The Product of the MLD Gene Is a Member of the Membrane Fatty Acid Desaturase Family: Overexpression of MLD Inhibits EGF Receptor Biosynthesis[†,‡]

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Received January 16, 1997; Revised Manuscript Received March 25, 1997[⊗]

ABSTRACT: Membrane fatty acid desaturases are responsible for inserting double bonds into specific positions in fatty acids. We have cloned a new member of the human membrane fatty acid (lipid) desaturase gene family, MLD. The derived amino acid sequence of MLD contains three consensus motifs, HX_3H, HX_2HH, and HX_2HHXFP, that are characteristic of a group of membrane fatty acid desaturases. MLD is predicted to be a multiple membrane-spanning protein and is found to be extractable from particulate fractions with detergent but not salt or urea. MLD is widely expressed in human tissues and is localized to the endoplasmic reticulum. Cotransfection of MLD with the epidermal growth factor (EGF) receptor resulted in decreased expression of the receptor but did not affect platelet-derived growth factor receptor expression. MLD overexpression inhibited biosynthesis of the EGF receptor, suggesting a possible role of a fatty acid desaturase in regulating biosynthetic processing of the EGF receptor.

Plasma membranes of cells provide boundaries that segregate intracellular components from the surrounding environment, allowing regulation of intracellular responses to extracellular signals. Intracellular membranes further serve to compartmentalize highly specialized functions in organelles of eukaryotic cells. The fluid mosaic model (*1*) predicts that proteins are embedded in a lipid bilayer, allowing proteins to diffuse laterally unless restricted by cytoskeletal attachments. The lipid composition of membranes must accommodate this requirement for "membrane fluidity". This is accomplished by incorporating unsaturated fatty acids, including polyunsaturated fatty acids, into membrane lipids, thus lowering the phase transition of membranes relative to saturated fatty acids (*2*). For example, the level of fatty acid desaturation is critical in regulating membrane fluidity and chilling tolerance in cyanobacteria and higher plants (*3*). Furthermore, the level and type of polyunsaturated fatty acid incorporated into membranes affects the activity of some integral membrane proteins (*2*), suggesting that the lipid composition of localized domains within the membrane may serve to regulate the function of membrane proteins.

The relative amounts of saturated and unsaturated fatty acids available for incorporation into membranes are determined by the activities of enzymes involved in fatty acid biosynthesis and metabolism. The level of fatty acid desaturation is regulated by the fatty acid desaturases, enzymes that insert double bonds into specific positions in the hydrocarbon chain of fatty acids (*4*). The desaturases require molecular oxygen and reduced pyridine nucleotide to catalyze the desaturation of fatty acids. In eukaryotes, the desaturases are localized primarily in the endoplasmic reticulum and, in plants, chloroplasts (*5*). In mammals, several fatty acid desaturase activities have been identified, including the Δ9-desaturase that functions in the conversion of saturated to monounsaturated fatty acids, in particular the critical reaction converting stearic acid to oleic acid (*6*). Other activities identified in mammalian cells include the Δ6 and Δ5 fatty acid desaturases, which function in the synthesis of polyunsaturated fatty acids such as arachidonic acid, and Δ8 and Δ4 desaturases (*4*). To date, only the Δ9 desaturase gene has been cloned in mammals (*6*). We report the identification of a gene encoding a putative fatty acid desaturase and find that it modulates biosynthesis of the transmembrane receptor for EGF.[1]

The EGF receptor is a 170 kDa type I transmembrane glycoprotein with an N-terminal extracellular ligand binding domain and an intracellular tyrosine kinase domain that is responsible for mitogenic signaling (*7*). The core tyrosine kinase domain of the EGF receptor (amino acid residues 663−958) was used in a yeast two-hybrid screen to isolate a sorting nexin, SNX1, that functions in the sorting of EGF receptor to lysosomes (*8*). Using the same approach, we have cloned a new member of the *m*embrane fatty acid (*l*ipid) *d*esaturase gene family, MLD. MLD contains three conserved His motifs that are characteristic of the membrane fatty acid desaturases and is predicted to be a multiple membrane-spanning protein. MLD has the properties of an integral membrane protein of the endoplasmic reticulum.

[†] This work was supported by funds provided by the Breast Cancer Fund of the State of California through the Breast Cancer Research Program of the University of California, Grants 1KB-0140 (D.L.C.) and 1FB-0314 (R.C.K.), and by NIH Grants CA58689 (G.N.G.) and F32DK08666 (R.C.K.).

[‡] The sequence of MLD has been deposited in GenBank (Accession No. AF002668).

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[⊗] Abstract published in *Advance ACS Abstracts,* May 15, 1997.

[1] Abbreviations: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetrataacetic acid; PMSF, phenylmethanesulfonyl fluoride; PBS, phosphate-buffered saline; RACE, rapid amplification of cDNA ends; BiP, immunoglobulin heavy chain-binding protein.

