Novel Membrane Protein Containing Glycerophosphodiester Phosphodiesterase Motif Is Transiently Expressed during Osteoblast Differentiation*

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Osteoblast maturation is a multistep series of events characterized by an integrated cascade of gene expression that are accompanied by specific phenotypic alterations. To find new osteoblast-related genes we cloned differentially expressed cDNAs characteristic of specific differentiation stages in the mouse osteoblast-like MC3T3-E1 cells by a differential display method. We identified a novel cDNA encoding a putative glycerophosphodiester phosphodiesterase, GDE3, which specifically was expressed at the stage of matrix maturation. Interestingly, the deduced amino acid sequence contains 539 amino acids including seven putative trans-membrane domains and a glycerophosphodiester phosphodiesterase region in one of the extracellular loops. Northern blot analysis revealed that GDE3 was also expressed in spleen as well as primary calvarial osteoblasts and femur. We next transfected HEK293T cells with GDE3 with green fluorescent protein fused to the C terminus. The green fluorescent protein-fused protein accumulated at the cell periphery, and the transfected cells overexpressing the protein changed from a spread form to rounded form with disappearance of actin filaments. Immunofluorescence staining with GDE3 antibody and phalloidin in MC3T3-E1 cells indicated that endogenous GDE3 might be co-localized with the actin cytoskeleton. To identify a role for GDE3 in osteoblast differentiation, MC3T3-E1 cells stably expressing the protein were constructed. Expression of GDE3 showed morphological changes, resulting in dramatic increases in alkaline phosphatase activity and calcium deposit. These results suggest that GDE3 might be a novel seven-transmembrane protein with a GP-PDE-like extracellular motif expressed during the osteoblast differentiation that dramatically accelerates the program of osteoblast differentiation and is involved in the morphological change of cells.

The osteoblasts are mesenchymal cells derived from bone marrow stromal cells, periosteum, or undifferentiated stem cells in connective tissue. Based on morphological and histological studies, osteoblast cells develop in a presumed linear sequence progressing from osteoprogenitors to preosteoblasts, osteoblasts, and lining cells or osteocytes (1, 2). The process also has been subdivided into three developmental time stages: proliferation, extracellular matrix development, and mineralization. Numerous investigations have been directed at elucidating factors that regulate osteoblast differentiation, and have shown many genes encoding either transcription factors or growth factors essential for bone formation (3, 4). It was well known that the transcription factor, core-binding factor-α1 (Cbfa1)1, which belongs to the runt domain gene family, regulates the expression of multiple genes in the osteoblasts, is involved in osteoblast differentiation (5, 6). In fact, Cbfa1 is reported to have a direct causal relationship with cleidocranial dysplasia syndrome (7). Although Cbfa1 expression is a pivotal event in endochondral bone formation (but it alone is not sufficient), the identification of genes induced prior to and after Cbfa1 will be essential to elucidate other factors, the interaction of which is required for normal skeletal differentiation.

When primary or clonal osteoblast cells, such as MC3T3-E1 cells derived from newborn mice calvaria, are cultured with β-glycerophosphate and ascorbic acid, the cells undergo a differentiation process and eventually accumulated calcium contents (8). In the current study, we employed the differential display method to isolate the components involved in osteoblast differentiation using MC3T3-E1 cells. We identified a novel protein, GDE3, which shares significant homology with bacterial glycrophosphodiester phosphodiesterases (GP-PDEs or GlpQs). Bacterial GP-PDEs are previously reported to play important roles in hydrolysis of deacylated phospholipid glycrophosphodiesterases, such as glycrophosphocholine and glycrophosphoethanolamine, to sn-glycerol 3-phosphate (Gly-3-P), which is a major carbon and phosphate source (9, 10). This reaction also generates the corresponding alcohols such as choline and ethanolamine that act as essential growth factors for bacteria. Previously, a mammalian homolog, GDE1/MIR16, which interacts with RGS16, was identified and characterized

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The abbreviations used are: Cbfa1, core-binding factor-α1; GP-PDE, glycrophosphodiester phosphodiesterase; MBP, maltose-binding protein; GFP, green fluorescent protein; PTH, parathyroid hormone; GDE3, glycrophosphodiester phosphodiesterase 3; PCS, fetal calf serum; PBS, phosphate-buffered saline; MOPS, 4-morpholinepropanesulfonic acid.

