple human tissue cDNA panel (Clontech) and primers designed from the h-MOLT sequence. As shown in Fig. 3, h-MOLT mRNA was highly expressed in the brain, but was also detected in the lung, pancreas, and testis. We also confirmed the m-MOLT expression in the mouse brain and lung by Northern blot analysis (data not shown).

Rabbit Anti MOLT Polyclonal Antibody (Ab238) Reacts with m-MOLT—To identify translation products of the m-MOLT mRNA, we raised polyclonal antibody Ab238, which was specific for a portion of the mouse sequence, 238CAKEDSRKSGGTPAGHNDVDE258. The specificity of the antibody was confirmed by Western blot analysis. Adult mouse whole brain extracts, cell extracts prepared from the human and mouse MOLT transfectants (hMCT and mMCT, respectively) and parental COS-7 cells were used as samples for SDS-PAGE. On Western blotting with anti-V5 mAb (Fig. 4B), two bands of 150–160 kDa bands were detected from the mMCT extract (Fig. 4B, lane 2) and hMCT extract (Fig. 4B, lane 3) but not the COS-7 extract (Fig. 4B, lane 4). The size of these bands was as expected considering the fused tag sequence to MOLTs and predicted glycosylation, since the calculated molecular mass of both h-MOLT and m-MOLT was approximately 130 kDa. The presence of the two bands might represent different forms of post-translational modification. Western blotting with Ab238 (Fig. 4A) revealed almost the same bands of 150–160 kDa (Fig. 4A, lane 2) from the extract of mMCT, but not from the extract of hMCT (Fig. 4A, lane 3) or COS-7 (Fig. 4A, lane 4). These results indicate that Ab238 antibody can specifically react with m-MOLT. To detect m-MOLT protein from mouse brain, we performed Western blotting with Ab238. A faint but substantial band around 150 kDa was detected from the mouse brain extract (Fig. 4A, lane 1), which indicated that m-MOLT was expressed in mouse brain.

Distribution of MOLT in the Adult Mouse Brain—The location of MOLT in the brain was identified by immunohistochemistry with Ab238 and with GA5, a monoclonal antibody against GFAP (Fig. 5). Cells positively stained by Ab238 in the mouse brain parenchyma were multipolar and often located next to astrocytes in the white matter and the gray matter of cerebral cortex. In the gray matter they appeared as OLs with radially oriented processes (Fig. 5A). The immunopositive cells in the white matter looked like OLs lying within the corpus callosum and bearing longitudinally oriented processes (Fig. 5B). Positive cells in the white matter and the gray matter showed a ratio of approximately 5:1 as counted within the same area, and so they were much more abundant in the corpus callosum. MOLT-positive cells were also observed in white matter in the spinal cord and in the optic nerve (data not shown). When we double-stained them with Ab238 and antibody against a microglial marker, Mac1, or against neurofilament, a marker for neurons, the MOLT-positive cells were negative for both of these other markers (data not shown). The amino acid

