Molecular Cloning of POEM
A NOVEL ADHESION MOLECULE THAT INTERACTS WITH $\alpha_5\beta_1$ INTEGRIN*

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Cell adhesion molecules are involved in a number of biological functions, such as cell survival, cell differentiation, tissue repair, and development. A novel molecule, POEM (preosteoblast epidermal growth factor-like repeat protein with meprin, $\alpha_5$ protein, and receptor protein-tyrosine phosphatase $\mu$ domain), was isolated by reverse transcription-polymerase chain reaction using a set of degenerate primers designed after other known epidermal growth factor (EGF)-like motifs. From its structure, POEM was suggested to be a novel adhesion molecule with five EGF-like domains, an Arg-Gly-Asp (RGD) cell binding motif, and a meprin, $\alpha_5$ protein, and receptor protein-tyrosine phosphatase $\mu$ (MAM) domain. By in situ hybridization using embryonic day 16.5 (E16.5) mouse embryos, strong expression of POEM mRNA was observed in developing kidney renal tubules, parathyroid and thyroid glands, developing bone, tooth germ, and endocrine organs of the brain. The inner ear, skeletal muscle, smooth muscle (except for the vascular system), and skin were also positive for POEM expression. Bacterial recombinant POEM protein containing the RGD sequence and MAM domain showed strong cell adhesion, spreading, and survival-promoting activities. By mutating the RGD sequence to RGE, the cell spreading and survival activities were significantly decreased, but the MAM domain was shown to contribute only to cell adhesion and not to cell spreading and survival-promoting activities. The distribution of POEM in several tissues was close to that of $\alpha_5\beta_1$ integrin. Therefore, we conducted cell adhesion assays using KA8 cells, a K562 leukemia clone stably expressing $\alpha_5$ integrin. Parental K562 cells, which expressed $\alpha_5\beta_1$ integrin, bound to fibronectin but not to POEM. On the other hand, KA8 cells showed strong binding and spreading on both fibronectin and POEM. These results suggest that POEM is a novel ligand for $\alpha_5\beta_1$ integrin and that POEM may be involved in the development and function of various tissues, such as kidney, bone, muscles, and endocrine organs.

The epidermal growth factor (EGF)-like repeat structure is present in a number of extracellular matrix (ECM) proteins and cell surface receptors (1). Fibrülin (2) and laminin (3) are typical ECM proteins with multiple EGF-like repeats. Notch and Delta are cell surface molecules that act as a receptor and ligand, respectively, and interact via their EGF-like domains to determine cell fates (4). Transforming growth factor $\alpha$ (5) and hepatrin-binding EGF-like growth factor (6) are generated by cleavage of transmembrane precursors and act through the EGF receptor. Recently, small matrix proteins with several EGF-like repeat structures have been reported. For example, Pref-1 is produced as a membrane protein and controls adipocytic cell differentiation (7). Del-1 is an adhesion molecule with an Arg-Gly-Asp (RGD) sequence and induces angiogenesis (8). DANCE is another secreted molecule with an RGD cell binding motif; it is expressed predominantly in developing arteries and supposed acts as a cell adhesion molecule in tissue development and repair (9).

Interactions between cells and ECM play roles in morphogenesis, tissue homeostasis, and remodeling. The ECM presents much information to the cells by working as a multifunctional ligand for cell adhesion receptors (10, 11). Integrins are a large family of heterodimeric cell surface proteins that serve as receptors for various ECM proteins (12–14). Integrins are involved in tissue repair, development, and immune responses. Integrins also serve important functions in bone development and remodeling. Recently, it was reported that $\beta_1$ integrin was significantly involved in osteoblastic function (15). Expression of dominant negative $\beta_1$ subunit in osteoblasts significantly reduced the bone-forming activity of osteoblasts. Matrix proteins produced by osteoblastic cells are the major target molecules not only for osteoblasts themselves but also for osteoclastic cells. Therefore, identification of a novel adhesion molecule produced by osteoblastic cells provides a new insight into the biology and development of bone tissue.

In this study, we screened a cDNA library derived from preosteoblastic MC3T3-E1 cells and isolated a cDNA clone coding for a novel adhesion protein, POEM.

