The glycerophosphodiester phosphodiesterase enzyme family involved in the hydrolysis of glycerophosphodiesters has been characterized in bacteria and recently identified in mammals. Here, we have characterized the activity and function of GDE3, one of the seven mammalian enzymes. GDE3 is up-regulated during osteoblast differentiation and can affect cell morphology. We show that GDE3 is a glycerophosphoinositol (GroPIns) phosphodiesterase that hydrolyzes GroPIns, producing inositol 1-phosphate and glycerol, and thus suggesting specific roles for this enzyme in GroPIns metabolism. Substrate specificity analyses show that wild-type GDE3 selectively hydrolyzes GroPIns over glycerophosphocholine, glycerophosphoethanolamine, and glycerophosphoserine. A single point mutation in the catalytic domain of GDE3 (GDE3R231A) leads to loss of GroPIns enzymatic hydrolysis, identifying an arginine residue crucial for GDE3 activity. After heterologous GDE3 expression in HEK293T cells, phosphodiesterase activity is detected in the extracellular medium, with no effect on the intracellular GroPIns pool. Together with the millimolar concentrations of ethanolamine, and glycerophosphoserine. A single point mutation in the catalytic domain of GDE3 (GDE3R231A) leads to loss of GroPIns enzymatic hydrolysis, identifying an arginine residue crucial for GDE3 activity. After heterologous GDE3 expression in HEK293T cells, phosphodiesterase activity is detected in the extracellular medium, with no effect on the intracellular GroPIns pool. Together with the millimolar concentrations of ethanolamine, and glycerophosphoserine. A single point mutation in the catalytic domain of GDE3 (GDE3R231A) leads to loss of GroPIns enzymatic hydrolysis, identifying an arginine residue crucial for GDE3 activity. After heterologous GDE3 expression in HEK293T cells, phosphodiesterase activity is detected in the extracellular medium, with no effect on the intracellular GroPIns pool. Together with the millimolar concentrations of ethanolamine, and glycerophosphoserine. A single point mutation in the catalytic domain of GDE3 (GDE3R231A) leads to loss of GroPIns enzymatic hydrolysis, identifying an arginine residue crucial for GDE3 activity. After heterologous GDE3 expression in HEK293T cells, phosphodiesterase activity is detected in the extracellular medium, with no effect on the intracellular GroPIns pool. Together with the millimolar concentrations of ethanolamine, and glycerophosphoserine. A single point mutation in the catalytic domain of GDE3 (GDE3R231A) leads to loss of GroPIns enzymatic hydrolysis, identifying an arginine residue crucial for GDE3 activity. After heterologous GDE3 expression in HEK293T cells, phosphodiesterase activity is detected in the extracellular medium, with no effect on the intracellular GroPIns pool. Together with the millimolar concentrations of ethanolamine, and glycerophosphoserine. A single point mutation in the catalytic domain of GDE3 (GDE3R231A) leads to loss of GroPIns enzymatic hydrolysis, identifying an arginine residue crucial for GDE3 activity. After heterologous GDE3 expression in HEK293T cells, phosphodiesterase activity is detected in the extracellular medium, with no effect on the intracellular GroPIns pool. Together with the millimolar concentrations of ethanolamine, and glycerophosphoserine. A single point mutation in the catalytic domain of GDE3 (GDE3R231A) leads to loss of GroPIns enzymatic hydrolysis, identifying an arginine residue crucial for GDE3 activity. After heterologous GDE3 expression in HEK293T cells, phosphodiesterase activity is detected in the extracellular medium, with no effect on the intracellular GroPIns pool. Together with the millimolar concentrations of ethanolamine, and glycerophosphoserine. A single point mutation in the catalytic domain of GDE3 (GDE3R231A) leads to loss of GroPIns enzymatic hydrolysis, identifying an arginine residue crucial for GDE3 activity. After heterologous GDE3 expression in HEK293T cells, phosphodiesterase activity is detected in the extracellular medium, with no effect on the intracellular GroPIns pool. Together with the millimolar concentrations of ethanolamine, and glycerophosphoserine. A single point mutation in the catalytic domain of GDE3 (GDE3R231A) leads to loss of GroPIns enzymatic hydrolysis, identifying an arginine residue crucial for GDE3 activity. After heterologous GDE3 expression in HEK293T cells, phosphodiesterase activity is detected in the extracellular medium, with no effect on the intracellular GroPIns pool.
text of Ras-transformed cells (10). They are present in virtually all cell types, where their intracellular levels can also be modulated according to cell activation, differentiation, and development (Refs. 11 and 12 and references therein). Recently, glycerophosphoinositol (GroPIns) was characterized as a mediator of purinergic and adrenergic regulation of PCCl3 thyroid cell proliferation (13), while GroPIns 4-phosphate (GroPIns4P) has been shown to induce reorganization of the actin cytoskeleton in fibroblasts and in T-lymphocytes, by promoting a sustained and robust activation of the Rho GTPases (14–16).