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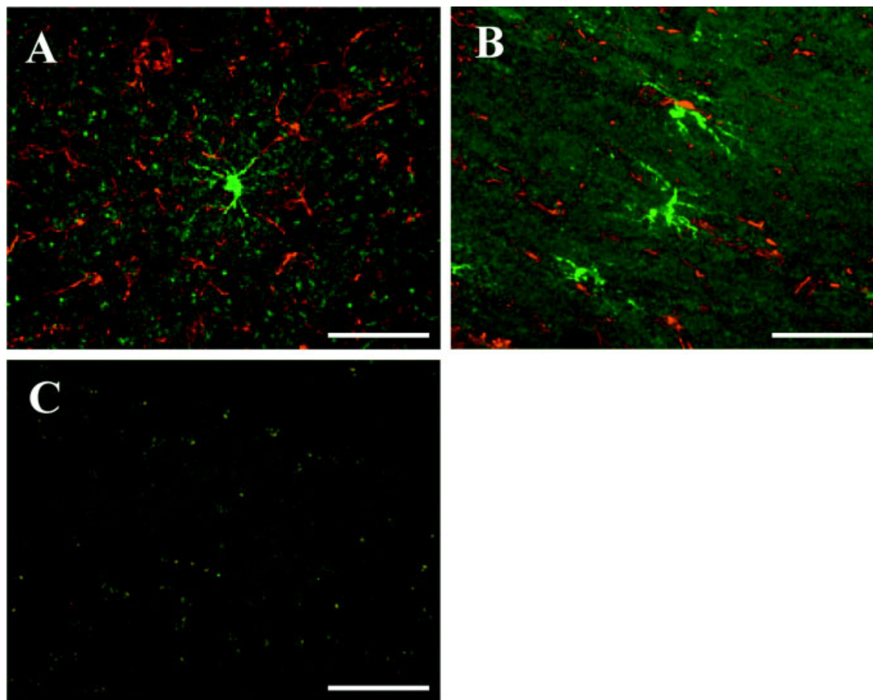

Fig. 5. **Localization of m-MOLT in cryostat sections of adult mouse brain.** Cryostat coronal sections (10 μm) of fresh adult murine cerebrum cortex were doubly immunostained with Ab238 (green fluorescence) and GA5 (red fluorescence). (A) An Ab238-positive cell within the gray matter. It appeared as OL that possessed radially oriented processes that extended toward astrocytes. (B) A few Ab238 positive-cells within the white matter (corpus callosm). They also looked like OLs that had longitudinally oriented processes. (C) Without 1st antibody. Scale bar = 50 μm.

sequence of the antigen peptide region of Ab238 is almost the same as that of the corresponding region of r-MOLT, and Ab238 was able to stain adult rat brain in a similar pattern.

MOLT Is Expressed by Mature Oligodendrocytes but Not by Oligodendrocyte Precursor Cells—To analyze whether cultured OLs expressed MOLT after their *in vitro* differentiation, we prepared rat OL lineage cells and induced the OPCs to differentiate into OLs by incubating them with triiodothyronine, thyroxine, and neurotrophin-3 but without PDGF (see "MATERIALS AND METHODS"). To confirm the quality of OL lineage cells, we performed immunostaining at 1 d *in vitro* (DIV) and 5 DIV (Fig. 6A). Of the cells at 1 DIV, 98% were positive for anti-NG2 polyclonal antibody, a marker of OPCs; and of those at 5 DIV, 97% were positive for O1, a marker of mature OLs, indicating that the cells differentiated from OPCs into mature OLs in this culture system, as reported earlier (*19*). OL lineage cells were harvested at 1, 3, 5 DIV, and their mRNAs were subjected to RT-PCR and used in PCR experiments to detect the presence of r-MOLT mRNA (Fig. 6B). r-MOLT mRNAs were detected at 3 and 5 DIV by RT-PCR, but were not at 1 DIV. This result demonstrates that OLs started expressing MOLT mRNA in the course of maturation.

Further, r-MOLT expression was also examined by double immunostaining of primary rat OL cultures with Ab238 and O1 (Fig. 6C). Multipolar cells were doubly

