EMR4, a Novel Epidermal Growth Factor (EGF)-TM7 Molecule
Up-regulated in Activated Mouse Macrophages, Binds to a Putative
Cellular Ligand on B Lymphoma Cell Line A20*

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A novel member of the EGF-TM7 family, mEMR4, was identified and characterized. The full-length mouse EMR4 cDNA encodes a predicted 689-amino acid protein containing two epidermal growth factor (EGF)-like modules, a mucin-like spacer domain, and a seven-transmembrane domain with a cytoplasmic tail. Genetic mapping established that mEMR4 is localized in the distal region of mouse chromosome 17 in close proximity to another EGF-TM7 gene, F4/80 (Emr1). Similar to F4/80, mEMR4 is predominantly expressed on resident macrophages. However, a much lower expression level was also detected in thioglycollate-elicited peritoneal neutrophils and bone marrow-derived dendritic cells. The expression of mEMR4 is up-regulated following macrophage activation in Biogel and thioglycollate-elicited peritoneal macrophages. Similarly, mEMR4 is over-expressed in TNF-α-treated resident peritoneal macrophages, whereas interleukin-4 and -10 dramatically reduce the expression. mEMR4 was found to undergo proteolytic processing within the extracellular stalk region resulting in two protein subunits associated noncovalently as a heterodimer. The proteolytic cleavage site was identified by N-terminal amino acid sequencing and located at the conserved GPCR (G protein-coupled receptor) proteolytic site in the extracellular region. Using multivalent biotinylated mEMR4-mFc fusion proteins as a probe, a putative cell surface ligand was identified on a B lymphoma cell line, A20, in a cell-binding assay. The mEMR4-ligand interaction is Ca²⁺-independent and is mediated predominantly by the second EGF-like module. mEMR4 is the first EGF-TM7 receptor known to mediate the cellular interaction between myeloid cells and B cells.

The G protein-coupled receptors (GPCRs), with more than 1000 members identified to date, constitute one of the largest protein superfamilies in nature (1, 2). By coupling heterotrimeric G proteins to their characteristic seven-transmembrane (TM7) and cytoplasmic regions, GPCRs mediate the signal transduction of an extensive array of exogenous stimuli including hormones, cytokines, peptides, amino acid derivatives, ions, neurotransmitters, light, taste, and odors (1, 3). A total of five classes of GPCRs are categorized based upon the similarity of their TM7 sequences (4). In recent years, a rapidly growing subfamily of class-B GPCRs with an unusual long N-terminal extracellular region has been identified (LNB-TM7; for review see Refs. 5 and 6). Among them, the epidermal growth factor (EGF)-TM7 receptor subfamily has been studied with great interest by us and others (for review see Refs. 7 and 8). At present, eight members of the EGF-TM7 family including human EGF module-containing mucin-like hormone receptor 1 (EMR1), F4/80 (mouse Emr1), EMR2, EMR3, human and rat EGF-TM7-latrophilin-related protein (ETL), and human and mouse CD97 have been reported (9–18). The EGF-TM7 molecules are distinguished by a novel hybrid structure that contains various numbers of N-terminal EGF-like modules connected to a TM7 domain by a mucin-like spacer (7, 8). Characterized by a set of 6 conserved cysteine residues typically disulfide bond in a 1–3, 2–4, 5–6 arrangement, the tandemly arrayed EGF-like modules of the EGF-TM7 molecules belong to the class I EGF-like modules often found in connective tissue proteins such as fibrillin-1 and -2 and fibulins (19). The majority of these EGF-like modules also contain a consensus sequence associated with calcium binding (cb), (D/N)(E/Q)Xₙ(DB/ND)Xₘ(Y/F), where m and n are variable and * indicates possible β-hydroxylation (19, 20). Calcium performs a key role in the orientation of cbEGF pairs by restricting con-

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† The abbreviations used are: GPCR, G protein-coupled receptor; EGF, epidermal growth factor; TM7, seven transmembrane; mEMR4, mouse epidermal growth factor module-containing mucin-like receptor 4; cbEGF, calcium-binding EGF module; GSP, GPCR proteolytic site; ETL, EGF-TM7-latrophilin-related; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction; Mb, macrophages; PMN, polymorphonuclear cells; DC, dendritic cells; RPMβ, resident peritoneal macrophages; Biomβ, Biogel-elicited peritoneal macrophages; Thiomβ, thioglycollate-elicited peritoneal macrophages; ThioPMN, thioglycollate-elicited neutrophils; BMMβ, bone marrow-derived macrophages; BMDC, bone marrow-derived dendritic cells; mAb, monoclonal antibodies; Fc, crystallizable fragment; mFc, mouse Fc; FACS, fluorescence-activated cell sorting; HRP, horseradish peroxidase; Chr, chromosome; IL, interleukin; TNF-α, tumor necrosis factor-α; bi-Tris, 2-[bis(2-hydroxyethyl)aminol]-2-hydroxymethylpropane-1,3-diol; MES, 4-morpholinoethanesulfonic acid; HBSS, Hank’s balanced salt solution; BSA, bovine serum albumin; contig, group of overlapping clones; FIRE, F4/80-like receptor.
formational flexibility of the interdomain linkage that is important in presenting a specific surface for protein-protein interaction. Genetic mutations causing amino acid changes in the cEGF modules have been found in diseases such as familial hypercholesterolemia, hemophilia B, protein S deficiency, and the Marfan syndrome (21–24). Following the EGF-like modules, a Ser/Thr-rich, mucin-like spacer region of ~200–250 amino acids precedes the TM7 region. Within this region, a highly conserved Cys-rich domain located immediately before the first TM segment has been identified in all EGF-TM7 proteins. This domain, termed the GPCR proteolytic site (GPS) domain, is characterized by four invariant Cys, one Cys, and two conserved Trp residues spaced in restricted positions (25). The GPS domain was initially identified in a sperm receptor for egg jelly from sea urchins (Strongylocentrotus purpuratus) (26) and later found in a number of LNB-TM7 receptors including HEK26 (27), GPR56 (28, 29), BA11 (30), CL1–3 (25, 31–33), Celsr1 (34, 35), Ig-Hepta (36), and the flamingo (fmi) gene product from Drosophila (37, 38). Several of these proteins, notably CL1 (calcium-independent receptor for latrotoxin (CIRL)/latrophilin), ETL, and CD97, have been shown to be proteolytically cleaved within this domain, resulting in two subunits that remain noncovalently associated as a heterodimer (14, 16, 25). Although its significance is still unknown, this unusual yet highly conserved post-translational protein modification is likely to have a physiological function for the EGF-TM7 receptors.