The GPs appear to rapidly equilibrate across the plasma membrane when added exogenously to cells, to exert their actions within the cell (12). The plasma membrane transporter for GroPIns characterized in yeast is the protein GIT1 (17), with one of its orthologs in mammalian cells identified as the human permease Glut2 (18). This specific transporter has been proposed to mediate both GroPIns uptake and release, which depends on the GroPIns concentration gradient across the plasma membrane. Under physiological conditions, this gradient can arise from the formation of GPs from the phosphoinositides inside cells following activation of a specific isoform of phospholipase A2, PLA2IVα (13, 19).

The release of the GPs into the extracellular medium can affect their paracrine targets (16) or initiate their catabolism. This is supported by our characterization of GDE1 activity, and now of GDE3 activity, both of which show a substrate selectivity toward GroPIns, and catalytic activity after heterologous expression that can only be monitored in the extracellular space. Interestingly, GDE3 activity appears to be related to modulation of osteoblast functions, delineating a role for GDE3 in promoting osteoblast differentiation, and mainly regulating osteoblast proliferation.

**EXPERIMENTAL PROCEDURES**

**Materials—**Dulbecco’s modified Eagle’s medium (DMEM), Minimal Essential Medium α (MEMα), fetal calf serum (FCS), Opti-MEM, phosphate-buffered saline (PBS), bovine serum albumin, and Hank’s Balanced Salt Solution with calcium and magnesium (HBSS+) were from Invitrogen Brl (Grand Island, NY). [3H]GroPIns was prepared from 1-α-[3H]phosphatidylinositol (314.5 GBq/mmol; PerkinElmer, Boston, MA) by deacylation, according to the original procedure of Clarke and Dawson (20). [6-3H]Thymidine (18.4 Ci/mmol) was from PerkinElmer. GroPIns was purchased from Euticals S.p.A. (Lodi, Italy) as its calcium salt, and from Calbiochem (La Jolla, CA) as its lithium salt. All other reagents were of the highest purity available and were obtained from Sigma, unless otherwise specified.

**Plasmids, Cell Culture, Transfection, and Proliferation Assays—**Full-length mouse GDE3 cDNA was subcloned into the expression vector pCMV-EGFPN1 (pEGFP-GDE3wt), as reported previously (8). An Arg→Ala mutant (pEGFP-GDE3R231A) was produced using the QuikChange™ site-directed mutagenesis kit (Strategene), as a set of PCR primers (5’-CAGCAGGCGGGCCCCAGGGCAACGCC3’ and 5’-GGGCTGGTGAGGAGCAAGCTGAGC-3’), and pEGFP-GDE3 as a template, according to the manufacturer’s protocol. The mutation was verified by DNA sequencing using an ABI PRISM 310 Genetic analyzer (Applied Biosystems).

HEK293T cells (American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, in a humidified atmosphere of 5% CO2 in air, at 37 °C. Chinese hamster ovary (CHO) cells were maintained in DMEM supplemented with 10% FCS, 58 μg/ml proline, 53 μg/ml l-aspartic acid, 60 μg/ml l-asparagine, 100 units/ml penicillin, and 100 μg/ml streptomycin. HEK293T and CHO cells were transiently transfected with 4 μg of pEGFP-GDE3wt, pEGFP-GDE3R231A, or vector pEGFP (control) using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions.

Stable clones of the MC3T3-E1 murine osteoblastic cell line (Dainippon Pharmaceutical Co., Osaka, Japan) were transfected with the empty vector (pEF/neo) or with pEF-GDE3 (MC3T3-E1-C115), and were prepared as reported previously (8). The cells were maintained in MEMα supplemented with 10% FCS, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, in a humidified atmosphere of 5% CO2 in air at 37 °C, in the presence of the selection antibiotic G418 (500 μg/ml).