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to localize on the plasma membrane and intracellular membrane (11). Very recent study has shown that GP-PDE activity of GDE1/MIR16 is regulated by stimulation of G protein-coupled receptors (12). However, the biological functions of mammalian GP-PDEs remain unclear. Here, we demonstrated that transiently overexpressed GDE3 in HEK293T cells induced cell rounding accompanied with disappearance of actin filaments, and transient expression of GDE3 to MC3T3-E1 cells dramatically stimulated both osteoblast differentiation and mineralization, suggesting that GDE3 exerts its simulative effect on osteoblast differentiation via rearrangement of actin filaments.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases and DNA modifying enzymes were purchased from Takara Shuzo (Kyoto, Japan). Mouse monoclonal preosteoblast MC3T3-E1 cells and HEK293T cells were obtained from Dainippon Pharmaceutical Co. (Osaka, Japan). Dulbecco’s modified Eagle’s medium, α-modification of Eagle’s medium, and fetal calf serum (FCS) were obtained from Invitrogen. Ascorbic acid was purchased from Wako Pure Chemical Industries (Japan). β-Glycerophosphate was from Nacalai tesque (Japan). 4-[6-(4-Chloro-7-oxo-1,2,3,4-tetrahydro-6-quinolinyl)pyrimidinyl]-2-picolinic acid was from Sigma-Aldrich (St. Louis, MO). The cDNA insert DNA was determined. To isolate the complete GP-PDE cDNA from true positive clones were sequenced by the dideoxy chain termination methods using an Applied Biosystems model 373A. Reamplified cDNA fragments were cloned into pGEM-T vector (Promega). After separation by 5% polyacrylamide gel electrophoresis, cDNA bands that appeared to be up-regulated after 11 days were recovered and reamplified with the corresponding primer. 32P-Labeled cDNA fragments encoding the murine osteoblastic cell line MC3T3-E1 was cultured in a growth medium, α-modification of Eagle’s medium supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin, under a humidified atmosphere of 5% CO2 in air at 37 °C. For studies examining the sequence of osteoblast differentiation, cells were seeded at 5,000 cells/cm2 in 24-well plates (for alkaline phosphatase and calcium content assays) or 100-mm dishes (for RNA isolation). Four days after MC3T3-E1 cells reached confluence (day 0), the medium was replaced with osteogenic medium, growth medium supplemented with 50 μg/ml ascorbic acid and 5 mM β-glycerophosphate. The medium was replaced every 3–4 days. The cells were grown in osteogenic medium for periods ranging from 7 to 28 days. A population of osteoblast cells was isolated from calvariae of newborn Wistar rats as previously reported (13). These cells were cultured in osteogenic medium. During subculture, the medium was replaced every 3 or 4 days. HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS under a humidified atmosphere of 5% CO2 in air at 37 °C.

Differential Display—Total RNA was isolated from MC3T3-E1 cells at 0 and 11 days, and the differential display was performed as previously described (14). Briefly, total RNA (3 μg) was converted to cDNA with reverse transcriptase (Invitrogen). Subsequently, each pool of cDNA was amplified by PCR with 104 different arbitrary primers. After separation by 5% polyacrylamide gel electrophoresis, cDNA bands that appeared to be up-regulated after 11 days were recovered and reamplified with the corresponding primer. Reamplified cDNA fragments were cloned into pGEM-T vector (Promega). The cDNA inserts from true positive clones were sequenced by dideoxy chain termination methods using an Applied Biosystems model 373A automated sequencer and a dye terminator cycle sequencing kit (PE Biosystems).