S0006-2960(97)00091-3 CCC: $14.00

When overexpressed in transfected cells, MLD specifically inhibited biosynthesis of the EGF receptor, implicating a functional role for membrane fatty acid desaturases in regulating biosynthesis of specific proteins.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Library Screen. A plasmid derived from pEG202 was generated to contain nucleotides corresponding to amino acids 663−958 of the EGF receptor fused to the lexA DNA binding domain (*8*). Proteins that interact with EGF receptor residues 663−958 were obtained by screening a HeLa library in pJG4−5 using galactose-inducible leucine auxotrophy and β-galactosidase activity in yeast (*9*). The 5′ ends of isolated cDNAs were sequenced by cycle sequencing and the sequence was used to search the nucleotide sequence databases of the National Center for Biotechnology Information using the BLAST algorithm (*10*). The sequence of the remainder of the insert was determined by transposon-mediated fragmentation following cloning into pMOBII (Gold Biotechnology, St. Louis, MO). The 5′ end of MLD cDNA was obtained using RACE with the Marathon cDNA amplification kit (Clontech, Palo Alto, CA) and the MLD specific primer ACATCGACGCCATCAGCTCCAAG-GT. The 5′ fragment was digested with *Not*I and *Kpn*I and subcloned into pBluescript (Stratagene, San Diego, CA) containing the original MLD insert to generate full-length MLD cDNA, which was confirmed by sequencing. The MLD cDNA was digested with *Not*I and *Xho*I and cloned into pCEP4 (Invitrogen, San Diego, CA) to generate a mammalian expression vector.

Northern Analysis. The size of MLD mRNA was determined by Northern analysis. An MLD fragment was generated by digestion with *Eco*RI and *Sca*I and ^{32}P-labeled in a random priming reaction. Human multiple tissue Northern blots were purchased from Clontech (catalogue nos. 7760-1 and 7759-1). Membranes were prehybridized in ExpressHyb (Clontech) for 1 h at 68 °C and hybridized in the presence of labeled probe for 1 h at 68 °C. Membranes were washed in four changes of 2 × SSC/0.1% SDS at room temperature and two changes at 50 °C followed by two changes of 0.1 × SSC/0.1% SDS at 50 °C and exposed to film.

Subcellular Fractionation. Confluent plates of HeLa human cervical carcinoma cells were placed on ice and rinsed with cold PBS containing 1 mM EDTA and 1 mM EGTA. Cells were scraped in cold extraction buffer (10 mM HEPES, pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, 1mM EDTA, and 1 mM EGTA) plus inhibitors (1 mM sodium vanadate, 10 mM NaF, 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 10 μg/mL antipain) and homogenized in a Dounce homogenizer. Homogenates were loaded onto a 1 mL cushion of 1 M sucrose in extraction buffer and centrifuged in an SW60 rotor at 1600*g* for 10 min. The pellet is designated P1. The interface at the sucrose cushion was diluted 1:1 with extraction buffer to make 0.5 M sucrose and centrifuged in an SW60 rotor at 150000*g*. The pellet is designated P2. The supernatant above the interface was centrifuged at 150000*g*. The supernatant from this spin is designated cytosol and the pellet is designated P3.

Confluent plates of CV1 African green monkey kidney cells were fractionated as above in extraction buffer plus inhibitors (1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 10 mM benzamidine). The pellet fractions were resuspended in extraction buffer and divided into 50 μL aliquots. Pellets were resuspended in 1 mL of extraction buffer (including inhibitors) or extraction buffer containing either 0.75 M NaCl, 6 M urea, or 1% Triton X-100 and incubated on ice for 30 min. Samples were centrifuged in a microfuge for 15 min at 4 °C and the supernatant was removed. Pellets were resuspended in SDS sample buffer and fractions were analyzed on SDS−acrylamide gels. The location of MLD was determined by probing immunoblots with an affinity-purified anti-peptide antibody (3906) against the C-terminal 14 amino acids.

Immunofluorescence. CV1 cells were fixed in 3.6% paraformaldehyde and permeabilized with 0.024% saponin. Cells were stained with MLD antibody (3906) followed by Texas Red-conjugated goat anti-rabbit IgG. The cells were costained with the endoplasmic reticulum marker rat GRP78 (BiP) and visualized with monoclonal antibody SPA-827 (StressGen Biotechnologies, Victoria, BC, Canada) followed by fluoroscein-conjugated goat anti-mouse IgG. Staining was detected using epifluorescence illumination.

Transient Transfection and in Vivo Labeling. For transient transfection experiments, 293 EBNA cells (Invitrogen), human embryonic kidney cells that stably express the Epstein-Barr virus EBNA-1 protein for high expression of the multicopy plasmid pCEP4, were seeded at 600 000−700 000 cells on 6 cm dishes and transfected by calcium phosphate coprecipitation (*11*). Cells were cotransfected with 0.5 μg of pCMV-βGal, 1 μg of pRcK$^+$ expressing the EGF receptor (*8*), the indicated concentrations of control pCEP4 vector or vector containing MLD (1−4 μg), and pUC18 DNA to a total of 10 μg for 4 h. Alternatively, the same conditions were used except that 1 μg of pPDGFR (Shi-Hsiang Shen, National Research Council of Canada) (*12*) was used instead of pRcK$^+$. In all of these vectors, protein expression was driven by the cytomegalovirus (CMV) promoter. Cells were harvested 48 h after transfection by placing on ice, washing with cold PBS containing 1 mM EDTA and 1 mM EGTA, and scraping in 150 μL of lysis buffer (50 mM HEPES, pH 7.4, 1% Triton X-100, 10% glycerol, 75 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 mM benzamidine, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 mM NaF, 1 mM sodium vanadate, and 10 μM ammonium molybdate). Samples were divided and a portion was denatured in SDS sample buffer for gel analysis. Aliquots of 50 μL were transferred and centrifuged in a microfuge and the supernatant was saved for determination of β-galactosidase activity (*13*). Samples were normalized on the basis of β-galactosidase activity and analyzed by immunoblotting with either anti-C-terminal MLD peptide antibody 3906, anti-EGF receptor antibody 1964, or anti-PDGF receptor antibody (Transduction Laboratories, Lexington, KY) and visualized using chemiluminescence. The effect of MLD on expression of receptors was estimated by scanning film using a laser densitometer (LKB Ultroscan XL).

