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The Developmentally Regulated Osteoblast Phosphodiesterase GDE3 Is Glycerophosphoinositol-specific and Modulates Cell Growth*[S]

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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 calcium required for GDE3 activity, this predicts an enzyme topology with an extracellular catalytic domain. Interestingly, GDE3 ectocellular activity is detected in a stable clone from a murine osteoblast cell line, further confirming the activity of GDE3 in a more physiological context. Finally, overexpression of wild-type GDE3 in osteoblasts promotes disassembly of actin stress fibers, decrease in growth rate, and increase in alkaline phosphatase activity and calcium content, indicating a role for GDE3 in induction of differentiation. Thus, we have identified the GDE3 substrate GroPIns as a candidate mediator for osteoblast proliferation, in line with the GroPIns activity observed previously in epithelial cells.

The glycerophosphodiester phosphodiesterases (GP-PDEs)[5] were initially characterized in bacteria, where they have functional roles for production of metabolic carbon and phosphate sources from glycerophosphodiesters (1, 2) and in adherence to and degradation of mammalian host-cell membranes (3). The GP-PDEs have a catalytic region of 56 amino acids (4). After their characterization in bacteria, mammalian glycerophosphodiesterases were identified, with the definition of a family of seven members (5). The first of these, GDE1, is an interactor of regulator of G-protein signaling (RGS)16, and was subsequently defined as a GP-PDE regulated by G-protein signaling (4). Indeed, GDE1 expression in HEK293T cells showed increased enzymatic activity upon α/β-adrenergic and lysophospholipid receptor stimulation (4). The second member, GDE2, was isolated by homology searches in neuronal tissues and its physiological role involves neuronal differentiation (6, 7). In contrast, GDE3 has been characterized as a marker of osteoblast differentiation and was isolated through a differential display method (8). GDE4 was isolated only recently with three-dimensional modeling defining it as a GP-PDE, although no functional activity has been correlated to its expression (9). The remaining members were cloned following data base searches, with further studies required for the definition of their properties (5). The diversity among these family members, in terms of tissue distribution, subcellular localization, and substrate specificity, suggests they selectively regulate biological functions and have distinct physiological roles (5).

The only GP-PDE activity that has been biochemically characterized to date followed GDE1 overexpression in HEK293T cells, which showed a selectivity for the glycerophosphoinositols (GPIs) as substrate (4), in contrast to the bacterial GP-PDEs that show broad substrate specificities with respect to the alcohol moiety of the glycerophosphodiesterases (1, 2). The GPIs are naturally occurring, biologically active metabolites of the phosphoinositides that were originally investigated in the con-

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[5] The abbreviations used are: GP-PDE, glycerophosphodiester phosphodiesterase; RGS16, regulator of G-protein signaling 16; GPIs, glycerophosphoinositols; GroPIns, glycerophosphoinositol; GroPIns4P, glycerophosphoinositol 4-phosphate; $PLA_2IV\alpha$, phospholipase A_2 type IVα; GroPCho, glycerophosphocholine; Ins1P, inositol 1-phosphate; CHO cells, Chinese hamster ovary cells; HEK293T, human embryonic kidney 293T cells; HPLC, high performance liquid chromatography; FCS, fetal calf serum; PBS, phosphate-buffered saline; GFP, green fluorescent protein; wt, wild type.

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 $PCCl_3$ 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 GPIs 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 GPIs from the phosphoinositides inside cells following activation of a specific isoform of phospholipase A_2, $PLA_2IV\alpha$ (13, 19).