Screening of cDNA Library.—To isolate the complete cDNA of GDE3, library screening was performed according to the standard procedure (15) using a 390-base fragment of cDNA encoding the murine osteoblastic cell line MC3T3-E1 as a probe in the MC3T3-E1 cDNA library. The PlasmidScript (+) plasmid containing an insert DNA was isolated from a positive plaque by in vivo excision method (Stratagene), and nucleotide sequence of the insert DNA was determined. To isolate the complete cDNA of human GDE3, library screening was performed using full-length cDNA as a probe in the human spleen cDNA library. Nucleotide sequences were analyzed by searching the GenBank™ data bank with BLAST.

Northern Blot Analysis.—Total RNAs were isolated using ISOGEN (Nippon Gene, Toyama, Japan), and poly(A)+ RNAs purified by oligo(dT)-cellulose column chromatography using an mRNA separator kit (Clontech) were fractionated in a 1% agarose gel containing 0.66 M formaldehyde and 0.02 M MOPS (pH 7.0). Fractionated RNAs were transferred onto a nylon filter by capillary blotting and then cross-linked by ultraviolet irradiation. A mouse multiple tissue Northern blot was obtained from Clontech. 32P-Labeled cDNA fragments encoding mouse GDE3, mouse osteopontin, mouse osteocalcin, mouse osteonectin cDNAs, and mouse collagen I and mouse collagen IV cDNAs were used for Northern blotting hybridization as probes. Hybridization was performed in 6 × SSC, 0.5% SDS, 5 × Denhardt’s solution, and 100 μg/ml salmon sperm DNA at 65 °C for 16 h with the probe. The membrane was washed finally with 0.1× SSC and 0.5% SDS at 65 °C for 1 h. Northern blots with poly(A)+ RNAs from mouse tissues including bone tissues were exposed to x-ray films at −70 °C for 2 days.

Stable Transfection.—1.7 kilobases of the GDE3 cDNA were subcloned into the expression vector pEF/neoI (17) containing the EF1α promoter (17) and the neomycin selectable marker gene, to yield pEF/ GDE3. For stable transfection of MC3T3-E1 cells, the cells were electroporated at 0.25 kV and 850 microfarads using 10 μg of pEF-GDE3 or vector alone (control). After transfection, the complexes were resuspended with 500 μg/ml G418 for 14 days. G418-resistant colonies were identified, and independent colonies were re-seeded at 5,000 cells/cm2 in growth medium, respectively. After 3 days, the medium was replaced with osteogenic medium, growth medium supplemented with 5 μg/ml β-glycerophosphate and 50 μg/ml ascorbic acid (day 0). Transfected MC3T3-E1 cells were subjected to alkaline phosphatase assays at day 7, and to calcium content assays at day 14.

Alkaline Phosphatase Activity.—Transfected MC3T3-E1 cells from individual wells of a 24-well plate (1.9 cm2/well) were washed twice with PBS, scraped in alkaline phosphatase buffer (50 mM Tris-HCl, pH 8.0, 0.1% Triton X-100), and then sonicated on ice by a handy sonic (TOMY Seiko, Japan). Alkaline phosphatase activity was assayed by the phosphatase substrate system for EIA (Kirkegaard and Perry Laboratories) using the supernatant of the cell lysate. Aliquots of supernatants were subjected to protein assay with a Bio-Rad kit (Bio-Rad). Activity was corrected for protein concentration. Statistical significance was determined by the unpaired Student’s t test.

Mineralization Assay.—Transfected MC3T3-E1 clones cultured in osteogenic medium for 14 days were washed twice with PBS and then lysed with saline solution containing 10 mM Tris-HCl (pH 7.8) and 0.2% Triton X-100. An aliquot was removed for protein determination using a Bradford assay. Thereafter, 0.5 ml of 0.5 × HCl was added to the lysates and the mineralized materials were dissolved with overnight gentle sonication. The calcium content in the sample was quantitated by the o-cresolphthalein complexone method with Calcium C-Test (Wako Pure Chemical Industries). Results are expressed as calcium per protein.