MATERIALS AND METHODS

Cell Lines and Culture—A cell population enriched in osteoblasts was prepared from the calvaria of a 4-week-old C57BL/6 mouse as described previously (16). MC3T3-E1, a mouse calvaria-derived osteoblast-like cell line, was maintained in α-modified essential medium

1 The abbreviations used are: EGF, epidermal growth factor; ECM, extracellular matrix; PCR, polymerase chain reaction; MAM, meprin, A5 protein, and receptor protein-tyrosine phosphatase $\mu$; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell-sorting; MBP, maltose-binding protein; POEM, preosteoblast epidermal growth factor-like repeat protein with meprin, A5 protein, and receptor protein-tyrosine phosphatase $\mu$ domain.
(Life Technologies, Inc.). COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.). K58 cells, which express the chicken integrin α5 subunit (17), and control K562 cells were kindly provided by Dr. Louis F. Reichardt (University of California, San Francisco, CA). K58 cells were cultured in RPMI 1640 medium containing 10% FBS. POEM clones 4141 (Calbiochem-Novabiochem Corp., La Jolla, CA), and K562 cells were cultured in the same medium but without the G418. Each medium was supplemented with 10% fetal calf serum (Roche Molecular Biochemicals) and antibiotic antimycotic solution (Life Technologies, Inc.). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO2 in air.

cDNA Cloning and Sequencing—A pair of degenerate primers used for polymerase chain reaction (PCR) amplification was designed based on the conserved amino acid sequences found in EGF-repeat structures (18). Complementary DNA was synthesized from the mRNA of MC3T3-E1 cells and amplified by use of a reverse transcription-PCR kit (Stratagene, La Jolla, CA). The PCR program consisted of 30 cycles of 94 °C for 1.5 min, 48 °C for 2 min, and 72 °C for 2 min. The amplified PCR products were subcloned into pBluescript plasmid (Stratagene) and subjected to sequencing by the dyeoxy-chain termination method with an automatic DNA sequencer (Model 373; Applied Biosystems, Foster City, CA). A cDNA library was constructed from the mRNA of MC3T3-E1 cells cultured for 6 days by using a UniZAP cDNA library construction kit (Stratagene).

DNA fragment of a gene that was not represented in the GenBankTM data base was obtained and used to screen a MC3T3-E1 (cultured for 6 days) cDNA phage library constructed in UniZAP (Stratagene). The single positive plaque was subjected to in vivo excision of the pBluescript SK(−) phagemid as described by the manufacturer. A homology search was performed with BLAST and FASTA against public sequence data bases, and a motif search was performed on-line with PROSITE. The nucleotide sequence data reported in this study will appear in the DDBJ/EMBL/GenBankTM nucleotide sequence data base with the accession number AB050956.

RNA Preparation and Northern Blotting Analysis—Total RNA was prepared from brain, kidney, liver, spleen, testis, and thymus of 4-week-old mice. In addition, 14.5, 16.5, and 18.5 days post coitus whole embryos and newborn mice were also used for RNA preparation. The total RNA was prepared by the acid guanidinium thiocyanate-cesium method (19) or with ISOGEN (Nippon Gene, Tokyo, Japan). Polyadenylated RNA samples were extracted from cells by using a Quick Prep Micro mRNA Purification Kit (Amersham Pharmacia Biotech). The RNA samples were electrophoresed in 1.2% agarose gel and then transferred to nylon membranes (Hybond-N+; Amersham Pharmacia Biotech). The blots were hybridized with radiolabeled probes and washed twice in 2× SSC, 0.1% SDS at room temperature followed by 0.2× SSC, 0.1% SDS at 55 °C. The equivalent loading of the RNA samples was confirmed by probing with a human β-actin probe.

In Situ Hybridization—Both sense and antisense probes were labeled by transcription from full-length mouse POEM cDNA with a digoxigenin RNA labeling kit (Roche Molecular Biochemicals). The labeled probes were dried-hydrated in 70% ethanol, and then redried.

Kidney, liver, and spleen were collected from 22-day-old mice, and spleen was collected from adult mice. These specimens and 16.5-day-old embryos were fixed in 4% paraformaldehyde, embedded in Tissue-Tec OCT (Miles, Inc., Elkhart, IN), frozen in a liquid nitrogen, and cryosectioned at 8-μm thickness. Three serial sections were made (two for hybridization with sense or antisense probe and one for staining with hematoxylin and eosin).

In situ hybridization with digoxigenin-labeled sense and antisense RNA probes was performed as described previously with a slight modification. In brief, sections were permeabilized with proteinase K (3 μg/ml) in phosphate-buffered saline (PBS) for 30 min at 37 °C and hybridized with probes (0.3 μg/ml) overnight at 45 °C. The sections were then washed with 1% blocking buffer, washed twice in 2× SSC, 0.1% SDS at room temperature, and developed for 6 h at room temperature in coloring solution (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) containing levamisole (10 μg/ml).