The EGF-TM7 receptors are predominantly expressed in myeloid cells (F4/80, EMR1, EMR2, EMR3, and CD97) and smooth muscle cells (ETL and CD97) (9–16, 39–41). Based upon their unique protein structure and restricted expression patterns, it has been suggested that the EGF-TM7 molecules may play a role in the cellular functions of myeloid leukocytes and cardiac muscle differentiation by interacting with other cell surface proteins or extracellular matrix proteins, leading to intracellular signaling. Indeed, the presence of cellular ligands has been demonstrated for CD97 (42), EMR3 (13), and EMR2.2. CD97 is the first EGF-TM7 molecule shown to interact with a defined cellular ligand, CD55 (decay-accelerating factor; DAF) (42, 43). We have further demonstrated that CD97 interacts with CD55 solely by the EGF-like modules and that the interaction is characterized by a low affinity (86 μM) and rapid off-rate (t0.6 s−1) (44). Likewise, by employing soluble multivalent EMR3 probes in a sensitive cell-binding assay, a putative EMR3 ligand has been found on the surface of human monocyte-derived macrophages and activated human neutrophils (13). However, it is currently not known whether these protein-protein interactions can lead to signal transduction via the TM7 moiety of these receptors.

In addition to the strong structural-functional similarity, the EGF-TM7 molecules are also closely linked at the genomic level. With the exception of ETL, which maps to human chromosome (Chr) 1, all other human EGF-TM7 members are located within Chr 19p13 region with EMR1 on 19p13.3, and CD97, EMR2, and EMR3 on 19p13.1 (9, 12, 13, 15). Furthermore, the mouse homologues of EMR1 (F4/80) and CD97 were mapped to the corresponding syntenic regions of mouse Chr 17 and Chr 8, respectively (11, 45, 46). More significantly, the genomic organization of CD97 and EMR2 was found to be identical in that each EGF-like module is encoded by a single exon and the TM7 region by a total of five exons (12, 47). These findings strongly indicate that the EGF-TM7 genes are derived from a common ancestral gene through gene duplication and are highly conserved among vertebrate species. It is therefore of great interest to decipher how individual EGF-TM7 genes evolve and function.

Herein, we describe the molecular and functional characterization of a new EGF-TM7 molecule, mouse EMR4. The mEMR4 gene maps to the distal region of mouse Chr 17 in close proximity to Emr1 and encodes a cell-surface receptor restrictively expressed in macrophages (Mφ), neutrophils (PMN), and dendritic cells (DC). The regulation of mRNA expression, the biochemical-structural characteristics, and the ligand-binding properties of mEMR4 are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals and reagents were obtained from Sigma unless otherwise specified. Cell culture media and supplements were purchased from Invitrogen. Cell lines were provided by the cell bank at the Sir William Dunn School of Pathology, University of Oxford. Laboratory-bred mice were housed and provided by the animal facility at the Sir William Dunn School of Pathology under standard pathogen-free conditions with access to food and water ad lib. Recombinant murine IL-4, IL-10, TNF-α, and interferon-γ were obtained from R&D Systems.

**Molecular Cloning of the mEMR4 cDNA**—The mEMR4 full-length cDNA was amplified by rapid amplification of cDNA ends (RACE)-polymerase chain reaction (PCR) using a mouse spleen Marathon Ready cDNA library (CLONTECH). Two rounds of PCR reactions were conducted to amplify visible mEMR4 cDNA fragments. Marathon adapter primers, AP-1 (5'-CCTACTAATACGACTATAGGGC-3') and AP-2 (5'-ACTACTATAGGGGCTACCGGCG-3') were paired individually with nested mEMR4-specific primers, 5'-1 (5'-TTGAGAGAAAGGTTTGCTCTCTCAAA-3') and 5'-2 (5'-CGGTGGCCCCCAGGAGGTTGAGCTCAGTAA-3') as well as 3'-1 (5'-CTTGGAGGCTACATCTTTCTTCACT3') and 3'-2 (5'-CGGAGGTTCAAGAGGTTGCTGTGTA-3') to generate S'-RACE and 3'-RACE fragments, respectively. mEMR4 cDNA fragments were separated on a 1% agarose gel, purified, subcloned, and sequenced using standard molecular techniques.

**Sequence Analysis**—All cDNA fragments and expression constructs were subjected to DNA sequencing to confirm the integrity of DNA sequencing reactions were performed using the BigDye™ Terminator DNA sequencing kit (PE Applied Biosystems). Samples were electrophoresed on an ABI 373A DNA Sequencer and analyzed by ABI Prism Model Version 2.1.1 software (PE Applied Biosystems). An homologous DNA sequence search was carried out using the BLAST algorithm against DNA sequences in GenBank™/EMBL data bases. Protein alignment and alignment of consensus sequences were analyzed using ClustalW software.