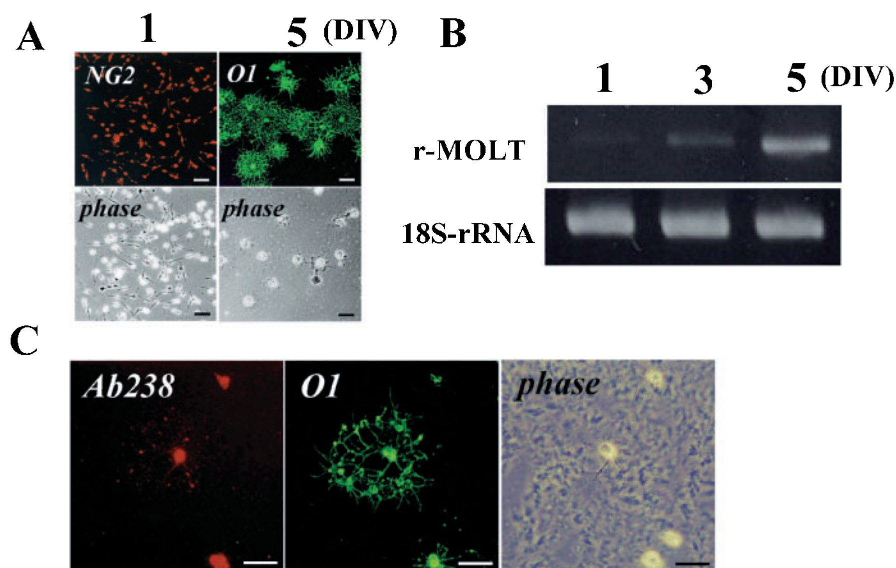

Fig. 6. **Mature OLs express r-MOLT *in vitro*.** (A) OPCs prepared from E16 rat cerebrum were incubated for differentiation. Cells at 1 DIV and 5 DIV were stained for anti-NG2 antibody, an OPC marker, and for O1, an OL marker. Phase-contrast images of each panel are shown. Note that most of the 1 DIV and 5 DIV cells were positive for anti-NG2 antibody and O1, respectively. We could not detect astrocytes or neuronal cells when the cultures were stained for specific markers (data not shown). Scale bar = 20 μm. (B) Ethidium bromide staining of RT-PCR samples generated from OL lineage cells at 1 DIV, 3 DIV, and 5 DIV. Note that r-MOLT mRNA was detected by RT-PCR in 3 DIV and 5 DIV samples, but not in the 1 DIV one. (C) r-MOLT was detected in mature OLs *in vitro*. r-MOLT expression was also examined by double immunostaining of primary rat OL cultures at 5 DIV with Ab238 and O1. Note that the three cells reacted positively with both antibodies. Scale bar=20 μm.

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immunolabeled with Ab238 and O1. With anti-NG2 polyclonal antibody and Ab238, however, they were positive only with Ab238 antibody (data not shown). These results also demonstrate that r-MOLT was expressed by mature OLs, not by OPCs.

DISCUSSION

Characterization and Expression of MOLT—We isolated a novel protein sequence by cDNA isolation and designated it as MOLT. Coding sequences deduced from our isolated mouse, rat and human cDNAs contained signal peptides and transmembrane domains but other functional domain structures were not evident. An extensive computer search did not show any protein family corresponding to MOLT, and therefore the function of MOLT is not yet known. We predicted the genomic structure of h-MOLT based on its cDNA sequence by using the BLAST search against the human genome draft sequences (http://www.ncbi.nlm.nih.gov/BLAST/). The h-MOLT gene was composed of 9 exons, and its chromosomal location was 12q24.3. It is interesting to note that the gene encompasses a stretch of 850 kb, although the related disease loci have not been found within this stretch or in a neighboring region.

Earlier, a part of the h-MOLT sequence was deposited in the GenBank/EMBL/DDBJ databases as KIAA1944 protein (*23*), which had been cloned as an extension of the human cDNA project for accumulating sequences of unidentified genes. The source of mRNA for this cDNA of KIAA1944 was human fetal brain, where more OPCs and fewer OLs would be present.

MOLT was found to be expressed in the brain, lung, pancreas, and testis by RT-PCR as shown in Fig. 3. KIAA 1944 seemed to be brain-specific from the results of ELISA RT-PCR (http://www.kazusa.or.jp/huge/gfpage/KIAA1944/). This difference in specificity could be due to difference in sensitivity in the two experiments, both of which employed RT-PCR.