Transient Expression and Immunofluorescence Microscopy.—HEK293T cells were transiently transfected with the full-length GDE3 cDNA in the expression vector pCMV-ZEPFP11(pGFP-GDE3) by LipofectAMINE PLUS (Invitrogen), according to the manufacturer’s instructions. After 24 h, the cells were directly fixed with 4% paraformaldehyde and 4% sucrose in 0.1 M NaPO4 (pH 7.2), for 30 min and blocked with 1.5% normal goat serum in PBS for 1 h at room temperature. Cells were treated with rhodamine phalloidin (Molecular Probes) for 1 h. MC3T3-E1 cells grown on glass coverslips were directly fixed with 4% paraformaldehyde and 4% sucrose in 0.1 M NaPO4 (pH 7.2) for 30 min and blocked with 1.5% normal goat serum in PBS for 1 h at room temperature, followed by incubation with anti-GDE3 antibody (diluted 1:1000 in PBS with 0.1% Tween 20) for 3 h at 4 °C. The primary antibody was visualized with fluorescein isothiocyanate-labeled goat anti-rabbit IgG (BD Biosciences).

Preparation of Recombinant GDE3 Protein and Antibody.—A cDNA encoding part of GDE3 (carnine acids 210–332) was subcloned into the site of the maltose-binding protein (MBP) expression vector pMAL-c (New England Biolabs), generating pMAL-GDE3. The MBP fusion protein construct pMAL-GDE3 was introduced into the bacterial strain JM109 (TOYOBO, Japan). An overnight culture in LB medium was diluted 1:100 into 100 ml of fresh LB medium and incubated at 37 °C in a shaking incubator for 2 h. After isopropyl-1-thio-β-D-galactopyranoside was added to the culture to a final concentration of 0.2 mM, the culture was incubated for an additional 2 h. The cells were washed once with ice-cold soluble buffer (50 mM Tris-HCl, pH 8.0, and 1 mM EDTA) and homogenized in glass coverslips and then cell lysis was performed with 10 mM maltose for 10 min at 4 °C to elute MBP-GDE3 fusion protein from the beads. After centrifugation, the supernatant was dialyzed against PBS. Polyclonal antibody raised against GDE3 was obtained by injecting rabbits with MBP-GDE3 fusion protein in Freund’s
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Identification of Novel Osteoblast Differentiation Specific cDNA by Differential Display—To identify new genes that are differentially expressed or regulated in differentiating preosteoblasts, we employed an RNA differential display procedure. Total RNA was prepared from undifferentiated (day 0) and differentiated (day 11) MC3T3-E1 cells conditioned with β-glycerophosphate and ascorbic acid. cDNAs derived from the RNAs were subsequently assayed for differential display PCR reaction in the presence of different sets of primers arbitrary in sequence. We focused on a cDNA fragment, termed fragment No. 45 found to be only amplified to a greater degree from the differentiated MC3T3-E1 RNA pool. The No. 45 fragment was excised, reamplified, and subcloned into a TA cloning vector, pGEM-T. Northern blots, probed with this subcloned cDNA, detected a single mRNA transcript of 2.4 kb, which was induced after 11 days of treatment with β-glycerophosphate and ascorbic acid.

Molecular Cloning of the Entire Coding Sequence of GDE3 and Deduced Amino Acid Sequence—The full-length cDNA of fragment No. 45 was obtained by screening a λ-phage cDNA library from differentiated MC3T3-E1 preosteoblasts by a conventional hybridization method with the differential display-derived cDNA fragment. Complete nucleotide sequence of the resulting cDNA was composed of 1617 nucleotides that encodes a polypeptide of 539 amino acids with a calculated molecular mass of 61 kDa (the DNA sequence data is available from DDBJ/EMBL/GenBankTM data bases under accession number AB048364). We also cloned the full-length cDNA of the human homolog from a human spleen cDNA library (accession number AB048363) (Fig. 1A).