For *in vivo* labeling, 293 EBNA cells were transfected with 0.5 μg of pCMV-βgal, 4 μg of either pCEP4 vector or vector containing MLD, and pUC18 to a total of 10 μg. Approximately 40 h after transfection, cells were incubated for 2 h in methionine-deficient medium containing 10% dialyzed calf serum. Cells were subsequently incubated in the presence of 1 mCi/mL ^{35}S-Trans Label (ICN, Irvine, CA)

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        10         20         30         40         50         60         70
MGSRVSREDF EWVYTDQPHA DRRREILAKY PEIKSLMKPD PNLIWIIIMM VLTQLGAFYI VKDLDWKWVI

        80         90        100        110        120        130        140
FGAYAFGSCI NHSMTLAIHE IAHNAAFGNC KAMWNRWFGM FANLPIGIPY SISFKRYHMD HHRYLGADGV

       150        160        170        180        190        200        210
DVDIPTDFEG WFFCTAFRKF IWVILQPLFY AFRPLFINPK PITYLEVINT VAQVTFDILI YYFLGIKSLV

       220        230        240        250        260        270        280
YMLAASLLGL GLHPISGHFI AEHYMFLKGH ETYSYYGPLN LLTFNVGYHN EHHDFPNIPG KSLPLVRKIA

       290        300        310        320
AEYYDNLPHY NSWIKVLYDF VMDDTISPYS RMKRHQKGEM VLE
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FIGURE 1: Predicted amino acid sequence of MLD, derived from the MLD cDNA. The His residues conserved in membrane fatty acid desaturases and membrane hydrocarbon hydroxylases are indicated in boldface and double-underlined. Predicted transmembrane hydrophobic regions are underlined.

for 30 min at 37 °C. Cells were rinsed two times with unlabeled medium and one set of plates was set aside (0 min). Unlabeled medium (5 mL) was added to the remaining plates and the cells were incubated at 37 °C for various times. Cells were placed on ice and rinsed in cold PBS containing 1 mM EDTA and 1 mM EGTA. Cells were harvested in lysis buffer and the EGF receptor was immunoprecipitated with monoclonal antibody 13A9 (*14*) and protein G−Sepharose. The immunoprecipitated EGF receptor was resolved on 6% acrylamide−SDS gels. The gel was equilibrated in 1 M sodium salicylate, dried and exposed to film. For quantitation of ^{35}S-incorporation, replicate transfections were labeled for 30 min, rinsed and harvested for immunoprecipitation. Bands resolved on 6% acrylamide−SDS gels were excised for scintillation counting and relative ^{35}S-incorporation was determined by normalizing counts to β-galactosidase activity (average of four sets of transfected plates).

Computer Sequence Analysis. The MLD sequence was examined using PSORT (*15*) to assess potential protein localization sites. The positions of predicted transmembrane sequences were determined using TMAP (*16*) and PHDhtm (*17*) of PredictProtein (*18*). Amino acid sequences of membrane fatty acid desaturases and membrane hydrocarbon hydroxylases were obtained from GenBank and SwissProt. Sequence alignment and identification of homologous regions was performed using MACAW (*19*).

RESULTS

Sequence of MLD and Homology with Membrane Fatty Acid Desaturases. A portion of MLD was isolated using a yeast two-hybrid screen. The 5′ end of the gene was isolated by RACE and used to generate a full-length cDNA containing an open reading frame encoding a 323 amino acid protein with a calculated molecular mass of 38 kDa (Figure 1). A BLAST (*10*) search of sequence databases indicated that MLD was a novel human gene and identified two short domains with 40−60% identity to yeast Δ9 desaturase (*20*) and β-carotene hydroxylase (ketolase) (*21*). One of these domains included conserved residues of the signature motif for a group of membrane fatty acid desaturases, GEXF/YHNF/YHHXFPXDY. The membrane fatty acid desaturases and membrane hydrocarbon hydroxylases contain three His motifs, $HX_{(3-4)}H$, $HX_{(2-3)}HH$, and $HX_{(2-3)}HH$ designated Ia, Ib, and II, respectively (*22*). MLD was aligned with representatives of various families of membrane fatty acid desaturases and membrane hydrocarbon hydroxylases (Figure 2). All three His-containing motifs are present in MLD. Outside the His regions, amino acids are conserved within families but are not conserved between families. MLD shares few amino acid identities with other fatty acid desaturases outside regions Ia, Ib, and II.