The entire nucleotide sequence corresponding to m-MOLT was defined by the RIKEN Mouse Gene Encyclopedia Project and made available to the public through the FANTOM database (http://fantom.gsc.riken.go.jp/). It contains more than 99.9% identity to our m-MOLT, and therefore we think that they are identical. It was expressed in the adult hippocampus, and the mouse gene contains 9 exons and was assigned to chromosome 5.

Ab238 Antibody—Earlier investigators reported some antibodies that were able to stain the cytoplasm of OLs, but these antibodies were not necessarily useful. These include antibodies against galactocerebroside, the major galactosphingolipid of myelin, for instance, R-mAb (*24*) and O1 (*14*, *15*). They stained cultured OLs and frozen sections but were not good for staining embedded tissues. Antibodies against carbonic anhydrase II stained rat OLs (*25*), and monoclonal antibodies against RIP (*26*) and transferrin (*27*) seemed to stain the cytoplasm of OLs from rat tissues. However, antibodies against MBP, MAG, PLP, 2′,3′-cyclic nucleotide-3′-phosphohydrolase, and MOG strongly stained myelin, and therefore they covered the positively-stained processes and cell body of OLs. We tried to double-stain brain tissues using antibodies against O1, carbonic anhydrase II, and RIP with Ab238, but this failed because the necessary conditions for Ab238 staining were not suitable for the other antibodies. We also tried with antibodies against MBP and PLP, but they reacted so strongly with material in the white matter, as expected, that we could not recognize the positive fluorescence staining for Ab238. However, when we used Ab238 alone, it specifically stained OLs and showed a low background, making it a good immunoreagent to detect processes and cytoplasm of OLs. It was possible to double-stain with Ab238 and antibodies against an astrocyte marker, GFAP, a microglial marker, Mac1 and a marker for neurons, neurofilament. The results showed that the Ab238 staining was distinct from that with anti-GFAP, Mac1, or neurofilament, and so we consider that it was possible to rule out the staining of glial cells with Ab238.

It is interesting to know exactly which population of OLs is positive for Ab238. The present result showed that Ab238-positive OLs in adult mouse brain seem to be limited to those positive for antibodies to myelin protein, for example, anti-2′,3′-cyclic nucleotide-3′-phosphohydrolase (*28*). The Ab238 antibody could recognize a specific population among OLs, and extended studies *in vivo* using this antibody may contribute to new knowledge of OL biology.

Oligodendrocyte Differentiation and MOLT Expression—In previous studies on the origin and differentiation of OLs, glial progenitor cells or clonal analysis were used to examine OL development (*5*–*7*, *19*). OPCs isolated from rat optic nerve or brain were cultured under different conditions to allow them to produce marker proteins. Continued maturation of OPCs resulted in elevated expression of the major MBP, PLP, MAG, and MOG (*1*). In the present study, we identified the previously unknown protein MOLT, which was specific to OLs and whose expression increased as OPCs developed into OLs. It is interesting to note that MOLT seems to locate not with myelin proteins but at the cell surface of OLs. Myelination takes place in mature OLs at the terminal differentiation stage, where expression of MBP and PLP genes are usually upregulated (*29*). Myelination requires a number of sequential steps in the maturation of OL cell lineage (*1*), which are accompanied by a coordinated change in the expression of cell-surface antigens often recognized by monoclonal antibodies such as O1. Although we succeeded in dual staining for r-MOLT and a mature OL marker, O1 antigen, not all cells positive for O1 antigen were stained with MOLT antibody. We do not yet know which population of mature OLs was MOLT-positive. Approximately 60% of the cultured cells at 5 DIV were positive for MBP and PLP (*19*). MOLT was expressed by these OLs at 5 DIV, and thus we think that the mature OLs are able to produce MOLT RNA as well as protein. Although further characterization of MOLT is needed, this protein that is specifically expressed in mature OLs can now be utilized together with other markers such as MBP and PLP to investigate the mechanism of OL differentiation. Extensive research will be needed to investigate the function of MOLT and determine if it is involved in the myelin repair process after dysmyelination or demyelination.

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