The release of the GPIs 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). [^{3}H]GroPIns was prepared from L-α-[^{3}H]phosphatidylinositol (314.5 GBq/mmol; PerkinElmer, Boston, MA) by deacylation, according to the original procedure of Clarke and Dawson (20). [6-^{3}H]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 purities 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 (Stratagene), a set of PCR primers (5′-CAGCATGGGGGCCCCTGCGTGTCCCACCAGCCC-3′ and 5′-GGGCTGGTGGGACACGCAGGGGCCCCCATG-CTG-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% CO_2 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/neoI) or with pEF-GDE3 (MC3T3-E1-Cl15), 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% CO_2 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 (2×10^4 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 [^{3}H]thymidine incorporation assay, MC3T3-E1 and MC3T3-E1-Cl15 cells were seeded in 96-well plates at a density of 5×10^3 cell/well in complete growth medium. After 12 h, the cells were treated with 250 μM GroPIns (calcium salt) or the equimolar 125 μM $CaCl_2$ and, when indicated, two further additions followed after 36 and 60 h. After 72 h, a pulse of [^{3}H]thymidine (1 μCi/well) was given 4 h before stopping the reaction by washing twice with $HBSS^{++}$. The [^{3}H]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 pepstatin, 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,

which included 100 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 mg/ml fatty-acid-free bovine serum albumin, 30,000 dpm [^{3}H]GroPIns, [^{3}H]GroPIns4P, or [^{3}H]glycerophosphocholine (GroPCho), unlabeled GroPIns, GroPIns4P, or GroPCho (as indicated), without or with addition of competing glycerophosphodiesters. Incubations were in the presence of 5 mM Ca^{2+} or as otherwise specified. Initial analysis of incubation conditions 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 Ca^{2+}, GroPIns substrate as the Li^+ salt (from 100 μM to 500 mM), 10 μg of postnuclear lysate protein, and a 2-h incubation at 37 °C. GroPIns hydrolysis was determined as that measured for GFP-GDE3wt over the background GFP postnuclear preparation control, 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 glycerophosphodiesters.

For the *in vivo* extracellular GP-PDE assays, cells were plated in 6-well plates and 100,000 dpm/well [^{3}H]GroPIns, [^{3}H]GroPIns4P, or [^{3}H]GroPCho were added in 2.5 ml of growth medium. At specific times, 500 μl of medium was analyzed, 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 (aqueous) 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 ^{3}H-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 0.2 M $NaPO_4$ (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 [^{3}H]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-[^{3}H]inositol (5 μCi/ml). Following labeling, the cells were washed twice with $HBSS^{++}$ and 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 chloroform (final, 1:1:0.5). After separation of aqueous and organic extraction phases, the [^{3}H]inositol-labeled water soluble metabolites were separated by anion exchange HPLC on a Partisil 10-SAX column using a non-linear water/1 M ammonium phosphate, pH 3.35 (phosphoric acid) gradient. Radioactivity associated with the ^{3}H-labeled compounds was analyzed by an on-line flow detector (Packard FLO ONE A-525). GroPIns levels are given as percentages of total aqueous ^{3}H-labeled compounds. 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). Alkaline phosphatase activity was assayed by the phosphatase substrate 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 experiments, 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 phosphodiesterase 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. 1*A*, *underlined*). To investigate the GP-PDE activity of GDE3, HEK293T cells were transiently transfected with cDNAs coding 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. 1*B*).

Ten micrograms postnuclear protein from HEK293T cells overexpressing GFP-GDE3wt show a GroPIns inositol phosphodiesterase 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. 1*D*, 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 experimental conditions.

To determine divalent cation requirements for this GDE3 GP-PDE activity, the GFP-GDE3wt lysate was incubated with