The osteoblast growth rate was evaluated by cell counting. The different clones were plated into 6-well plates (2 × 104 cell/well) in growth medium. At the indicated times, the cells were detached by trypsinization, recovered by centrifugation, and put through two independent and blinded cell counts (Neubauer cell-counting chamber).

For the [3H]thymidine incorporation assay, MC3T3-E1 and MC3T3-E1-C115 cells were seeded in 96-well plates at a density of 5 × 103 cells/well in complete growth medium. After 12 h, the cells were treated with 250 μM GroPIns (calcium salt) or the equimolar 125 μM CaCl2 and, when indicated, two further additions followed after 36 and 60 h. After 72 h, a pulse of [3H]thymidine (1 μCi/well) was given 4 h before stopping the reaction by washing twice with HBSS++. The [3H]thymidine incorporation into trichloroacetic acid-insoluble material was evaluated as previously described (13, 21).

**Postnuclear Lysate Preparation and Western Blotting—**Twenty-four hours after transfection, cells were washed twice with cold PBS and scraped into homogenization buffer containing protease inhibitors (0.5 μg/ml leupeptin, 2 μg/ml aprotinin, 0.5 mM phenanthroline, 2 μM peptatin, and 1 mM phenylmethylsulfonyl fluoride), and 5 mM EDTA in TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl). Following gentle homogenization by 10 passages through a 28 1/2-gauge needle, postnuclear supernatants were prepared by removing nuclei and unbroken cells by centrifugation (600 × g for 3 min, at 4 °C) according to Ref. 4. Eighty micrograms of supernatant protein was subjected to SDS-PAGE, and Western blotting was performed with a polyclonal anti-GFP antibody (a kind gift of G. Di Tullio, Consorzio Mario Negri Sud, Italy) and a polyclonal antibody against GDE3 (epitope: amino acids 210–332) (8). Western blots were developed using the chemiluminescent method (ECL, Amersham Biosciences).

**GP-PDE Activity Assays—**Incubations were routinely at 37 °C with postnuclear supernatants in a final volume of 50 μl,
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which included 100 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 2 mg/ml fatty-acid-free bovine serum albumin, 30,000 dpm [³H]GroPIns, [³H]GroPIns₄P, or [³H]glycerophosphocholine (GroPCho), unlabeled GroPIns, GroPIns₄P, or GroPCho (as indicated), without or with addition of competing glycerophos-
phodiester. Incubations were in the presence of 5 mM Ca²⁺ or as otherwise specified. Initial analysis of incubation con-
tions for the GP-PDE assay indicated that with 10 mM GroPIns as substrate, increasing amounts of postnuclear protein preparations (2, 10, 30 μg) from HEK293T cells overexpressing GFP-GDE3wt maintained linear activities in a 2-h incubation at 37 °C. Thus, 10 μg of postnuclear protein preparations were routinely used, unless otherwise specified. The GroPIns dose-response analysis was performed using 5 mM Ca²⁺, GroPIns substrate as the Li⁺ salt (from 100 μM to 500 mM), 10 μg of postnuclear lysate protein, and a 2-h incub-
ation at 37 °C. GroPIns hydrolysis was determined as that measured for GFP-GDE3wt over the background GFP post-
incubation preparation control, as expressed as nanomoles. In the GP-PDE competition assays, the substrate concentration was 1 mM GroPIns, with addition of a 10-fold excess (10 mM) of competing glycerophosphodiester.

For the in vivo extracellular GP-PDE assays, cells were plated in 6-well plates and 100,000 dpm/well [³H]GroPIns, [³H]GroPIns₄P, or [³H]GroPCho were added in 2.5 ml of growth medium. At specific times, 500 μl of medium was ana-
yzed, as indicated below. The incubations were terminated by addition of cold methanol (−20 °C), followed by two-phase extraction, and the lyophilizing of the resultant upper (aque-
ous) phase (further details in Ref. 22). For the dose-response curves, GP-PDE activities were calculated from the known cold GroPIns in each assay (pmol) and the level of postincubation GroPIns hydrolysis, as seen by HPLC analysis of the ³H-labeled inositol, inositol 1-phosphate (Ins₁P), and GroPIns (further details in Ref. 22).