GDE3 Amino Acid Sequence Analysis and Comparisons—At the amino terminus of the predicted protein, there is no apparent signal peptide sequence. As determined by hydropathy analysis by the SOSUI program and motif analysis by the GenetyxTM program, the peptide consists of three regions: a large extracellular domain of 225 amino acids, a 7-transmembrane region of 161 amino acids, an intracellular cytoplasmic tail of 40 amino acids, and a short extracellular cytoplasmic tail of 24 amino acids (Fig. 1A) (18). There are predicted N-linked glycosylation sites present in the extracellular domains. A search of the GenBank™ data bank using the nucleotide and protein sequence revealed that no identical proteins have been reported. However, very interestingly, one of the predicted extracellular regions between amino acid residues 218 and 442 shares significant similarity with bacterial GP-PDEs from Escherichia coli (9, 10), Haemophilus influenzae (19), and Bacillus subtilis. It was recently reported that GDE1/MIR16, the membrane interacting protein of RGS16, has significant homology with bacterial GP-PDEs and one membrane-spanning region (11), whereas the GDE3 protein contains 7 transmembrane-spanning regions.

Expression of GDE3 in Comparison to Other Osteoblast mRNAs in Differentiating MC3T3-E1 Cells—Next, we characterized the expression pattern of GDE3 mRNA in comparison to other osteoblast marker mRNAs in MC3T3-E1 development (Fig. 2A). Total RNA was extracted from MC3T3-E1 cells cultured in the presence of β-glycerophosphate and ascorbic acid for 0, 4, 7, 11, and 15 days. This conditioned culture induced differentiation as indicated by increased osteopontin, osteocalcin, type I collagen, and cbfa1 mRNA, standard markers of mature osteoblasts. The GDE3 transcript was first detectable at day 4 and peaked at day 7. Additionally, a very weak hybridization signal was also observed at days 11 and 15. Because osteopontin and osteocalcin mRNAs, considered mature osteoblast markers, were increased at days 11 and 15, GDE3 mRNA was specifically expressed in premature stages of MC3T3-E1 development.

Pattern of GDE3 mRNA Expression in Various Mouse Tissues—To investigate whether the expression of this transcript was specific to bone or not, Northern blot analysis was performed using poly(A)+ RNA derived from various mouse tissues and the GDE3 cDNA fragment as a probe (Fig. 2, C and D). The mRNA corresponding to GDE3 was expressed in femur and calvaria. As noted also, a strong hybridization signal was observed in spleen. Furthermore, we determined that GDE3 mRNA was also expressed in the rat fetal calvaria cells (Fig. 2E).

Preparation of Anti-GDE3 Antibody and Detection of Endogenous GDE3 Protein in MC3T3-E1 Cells—Next, we prepared a polyclonal antibody against a putative GP-PDE domain in a large putative extracellular loop of mouse GDE3 using a bacterially synthesized MBP-fused fragment corresponding to amino acids 219–332. The specificity of the purified anti-GDE3 antibody was tested by Western blot analysis of total proteins from HEK293T cells transfected with an expression vector encoding GFP-tagged GDE3(GFP-GDE3). Although no signal was observed in the mock-transfected HEK293T cells, a specific band was detected in the cells transfected with a plasmid encoding GFP-GDE3 using either anti-GDE3 (Fig. 2B, left panel) or anti-GFP antibody (data not shown). Next, to determine whether the GDE3 mRNA detected in osteoblasts is indeed translated into a protein, Western blot analysis was performed with membrane protein from MC3T3-E1 cells. The anti-GDE3 antibody recognized an immunoreactive polypeptide of 65 kDa in the membrane fraction of MC3T3-E1 cells, and that the GDE3 protein level was up-regulated at day 7 (Fig. 2B, right panel), paralleled with the increase in GDE3 mRNA level.