**Chromosome Mapping Analysis**—To determine the chromosomal position of the mEMR4 gene, the segregation of sequences detected by a full-length mEMR4 cDNA probe was followed in 118 progeny of a Mus musculus × Mus spretus interspecific back-cross: (C3Hf/R1-Mg-spretus × M. spretus) × C57BL/6j. The mEMR4 was localized to fragments of ~4.8, 4.5, 4.38, 4.2, 3.9, 1.9, 1.8, and 0.85 kb in HincII-digested M. spretus (S) DNA and fragments of ~4.8, 4.5, 4.38, 4.2, 3.9, 3.45, 3.05, 1.55, and 0.8 kb in HincII-digested C3Hf inbred mouse (M) DNA. Other variant fragments were described recently (48) and were traced as follows: Nfya, BamHI (S) 1.6 kb (M) 3.2 kb; Cs, PvuII (S) 4.6, 6.2 kb (M) 3.3 kb; Mllt1, EcoRI (S) 1.3 kb (M) 0.8 kb; Emr1, BamHI (S) 7.7, 5.2 kb (M) 15.0, 9.8 kb; Vov, Tq1 (S) 5.0, 5.5 kb (M) 5.6 kb; Rf2a, Tq1 (S) 2.7 kb (M) 3.6 kb. Gene linkage, order, and intergenic distances with associated standard errors were calculated according to standard statistical methods with the aid of the Map Manager data analysis programs (49, 50).

**Cell Culture**—All culture media were supplemented with 10% heat inactivated fetal calf serum, 2 mM l-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. All cells were incubated at 37 °C in a 5% CO2, 95% humidity incubator. A20 cell line was cultured in RPMI 1640 medium. HER293T cells were grown in Dulbecco’s modified Eagle’s medium. Mouse primary cells were obtained from 8–10-week-old C57/B16 or BALB/c mice. Mouse bone marrow-derived Mφ (BMMφ) were cultured in RPMI 1640 medium supplemented with 15% L-cell-conditioned medium as described previously (51). Mouse resident peritoneal Mφ (RPmφ), Biogel-elicited peritoneal Mφ (BioMφ), thyroglycollate-elicited peritoneal Mφ (BioMφ), and thyroglycollate-elicited peritoneal neutrophils (ThioPMN) were obtained using standard protocols described previously (52, 53). For the cytokine treatment of RPmφ, cells were cultured for 1 h for adherence, washed, and then treated with or without IL-4 (20 ng/ml), IL-10 (10 ng/ml), TNF-α (10 ng/ml), and inter-
feron-γ (250 μU/ml) for 36 h prior to the isolation of total RNA. Mouse spleen B and T cells were isolated from a single cell suspension by magnetic separation of B220 mAb-coated Dynabeads (Dynal A.S., Oslo, Norway) and by nylon wool purification, respectively (54). Mouse bone-marrow-derived dendritic cells (BMDC) were obtained by incubating bone marrow cells from bone marrow/marrow colony stimulating factor as described previously (55).

**RNA Blot and RT-PCR Analysis**—Total RNA was prepared from mouse primary cells and cell lines using the acid guanidinium thiocyanate-phenol-chloroform method (56). Total RNA (10 μg) was electrophoresed on a 1% formaldehyde agarose gel, blotted onto a nylon membrane (Durafilm UV, Shandon, Xenogen), and hybridized with radiolabeled probes as described previously (11). Similarly, a commercially available mouse multiple tissue Northern blot (OriGene Technologies, Inc) was hybridized with a full-length mEMR4 CDNA probe according to the manufacturer’s instructions. Hybridized blots were washed in 0.5× SSC (0.15 M NaCl and 0.015 m sodium citrate), 1% SDS at 55 °C for 30 min and exposed to x-ray films (X-Omat, Kodak) at -80 °C for 2–4 days. Radioactive probes were stripped from RNA blots by washing in 0.1× SSC, 1% SDS for 15 min at 95 °C, and the blots were rehybridized with a mouse β-actin cDNA probe to compare the amount of RNA loaded in each lane. RT-PCR analysis was conducted using RNA isolated from mouse spleens to study whether mEMR4 is alternatively spliced. Primers used in the analysis include 5′-CTCCAGAAATTGTTGAGGGACGCA-3′ and 5′-ACCCCCATGGAGACTTGCA-3′ (coding for the 5′-end extracellular domain and 5′-CTGGAACCTGTAACTCTGACTTTACAAGGAAA-3′ and 5′-CAATCTCAATAGTGTGCTGCTGAGTCA-3′ (for the 3′-end TM7 domain). The PCR reactions were carried out using the Advantage HF PCR kit (CLONTECH) for 30 cycles of denaturation at 95 °C for 30 s, 72 °C for 1.5 min. 

**Construction of Expression Vectors**—All mEMR4 expression vectors were constructed on pCDNA3.1 (+) or pCDNA3.1-V5-His (Invitrogen). For the construction of V5-His-tagged mEMR4 vector, pMV-EMR4-V5-His, the entire mEMR4 open reading frame was amplified by PCR using mEMR4 primers and the PSV-169/mIgG2b-322 plasmid as a template (52) using 5′-CAGTA) and 3′-ACCCCCATGGAGACTTGCA) and subcloned into the pcDNA3.1-V5-His vector via HindIII and BamHI sites. For the construction of vectors expressing mouse Fc (mFc) fusion proteins, two mEMR4 DNA fragments containing either the entire extracellular domain (1–341 amino acids) or the EGFR-like modules (1–147 amino acids) were generated by PCR using 5′-CTGCATTCTGCACGCTGCACGGAAG-3′ and 5′-TCTCTTCTAGATTTACCCGGAGACGTTGCAA-3′ respectively. A truncated mFc DNA fragment containing the hinge and constant regions (6C, 2–3 of 1069CACACCATCCTCCTCATGGGG, 5′-GCTAAGCTT-3′) of mEMR4, immunoglobulin domain V (mIgD), was cloned into pcDNA3.1 vector by PCR using 5′-CGGGA primers and the PSV-169/mIgG2b-322 plasmid as a template (53). In the construction of mFc fragments and the biotinylation signal fragments into the pSecTag2 vector (Invitrogen) immediately after the leader peptide of the immunoglobulin γ-chain.