Quantitative Cell Spreading Assay and Immunofluorescence Analysis—HEK293T cells were transiently transfected with pEGFP-GDE3wt, pEGFP-GDE3R231A, or pEGFP vector (control), as described above. After 24 h, the cells were directly fixed with 4% paraformaldehyde and 4% sucrose in 0.2M NaPO₄ (pH 7.2), for 30 min. At least three images from different regions of the dish were captured in bright field mode. The edges of individual cells were traced by hand, and the area enclosed by the trace was measured using Scion Image software (Scion Corp.). Each data point represents a mean of at least 100 individual area measurements. Immunofluorescence analysis was performed as reported in Ref. 14.

Analysis of [³H]inositol-containing Phospholipids—HEK293T and CHO cells were grown in 6-well plates and transfected, and 4 h after transfection they were labeled for 24 h (to isotopic equilibrium) in Medium 199, with 5% FCS containing myo-
[³H]inositol (5 μCi/ml). Following labeling, the cells were washed twice with HBSS⁺⁺ without preincubated for 15 min in HBSS⁺⁺ containing 10 mM LiCl (pH 7.4) at 37 °C, prior to addition of ATP, as required. Incubations were terminated by medium aspiration and addition of methanol/1 M HCl (1:1, −20 °C), with extraction by addition of a half volume of chlo-
roform (final, 1:1:0.5). After separation of aqueous and organic extraction phases, the [³H]inositol-labeled water soluble metabolites were separated by anion exchange HPLC on a Part-
tisil 10-SAX column using a non-linear water/1 M ammonium phosphate, pH 3.35 (phosphoric acid) gradient. Radioactivity associated with the [³H]-labeled compounds was analyzed by an on-line flow detector (Packard FLO ONE A-525). GroPIns lev-
els are given as percentages of total aqueous [³H]-labeled compo-
ounds. For additional details see Ref. 22.

Alkaline Phosphatase Activity and Mineralization Assay—Transfected MC3T3-E1 cells from individual wells of a 24-well plate were washed twice with PBS, scraped into alkaline phosphatase buffer (50 mM Tris-HCl, pH 8.0, 0.1% Triton X-100), and sonicated on ice (Handy Sonic; TOMY Seiko, Japan). Alkal-
ine phosphatase activity was assayed by the phosphatase sub-
strate system for EIA (Kirkegaard and Perry Laboratories, Gaithersburg, MD) using the cell lysate supernatant. Activities were corrected for protein concentrations and expressed as nmol/min/mg protein. Transfected MC3T3-E1 cells cultured in osteogenic medium for 7 days were washed twice with PBS and lysed with saline solution containing 10 mM Tris-HCl, pH 7.8, and 0.2% Triton X-100. Thereafter, 0.5 ml 0.5 N HCl was added to lysates and the mineralized materials were dissolved with gentle overnight shaking. The calcium contents were quantitated by the o-cresolphthalein complexone method with the Calcium C-Test (Wako Pure Chemical Industries). Protein concentrations were measured with a Bio-Rad kit.

Statistical Analysis—The data are expressed as means ± S.D./± S.E., as specified, of two to four independent experi-
ments, each performed in duplicate. Statistical analysis was by Student’s t test.

RESULTS

GDE3 is a Glycerophosphoinositol Inositol Phosphodiesterase—We have previously shown that GDE1 is a GroPIns phosphodi-
esterase that selectively hydrolyzes GroPIns over GroPCho (4). In addition, we cloned the GDE3 protein in osteoblasts (8), and its alignment with GDE1 and bacterial phosphodiesterases shows conserved amino acids in the catalytic region, such as an arginine believed to be relevant for GP-PDE activity (Fig. 1A, underlined). To investigate the GP-PDE activity of GDE3, HEK293T cells were transiently transfected with cDNAs codi-
ing for wild-type GDE3 with a C terminus green fluorescent protein (GFP) tag (GFP-GDE3wt), a GFP-tagged GDE3 with a catalytic domain point mutation (R231A; GFP-GDE3R), or GFP alone (mock-transfected control) (Fig. 1B).

Ten micrograms postnuclear protein from HEK293T cells overexpressing GFP-GDE3wt showed a GroPIns inositol phos-
phodiesterase activity, with hydrolysis of GroPIns to Ins₁P and glycerol (see “Experimental Procedures,” and Fig. 1, C and D). In comparison, following transfection with pEGFP alone and with pEGFP-GDE3R231A, postnuclear cell lysates showed no background GP-PDE activity over the no-lysate control (Fig. 1D, w/o). There was no evidence of a GDE1-like activity (i.e. hydrolysis of GroPIns to inositol and glycerol phosphate) correlated to GFP-GDE3wt overexpression under these experi-
mental conditions.