Morphological Changes by Transiently Overexpressed GDE3 Proteins in HEK293T Cells—To examine the physiological role of cellular localization of GDE3, we next transfected the GDE3 overexpressing plasmid GFP-GDE3 into HEK293T cells. Fluorescence microscopy showed that wild-type GFP was distributed evenly over the entire cytoplasm in HEK293T cells when...
transfected with plasmid DNA encoding the wild-type GFP (Fig. 3A). On the contrary, GDE3-fused GFP was concentrated at the cell periphery and also at lamellipodial extension (Fig. 3D). Surprisingly, significant changes of HEK293T morphology were observed: transient overexpression of GDE3 resulted in the cells rounding up (Fig. 3B), which correlates with disappearance of actin filaments as visualized with rhodamine phalloidin at the cell periphery (Fig. 3E). Whereas the wild type HEK293T cells were spread and squamous (Fig. 3A). These findings strongly suggested that GDE3 is involved in osteoblast differentiation by rearrangement of actin filaments and by resulting morphological changes of osteoblasts. In addition, FACS analysis showed that the purified anti-GDE3 antibody strongly binds to living HEK293T cells expressing GDE3 compared with normal rabbit IgG (Fig. 4), indicating that the putative GP-PDE domain faces lumen/extracellular space.

**Cellular Localization of GDE3 in MC3T3-E1 Cells**—To determine the cellular localization of GDE3 in osteoblastic cells, MC3T3-E1 cells were immunostained with the anti-GDE3 antibody described above. Much brighter staining was seen along with the plasma membrane and cytoskeletal filaments indicating co-localization of the GDE3 protein with actin fibers. To know whether GDE3 interacts with actin, we immunostained MC3T3-E1 cells with rhodamine-conjugated phalloidin to visualize F-actin filaments. Resulting double staining showed that most endogenous GDE3 products were co-localized with the F-actin cytoskeleton in MC3T3-E1 cells (Fig. 5C).

**Constitutive Expression of GDE3 Dramatically Promoted MC3T3-E1 Osteoblastic Differentiation**—Because both GDE3 mRNA and protein were expressed during ascorbate- and β-glycerophosphate-induced differentiation of osteoblastic MC3T3-E1 cells, we investigated whether this novel gene expression could alter the program of osteoblast differentiation. These cells were stably transfected with the full-length coding region of GDE3 (MC3T3-E1 overexpression) or with the empty vector (MC3T3-E1 control) and selected markers of osteoblast differentiation.
Overexpression of GDE3 increases the alkaline phosphatase activity at day 7 and calcium content at day 14 (with 4.0- and 93.4-fold increases, respectively, Fig. 6A), compared with the MC3T3-E1-control pooled clones. GDE3 expression in stable clones was analyzed by Northern blot and Western blot analyses. Because clone S15 cells expressed GDE3 mRNA and protein at the highest levels, and could differentiate fastest, S15 cells were used as a representative clone throughout this study. Northern blot analysis has shown that expression of osteopontin and osteocalcin mRNAs was up-regulated in S15 cells (Fig. 6D). Furthermore, S15 cells highly expressing GDE has shown changes of cell shape when compared with control MC3T3-E1 cells (Fig. 6E). Interestingly, actin stress fiber was significantly decreased in S15 cells when compared with control cells (Fig. 6F), which is suggested to induce morphological changes of MC3T3-E1 cells.

**Inhibition of MC3T3-E1 Osteoblastic Differentiation by Anti-GDE3 Antibody**—It is also of interest that treatment of anti-GDE3 antibody in the cultured medium caused a 72.5% decrease in alkaline phosphatase activity, compared with those of normal rabbit IgG (Fig. 7). Because the antibody specifically recognizes the putative GP-PDE domain in the extracellular loop, this antibody might have a neutralizing effect on the GP-PDE activity. Taken together, these results suggested that GDE3 could accelerate the program of osteoblast differentiation of MC3T3-E1 cells.