Construction of Expression Vectors and Immunoblotting—A cDNA fragment (nucleotide positions 1441–1511 as shown in Fig. 1) coding amino acids 486–561 of POEM was amplified by PCR and purified using primers 5′-CCACAGCTTGGTGGCAGCAGCGCCCGG-3′ (forward direction) and 5′-GAAGATCTGACGGACTCTTCTTTT-3′ (reverse direction). Another cDNA fragment corresponding to nucleotide positions 1441–1511 (amino acids 1–421; POEMΔMAM) of POEM was also amplified using the same forward primer and reverse primer 5′-GAAGATCTGTTCTGTTGAGAATACCTG-3′. PCR products were digested with HindIII and BglII and used to replace the osteopontin signal sequence and mouse CTLA4 cDNA of expression vector pcDNA3-OSM-mCTLA4-Ig (kindly provided by Dr. R. Abe; Science University of Tokyo, Tokyo, Japan) to generate expression vectors for fusion proteins of POEM and human immunoglobulin G (IgG) constant region. A single amino acid substitution (RGD→RGE) was introduced into POEM cDNA by PCR using a primer containing a single transition vector for maltose-binding protein (MBP)+POEM fusion protein pMAL-c2x-POEM-MBP. MBP-POEMcRGE (POEM with a single amino acid substitution at amino acid position 384 converting the RGD sequence to RGE) was constructed by replacing the BamHI-HindIII fragment of cDNA with a 378-bp XbaI-XbaI fragment from pMAL-c2x vector (New England Biolabs, Beverly, MA) to generate an expression vector for maltose-binding protein (MBP)+POEM fusion protein pMAL-c2x-POEMcRGE (POEM with a single amino acid substitution at amino acid position 384 converting the RGD sequence to RGE).

FACS Analysis—COS-7 cells (5 × 104 cells) were transfected with POEM-Fc vector or other POEM mutant-Fc vectors by using TransIT-LT1. Two days later, the cells were washed with PBS and harvested with 0.2% EDTA in PBS. The cells (1 × 106) were washed with PBS containing 2% fetal calf serum and 0.02% NaN3 and incubated with a fluorescein isothiocyanate-conjugated anti-human IgG antibody (Southern Biotechnology Associates, Birmingham, AL). The membrane was exposed to x-ray film with chemiluminescence enhancement (Amersham Pharmacia Biotech). A homology search was performed with BLAST and FASTA against public sequence data bases, and a motif search was performed on-line with PROSITE. The nucleotide sequence data reported in this study will appear in the DDBJ/EMBL/GenBankTM nucleotide sequence data base with the accession number AB050956.

Bacterial Expression of POEM and Cell Adhesion Assay—The BamHI-HindIII fragment of POEM cDNA (nucleotide positions 1376–2887) encoding the C terminus portion of POEM protein (amino acids 377–561; POEMc) was cloned into the pMAL-c2 bacterial expression vector (New England Biolabs, Beverly, MA) to generate an expression vector for maltose-binding protein (MBP)+POEM fusion protein pMAL-c2x-POEMcRGE. MBP-POEMcRGE (POEM with a single amino acid substitution at amino acid position 384 converting the RGD sequence to RGE) was constructed by replacing the BamHI-KpnI fragment of cDNA with a 378-bp XbaI-XbaI fragment from pMAL-c2x vector with the BamHI-KpnI fragment of POEM (Bacterio-POEMcRGE). Recombinant MBP-POEMcRGE, MBP-MBPcRGE, and MBP proteins were produced by Escherichia coli strain XL1-Blue transformed with pMAL-c2x-POEMc, pMAL-c2x-POEMcRGE, or pMAL-c2x, respectively. The recombinant proteins were purified by amylase resin column chromatography and analyzed by Coomasie Brilliant Blue staining after SDS-polyacrylamide gel electrophoresis. The protein concentration was determined with a Coomasie Plus Protein Assay Reagent (Pierce) by comparing to a bovine serum albumin standard.