To determine divalent cation requirements for this GDE3 GP-PDE activity, the GFP-GDE3wt lysate was incubated with
The specificity of GDE3 for GroPlins as substrate was then investigated in competition assays, with the GroPlins concentration reduced to 1 mM to allow addition of a 10-fold excess (10 mM) of unlabeled competing glycerophosphodiesters: GroPCho, glycerophosphoethanolamine, and glycerophosphoserine. Under these conditions, GDE3 GroPlins inositol phosphodiesterase activity was not inhibited (supplemental Fig. S1 SF1). We also tested other GPIs in this competition assay of GDE3, and 10 mM GroPlins4P and 10 mM GroPlins 4,5-bisphosphate competed with GroPlins hydrolysis at least in part (45 and 55% inhibition, respectively) (Fig. 2E).

**Cells Overexpressing GDE3 Do Not Have Modified Intracellular GroPlins Levels—Glycerophosphodiesterase activity was also tested intracellularly with the HEK293T cells overexpressing GFP-GDE3wt, GFP-GDE3R, and GFP: these transfectants showed comparable intracellular levels of GroPlins (0.52 ± 0.08%, 0.52 ± 0.01%, 0.53 ± 0.07% of total aqueous radioactivity, respectively; n = 3; see “Experimental Procedures”).**

Similar results were obtained with CHO cells, where intracellular GroPlins levels can be modulated by hormone stimulation. Here, addition of 100 μM ATP for 15 min induced about a 2-fold increase in intracellular GroPlins levels. Overexpression of GFP-GDE3wt, the GFP-GDE3R mutant, and GFP did not modify either mean basal (20.7 ± 1.4%, 19.7 ± 1.6%, 18.8 ± 1.8% of total aqueous radioactivity; respectively) or mean ATP-stimulated intracellular GroPlins levels (about 2-fold the basal). These data show that GDE3 does not affect intracellular levels of GroPlins, supporting the idea that the catalytic domain of GDE3 is oriented extracellularly, at least in these cell systems.

**An Extracellular GroPlins Inositolphosphodiesterase Activity Is Present in HEK293T Cells Overexpressing GDE3**—The glycerophosphodiesterase activity was also tested extracellularly, adding the GroPlins substrate to the cell medium, which was then sampled and analyzed at different times. As for the in vitro assays, GroPlins was hydrolyzed to Ins1P in the medium of cells overexpressing GFP-GDE3wt (Fig. 3, A and C), while cells overexpressing the GFP transfectant showed low background levels of extracellular GroPlins hydrolysis (Fig. 3, B and C). Also in agreement with the in vitro assays, cells overexpressing the...
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GFP-GDE3R mutant did not show extracellular GroPIns inositolphosphodiesterase activity (Fig. 3C). Furthermore, in cells overexpressing GFP-GDE3wt, addition of GroPCho and GroPIns4P to the medium under identical conditions did not demonstrate hydrolytic GDE3 activity. In conclusion, an extracellular GDE3 activity specific for GroPIns was confirmed for HEK293T cells overexpressing GFP-GDE3wt under these conditions.

Morphological Changes Induced by GDE3 Expression in HEK293T Cells—Our previous study demonstrated that HEK293T cells overexpressing GDE3 changed from a spread form to a rounded form (8). The transient overexpression of GDE3wt in this study also resulted in cell rounding, while HEK293T cells overexpressing the catalytically inactive GFP-GDE3R mutant (see above) did not show morphological changes (Fig. 3D). Quantitative analysis of cell spreading showed no significant differences in spreading area between HEK293T cells expressing GFP-GDE3R and GFP (control), while with GFP-GDE3wt, there was a ~60% lower spreading area compared with GFP-transfected cells (Fig. 3E). The protein expression levels of the GFP-GDE3R mutant were similar to those of GFP-GDE3wt (data not shown).