**Transient Transfection of Cells and Protein Analysis**—The mEMR4 expression constructs were transfected into HEK293T cells using LipofectAMINE (Invitrogen) according to the manufacturer’s protocol. Consideration of the presence and absence of biotinylated mEMR4-mFc fusion proteins was determined by 48 h post-transfection, and protein concentrations were determined by a BCA Protein Assay Kit (PerkinElmer Life Sciences). For immunoprecipitation, proteins were biotinylated with EZ-Link sulfo-NHS-LC-biotin (Pierce) at room temperature for 30 min, washed extensively with phosphate-buffered saline, and lysed with cell lysis buffer. Cell lysates were precleared with protein G-Sepharose beads prior to the incubation with an anti-V5 mAb at 4 °C for 1 h followed by incubation with protein G-Sepharose beads. After extensive washing, immunoprecipitated complexes were resuspended in electrophoresis sample buffer and subjected to standard gel electrophoresis and Western blotting using Extravidin-HRP (1:5000) for detection. For the production of soluble mEMR4-mFc fusion proteins, HEK293T cells were transfected with 40 μg of DNA (2375 bp) encoding an mKv1.5 mAbs as described previously (13, 44). The medium was replaced with 25 ml of serum-free Opti-MEM I 16–18 h post transfection and incubated for a further 72 h. Conditioned medium was collected, spun, and passed through a 0.45 μm filter followed by protein A-Sepharose 4 Fast Flow (Amersham Biosciences) column purification according to the manufacturer’s protocols. For N-glycosidase F treatment, 5 μg of the purified mEMR4-mFc fusion protein was incubated with or without 3 units of N-glycosidase F (Roche Molecular Biochemicals) in 20 μl sodium phosphate buffer, pH 7.0, at 37 °C for 20 h. Samples were then reduced, run on an 8% SDS-PAGE, and stained with Coomassie Brilliant Blue. For N-terminal amino acid sequencing, the purified mEMR4-mFc fusion protein was reduced and run on a 10% NuPAGE gel (Invitrogen) at 200 mA/gel using MES buffer. The gel was electroblotted to a Novex 0.2 μm polyvinylidene difluoride membrane (Invitrogen) and stained with Coomassie Brilliant Blue. The desired ~40-kDa band was excised, washed extensively with 10% methanol, and subjected to sequencing on an Applied Biosystems 494A Procise protein sequencer (PerkinElmer Life Sciences, Applied Biosystems Division, Warrington, UK) using standard sequencing protocols (63).

**In Vitro Biotinylation of mEMR4-mFc Fusion Proteins**—Purified mFc fusion proteins were biotinylated in 10 μl Tris-HCl, pH 8.0, by dialysis and incubated with 1 μl of BirA enzyme (Avdiity, Denver, CO) overnight at room temperature. Excess biotin was subsequently removed by dialysis with 10 μl Tris-HCl, pH 7.3, containing 10 mM CaCl2 and 100 mM NaCl. Following the confirmation of integrity of the biotinylated proteins using Western blotting probed with Extravidin-HRP (Sigma), the proteins were quantified by dot-blot analysis using myelin basic protein-biotin (Avdiity, Denver, CO) as standard and stored at -80 °C.

**Cellular Ligand-binding Assay**—To search for the putative cellular ligand of mEMR4, various mouse cell lines were subjected to a FACS-based cell-surface binding analysis using a well characterized monoclonal antibody (mAb) directed against a protein-protein interaction (44, 59). In brief, 20 μl of avidin-coated fluorescent beads (Spherotech, Inc., Libertyville, IL) were washed twice and added to 2 μg of biotinylated protein in Hanks’ balanced salt solution (HBSS) containing 0.5% bovine serum albumin (BSA) (HBSS/BASA) in a total volume of 50 μl. The bead-protein mixture was sonicated at 20% power for 1 min (Heat Systems Sonicor) and then incubated at 4 °C for 1 h. Nonbinding proteins were removed by washing twice with HBSS/BASA and the beads were resuspended in 50 μl of HBSS/BASA. The bead-protein complex was sonicated again immediately before its addition to single cell suspensions in a 96-well plate (1 × 105 cells/50 μl beads/well). The cell-bead mixture was spun at 250 × g at 4 °C for 20 min, incubated for a further 40 min at 4 °C, and subsequently resuspended in 500 μl of HBSS for FACS analysis. Where necessary, 10 μg EDTA was added in the reaction.

**RESULTS**

mEMR4 Is a New Member of the EGF-TM7 Family—In an effort to identify the murine homologues of human EMR2 and...
EMR3 genes previously characterized by us (12, 13), a homology search in the data bases was carried out, and two mouse EST clones (GenBank™ accession numbers BG080641 and AA823656) containing sequences homologous to the EGF-TM7 genes were identified. Using oligonucleotide primers derived from these clones, 5′-end and 3′-end RACE reactions were performed to obtain the full-length cDNA, which was similar but distinct from EMR2 and -3 and was therefore designated mEMR4. The composite 2177-bp mEMR4 RACE product contains an open reading frame of 2067 bp encoding a 689 amino acid protein (Fig. 1A). A Kyte-Doolittle hydrophyt profile of the predicted protein revealed a hydrophobic signal peptide at the N terminus and seven hydrophobic segments at the C terminus (data not shown). A predicted signal peptide cleavage site at residue Met31 of the precursor protein indicated that the resulting mature mEMR4 protein is a cell surface molecule containing a long N-terminal extracellular region of 310 amino acids, a 7TM region of 254 amino acids, and a cytoplasmic tail of 94 amino acids (Fig. 1, A and B). The estimated molecular mass for the mature mEMR4 protein is 74 kDa.