Non-tissue culture 96-well plates were coated with 1–5 μg of recombinant MBP-POEMc, MBP-POEMcRGE, or MBP proteins diluted with PBS and incubated for 24 h at 4 °C. Bovine fibrinectin (Sigma) was used as a positive control, and MBP and bovine serum albumin were used as negative controls. Coated wells were washed with PBS and blocked for 1 h at 37 °C with a solution of 10 mg/ml bovine serum albumin in PBS. Subconfluent MC3T3-E1 cells were harvested by trypsin-EDTA, suspended in serum-free medium (3 × 104 cells/100 μl), and placed in the wells. After incubation for 90 min at 37 °C, the wells were washed several times with serum-free medium until almost all of the cells had been washed out of the negative control wells. Attached cells were fixed and stained with 0.1 ng/ml 4′,6-diamidino-2-phenylindole dihydrochloride (Roche Molecular Biochemicals) in methanol, and the cell number was counted as the number of fluorescent nuclei seen under a fluorescence microscope. Experiments were performed in duplicate.

For the cell adhesion assay using K562 and KA8 cells, glass-bottomed dishes (Mastunami, Osaka, Japan) were coated with recombinant proteins, bovine fibrinectin, or MBP at a concentration of 5 μg/ml at 4 °C overnight and then blocked as described above. These cells were then incubated in a 3% fetale κ serum/0.2% EDTA in PBS at 37 °C, and observed under a phase-contrast microscope.

Reverse Transcription-PCR for Integrin α5 Subunit—A 505-base pair fragment of mouse integrin α5 sequence corresponding to 2507–3011 of GenBankTM accession number AF401409 was amplified using the following primers: 5′-GCTCGCTTCCACCGAGGATTACG-3′ (forward direction) and 5′-GTCAGTGTCTCCGTCCTGCTAC-3′ (reverse direction).
The reverse transcription-PCR amplification was conducted as described above using cDNA of MC3T3-E1 cells as a template. The PCR program consisted of 30 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min. The amplified PCR products were electrophoresed on a 2% agarose gel and visualized with ethidium bromide staining.

RESULTS

Molecular Cloning of a Gene Encoding a Novel EGF-like Repeat Protein from MC3T3-E1

MC3T3-E1 is a cell line with preosteoblastic phenotypes. We generated a pair of degenerate PCR primers based on the EGF-like repeat structures found in various ECM and receptor proteins (18). Using a single-strand cDNA of MC3T3-E1 (cultured for 3 days) as a template, we obtained a clone, OB-9, whose sequence was not found in the GenBank™ data base. By screening a MC3T3-E1 cDNA library with OB-9 partial cDNA as a probe, we isolated four independent and overlapping clones. By restriction enzyme mapping and DNA sequencing, we isolated a full-length 3.6-kilobase cDNA with an open reading frame of 561 amino acid residues (Fig. 1). The translation initiation site, Met, was assigned at nucleotide positions 249–251 according to the Kozak consensus sequence (21). The deduced amino acid sequence of this protein had an N-terminal hydrophobic domain, which was presumed to be a signal sequence, but no other hydrophobic regions that could serve as transmembrane domains were found, suggesting that OB-9 is a secreted protein. Additionally, five EGF-like repeat domains, a proline-rich domain, one putative Asn glycosylation site, an RGD cell adhesion sequence motif, and a MAM domain were found. Therefore, we named this potential protein POEM (preosteoblast EGF-like repeat protein with MAM domain).

The overall POEM sequence showed significant similarity to mouse MAEG (GenBank™ accession number AJ245672) or its human counterpart, EGFL6 (GenBank™ accession number AF186084), which contains a predicted signal sequence, an