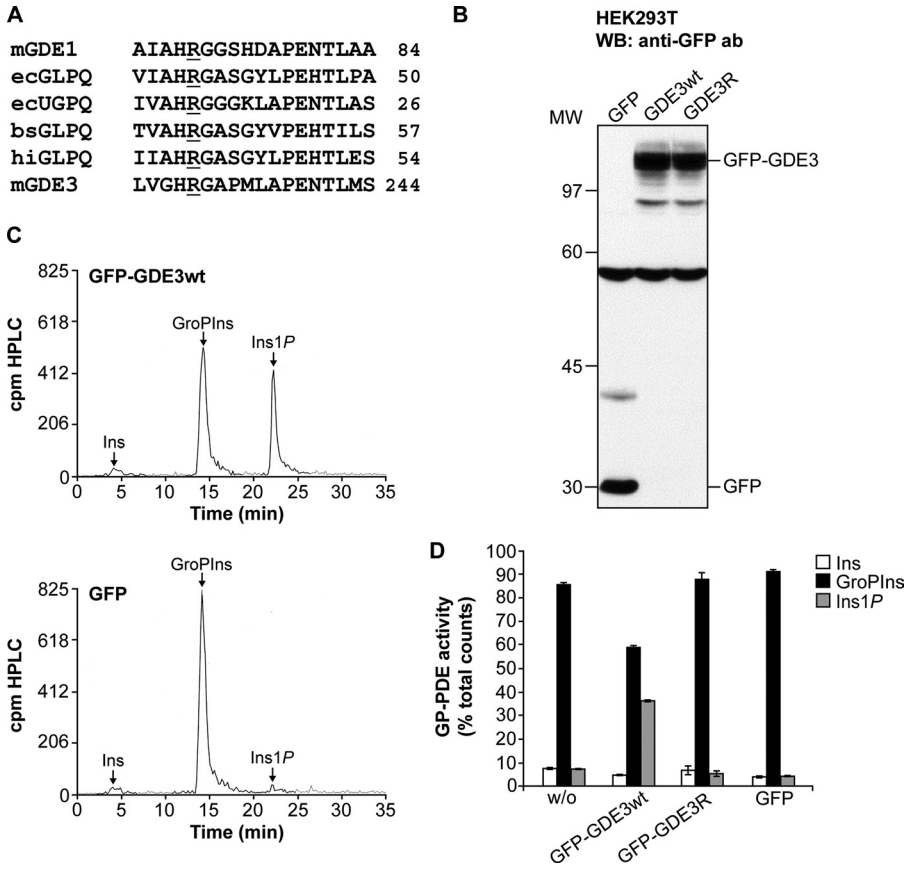

FIGURE 1. **GDE3 is a GroPIns inositol phosphodiesterase.** *A*, alignment of the catalytic domains of mouse GDE1 and GDE3, and bacterial GP-PDEs (GLPQ and UGPQ). The highly conserved arginine (*R*) residue is *underlined*. *m*, *Mus musculus*; *ec*, *Escherichia coli*; *bs*, *Bacillus subtilis*; *hi*, *Haemophilus influenzae*. The *numbers* indicate the final amino acid position. *B–D*, postnuclear lysates were prepared from HEK293T cells overexpressing GFP-GDE3wt, the GFP-GDE3R mutant, and GFP (see "Experimental Procedures"). *B*, representative Western blot with an anti-GFP antibody shows specific bands in the cell lysates for a molecular mass of ~116 kDa in GFP-GDE3wt-transfected and GFP-GDE3R-transfected cells, and of ~27 kDa in GFP-transfected cells. The band at ~60 kDa is a nonspecific band arising from the anti-GFP antibody. *MW*, molecular weight (kDa) markers. *C*, HPLC analysis of aqueous extracts following *in vitro* GP-PDE assays with postnuclear lysates from GFP-GDE3wt- and GFP-transfected cells, as indicated. Elution peaks for [^{3}H]inositol (*Ins*), [^{3}H]GroPIns, and [^{3}H]Ins1P as indicated. *D*, quantification of GP-PDE activities as indicated, on 10 mM GroPIns (Ca^{2+} salt) for 2 h at 37 °C in the absence (w/o) and the presence of postnuclear lysates (see "Experimental Procedures"). *Black bars*, GroPIns (as substrate); *white bars*, inositol (*Ins*); *gray bars*, Ins1P. GP-PDE activity is given as percentages of total [^{3}H]GroPIns counts added (25,000 dpm on HPLC) for each component. The data are from a single experiment carried out in duplicate (mean ± S.D.) and are representative of four independent experiments.