GDE3 Activity in Osteoblasts—GDE3 was originally cloned in a search for genes involved in osteoblast differentiation, where it was transiently expressed at the stage of extracellular matrix maturation (8). Here, we have characterized the glycerophosphodiesterase activity in wild-type osteoblast MC3T3-E1 cells and in a clone overexpressing GDE3wt (MC3T3-E1-C115). In postnuclear preparations from MC3T3-E1 cells, there were low levels of activity after the standard 2-h incubation at 37 °C (Fig. 4A). For the MC3T3-E1-C115 clone, this activity showed a significant ~60% increase in Ins1P production, compared with the parent MC3T3-E1 cell line (Fig. 4A). Similarly, for extracellular GroPIns inositolphosphodiesterase activity in intact cells, the MC3T3-E1-C115 clone produced a significant ~50% increase in Ins1P production over 24 h, compared with the parent MC3T3-E1 cell line (Fig. 4B).

We also quantified intracellular levels of GroPIns in these two osteoblast cell lines by mass spectrometry (23), which showed no correlation between intracellular levels of GroPIns and GDE3 expression, further supporting the extracellular activity of GDE3.

To determine whether expression of GDE3 also affects functional parameters, we followed morphology, proliferation, and selected markers of differentiation in these cell lines. First, F-actin was monitored by phalloidin staining, as a read-out for cytoskeleton organization. Under normal growth conditions, wild-type MC3T3-E1 cells showed a clear stress fiber network,
which was completely absent in the MC3T3-E1-Cl15 clone (supplemental Fig. S2 SF2). Interestingly, this phenotype was reminiscent of that in HEK293T cells overexpressing GDE3 (8). In parallel, the MC3T3-E1-Cl15 clone showed a decreased growth rate (40%; Fig. 4, C and D compared with wild-type osteoblasts).

[3H]Thymidine incorporation assays were carried out to investigate cell proliferation effects more specifically: following extracellular addition of 250 μM GroPIns (calcium salt; or equimolar 125 μM CaCl₂) either as a single application 24 h after cell plating, or as repeated applications 24, 36, and 60 h compared with wild-type osteoblasts). Here, single application of 250 μM GroPIns stimulated cell growth only in wild-type MC3T3-E1 osteoblasts (~1.3-fold over control); repeated GroPIns applications showed increased cell growth both in wild-type MC3T3-E1 osteoblasts and, to a lesser extent, in the MC3T3-E1-Cl15 clone (~3.7- and 2.2-fold over control, respectively), with no effects seen for the parallel CaCl₂ addition (Fig. 4E). The difference between the two cell lines following a single GroPIns application was confirmed also by growth curve analysis (Fig. 4D). These data suggest first that these effects arise directly from the GroPIns, and not the added Ca²⁺ ions, and then that the decreased proliferation of MC3T3-E1-Cl15 clone will arise from their increased catabolism of this osteoblast growth activator, GroPIns.

Finally, markers of cell differentiation were also monitored: alkaline phosphatase activity at day 4, and calcium content at day 7. These were both increased in the MC3T3-E1-Cl15 clone (by 23.2-fold and 19.9-fold, respectively; Fig. 4F), compared with the parental MC3T3-E1 cell line. These data thus indicate that increased GDE3 levels accelerate the program of osteoblast growth and differentiation.

**DISCUSSION**

Our study defines GDE3 as a membrane enzyme with particular characteristics among the mammalian GP-PDE family members: it is a specific GroPIns inositolphosphodiesterase (EC 3.1.4.43) with an ectocellular activity in the HEK293T heterologous expression system and the more physiological MC3T3-E1 cell line, where GDE3 induces osteoblast differentiation.