![Fig. 1. Nucleotide and deduced amino acid sequences of mouse POEM. A, nucleotide sequence of complementary DNA and deduced amino acid sequences of POEM. To the right of each line, non-bold numbers indicate the position of nucleotides, and bold numbers indicate the position of the amino acids. A putative cleavage site of the signal peptide is indicated by an arrowhead. Arrows show the five EGF-like repeats. Broken underline indicates the proline-rich region. A cell adhesion RGD sequence is boxed. A potential N-glycosylation site is indicated by an asterisk. The MAM domain is double-underlined. B, alignment of five EGF-like repeats of POEM. Conserved amino acids are shown by bold letters. FBN1, mouse fibrillin 1; FBN2, mouse fibrillin 2; CD97, human leucocyte antigen CD97; LT-TGFβ1, rat latent transforming growth factor β-binding protein 1; NTC1, mouse Notch1; DLL, mouse Delta-like 1. Numbers indicate amino acid positions. C, alignment of the MAM domain of POEM with that found in other known protein sequences. Conserved amino acids are shown by bold letters. mpr-α, mouse meprin α; A5, chicken A5 protein; RPTPα, mouse receptor protein-tyrosine phosphatase α; Zonadhesin/MAM1, the first MAM domain repeat of mouse Zonadhesin; ϕ, hydrophobic or aromatic amino acids.](http://www.jbc.org/leh glab JBC2018)
putative human counterpart of POEM (hCG20771, hCP38387) the proline-rich domain found in POEM. Recently, we found a amino acid sequence level, and MAEG/EGFL6 did not contain domain (Fig. 2). However, homology was about 39% at the array of five EGF-like repeats, an RGD motif, and a MAM (Fig. 3), suggesting that POEM is involved in embryonic 11.5 of gestation (data not shown) and subsequently increased expression of POEM mRNA could be detected as early as day Northern blotting (data not shown). In mouse development, exceeded. Expression of POEM was also detected in primary at the proliferation stage of MC3T3-E1 cells (Fig. 3). Approximately 4-kilobase POEM mRNA was highly expressed by in situ hybridization using frozen sections of 16.5-day-old embryos. Two µg of polyadenylated RNA from MC3T3-E1 cells of each culture day (A) and 20 µg of total RNA of each embryo (B) were blotted onto nylon membrane and hybridized with a radioactive POEM cDNA probe. Migratory positions of 18 S and 28 S ribosomal RNA are indicated. Migratory positions of POEM and ß-actin mRNA are indicated by arrows.

Hybridization of tissue sections with the sense probe of POEM showed no positive reaction (Fig. 4A). In 22-day-old mice, POEM expression was also observed in many tubules of the kidney (data not shown).

In the parathyroid gland, remarkable expression of POEM mRNA was observed in the developing parenchymal cells of cell cords (Fig. 4, C–E). However, in the capsule, no expression was detected (Fig. 4, C and D).

Thyroid tissue of 16.5-day-old embryos showed disordered cords of cells without obvious organization into follicles or the presence of colloid. However, some follicular structures were becoming clearly defined at the same time (Fig. 4E). The relatively high level of POEM mRNA was restricted to the developing follicular epithelial cells and potential parafollicular cells of the thyroid gland (Fig. 4, C and D).

A significant level of POEM mRNA was also detected in the tooth germs (Fig. 4, F–H). POEM mRNA expression was detected in the outer enamel epithelium. Higher expression was frequently observed in the outer part on the frontal side of the outer enamel epithelium and its stalk region. The entire region of the dental papilla was negative. POEM expression was also observed in some of the inner enamel epithelium and in the stellate reticulum.

In the intramembranous ossification sites, such as the ramus of the mandible (Fig. 4, I–K), the membranous primordium of the frontal bone, and calvaria, osteoblasts were identified adjacent to the mineralized tissues as cuboidal cells. Weak expression of POEM mRNA was detected in these mature osteoblasts lining the bone matrix, however, more intensive expression was observed in the condensed mesenchymal cells, surrounding developing bones in both the ramus of the mandible (Fig. 4, I and J) and the calvaria (data not shown). In the case of the intracartilaginous ossification site in vertebral bones, weak expression of POEM mRNA was detected both in cuboidal osteoblasts on the trabecular bone surface and in cells surrounding the bony collar showing the early stage of osteogenesis (Fig. 5, A–C).

POEM mRNA was also detected in the cells covering the outer side of the Eustachian tube and tubo-tympanic recess (Fig. 5, D–F). In the cochlea, two types of cells were recognized: support cells and sensory epithelium (hair cells; Fig. 5J). POEM mRNA was localized apically in some of the developing hair cells next to the cluster of mature hair cells as seen in a sagittal in the plane (Fig. 5, G and H).

POEM was widely expressed in the skeletal muscles (Fig. 5, J–O). Generally, the expression was relatively high around the nucleus in the skeletal muscles and near the tendon. POEM expression was not detected in the tendon. In the lingua (Fig. 5, M–O), POEM was detected not only in the lingual muscle but...
also in the mucosal epithelium (Fig. 5, M and N). In the lingual muscle, POEM expression was also high around the nucleus, especially in the region just under the lingual aponeurosis and near the lingual septum, where the muscle made contact. In the mucosal epithelium, a relatively low level of POEM was expressed and restricted to the upper surface of the lingua. POEM expression could not be observed in the lingual aponeurosis or lingual aorta.