A hydropathy plot indicated that MLD has significant hydrophobic character interspersed throughout the sequence (Figure 3A). Sequence analysis designed to assess the presence of likely protein localization sites predicted that MLD was a type II multiple membrane-spanning protein. Shanklin et al. (*22*) noted that, in the membrane fatty acid desaturases and membrane hydrocarbon hydroxylases, the distance between the His-containing motifs and the end of the previous hydrophobic segment was conserved, with region Ia located within 10 residues, region Ib between 37 and 44 residues, and region II between 29 and 52 residues of the proximal N-terminal transmembrane segment. In MLD, the distance between the predicted transmembrane domains and regions Ia, Ib, and II is also conserved. Sequence analysis identifying His-containing motifs and prediction of multiple hydrophobic transmembrane domains supports the assignment of MLD to the membrane fatty acid desaturase gene family.

On the basis of the homology with membrane fatty acid desaturases, the predicted multiple membrane-spanning properties of MLD, and the predicted topological model of the membrane fatty acid desaturase and membrane hydrocarbon hydroxylase families (*22*), MLD is predicted to be a type II multiple membrane-spanning protein localized to the endoplasmic reticulum (Figure 3B). Additional support for this proposed topology is based on the "positive inside" rule, which states that positively charged residues are preferentially located on the cytoplasmic face of the membrane in multiple membrane-spanning proteins (*23*). The N-terminal hydrophilic domain of MLD contains eight Lys and Arg residues that would, on the basis of the "positive inside" rule, impose a preference for this polypeptide segment to remain on the cytoplasmic side of the membrane. The location of the N-terminal domain on the cytoplasmic face also places the conserved His-containing motifs on the cytoplasmic face of the membrane, consistent with the topology predicted for the membrane fatty acid desaturases (*22*). The model depicted in Figure 3B is thus a reasonable representation of the likely orientation of MLD based on analysis of its sequence and homology to membrane fatty acid desaturases.

MLD Is Widely Expressed and Localized to the Endoplasmic Reticulum. The expression of MLD mRNA was