At the most N-terminal portion of the mature EMR4 protein, two tandemly arrayed EGF-like modules were identified. Protein sequence analysis indicated that the first EGF-like module is a non Ca2+-binding EGF (cEGF) domain, whereas the second is a cEGF domain (Fig. 2). Immediately C-terminal to the EGF-like modules is a Ser/Thr-rich, mucin-like spacer region within which a highly conserved Cys-rich GPS domain was identified right before the first TM region (Fig. 2) (5, 6, 64). The TM7 region of the mEMR4 is most similar to those of EMR3 and EMR2 (60 and 59% identical, respectively) and hence could indicate identity with the up-regulation of mEMR4 in primed/activated M cells. Indeed, among various primary cells tested, the expression of mEMR4 was strongly detected in resident peritoneal macrophages (RPMϕ), BioMϕ, and ThioMϕ (Fig. 4B). BMDC and ThioPMN showed lower levels of expression, although we could not rule out the possibility of Mϕ contamination in ThioPMN preparation. Splenic B220+ B cells showed a very weak signal, and no signal was detected in splenic T cells and BMMϕ (Fig. 4B).

Interestingly, the expression was found to be up-regulated in BioMϕ and even more so in ThioMϕ, suggesting an induction of gene expression following macrophage activation (Fig. 4B). To investigate this further, RPMϕ were cultured with or without various pro- and anti-inflammatory cytokines for 36 h. In concert with the up-regulation of mEMR4 in primed/activated Mϕ such as BioMϕ and ThioMϕ, TNF-α-treated RPMϕ expressed a higher level of expression in comparison to that of untreated cells, whereas interferon-γ did not seem to induce further expression (Fig. 4C). In contrast, IL-10 treatment substantially reduced mEMR4 expression in RPMϕ and IL-4 seemed to abolish the expression (Fig. 4C). The expression of mEMR4 in RPMϕ therefore appeared to be highly regulated.

The majority of the EGF-TM7 genes are also characterized by extensive alternative splicing of mRNA. Alternative splicing occurs predominantly at the 5′-end of the transcripts resulting in multiple protein isoforms that contain different numbers and/or combinations of EGF-like domains (12, 16–18). RT-PCR analysis using different primer sets that cover the 5′- and 3′-end regions of the gene was employed to determine whether mEMR4 also expressed alternatively spliced transcripts. Fig. 4D shows that both 5′-end and 3′-end primer sets generate only one band of PCR product of expected sizes indicating that, unlike other EGF-TM7 members, mEMR4 is not alternatively spliced. Hence, only one protein species is predicted.

mEMR4 Maps to Mouse Chromosome 17—To date, every known human member of the EGF-TM7 family, with the exception of ETL, was located on mouse Chr 19p13 region, suggesting a strong evolutionary link among these molecules (9, 12, 13, 15). To determine the genetic map of the mEMR4 gene, the segregation pattern of sequences detected by a mEMR4 gene, the tight linkage of markers of C3, Emr1, Vav, and Rfx2 (Fig. 3). The location of mEMR4 was mapped at 10.43 (± 2.85) centimorgans and 0.86 (± 0.86) centimorgans from those flanking genes, respectively (Fig. 3). The C3, Emr1, Vav, and Rfx2 region of murine Chr 17, as determined by physical and comparative mapping, is known to be syntenic to the human Chr 19p13.3 locus (48). Interestingly, a search in the human genome data base identified a genomic contig of human Chr 19p13.3 (accession no. NT011169.4) containing the human EMR1 gene and another incomplete EGF-TM7 gene highly homologous to mEMR4 (82% identical on existing sequence; data not shown). This putative human homologue of mEMR4 is 20 kb proximal to EMR1, comparable with the genetic map of Emr1 and mEMR4 in the mouse genome, indicating that this region of the genome is highly conserved among species.

mEMR4 Is Expressed Predominantly in Resident Macrophages and Is Up-regulated following Macrophage Activation—The majority of the EGF-TM7 genes are expressed highly and restrictedly in myeloid cells including monocytes, macrophages, and granulocytes (7, 8). Northern blot analysis was carried out to examine the expression pattern of mEMR4 using RNA samples from multiple mouse tissues and various primary cells. Fig. 4A shows that a major mEMR4 transcript of approximate 3.3 kb was expressed abundantly in spleen and liver, whereas a low level of expression was found in lung and kidney and weakly in thymus. No signal was detected in brain, heart, skeletal muscle, skin, small intestine, stomach, or testis. The expression pattern is similar to that of other EGF-TM7 members and is consistent with that of subpopulations of resident macrophages. Indeed, among various primary cells tested, the expression of mEMR4 was strongly detected in resident peritoneal macrophages (RPMϕ), BioMϕ, and ThioMϕ (Fig. 4B).

To characterize the biochemical properties of the mEMR4 protein, the full-length mEMR4 open reading frame was subcloned into an expression vector and tagged with a V5-His epitope at the C-terminal end (see “Experimental Procedures”). Total cell lysates from HEK293T cells transiently transfected with or without this vector were analyzed by Western blotting using an anti-V5 mAb (Fig. 5A). Two specific bands of ~85–100 and 34 kDa were observed in the vector-transfected cells but not in mock-transfected cells. The 85–100 kDa band appeared to be dim and diffuse and might represent unprocessed mature protein species (see below). Alternatively, it might represent immature protein precursor as the sample contained total cell lysates. The smeared appearance also suggested that mEMR4 is glycosylated. The 34-kDa band is very intense, specific, but
FIG. 1. Primary amino acid sequence and predicted structure of mEMR4. A, the deduced amino acid sequence of mEMR4. The putative signal peptide is shown in boldface letters, and the first and second EGF-like module as well as the TM7 region are highlighted in yellow, orange, and gray backgrounds, respectively. The potential N-linked glycosylation sites are enclosed in boxes, and the GPS domain is underlined in green. The potential protein kinase C phosphorylation sites and casein kinase II phosphorylation sites are underlined once and twice, respectively. B, schematic structure of mEMR4. The EGF-like modules are represented by numbered and color-coded triangles, the GPS domain is shown as a green box, and the TM7 region is represented by membrane-spanning cylinders. Filled circles denote potential N-linked glycosylation sites.
much smaller than the expected mature mEMR4 protein. However, it is consistent with the predicted size of the TM7 and cytoplasmic tail regions. In light of the presence of the GPS domain in the stalk region of mEMR4, the possibility of proteolytic cleavage was investigated further using biotinylation of cell surface proteins followed by immunoprecipitation by an α-V5 mAb. Immunoprecipitated proteins were separated in an SDS-PAGE and detected by Extravidin-HRP by Western blot.