POEM expression was detected in the smooth muscle as well as in the skeletal muscle. In the digestive tract, POEM was expressed in the muscle layer of both the esophagus (data not shown) and stomach (Fig. 5, P–R). In the esophagus, expression was observed in both the inner and outer muscle layers. On the other hand, abundant expression was localized restrictively in the inner muscle layer of the stomach (Fig. 5, P and Q). Through the remaining part, i.e. from the duodenum to the rectum, expression was barely detected, even in the muscle layer. The smooth muscle layer under the skin (Fig. 6, J–L) and the trachea (Fig. 6, P–R) were also positive for POEM mRNA. Vascular smooth muscle of arteries and veins showed no expression of POEM mRNA. The expression was not detected in the endothelial cells of blood vessels either.

In the brain, expression of POEM was detected in the pineal body (Fig. 6, A–C), choroid plexus, hypophysis (Fig. 6, D–F),
**Fig. 5.** POEM expression in additional embryonic day 16.5 mouse tissues. Frozen sections were hybridized with digoxigenin-labeled sense (A, D, G, J, M, and P) or antisense probe (B, E, H, K, N, and Q) or stained with hematoxylin and eosin (C, F, I, L, O, and R). A–C, three consecutive sagittal sections through vertebral bone, showing expression of POEM in osteoblasts (Ob, arrowheads) and in the cells surrounding bony collar (BC, arrowes). T, trabecula. D–F, three consecutive coronal sections through the head. Arrowheads reveal expression of POEM in the outer side of epithelial cells in the Eustachian tube (ET) and tubo-tympanic recess (TTR). G–I, three consecutive sagittal sections through the cochlea. Poem expression is observed in the apical region of several developing hair cells (arrowheads) next to the hair cells (HC). SC, support cell. J–L, three consecutive sagittal sections through the dome of the diaphragm. Arrowheads point to expression in the skeletal muscle. M–O, three consecutive coronal sections through the lingua. Arroheades and arrowes show POEM expression in the mucosal epithelium and skeletal muscle, respectively. LA, lingual aponueosis. P–R, three consecutive sagittal sections through the stomach (ST) and duodenum (D). Expression is specifically present in the inner muscle layer (IML, arrowheads) of the stomach. No positive signals were detected in the consecutive sections hybridized with sense probe (A, D, G, J, M, and P). Scale bars, 100 μm.

In the skin, expression of POEM mRNA was located in the basal layer (Fig. 6, J–L). The most abundant expression was observed in the basal layer of the glans clitoris (Fig. 6, M–O), and the expression level decreased in the prepuce. In the clitoris, POEM expression was also observed in the corpus cavernosum clitoridis, and the expression was higher in the glans clitoridis than in the prepuce (Fig. 6, M and N). In the respiratory system, POEM expression was localized in the epithelial cells surrounding the lumen of the nasopharynx and trachea (Fig. 6, P–R). In the lung, expression was observed in the cells surrounding the developing alveoli (data not shown).

In the 16.5-day-old embryo, POEM mRNA was hard to detect in the spleen, and few cells, if any, were faintly stained. However, in 22-day-old mice, POEM was weakly expressed in unidentified cells present in the white pulp (data not shown). In adult mice, expression could not be detected in the spleen.

No obvious POEM expression was detected in the liver, pancreas, adrenal gland, blood-vascular system, atrium, ventricle, ovary, cartilage, thymus, spinal cord, or ganglion in the 16.5-day-old embryo. In 22-day-old mice, POEM expression was not observed in the liver.

**Subcellular Localization of POEM Protein**—Most of the EGF-like repeat-containing proteins reported thus far are extracellular or membrane-bound proteins. To characterize POEM, we generated an expression vector for a fusion protein...
of POEM and human immunoglobulin constant region (POEM-Fc) and transfected COS-7 and MC3T3-E1 cells with it for transient expression (Fig. 7).

For examination of the localization of POEM-Fc in these cells, Western blotting was performed on the conditioned medium, cell extract, and ECM fraction of COS-7 cells transfected with the POEM-Fc expression vector (Fig. 7A). An 80-kDa POEM-Fc protein was detected in both cell and ECM fractions. Very little POEM-Fc protein was found in culture medium, suggesting that POEM-Fc protein was trapped on the cell surface and/or in ECM. Interestingly, the absence of the RGD cell binding motif did not seem to affect the subcellular localization. Additionally, a significant portion of the POEM protein seemed to be dimerized or trimerized, as suggested by nonreducing gel electrophoresis and Western blotting of each fraction (data not shown).