**DISCUSSION**

In an attempt to seek novel bone-related factors, we have isolated a novel osteoblast cDNA encoding a differentiation stage-specific 539-amino acid protein, GDE3, with 7 putative transmembrane regions characterized by a large extracellular domain containing a putative glycerophosphodiester phosphodiesterase motif. Previous studies (9, 10) have demonstrated that *E. coli* GP-PDE, GlpQ, is a soluble periplasmic protein and...
has important functions to catalyze the hydrolysis of deacylated glycerophospholipids to glycerol phosphate and alcohol, which were utilized as a major carbon and phosphate source. On the other hand, *H. influenzae* incorporates choline obtained from environmental sources onto its lipopolysaccharide as phosphorylcholine. Very recently, *H. influenzae* GlpQ anchored to the outer membrane is shown to be essential to transfer of choline directly from epithelial cells to the bacterial cell surface (20). It is supposed that the difference in the cellular location of these GlpQs provides different physiological significance among these enzymes. In contrast, recent work describing a mammalian GP-PDE has demonstrated that MIR16 isolated as a protein that interacts with RGS16, regulator of G protein signaling, by the yeast two-hybrid screening has shown a significant homology with bacterial GP-PDEs (11). GDE1/MIR16 was reported to be an integral membrane-bound glycoprotein, and localized on the plasma membrane in rat liver and kidney and on intracellular membranes in pituitary cells. However, the potential functions of mammalian GP-PDEs, such as GDE1/MIR16, are still unknown. The current study is a first report about physiological functions of mammalian GP-PDEs, showing that GDE3 is involved in osteoblast differentiation and in actin cytoskeleton modulation.

The observation in the present study supposes that GDE3 might be itself a bone anabolic factor that can stimulate mineral deposition by promoting alkaline phosphatase expression directly or indirectly. Because bone-specific anabolic therapy is thought to be an ideal intervention with osteoporotic bone loss, there is significant effort applied to identification of new target classes in that area. Currently the only anabolic agent for treatment of osteoporosis is parathyroid hormone (PTH). Although PTH has anabolic effects when used intermittently in osteoporotic patients, further studies are in progress to confirm that it will have little adverse systemic effects. In this context, down-regulation of cyclin D1 expression might be necessary for increased membrane tension during osteoblast maturation, and for reorganization of actin filaments in multiple and slender osteocyte processes. Although we observed that endogenous GDE3 is co-localized with actin filaments in MC3T3-E1 cells, further study is needed to determine whether endogenous GDE3 is involved in the regulation of actin filaments.

Changes of the osteoblast morphology are considered to be associated with bone remodeling. The bone is in a dynamic state, being continually resorbed and remodeled by the coordinate actions of the osteoclasts and osteoblasts. Bone remodeling is the physiologic process for vertebrates to maintain a constant bone mass and to renew bone and also to regulate serum calcium level. When a concentration of calcium ion in blood is lowered, secretion of PTH from parathyroid tissue is increased, and PTH directly acts on bone, causing osteoclastic bone resorption to release calcium ions, followed by osteoblastic bone formation to repair the defect. This process is initiated by a dynamic morphological change of resting osteoblasts (bone

**Fig. 4.** Fluorescence activated cell sorter analysis. GDE3 was transiently expressed in HEK293T cells and recognition of the protein by purified anti-GDE3 antibody was analyzed by fluorescence activated cell sorter. Cells were incubated either with anti-GDE3 antibody against GP-PDE domain (black) or with normal rabbit IgG (dotted), and followed by incubation with R-PE-conjugated rabbit IgG (H+L). The shift of cell counts to higher fluorescence intensities was observed when using anti-GDE3 antibody.

**Fig. 5.** Intracellular localization of GDE3 protein in MC3T3-E1 cells. MC3T3-E1 cells were fixed and incubated with anti-GDE3 antibody. The primary antibody was visualized by fluorescein isothiocyanate-labeled goat anti-rabbit IgG (A). Actin filaments were stained with rhodamine phalloidin (B), followed by fluorescence microscopy. GDE3 was co-localized with actin filaments in MC3T3-E1 cells (arrows). GDE3 and actin filaments were overlaid (C).
MC3T3-E1 cells. 5
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pooled clones (phosphatase activity and calcium content of MC3T3-E1-GDE3 pooled clones (overexpression) are increased compared with the MC3T3-E1 control pooled clones (control). *, p < 0.05 compared with control. B, GDE3 mRNA was overexpressed in clone S15 cells (S15) when compared with control MC3T3-E1 cells. 5 μg of total RNA from clone S15 cells and control cells (C) was subjected to Northern blot analysis. C, whole cell extracts prepared from clone S15 cells (S15) and control MC3T3-E1 cells (C) were subjected to SDS-PAGE. The gels were analyzed by Western blot with anti-GDE3 antibody. D, expression of osteopontin and osteocalcin mRNAs, mature osteoblast markers, was up-regulated in clone S15 cells compared with control cells. 10 μg of total RNA from MC3T3-E1 cells cultured at the indicated days was subjected to Northern blot analysis. Filters were probed for osteopontin (OP) and osteocalcin (OC). E, S15 cells highly expressing GDE3 showed morphological changes (right panel) compared with control cells. F, actin filaments were visualized with rhodamine phalloidin. Actin stress fiber (arrow) was significantly decreased in S15 cells (right panel) when compared with control cells.