**FIG. 2.** Amino acid sequence alignments of mEMR4, hEMR3, and rETL. The amino acid sequences of mEMR4, hEMR3, and rETL proteins are aligned for maximal homology. Identical and similar residues are shaded in red and gray backgrounds, respectively. The EGF-like modules, the GPS domain, and the transmembrane regions are indicated individually. Asterisks indicate the cysteine, glycine, and tyrosine residues conserved in the GPS domain.

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**EGF-1**

mEMR4 | hEMR3 | rETL
---|---|---
MLMGATRNGSGRCYAVSHVPWMLLLESLIQMNMS..ASPYQPKKNLSFQ...SYKHEKFRGSGSNRRIEIP..HE
IQOPPLL..FICLCELSLLFQGAVTKQKTSQAK..SGPAKSTVNT..HETYNQ...YSGSGGKQFKTFPLE
KRLLIL..VALSSLN..HSTYQN...KRLFHEAKIVLDEVAHCHGSTG...XTGN..GITOR

**EGF-2**

mEMR4 | hEMR3 | rETL
---|---|---
KQDINEKLLKL..EDIKVCSVQ..KIKTVSEIK..VPFLNWNAGIINIDHPL...YVNEK...
CINQCTPSSYVGNAFKVPCCHRSHGQNSQPSNENTQDTSS
IESDRCN..ETS.V..GDHAV..ETAKG..GPSHKEG..EG..GQSGTGTKQPTPDNGS...QVDCENET..SVCGDHAVCENNTGG

**GPS domain**

mEMR4 | hEMR3 | rETL
---|---|---
...KTMTILEAG...NNITMKVDCTG...FEKH...NSSGDTAVHAIKLNLNSG.LFS...
ERKTFNIN...VQMNMDIRSCD...IIQC...DTQPSIAAFSISLONINAT...FFE
KN.TQFDMNSTDLALKVFVDHVKHYTHHPHMVDGGYKVSRRKAYDPENNYVIAVCLFQIUPLSSSEDDELGAQ

**TM1**

mEMR4 | hEMR3 | rETL
---|---|---
1KCPFSSSILV1KAPHEEGDV.LASVS1TYYVLSVLLCLLLELAA11TLCLCRPQNSSTTLLCRCSPICLPLAFLITT
20NSKGSSTAV1MALTSQ...DPTIVTYVYLYSVLLCLLLELAA11TLCLCKSNNS1LQSLCLFLHLPFL
STRTSITHAILNSPTSSILVDYKNLTI..TKIQ..AK1S1FTSHHIFMTWFFSESCRTKIC1KLSCCIFLPGCGV

**TM3**

mEMR4 | hEMR3 | rETL
---|---|---
SGRTKPV1LCST1AGCMLHVLYLAWKWMNLLEDPFHTVSNKKNVANYSNSGGRKFKMPYVGYGDFAPNVAIAAGHK
SIDRH1KVTSL1ACAAHYLAFTWNMLLEGHFLF2ARTNTTVYNNYSINLKMWPYMVGFVTVIAIAASWH
STININT1MLCST1AGCMLHVLYLAWKWMNLLEDPFHTVSNKKNVANYSNSGGRKFKMPYVGYGDFAPNVAIAAGHK

**TM5**

mEMR4 | hEMR3 | rETL
---|---|---
NGTTHHHRRISLSLRRGFPISLSLLLAA11A111LVFVFLWILRSLKSLSNKLSTCLQTKWTFPAVYFVYVSMIGL
LY7AD4CWL1LDQQFMI1SVGLPSVAFAALVLVFILVWFLKRLSSLSEVSITQTRMLAPKTACAGIFLMTCCL
YTV7TKTV5WESTENVFRHFDACLLIVLLAFGVIYKIVHPATAGLKRFSVCEYNIRSCGALALALGATTTC5

**TM7**

mEMR4 | hEMR3 | rETL
---|---|---
LFIPTEVGCSTVLALVYFLITIVLVLTVFVHLNQFRVME..KVKW..HRLKKEVESESTVESHSTTHTKMLSLNLENF
ILLGQV.PAACLAMFL1TSLQCG1FTLFLVLYLSSQVKKCQKQQKR...EIKVSKSBETFYTLSSLKHPG...
VLHVVHASVTYTL1V5..FAAMETFL1V1LVRSKR1QEYE.YALN...VFPC..FECUL
ting. Fig. 5B shows that a major band of ∼50 kDa was detected along with a minor band of ∼90–100 kDa. The latter was probably the uncleaved form of the receptor, whereas the earlier band is the cleaved extracellular domain of mEMR4. No visible bands were detected in mock-transfected samples. The proteolytic cleavage of mEMR4 was finally confirmed using a fusion protein containing the full-length mEMR4 extracellular domain (1–341 amino acids) and an mFc fragment, mEMR4-341mFc (see “Experimental Procedures”). Fig. 5C shows that when conditioned media collected from cells transfected with mEMR4-341mFc and a control mFc construct were probed with an anti-mFc mAb, both samples produced a band of approximately 40 kDa, which correlates to the size of the mFc protein. A very faint band of ∼80–100 kDa was also detected in the mEMR4-341mFc protein sample, which is likely to be the uncleaved full-length fusion protein. No signal was found in samples from mock-transfected cells. This result indicates that the majority of mEMR4-341mFc fusion protein is proteolytically cleaved within the extracellular stalk region very close to the C terminus of the GPS domain, thus generating a cleaved product the same size as the control mFc protein. Furthermore, it shows that the TM7 region is not required for the cleavage activity, as the fusion protein contains only the extracellular region. To characterize the proteolytic process further, we purified the mFc fusion protein by protein A affinity column chromatography and resolved it in an 8% SDS-PAGE (Fig. 5D). Three protein bands of approximate 80–100, 46–60, and 40 kDa were visible after Coomassie Brilliant Blue staining, indicating that mEMR4 is indeed a glycoprotein. Moreover, it