When expressed in MC3T3-E1 cells, POEM-Fc could hardly be detected in the medium fraction, as observed for the COS-7 cells. In the cell/ECM fraction, a significant amount of the POEM-Fc protein was localized on the cell surface, and the cell binding activity was not affected by the mutation introduced into the RGD sequence. However, the binding activity was significantly reduced by the deletion of the MAM domain, suggesting that this domain plays an important role in the localization of POEM at the cell surface.

**RGD Sequence of POEM Promotes Cell Attachment and Spreading via α₅β₁ Integrin**—To assess the role of the RGD motif and MAM domain in the cell adhesion and spreading activity, we produced recombinant MBP fusion protein of POEM C terminus portion (POEMc; amino acids 377–561) in *E. coli* and purified it by amylose resin column chromatography until it appeared as a single band by Coomassie Brilliant Blue staining on SDS-polyacrylamide gel electrophoresis gel (Fig. 9A). MBP-POEMc was detected at the expected size of 60 kDa. MC3T3-E1 cells adhered to and spread on MBP-POEMc protein and fibronectin in a dose-dependent manner (Fig. 9, B and C). To investigate the role of the RGD motif in this cell adhesion activity, we generated recombinant protein MBP-POEMc(RGE) encoding a mutant form of MBP-POEMc with a substitution of the RGD motif by RGE (Fig. 9A). Significantly less cell adhesion activity was observed with MBP-POEMc(RGE) (Fig. 9B). However, MBP-POEMc(RGE) stimulated cell attachment at a higher concentration (>5 μg/ml) without cell spreading activity (Fig. 9C), consistent with the result of FACS analysis (Fig. 8).
When the culture period was prolonged to 2 days in the absence of serum, MC3T3-E1 cells could survive on either MBP-POEMc or fibronectin, whereas the cells plated on MBP-POEMc(RGE) showed an apoptotic phenotype with condensed nuclei (Fig. 9C). These results indicate that not only the MAM domain but also the RGD motif in MBP-POEMc protein plays an important role in adhesion, spreading, and survival of MC3T3-E1 cell.

We suspected POEM to be a ligand for the α5β1 integrin receptor based on the following information. (a) The RGD motif is known as an α5β1 integrin-binding motif. (b) We confirmed that α5 integrin subunit was expressed in MC3T3-E1 cells by reverse transcription-PCR (data not shown). Furthermore, the expression is diminished with differentiation during primary osteoblasts cultured (24). This expression profile is similar to that of POEM observed in MC3T3-E1 cells (Fig. 2). (c) The mRNA expression profile of POEM in mouse embryos was similar to that of α5β1 integrin in chick embryos (25). (d) Localization of POEM in kidney and bone tissues was almost identical to that of postulated ligands for α5β1 identified as binding sites of α5β1–alkaline phosphatase fusion protein (26).

To confirm this possibility, we carried out a cell adhesion assay using KA8 cells, a K562 clone stably transfected with an α5 integrin expression vector and expressing both α5β1 and α5β1 integrins (17). Parental K562 cells, which were used as a control, express only α5β1. KA8 cells adhered remarkably to both MBP-POEMc and fibronectin (Fig. 10). In contrast, K562 cells adhered only to fibronectin. Neither KA8 cells nor K562 cells adhered to MBP-POEMc(RGE). These observations suggest that POEM is a specific ligand for α5β1 integrin, whereas fibronectin is a multiligand for both α5β1 and α5β1 integrins, as reported previously (17, 27).
Molecular Cloning of POEM

After the molecular cloning of the chick integrin α8 subunit, a number of biological functions of this molecule were reported. Mice deficient in α8 showed severe defects in kidney morphogenesis (28) and disorganization of inner ear cells (29). We found POEM to be abundantly expressed in kidney cells, especially in the glomerulus and epithelial cells of the urogenital sinus. This expression continued after birth, and these results indicate that POEM is involved in kidney morphogenesis and function. Expression in the inner ear is another unique feature of POEM. Integrin molecules are usually expressed as heterodimers of α and β subunits. The α8 integrin subunit associates with β1 subunit (25). Thus far, fibronectin, vitronectin, tenasin-C, and osteopontin have been shown to be ligands for α8β1 integrin (17, 28, 30, 31). Among these ligands, binding of α8β1 integrin to vitronectin showed great variability, depending on the preparation of the protein (17). Denda et al. (27) generated a soluble α8β1 integrin heterodimer-alkaline phosphatase fusion protein to search for ligands of α8β1 integrin. Far Western blotting of mouse embryo extracts with the α8β1-alkaline phosphatase fusion protein suggested the presence of several ligand molecules with molecular masses ranging from 65–85 kDa. These investigators also stained developing kidney and bone tissues with α8β1-alkaline phosphatase and found that localization of ligand molecules was similar to that of osteopontin. However, in a recent study (32), mice deficient in osteopontin did not show any significant defects in kidney morphogenesis, suggesting the presence of other ligand molecules for α8β1 integrin. Very interestingly, the predicted size of POEM and the distribution of POEM mRNA are quite similar to those of the ligand molecule suggested by using α8β1-alkaline phosphatase, and these facts indicate that POEM is a novel ligand molecule for α8β1 integrin. We confirmed this possibility by using KA8 cells expressing α8β1 integrin, and we also found that K562 cells, which express α8β1 (a fibronectin receptor), did not bind to POEM. Therefore, although the binding of POEM to other integrins has not yet been well investigated, POEM seems to have a unique binding activity distinct from that of other ligand molecules for α8β1 integrin. From these results, we conclude that POEM is one of the important ligand molecules for α8 integrins.