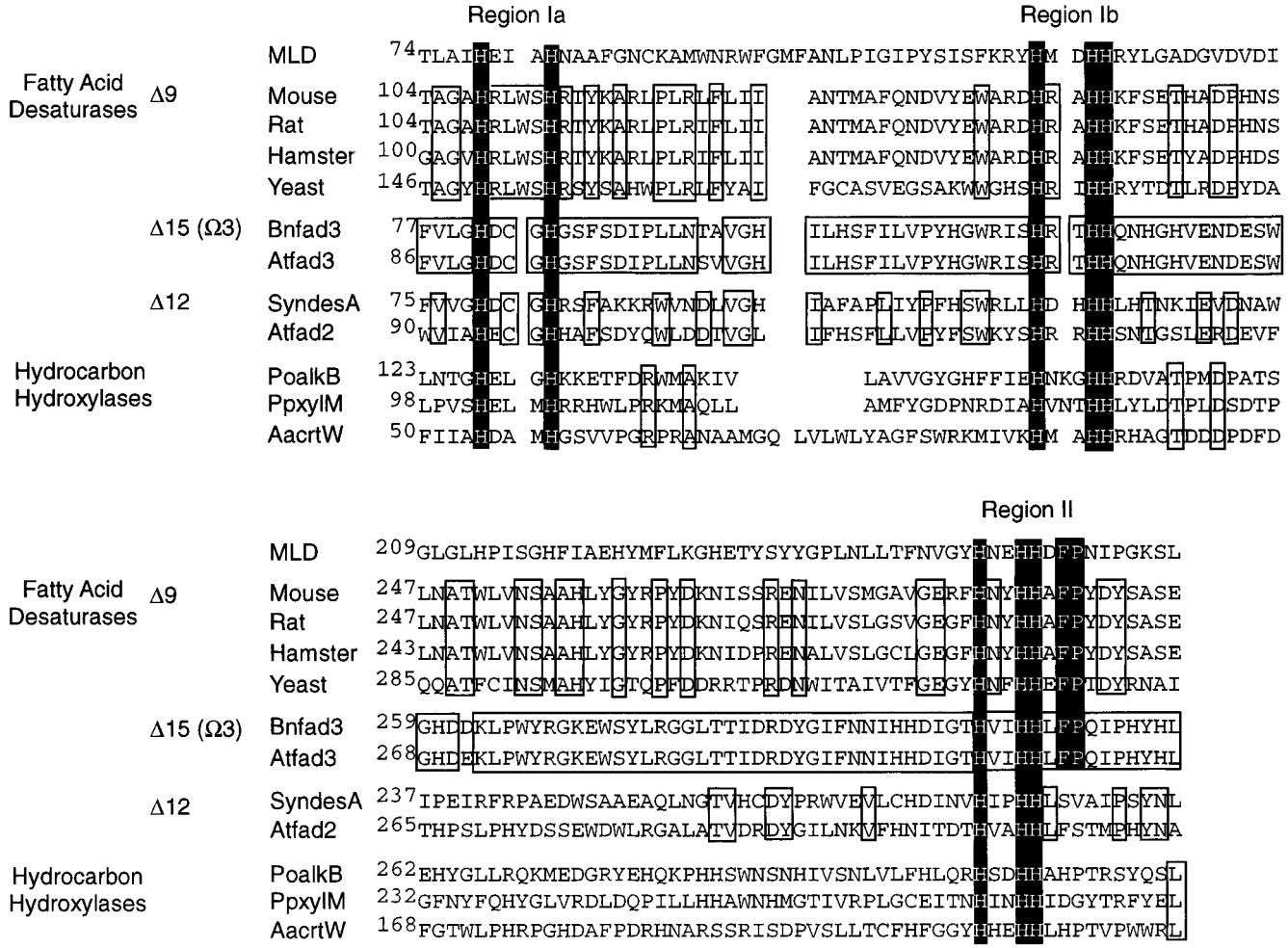

FIGURE 2: Sequence alignment of MLD with membrane fatty acid desaturases and membrane hydrocarbon hydroxylases. The MLD amino acid sequence was aligned with representative members of membrane fatty acid desaturase and membrane hydrocarbon hydroxylase families. The His residues conserved in regions Ia, Ib, and II and the FP sequence conserved in region II of a subset of membrane fatty acid desaturases are highlighted in black. Identical amino acids within family members of similar enzyme activity are shown in boxes. The position of insertion of double bonds in C_{18} fatty acid derivatives are indicated on the left. Mouse, *Mus musculus* Δ9 desaturase (*34*); rat, *Rattus norvegicus* Δ9 desaturase (*35*); hamster, *Mesocricetus auratus* Δ9 desaturase (*36*); yeast, *Saccharomyces cerevisiae* Δ9 desaturase (*20*); Bnfad3, *Brassica napus* endoplasmic reticulum Ω-3 desaturase (*37*); Atfad3, *Arabidopsis thaliana* endoplasmic reticulum Ω-3 desaturase (*37*); SyndesA, *Synechocystis* sp. strain PCC 6803 Δ12 desaturase (*38*); Atfad2, *Arabidopsis thaliana* endoplasmic reticulum Δ12 desaturase (*39*); PoalkB, *Pseudomonas oleovorans* alkane hydroxylase (*40*); PpxylM, *Pseudomonas putida* xylene monooxygenase (*41*); AacrtW, *Agrobacterium aurantiacum* β-carotene hydroxylase (*21*).

examined in human tissues by Northern blotting with a MLD-specific probe (Figure 4). Two bands of 2.2 and 1.9 kb were observed with approximately equal intensity. These likely correspond to alternative use of polyadenylation sites. MLD expression was detected in all tissues examined and was most highly expressed in prostate, ovary, heart, and placenta. Although the level of expression in different tissues varied somewhat, the relative amounts of the 2.2 and 1.9 kb bands were fairly constant. MLD mRNA is therefore widely expressed in a variety of human tissues.

HeLa cells were assayed for expression of MLD protein using affinity-purified anti-peptide antibody against the C-terminal 14 amino acids of MLD because the MLD cDNA was isolated from a HeLa library. A 34 kDa immunoreactive protein, similar in size to the 38 kDa protein predicted for MLD, was detected in the total cell extract (Figure 5A, lane T). In some experiments, MLD appeared as a doublet. Although 34 kDa is similar to the 38 kDa size predicted from the MLD cDNA sequence, the use of the C-terminal peptide antibody does not exclude the possibility that some proteolysis may be occurring at the N-terminus, perhaps contributing to the occasional appearance of a doublet. To determine the subcellular localization of MLD, HeLa cells were fractionated and analyzed by immunoblotting. MLD was located in the insoluble pellet fraction (Figure 5A, lane P) but not the soluble fraction (Figure 5A, lane S). Cells were further fractionated into cytosolic and three pellet fractions: P1, including nuclei and intact cells, P2, including mitochondria and lysosomes, and P3, including endosomes and endoplasmic reticulum. MLD was absent from the cytosolic fraction but present in all of the pellet fractions. Fractionation of CV1 cells showed a similar distribution (data not shown).

Sequence analysis of MLD and the homology to membrane fatty acid desaturases suggested that MLD was an integral membrane protein (Figure 3B). The location of MLD in particulate fractions was consistent with this possibility. To assess the likelihood that MLD is an integral membrane protein, CV1 cells were fractionated and the P1 fraction was extracted with various reagents (Figure 5B). MLD remained in the pellet fraction when extracted with NaCl or urea. Extraction with Triton X-100 resulted in a

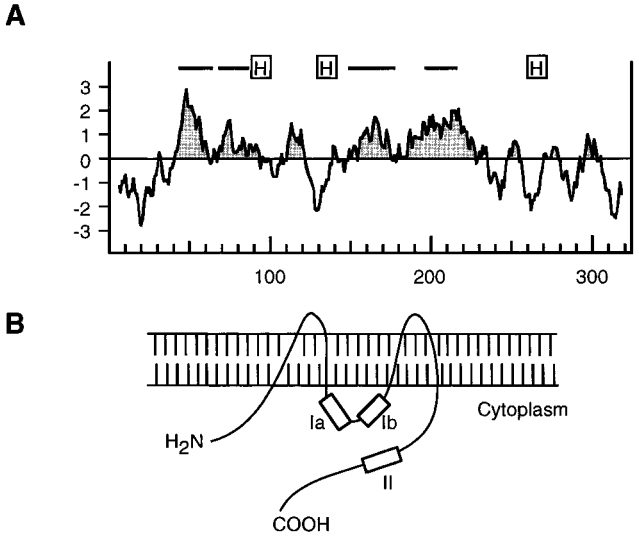

FIGURE 3: Hydropathy plot and predicted model of the topology of MLD. (A) A Kyte−Doolittle hydropathy analysis (*42*) was performed on the MLD amino acid sequence. The hydrophobic regions are shaded and the predicted transmembrane segments are indicated by bars. The positions of the His motifs are indicated by boxes. The amino acid sequence number is indicated below. (B) Model of the membrane topology of MLD. The conserved His motifs are indicated by boxes.