![Figure 3. Genetic mapping of mEMR4.](image)

**FIG. 3.** Genetic mapping of mEMR4. The linkage of mEMR4 to Nfya and Vav genes on mouse chromosome 17 was determined by Southern blot hybridization of HincII-digested DNA from 118 interspecific back-cross progeny. Black boxes represent the inheritance of a C3Hf allele for a particular set of loci, and white boxes represent the M. spretus allele. Gene linkage, order, and intergenic distance with associated standard errors were determined using Map Manager data analysis software.

![Figure 4.](image)

**FIG. 4.** The expression profile of mEMR4. A. Northern blots containing equal amounts (2 μg) of poly(A)+ RNA from the indicated mouse tissues were subsequently hybridized with 32P-labeled probes specific for mEMR4 and β-actin. An approximate 3.30-kb mEMR4 transcript is indicated. B and C, total RNA (10 μg/lane) from various mouse primary cells and cytokine-treated RPM was analyzed by Northern blotting as described under “Experimental Procedures.” The numbers indicate the positions of molecular weight markers. Ethidium bromide staining of the gels in the lower panels shows the equal loading and integrity of the RNA samples. D, RT-PCR analysis of mEMR4 using primer sets covering the 5’-end extracellular region and the 3’-end TM7 region shows that only a single band of PCR product is generated from the tissues examined. The numbers indicate the sizes of the PCR products.
shows clearly that the two cleaved protein subunits remain associated after proteolysis. To determine the cleavage site, the 40-kDa mFc band was excised for N-terminal amino acid sequencing. The resulting sequence, SSFAVLMALP, matches precisely to the mEMR4 stalk sequence (residues 327–336) and is consistent with the conserved GPS cleavage site (Fig. 5E). The cleavage therefore occurred at the peptide bond between Leu326 and Ser327.

mEMR4 Binds to a Putative Cellular Ligand on A20 Cells—To examine whether mEMR4 can mediate protein-protein interaction and to search for its potential cellular ligand, a previously described cell-binding assay was modified and employed (13, 44). The EGF-like modules of mEMR4 were fused with a truncated Fc region of a mouse immunoglobulin (IgG2b subtype) and a biotinylation signal to generate pmEMR4-147mFc (see “Experimental Procedures”) (Fig. 6A). The mFc region enabled us to purify large amounts of soluble proteins from transfected mammalian cells, whereas the biotinylation signal allowed efficient in vitro biotinylation of the purified proteins (Fig. 6B). By coupling the soluble biotinylated mEMR4-mFc fusion proteins to avidin-coated fluorescent beads, a multivalent probe with increased binding abilities and sensitivities are generated. A FACS-based cell-binding assay was then performed to screen for the presence of the mEMR4 ligand (see “Experimental Procedures”).

From a total of eight cell lines (J774, NIH3T3, RAW, P388D1, Wehi-231, Daudi, BW-1547, EL-4, and A20) tested, A20, a mouse B lymphoma cell line, showed a strong fluorescence signal, indicating that it expresses a putative mEMR4 ligand on the cell surface (Fig. 6C). The interaction between the multivalent mEMR4 probe and A20 cells is specific because no binding signal was found when a similar EMR2 (1,2)-mFc fusion protein was used in the same assay. Moreover, the interaction was shown to be Ca2+-independent because the addition of EGTA did not affect binding (Fig. 6C). The Ca2+-independent interaction suggested that the first EGF-like module might be involved in the binding because it is predicted to be a non-cbEGF (Figs. 1 and 2). To locate the ligand-binding domain, the first and second EGF-like modules of mEMR4 and EMR2 were exchanged by genetic engineering to generate chimeric protein probes (Fig. 6A). FACS analysis using the domain-swapping chimeras surprisingly but unequivocally showed that the interaction is mediated predominantly by the second EGF-like module of mEMR4 (Fig. 6D).

**DISCUSSION**

With two N-terminal EGF-like modules, a mucin-like spacer region and a class-B GPCR-related TM7 domain, mEMR4 is the ninth member of the EGF-TM7 family to be identified to date (Figs. 1 and 2). As with the majority of the EGF-TM7
genes, mEMR4 is expressed predominantly in myeloid cells including RPM/H9278, BioM/H9278, ThioM/H9278, and BMDC (Fig. 4), suggesting that it may play a functional role in these professional phagocytes and antigen-presenting cells. The up-regulation of mEMR4 expression in BioM/H9278, ThioM/H9278, and TNF-/H9251-treated RPM/H9278 further suggested that mEMR4 might participate in situations involving activated M/H9278 such as inflammation and infection. The regulated mEMR4 gene expression by pro- and anti-inflammatory cytokines indicates that the mEMR4 gene is highly controlled at the transcriptional level (Fig. 4C). Similar tightly regulated gene expression has also been found for other EGF-TM7 genes. For example, CD97, a T-cell activation marker, is rapidly up-regulated at a very early stage upon T cell activation, whereas F4/80 has been shown to be diminished from F4/80ve Langerhans cells to F4/80ve interdigitating DCs in response to local antigen exposure (15, 39, 65). Understanding the regulatory mechanisms governing the expression profiles of individual EGF-TM7 genes in the future will shed light on their temporal and lineage-specific expression patterns and possibly their specific functions.