10 mM GroPIns (Li^+ salt) in the absence and presence of 10 mM $MgCl_2$, and in the presence of 10 mM $MgCl_2$ plus increasing concentrations of $CaCl_2$ (0.1–10 mM). Unlike the enhancement of GDE1 activity with Mg^{2+} (4), this GDE3 activity did not require $MgCl_2$ (data not shown), but instead required addition of millimolar Ca^{2+} (Fig. 2*A*). With 5 mM Ca^{2+}, GroPIns hydrolysis by GFP-GDE3wt was essentially linear over 5 h at 37 °C (Fig. 2*B*), with no activity in the control GFP postnuclear preparation (Fig. 2*C*). The Eadie-Hofstee plot derived from GroPIns dose-response analysis (Fig. 2*D*) shows an apparent K_m for GDE3 of ~97.2 mM, and a V_{max} of ~1,900 pmol/mg/min for GroPIns (see "Experimental Procedures").

GDE3 Is Specific for Glycerophosphoinositols—To determine the specificity of GDE3, 10 mM GroPCho and GroPIns4P were also used as substrates in phosphodiesterase activity assays with the GFP-GDE3wt postnuclear preparation; neither of these glycerophosphodiesters were hydrolyzed by GDE3 under these conditions.

The specificity of GDE3 for GroPIns as substrate was then investigated in competition assays, with the GroPIns 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 GroPIns inositol phosphodiesterase activity was not inhibited (supplemental Fig. S1 SF1). We also tested other GPIs in this competition assay of GDE3, and 10 mM GroPIns4P and 10 mM GroPIns 4,5-bisphosphate competed with GroPIns hydrolysis at least in part (45 and 55% inhibition, respectively) (Fig. 2*E*).

Cells Overexpressing GDE3 Do Not Have Modified Intracellular GroPIns 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 GroPIns (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 GroPIns levels can be modulated by hormone stimulation. Here, addition of 100 μM ATP for 15 min induced about a 2-fold increase in intracellular GroPIns 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 GroPIns levels (about 2-fold the basal). These data show that GDE3 does not affect intracellular levels of GroPIns, supporting the idea that the catalytic domain of GDE3 is oriented extracellularly, at least in these cell systems.

An Extracellular GroPIns Inositolphosphodiesterase Activity Is Present in HEK293T Cells Overexpressing GDE3—The glycerophosphodiesterase activity was also tested extracellularly, adding the GroPIns substrate to the cell medium, which was then sampled and analyzed at different times. As for the *in vitro* assays, GroPIns 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 GroPIns hydrolysis (Fig. 3, *B* and *C*). Also in agreement with the *in vitro* assays, cells overexpressing the