GroPIns is a water-soluble compound that arises from the enzymatic deacylation of membrane phosphoinositides (reviewed in Ref. 11), which is mediated by phospholipase A₁IVα via receptor or oncogene activation (13, 19). GDE1 was the first mammalian enzyme identified here, and it converts GroPIns into inositol and glycerol phosphate (4). While a BLAST search revealed a striking similarity between the C-terminal portion of the GDE1 catalytic domain and the X-domain...
The GP-PDE activity of wild-type MC3T3-E1 osteoblasts is increased in the MC3T3-E1-Cl15 osteoblast clone overexpressing GDE3 wt. MC3T3-E1 osteoblasts and the MC3T3-E1-Cl15 osteoblast clone overexpressing GDE3 wt were used to prepare postnuclear lysates (A) and to monitor GP-PDE activity in the extracellular medium (B), and for growth curves (C and D), [3H]Thymidine incorporation (E) and alkaline phosphatase activity and calcium content (F) (see “Experimental Procedures”). A, quantification of GP-PDE activities of the cell lysates on 10 mM GroPIns for 2 h at 37 °C with 10 μM of postnuclear protein, as indicated (see “Experimental Procedures”). Black bars, GroPIns (as substrate); white bars, inositol (Ins); gray bars, Ins1P. GP-PDE activity is given as percentages of total [3H]GroPIns counts added (25,000 dpm on HPLC) for each component. The data are means (± S.E.) of three independent experiments, each carried out in duplicate. B, time courses of GP-PDE activity on GroPIns in the extracellular media, as indicated (see “Experimental Procedures”), with Ins1P production as a measure of GroPIns hydrolysis, expressed as percentages of total counts (90,000 dpm on HPLC), as means (± S.E.) of three independent experiments, each carried out in duplicate. MC3T3-E1 osteoblasts (black line); MC3T3-E1-Cl15 clone (gray line). C and D, representative growth curves, as indicated, of untreated cells (C) or those stimulated with 250 μM GroPIns (calcium salt) or the equimolar 125 μM CaCl2, added 12 h after plating (D). Data are percentages of the numbers of cells 12 h after plating and are means (± S.E.) of three independent experiments, each carried out in quadruplicate. MC3T3-E1 osteoblasts (black lines); MC3T3-E1-Cl15 osteoblast clone (gray lines). E, [3H]Thymidine incorporation in cells stimulated with 250 μM GroPIns (calcium salt) or the equimolar 125 μM CaCl2, added once 24 h after cell plating, or added three times at 24, 36, and 60 h after plating (see main text: single addition, triple addition). [3H]Thymidine incorporation was determined from 72 h (see “Experimental Procedures”). The data are from a single experiment carried out in quadruplicate (mean ± S.D.) and are representative of four independent experiments. F, alkaline phosphatase activity and calcium content as indicated, measured 4 and 7 days, respectively, after cell plating (see “Experimental Procedures”). Data are means (± S.D.) of two independent experiments, each carried out in triplicate. A–F, *, p < 0.05; **, p < 0.02, compared with the respective controls (paired Student’s t test).
of mammalian phosphoinositide-specific phospholipases C, GDE1 enzymatic activity resembles more a phospholipase D-like attack of the phosphodiester bond (4). Similar behavior has been reported for all bacteria GP-PDEs studied to date (1, 2). Intriguingly, the GDE1 catalytic domain contains an amino acid sequence resembling the known HKD signature of the phospholipase D family (Ref. 24; e.g. \(^{79}\)HRXXXD in mGDE1). The aspartic acid required for phospholipase D activity is not present in the GDE3 catalytic domain, which probably accounts for the GDE3 selectivity at the phosphodiester bond and for its phospholipase C-like activity (i.e. GroPlin hydrolysis to glycerol and Ins1P) (4). Thus, despite their different enzymatic activity and their expression patterns (4, 8), GDE1 and GDE3 both mediate GroPlin catabolism, leading to different products, potentially reflecting the specific function(s) of GroPlins in a given cell system/tissue.

In a previous study, we proposed the GDE1 topology of the N and C termini facing the cytoplasm, and the catalytic domain facing the extracellular space or the lumen of the endoplasmic reticulum (4). Our results here indicate that the GDE3 catalytic domain is also exposed and active toward the extracellular space, as a consequence of the requirement of calcium in the millimolar range for GDE3 activity and with the absence of hydrolysis of the intracellular GroPlins pool when GDE3 is overexpressed.

Based on hydropathy analysis, all of the glycerophosphodiesterases contain multiple transmembrane regions (5) and appear to be membrane-bound (25), except for GDE5, which is cytosolic. GDE5 therefore represents the only good candidate for regulating intracellular GroPlins levels. However, recent data obtained in our laboratory indicate that GDE5 does not hydrolyze GroPlins or GroPlin4P. Therefore, although both GDE1 and GDE3 show substrate specificity toward GroPlins, this is not a general feature of the GP-PDE family; furthermore, the evidence collected to date indicate their extracellular catabolism of GroPlins.