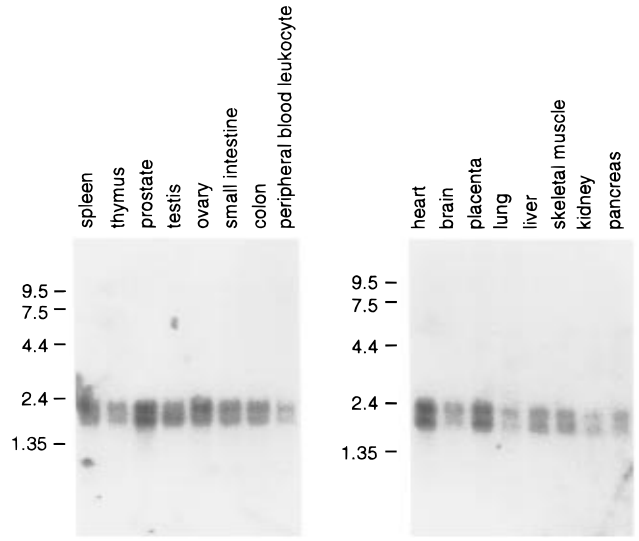

FIGURE 4: Northern analysis of MLD expression in various human tissues. RNA blots of various human tissues were hybridized with ^{32}P-labeled MLD-specific probe. The tissues are indicated above the blots. The positions of RNA size markers (in kilobases) are indicated on the left.

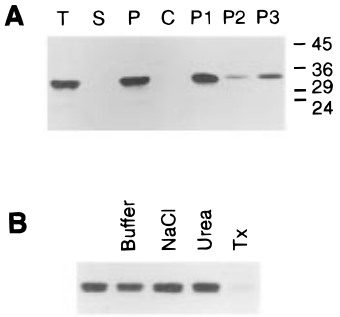

FIGURE 5: Subcellular distribution of MLD. (A) Subcellular fractionation of HeLa cells. HeLa cells were lysed and separated into soluble and insoluble fractions (T, total cell lysate; S, supernatant; P, pellet) or fractionated by differential centrifugation (C, cytosol; pellets P1, P2, and P3). Fractions were resolved on 12% acrylamide−SDS gels and immunoblotted with MLD C-terminal antibody. The sizes of protein standards are indicated (in kilodaltons) on the right. (B) Extraction of the P1 pellet fraction from CV1 cells. Aliquots of the P1 fraction (lane 1) were extracted with either buffer alone or buffer containing 0.75 M NaCl, 6 M urea, or 1% Triton X-100 as indicated. Pellets were analyzed for the presence of MLD by resolving on 12% acrylamide−SDS gels and immunoblotting with MLD C-terminal antibody.

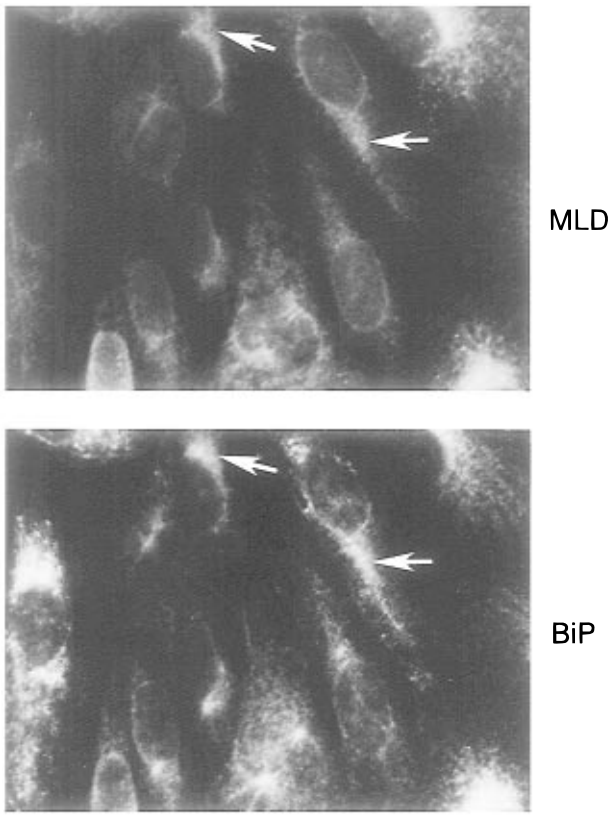

FIGURE 6: Colocalization of MLD with an endoplasmic reticulum marker in CV1 cells. Cells were fixed and stained with antibodies directed against MLD and the endoplasmic reticulum marker BiP followed by Texas Red-conjugated anti-rabbit and fluorescein-conjugated anti-mouse secondary antibodies. Cells were visualized by epifluorescence illumination. The protein visualized is indicated on the right. Arrows indicate representative areas where overlap of MLD and BiP staining is clearly visible.

decrease in MLD levels in the pellet fraction. Similar results were seen with the P2 and P3 fractions and with HeLa cell fractions (data not shown). The ability to extract MLD from particulate fractions with detergent but not high salt or urea indicates that MLD is an integral membrane protein.