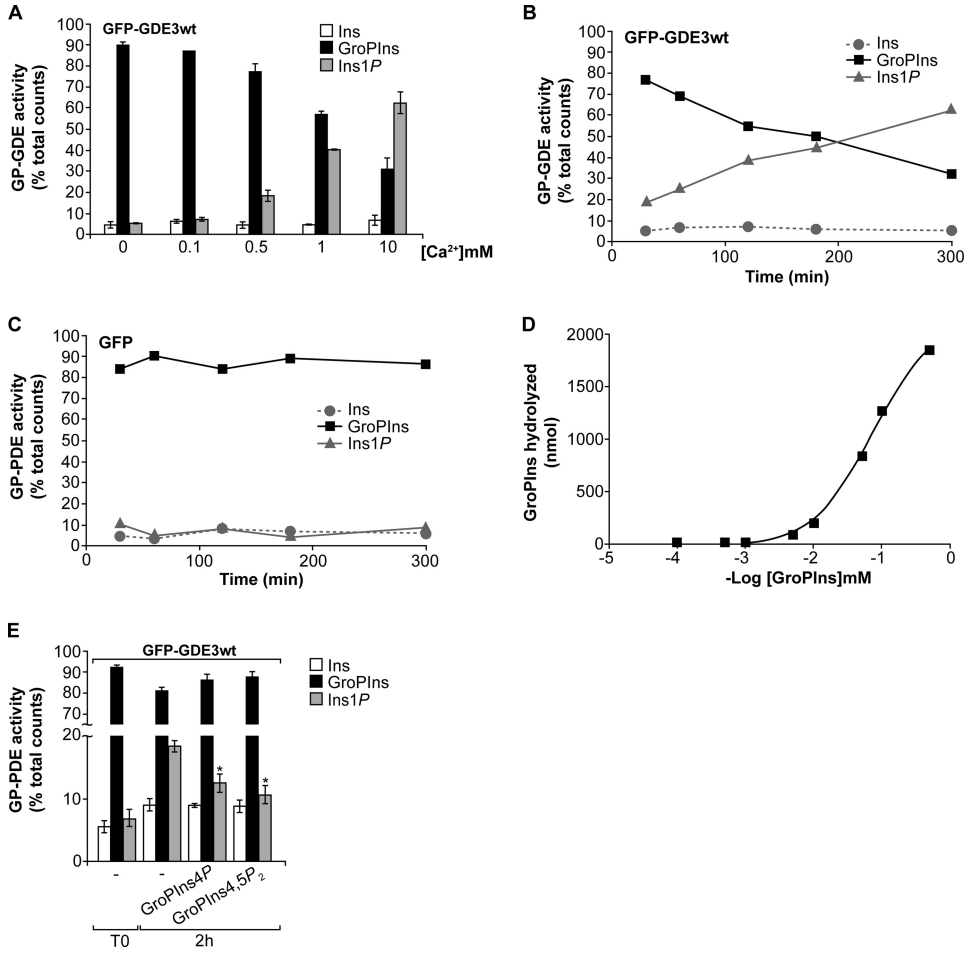

FIGURE 2. **Characterization of GDE3 activity on GroPIns.** Postnuclear lysates were prepared from HEK293T cells overexpressing GFP-GDE3wt and GFP (see "Experimental Procedures"). *A*, quantification of GP-PDE activities on 10 mM GroPIns (Li^+ salt) for 2 h at 37 °C with 10 μg of postnuclear GFP-GDE3wt protein in the absence (0) and presence of increasing $CaCl_2$ concentrations, 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 [^{3}H]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* and *C*, time courses of GDE3 activity on GroPIns with 10 μg of postnuclear GFP-GDE3wt (*B*) and GFP (*C*) protein. *Black line* (*squares*), GroPIns (as substrate); *dashed line* (*circles*), inositol (*Ins*); *gray line* (*triangles*), *Ins1P*. GP-PDE activity is given as percentages of total [^{3}H]GroPIns counts added (25,000 dpm on HPLC) for each component. The data are from a single representative experiment carried out in duplicate (mean ± S.D.), and are representative of three independent experiments. *D*, log dose-response curve of postnuclear GFP-GDE3wt activity (over the GFP control) as nmol of GroPIns hydrolyzed after 2 h at 37 °C. The data are from a single experiment carried out in duplicate (mean ± S.D.) and are representative of two independent experiments. *E*, GPIs competition assay of GDE3 activity toward GroPIns. Quantification of GP-PDE activities of 10 μg of GFP-GDE3wt postnuclear protein on 1 mM GroPIns with 10 mM phosphorylated GPIs, as indicated. The assays were stopped either immediately (T0 control) or after a 2-h incubation at 37 °C (2 h), in the absence (−) and presence of 10 mM of the indicated glycerophosphodiesters (see "Experimental Procedures"). The data are means (±S.E.) of three independent experiments, each carried out in duplicate. *Black bars*, GroPIns (as substrate); *white bars*, inositol (*Ins*); *gray bars*, Ins1P. GP-PDE activity is given as percentages of total [^{3}H]GroPIns counts added (25,000 dpm on HPLC) for each component. *, $p < 0.05$, compared with GroPIns alone (paired Student's *t* test).

GFP-GDE3R mutant did not show extracellular GroPIns inositolphosphodiesterase activity (Fig. 3*C*). 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. 3*D*). 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. 3*E*). 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-Cl15). In postnuclear preparations from MC3T3-E1 cells, there were low levels of activity after the standard 2-h incubation at 37 °C (Fig. 4*A*). For the MC3T3-E1-Cl15 clone, this activity showed a significant ~60% increase in Ins1P production, compared with the parent MC3T3-E1 cell line (Fig. 4*A*).