By amino acid alignment and data base search, POEM was found to encode several potential functional domains, such as five EGF-like repeats and a MAM domain, as well as an RGD integrin binding motif. These structures are commonly found in secreted proteins; however, by Western blotting and immunostaining, we found that the majority of the POEM proteins were localized on the cell surface or in the ECM, but not in the culture medium. Interestingly, this localization was not affected by the mutation of the RGD cell adhesion motif. This finding raised the possibility that POEM may interact not only with RGD-dependent α8β1 integrin but also with other cell surface molecules, including other integrins and/or other family receptor proteins. The FACS analysis showed that a mutant POEM-Fc molecule without the MAM domain detached from the cell surface. Moreover, this mutant POEM-Fc molecule without the MAM domain was detected mainly in the culture medium rather than in cell extracts when expressed in COS-7 cells (data not shown). Although there is a possibility that the deletion of the MAM domain affected the entire structure of POEM protein, these results suggest that the MAM domain plays a significant role for cell surface localization of POEM as well as the RGD motif in COS-7 cells. It has been reported that the MAM domain family, including meprin, A5 protein, neuropilin-1, neuropilin-2, and receptor protein-tyrosine phosphatases, mediates cell adhesion activities via homo- or heterophilic MAM domain interactions (33–37). This fact supports the hypothesis that MAM domain is involved in the cell surface binding via protein-protein interaction, and these molecules could serve as candidate receptor molecules for POEM. Additionally, MC3T3-E1 cells bound to MBP-POEMc(RGE), but K562 and KA8 cells did not. These results also suggest that the binding molecule for the MAM domain of POEM is expressed in a cell type-specific manner. The identification of this molecule will help us to prove the precise function of POEM.

In the developing mouse embryo, POEM was expressed in the endocrine organs (parathyroid gland, thyroid gland, hypophysis, and pineal organ). These endocrine organs are closely related to growth, bone metabolism, and calcium and phosphorus homeostasis. In the mouse embryo, POEM mRNA showed a unique distribution in and around the developing bone in the tooth germ, and in muscle. These data also suggest the relationship between POEM and calcium metabolism. Osteoblastic cells produce a number of ECM proteins. For example, type I collagen and osteopontin are up-regulated after osteoblast maturation (38). On the other hand, we noted that the expression of POEM was down-regulated as osteoblast differentiation proceeded. The distribution of POEM expression in the mouse embryo also suggested the role of POEM in the early stage of osteoblastic cell differentiation. POEM has an RGD cell adhesion motif, which is known to interact with integrins (13, 14). In this study, we found POEM to be a novel candidate
ligand molecule for $\alpha_{\beta_1}$ integrin. In osteoclasts, integrins have been shown to play important functional roles by regulating cell attachment and bone resorbing activity (39). POEM was preferably expressed in preosteoblastic cells, which do not seem to interact with directly osteoclastic cells; however, integrins have been shown to play significant roles not only in osteoclasts but also in osteoblasts (15). Mice deficient in the $\beta_1$ integrin subunit showed significantly less bone-forming activity. Morsci et al. (24) also reported that integrins, such as $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, and $\alpha_6\beta_1$, are critical for mineralized nodule formation and osteoblast differentiation. Therefore, POEM may play important roles in osteoblastic function by sending survival signals via $\alpha_2\beta_1$ integrin and mediating cell-cell interaction. POEM is a novel class of adhesion molecule with multiple motifs. Recently, a number of novel small EGF-like repeat proteins with a novel class of adhesion molecule with multiple motifs. Re-