The localization and extraction properties of MLD in subcellular fractions of HeLa and CV1 cells were identical. Because HeLa cells are transformed and less adherent, we used CV1 cells, which form a more fibroblastlike monolayer, to examine the subcellular localization of endogenous MLD using immunocytochemistry. Although MLD was localized in the P1 fraction that includes nuclei and unbroken cells (Figure 5A), immunofluorescence staining of CV1 cells clearly shows that MLD is excluded from the nucleus (Figure 6). Instead, MLD displays a reticular staining pattern. Double staining of cells with MLD and the endoplasmic reticulum marker immunoglobulin heavy chain binding protein (BiP) shows extensive overlap of expression and colocalization. A similar staining pattern is seen with another endoplasmic reticulum marker, calnexin (data not shown).

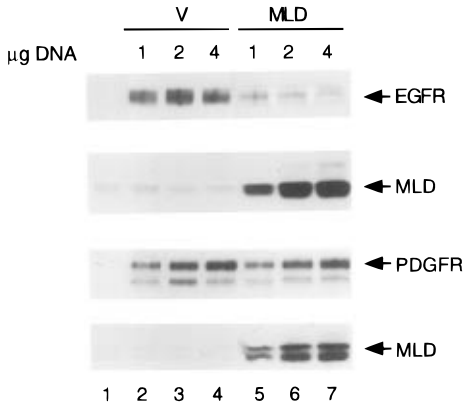

FIGURE 7: Cotransfection of 293 EBNA cells with MLD and EGF receptor. 293 EBNA cells were transiently cotransfected with EGF receptor cDNA (upper two panels) or PDGF receptor cDNA (lower two panels) with increasing amounts of vector (V; lanes 2−4) or MLD cDNA (lanes 5−7) as indicated. Mock transfections did not contain EGF receptor, MLD, or vector DNA (lane 1). Extracts were prepared 48 h after transfection and normalized aliquots were analyzed on 6% (EGFR and PDGFR) and 12% (MLD) acrylamide−SDS gels. Immunoblots were probed with either EGF receptor (EGFR), PDGF receptor (PDGFR), or MLD antibodies and visualized by chemiluminescence.

Immunocytochemistry indicates that MLD is localized to the endoplasmic reticulum.

MLD Inhibits Expression of EGF Receptor. To ensure that the identified open reading frame in the MLD cDNA was full-length, MLD was transiently transfected into 293 EBNA cells, a cell line known to give high levels of protein expression (*24*). Transfection with MLD cDNA resulted in increased expression of a 34 kDa protein identical in size to the endogenous MLD protein detected with an anti-C-terminal peptide antibody (Figure 7, second and fourth panels, compare lanes 1 and 5). We tested the effect of MLD on EGF receptor expression because MLD was originally isolated as a protein that interacted with the EGF receptor. EGF receptor cDNA was cotransfected in 293 EBNA cells with increasing amounts of MLD cDNA (Figure 7). The EGF receptor was readily detected in transfected 293 EBNA cells relative to mock-transfected cells that contain low levels of endogenous EGF receptor (Figure 7, compare lane 1 to lanes 2−7). Increasing amounts of MLD resulted in decreased expression of EGF receptor (50−75% inhibition) (Figure 7, lanes 5−7). In contrast, increasing amounts of vector alone had no effect on EGF receptor expression (Figure 7, lanes 2−4). Although an increase in PDGF receptor expression is observed with increasing amounts of MLD, a parallel increase is seen in vector-transfected cells (Figure 7, compare lanes 2 and 5, 3 and 6, and 4 and 7). This increase is likely due to the increasing amounts of CMV promoter provided by the multicopy plasmid pCEP4, which leads to a general suppression of the β-galactosidase activity used to normalize for transfection efficiency. Nevertheless, if PDGF receptor levels are compared with equivalent amounts of vector and MLD DNA, there is no significant change in PDGF receptor expression in response to MLD. Similar results were observed in at least four independent transfection experiments. Thus, the effect of MLD appears to have specificity, at least with respect to the receptors used in this study.

Overexpression of MLD markedly decreased the steady-state levels of transfected EGF receptor but did not alter

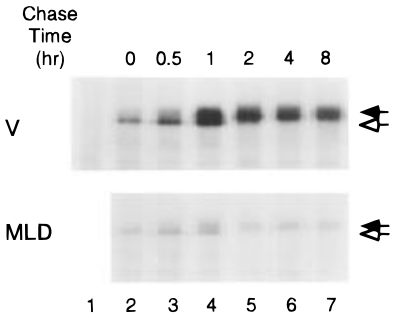

FIGURE 8: *In vivo* labeling and immunoprecipitation of EGF receptor in cells cotransfected with MLD. 293 EBNA cells were cotransfected with EGF receptor and either vector (V) or MLD cDNA. Mock-transfected cells did not contain EGF receptor, MLD, or vector DNA (lane 1). Cells were labeled for 30 min with ^{35}S-amino acids (lanes 1 and 2) and chased for the indicated times (lanes 3−7). The EGF receptor was immunoprecipitated and analyzed on 6% acrylamide−SDS gels. The position of the 160 kDa precursor (white arrow) and the mature 170 kDa (black arrow) EGF receptor is indicated on the right. The fluorogram was exposed for 4 h.