Similarly, for extracellular GroPIns inositolphosphodiesterase activity in intact cells, the MC3T3-E1-Cl15 clone produced a significant ~50% increase in Ins1P production over 24 h, compared with the parent MC3T3-E1 cell line (Fig. 4*B*).

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,

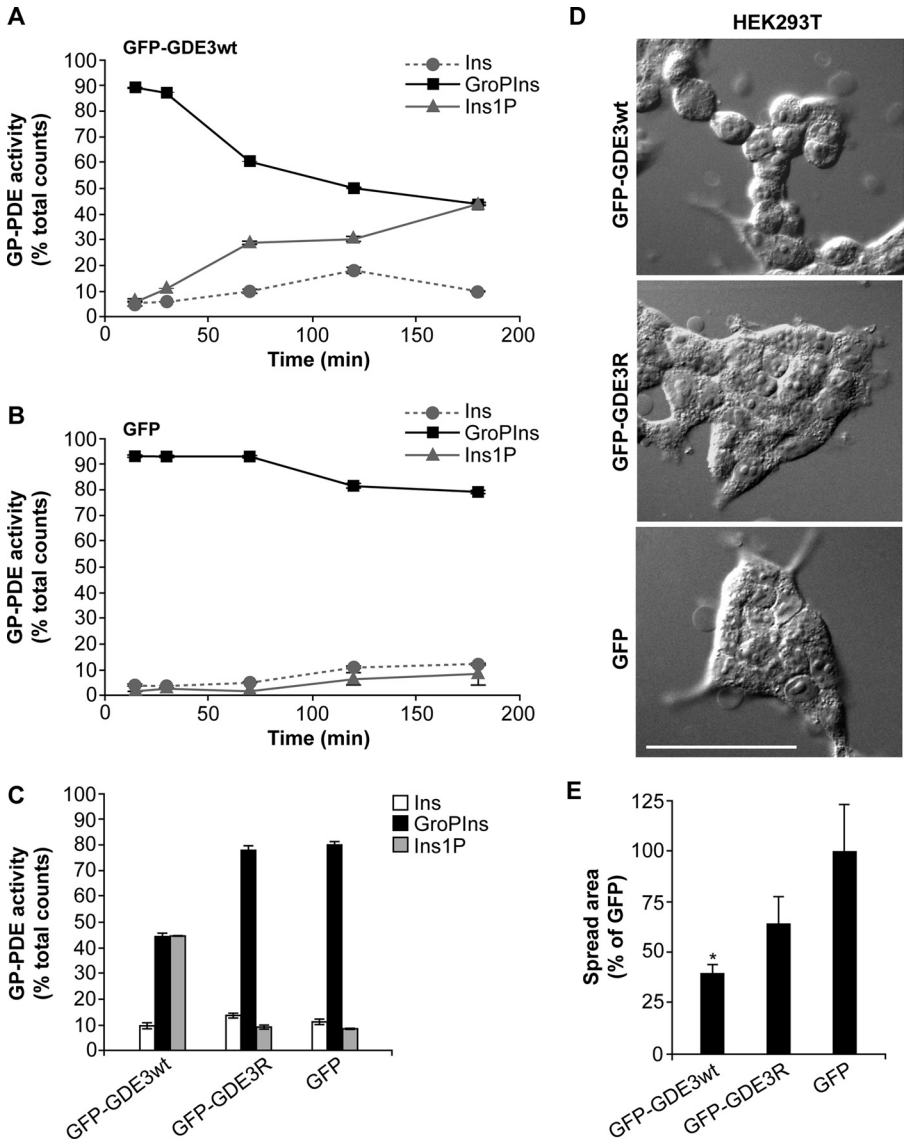

FIGURE 3. **GP-PDE activity in the extracellular space of intact cells transfected with GDE3wt.** HEK293T cells overexpressing GFP-GDE3wt (*A* and *C–E*), the GFP-GDE3R mutant (*C–E*) and GFP (*B–E*) were incubated with GroPIns added to the growth medium (*A–C*) or examined for morphology (*D*) or cell spreading (*E*) (see "Experimental Procedures"). *A* and *B*, time courses of GP-PDE activity on GroPIns in the extracellular medium. *Black line* (*squares*), GroPIns (as substrate); *dashed line* (*circles*), inositol (*Ins*); *gray line* (*triangles*), Ins1P. *C*, quantification of similar experiments carried out for 3 h at 37 °C. *Black bars*, GroPIns (as substrate); *white bars*, inositol (*Ins*); *gray bars*, Ins1P. For *A–C*, GP-PDE activity is given as percentage total [^{3}H]GroPIns counts added (90,000 dpm on HPLC) for each component, and the data are means (±S.E.) of three independent experiments, each carried out in duplicate. *D*, morphology of HEK293T cells, as indicated. *Bar*, 10 μm. *E*, quantitative analysis of cell spreading, expressed as percentages of GFP-transfected HEK293T cell area. The data are means (±S.D.) of two independent experiments, each carried out in duplicate. *, $p < 0.05$, GFP-GDE3wt compared with GFP-transfected cells (Student's *t* test).

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).

[^{3}H]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 $CaCl_2$) either as a single application 24 h after cell plating, or as repeated applications 24, 36, and 60 h after cell plating, with [^{3}H]thymidine added after 72 h. 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 $CaCl_2$ addition (Fig. 4*E*). The difference between the two cell lines following a single GroPIns application was confirmed also by growth curve analysis (Fig. 4*D*). These data suggest first that these effects arise directly from the GroPIns, and not the added Ca^{2+} 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. 4*F*), 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 A_2IVα 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