The proteolytic cleavage of mEMR4 at the GPS site, as revealed here by Western blot analysis and N-terminal amino acid sequencing (Fig. 5), clearly shows that mEMR4 is proteolytically processed from a precursor polypeptide resulting in an extracellular domain subunit and a TM7 subunit. In addition, the immunoprecipitation of biotinylated cell surface proteins by anti-V5 mAb further indicates that the cleaved extracellular domain subunit is associated with the TM7 domain subunit (Fig. 5B). The same protein modification has been demonstrated for other LNB-TM7 molecules and is possibly a common feature for all the GPS domain-containing proteins. Although the functional significance of this modification is still unclear, several modes of actions regarding intracellular signaling could be envisioned as a consequence of this well conserved modification. The tethered extracellular domain could be shed following ligand binding or cellular activation leading to the activation of the "unoccupied" TM7 receptor domain. Alternatively, the resulting 'unoccupied' TM7 receptor domain may become available to other peptide hormone-like ligands similar to those of the classical class B-GPCRs. In contrast, the shedding of the extracellular domain could possibly be a mechanism for receptor internalization/down-regulation leading to the termination of signaling. It is also possible that the proteolytic cleavage at the GPS site is prerequisite for the maturation and function of receptors, which may acquire certain conformational changes due to the cleavage. The detailed relationship between proteolytic processing and receptor functions awaits further investigation. In addition, the consensus GPS domain and proteolytic cleavage site found in the LNB-TM7 molecules strongly suggested the presence of a functionally conserved protease(s), the true identity of which is also of particular interest.

In concert with the similarity in structure, expression, and protein modification, the genetic mapping of mEMR4 to the distal region of mouse Chr 17 (Fig. 3) also points to a well conserved gene family located mainly in human Chr 19p13 region (and the syntenic mouse Chr regions) (9, 11–13, 15, 45, 46). Interestingly, the Chr 19p13 EGF-TM7 genes can be further subdivided into two distinct loci, with EMR1 and -4 on
EGF-TM7 Molecule mEMR4 Binds to a Cellular Ligand on A20 Cells

human Chr 19p13.3 (mouse Chr 17) and CD97, EMR2, and EMR3 on human Chr 19p13.1 (mouse Chr 8). Moreover, members of other gene families were also found closely linked with the EGF-TM7 genes within these two loci. For example, transcription factors RXF1 and RXF2, two closely related site-specific DNA-binding proteins important for immune system functions (66, 67), are tightly linked with CD97 and EMR1, respectively, both in human and mouse genomes (11, 46, 48). These findings have given rise to the notion that both sets of gene families were derived from an ancient gene duplication event encompassing their predecessors and subsequently separated during evolution (46). The identification and mapping of mEMR4 as well as the detailed genomic sequence information gradually emerging from the human and mouse genome projects will no doubt further delineate the extent of gene duplication and details of their evolution.

Although the significance of the interaction between mEMR4 and A20 cells (Fig. 6) has yet to be established, the predominant myeloid cell expression profile and the up-regulation of mEMR4 in activated Mφ suggested that the interaction could play an important role in modulating immune and inflammatory responses by allowing “cross-talk” between myeloid cells and the ligand-bearing cells. The ligation of the mEMR4 receptor could potentially activate Mφ via the TM7 moiety to amplify immune and inflammatory responses. On the other hand, the signaling could occur in the opposite direction to activate the ligand-bearing cells. A20 is a B cell lymphoma line derived from a spontaneous reticulum cell neoplasm (68). At present, it is not known whether the putative mEMR4 ligand is restricted only to B cell populations or is shared also by other cell lineages. Our preliminary results show that it is not detectable in single spleen cell suspension from naive mice but is present in the white pulp B cell zones of infected mice. If the ligand is indeed restricted to certain B cell subpopulations, the mEMR4-ligand interaction could potentially stimulate the proliferation or differentiation of the ligand-bearing B cells. The exact nature of the mEMR4 ligand is currently under investigation.

The EGF-like module is one of the most common structural motifs used by cell surface and extracellular matrix proteins and has been identified in proteins involved in cellular adhesion, cell fate determination, extracellular matrix structure, receptor-ligand interactions, and blood coagulation (20). Recent protein structure studies have revealed a major double-stranded β-sheet conformation for the EGF-like modules (19, 69), which mediate protein-protein interaction either via the intramolecular loops on the surface or via the Ca2+ ion chelated by their calcium-binding domains (20, 70). The finding that the interaction between mEMR4 and its ligand on A20 cells is Ca2+-independent but mediated predominantly by the Ca2+-binding second EGF-like module (Fig. 6) may reflect the fact that mEMR4 contains only one non-cbEGF and one cb-EGF module (Fig. 1). It is possible that in this setting calcium signaling could occur in the opposite direction to activate the immune and inflammatory responses. On the other hand, the TM7 moiety could potentially stimulate the proliferation or differentiation of the ligand-bearing cells. The exact nature of the mEMR4 ligand is currently under investigation.

The unique hybrid structure of the EGF-TM7 receptors is suggestive of a dual function in which the EGF-like modules carry out cellular adhesion/interaction while the TM7 moiety transmits intracellular signaling messages (7, 8). The recent demonstration of the presence of cell surface ligands for CD97, EMR3, EMR2, and mEMR4 herein has further strengthened this hypothesis. As the first step toward definitively proving this hypothesis, the sensitive ligand-binding assay described above is currently being employed to identify molecularly the cognate cell surface ligands for the individual EGF-TM7 receptors. Once identified, future demonstrations of the signaling pathway of the EGF-TM7 receptors and the linkage between the dual adhesion-signaling function will be the keys to unraveling the cellular importance of these unusual TM7 receptors.

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