Alignment of the most conserved portion of the catalytic GP-PDE domain in mammalian, yeast, and bacterial glycerophosphodiesterases reveals universally conserved residues, including arginine 231 of mouse GDE3. We show that the single point mutation Arg \(\rightarrow\) Ala (GDE3R231A) completely abolishes the enzymatic activity on GroPlins in in vitro assays with post-nuclear preparations and in intact HEK293T cells, and it also completely reverses the round-shaped phenotype induced by GDE3wt overexpression. This highlights the relevance of this arginine for GDE3 enzymatic activity, and indicates that a catalytically active enzyme is required for the actin cytoskeleton modulation that leads to decreases in cell spreading area in HEK293T cells overexpressing GDE3wt. Again, and as for GroPlins substrate specificity, the effects of cell rounding after GDE3wt overexpression in HEK293T cells is not shared by all GDEs, since overexpression of the recently cloned GDE4 in the same system does not affect cell morphology (9).

Interesting data were obtained in osteoblasts, where GDE3 activity was indeed related to the physiology of these cells. Here, we show that GDE3 expression in MC3T3-E1 cells induces actin cytoskeleton disorganization, resulting in a clear disassembly of the stress fibers. Intriguingly, GroPlin4P is a well characterized modulator of the actin cytoskeleton in fibroblasts, where exogenous addition of GroPlin4P induces ruffle formation and stress fiber appearance (14, 15). Our biochemical characterization excluded GroPlin4P as a GDE3 substrate, however; in addition, neither GroPlins nor Ins1P (at concentrations and times of treatment up to 100 \(\mu\)M and 24 h, respectively) have any effects on the actin cytoskeleton in this osteoblast cell system, indicating that other substrates are involved in GDE3 activity for the actin cytoskeleton.

Interestingly, new substrates of GDE1 have been discovered recently among the glycerophospho-N-acyl ethanololamines, suggesting the physiological involvement of GDE1 in the biosynthesis of anandamide (N-arachidonoyl ethanolamine), an endogenous ligand for the brain cannabinoid receptor (25). In contrast to GDE1, the other membrane-associated GDEs, including GDE3, are not active on these ethanololamines (25). However, from our analysis of the different GDE3 substrates, the possibility that molecules other than GroPlins are physiologically relevant for GDE3 activity cannot be excluded, as might be the case for the phosphorylated GPls.

The most appealing aspect of this study are the data indicating a role for GDE3 in inducing the osteogenic process, whereas our previous report simply showed GDE3 as an early marker of osteoblast differentiation (8). GDE3 expression patterns during MC3T3-E1 development have shown that its mRNA levels peak at day 5–7 of culture in osteogenic medium (with \(\beta\)-glycerophosphate and ascorbic acid). Among the three osteoblast differentiation stages, this corresponds to the extracellular matrix development stage (8). Here, we show that stable expression of GDE3 in osteoblasts is sufficient to induce a decrease in cell growth rate. In addition, we provide evidence that GroPlins can stimulate osteoblast proliferation and that the decrease in growth rate in the GDE3-expressing clone correlates with increased catabolism of extracellular GroPlins. Several aspects of skeletal development are mechanistically linked, including lineage specification, and growth and differentiation of mesenchymal cells. In particular, temporal growth arrest is considered to have a critical role in triggering osteoblast differentiation. Recent reports have indicated that bone morphogenetic protein 2, which is a potent inducer of osteoblast differentiation and parathyroid hormone-related protein, which is involved in bone cell turnover, can induce cell growth arrest in differentiated osteoblasts (26, 27), suggesting a possible cellular function of GDE3 via negative growth control.

Indeed, this is the first report of GDE3 as an inducer of osteoblast differentiation, rather than just a marker, although further studies are needed to clarify the interplay between GDE3 and other well known regulators of osteoblast differentiation.

With a view to unraveling the mechanistic aspects of this study, we note that the decrease in growth rate in osteoblasts overexpressing GDE3wt, and the rescue of this inhibition with successive additions of extracellular GroPlins, suggest that GroPlins is indeed a mediator of osteoblast growth. Thus increased GDE3 expression accelerates GroPlins catabolism, decreasing osteoblast proliferation, and inducing cell differen-

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6 S. Mariggio and C. Iurisci, unpublished observations.
tiation. While involvement of alternative mediators in these processes cannot at present be ruled out, this GroPIns mediation of osteoblast growth is consistent with effects seen in our previous system of thyroid epithelial PCCl3 cells (13), reinforcing the link between GroPIns and the regulation of cell growth.

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