expression of the PDGF receptor. The localization of MLD in the endoplasmic reticulum, the site of EGF receptor and PDGF receptor biosynthesis, suggested the possibility that the decreased steady-state expression of EGF receptor could be due to an alteration in biosynthesis. To determine if MLD altered biosynthesis of EGF receptor, cells were labeled *in vivo* with ^{35}S-amino acids and chased for various times with unlabeled medium. Following immunoprecipitation with EGF receptor-specific monoclonal antibody 13A9 (*14*), the 160 kDa EGF receptor precursor can be detected after the 30 min labeling period (Figure 8, lane 2). The 160 kDa EGF receptor precursor observed at short labeling times (*25*) is present in both vector- and MLD-transfected cells, although the level of incorporation in MLD-transfected cells is lower. Chasing for various times revealed that there was no significant alteration in the rate of oligosaccharide processing during maturation of the EGF receptor (Figure 8, lanes 3−7). Quantitation by excising and counting bands and normalizing to cotransfected β-galactosidase activity indicated that ^{35}S incorporation was decreased 2-fold at the end of the 30 min labeling period in MLD relative to vector-transfected cells (average of four data sets), accounting for a significant amount of the inhibition of steady-state expression of EGF receptor observed in Figure 7. Thus, overexpression of MLD, a protein identified by its ability to bind to the EGF receptor, inhibits biosynthesis or a very early step in biosynthetic processing of the receptor.

DISCUSSION

We have identified MLD as a new member of the membrane fatty acid desaturase gene family. MLD contains all three of the His-containing signature motifs found in the membrane fatty acid desaturases and membrane hydrocarbon hydroxylases. The conserved His residues in regions Ia, Ib, and II have been proposed to function as iron ligands for the membrane fatty acid desaturases and membrane hydrocarbon hydroxylases (*22*), analogous to the soluble diiron-oxo protein stearoyl acyl carrier protein Δ^9 desaturase (*26*, *27*). The requirement of the conserved His residues for enzyme function has been shown by site-directed mutagenesis in rat Δ^9-CoA desaturase (*22*) and *Synechocystis* Δ^{12} acyl-lipid desaturase (*28*). The conservation of the His-

containing motifs in regions Ia, Ib, and II supports the proposal that these residues are likely to bind iron and are thus important for the function of MLD. The conservation of the FP in region II (HX_2HHXFP) suggests that MLD is likely to function as a fatty acid desaturase, although it is not homologous to the human Δ9 desaturase (*29*). The lack of conservation outside regions Ia, Ib, and II suggests that MLD is not a human homologue of any of the known fatty acid desaturase genes but instead represents a new family member, possibly one of the desaturase activities previously identified in mammals (*4*).

Overexpression of MLD decreased steady-state levels of EGF receptor expression and decreased biosynthesis of the receptor 2-fold relative to vector-transfected cells. MLD was originally isolated as a protein that interacts with the EGF receptor core tyrosine kinase domain and MLD is localized to the endoplasmic reticulum, positioning MLD in the same compartment as the site of EGF receptor biosynthesis. However, overexpression of MLD is not inhibiting receptor processing in the endoplasmic reticulum nonspecifically, since overexpression of MLD does not inhibit PDGF receptor expression. Therefore, it is likely that the binding of MLD to the EGF receptor detected in the yeast two-hybrid screen is contributing to the observed inhibition of EGF receptor biosynthesis, providing some level of specificity in the regulation of biosynthesis of proteins processed through the endoplasmic reticulum.

The 160 kDa EGF receptor precursor does not accumulate in cells overexpressing MLD but is processed to the mature glycosylated form at the same rate as in vector-transfected cells. In addition, decreased EGF receptor labeling is observed at the earliest time point (30 min), suggesting that the effect of MLD on EGF receptor expression must be occurring at an early stage, during receptor biosynthesis. The region of EGF receptor that interacts with MLD resides in the cytoplasm and, therefore, the cytoplasmic domains of MLD must recognize the EGF receptor. The effect of overexpression of MLD on EGF receptor biosynthesis may be due to a direct interaction, either by slowing the rate of translation or by causing selective rapid degradation of a portion of newly synthesized EGF receptor, perhaps by interfering with proper folding of the intracellular domain of the EGF receptor. Precedence for the release of misfolded proteins from the endoplasmic reticulum for degradation by proteasomes has been demonstrated (*30*, *31*). In addition, the fatty acid desaturase activity of MLD could alter membrane fluidity in a microenvironment of the endoplasmic reticulum in such a way as to facilitate rapid release of EGF receptor from the endoplasmic reticulum and subsequent rapid degradation.

Several of the fatty acid desaturase activities that have been studied in mammals were found to be regulated by exogenous signals, including the availability of fatty acids and differentiation signals (*32*, *33*). Although the effect of MLD on EGF receptor was observed when MLD was overexpressed, it is nevertheless interesting to speculate that due to its ability to alter EGF receptor biosynthesis or a very early step in biosynthetic processing, variable MLD expression could provide a mechanism to coordinate a fatty acid metabolic pathway with regulation of EGF receptor biosynthesis.

ACKNOWLEDGMENT

We thank Linda W. Jurata for assistance in isolation of full-length MLD cDNA, Roger Brent for providing the components of the yeast two-hybrid system, and Shi-Hsiang Shen for the pPDGFR vector.

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BI970091L