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Fig. 6. GDE3 promotes osteoblast differentiation. A, alkaline phosphatase activity and calcium content of pooled MC3T3-E1 cells. Alkaline phosphatase activity and calcium content of MC3T3E1-GDE3 pooled clones (overexpression) are increased compared with the MC3T3E1 control pooled clones (control). *, p < 0.05 compared with control. B, GDE3 mRNA was overexpressed in clone S15 cells (S15) when compared with control MC3T3-E1 cells. 5 μg of total RNA from clone S15 cells and control cells (C) was subjected to Northern blot analysis. C, whole cell extracts prepared from clone S15 cells (S15) and control MC3T3-E1 cells (C) were subjected to SDS-PAGE. The gels were analyzed by Western blot with anti-GDE3 antibody. D, expression of osteopontin and osteocalcin mRNAs, mature osteoblast markers, was up-regulated in clone S15 cells compared with control cells. 10 μg of total RNA from MC3T3-E1 cells cultured at the indicated days was subjected to Northern blot analysis. Filters were probed for osteopontin (OP) and osteocalcin (OC). E, S15 cells highly expressing GDE3 showed morphological changes (right panel) compared with control cells. F, actin filaments were visualized with rhodamine phalloidin. Actin stress fiber (arrow) was significantly decreased in S15 cells (right panel) when compared with control cells.

Fig. 7. Effect of anti-GDE3 antibody on osteoblast differentiation. Treatment with anti-GDE3 antibody for 4 days (i.e. from days 0–4) at the indicated concentrations suppressed alkaline phosphatase activity of MC3T9-E1 cells. **, p < 0.01 compared with control cells (C).

lining cells) to retracted active osteoblasts. Bone resorbing factors such as PTH act on lining osteoblasts to induce a retracted cell shape causing exposure of the bone matrix surface for a convenient access to osteoclasts. PTH also stimulates RANKL expression on the osteoblast cell surface that promotes osteoclast activation and differentiation (24). Previous reports have shown that the effect of PTH on osteoblast retraction is dependent on the cyclic AMP signaling pathway (25, 26). In addition, prostaglandin E2 induces a morphological change of osteoblasts with the breakdown of the actin microfilaments via an increase of intracellular cyclic AMP level (27). Based on the observation in the current study, there remains a possibility that GDE3 could act as a regulator of osteoblast retraction via actin cytoskeleton reorganization, indicating that GDE3 might play a pivotal role in bone resorption as well as bone formation. Although the detailed mechanism underlying the morphological change of osteoblasts remains unclear, bone remodeling progression is a potential molecular target for novel bone antiresorptive agents and the GDE3 regulation might be a possible intervention of osteoblastic metamorphosis in this process (28).

Although detection of GP-PDE activity was not successful by using overexpressed GDE3 protein in HEK293T cells and glycophosphorylcholine, a typical substrate for bacterial GP-PDEs, as a substrate (data not shown), the neutralizing effect of anti-GDE3 antibody, which specifically recognized the putative GP-PDE domain, on osteoblast differentiation strongly suggests the cellular functions of the putative GP-PDE domain of GDE3, including actin cytoskeletal modulation. The current study may provide useful information that modulation of the GDE3 function is a new potential pharmacological target for novel bone antiresorptive agents and the GDE3 regulation might be a possible intervention of osteoblastic metamorphosis in this process.

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