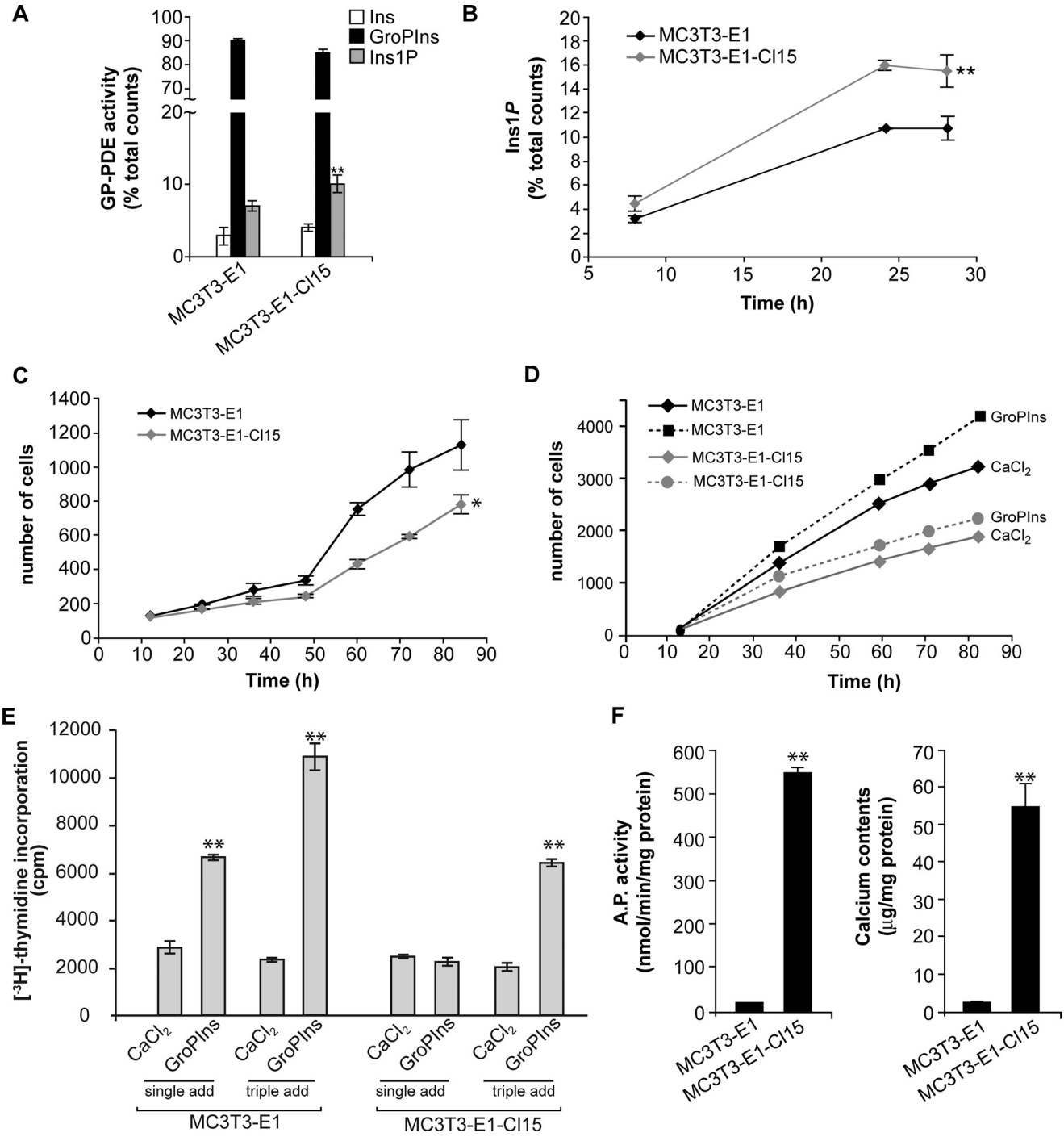

FIGURE 4. **The GP-PDE activity of wild-type MC3T3-E1 osteoblasts is increased in the MC3T3-E1-Cl15 osteoblast clone overexpressing GDE3wt.** MC3T3-E1 osteoblasts and the MC3T3-E1-Cl15 osteoblast clone overexpressing GDE3wt were used to prepare postnuclear lysates (*A*) and to monitor GP-PDE activity in the extracellular medium (*B*), and for growth curves (*C* and *D*), [^{3}H]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 μg 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 [^{3}H]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 $CaCl_2$, 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*, [^{3}H]thymidine incorporation in cells stimulated with 250 μM GroPIns (calcium salt) or the equimolar 125 μM $CaCl_2$, 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). [^{3}H]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 indicted, 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.* ^{70}HR*XXXX*D 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.* GroPIns hydrolysis to glycerol and Ins1P) (4). Thus, despite their different enzymatic activity and their expression patterns (4, 8), GDE1 and GDE3 both mediate GroPIns catabolism, leading to different products, potentially reflecting the specific function(s) of GroPIns 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 GroPIns 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 GroPIns levels. However, recent data obtained in our laboratory indicate that GDE5 does not hydrolyze GroPIns or GroPIns4P.[6] Therefore, although both GDE1 and GDE3 show substrate specificity toward GroPIns, this is not a general feature of the GP-PDE family; furthermore, the evidence collected to date indicate their extracellular catabolism of GroPIns.

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 → Ala (GDE3R231A) completely abolishes the enzymatic activity on GroPIns in *in vitro* assays with postnuclear 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 GroPIns 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, GroPIns4P is a well characterized modulator of the actin cytoskeleton in fibroblasts, where exogenous addition of GroPIns4P induces ruffle formation and stress fiber appearance (14, 15). Our biochemical characterization excluded GroPIns4P as a GDE3 substrate, however; in addition, neither GroPIns nor Ins1P (at concentrations and times of treatment up to 100 μM and 24 h, respectively) have any effects on the actin cytoskeleton in this osteoblast cell system,[6] 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 ethanolamines, 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 ethanolamines (25). However, from our analysis of the different GDE3 substrates, the possibility that molecules other than GroPIns are physiologically relevant for GDE3 activity cannot be excluded, as might be the case for the phosphorylated GPIs.

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 β-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 GroPIns can stimulate osteoblast proliferation and that the decrease in growth rate in the GDE3-expressing clone correlates with increased catabolism of extracellular GroPIns. 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 GroPIns, suggest that GroPIns is indeed a mediator of osteoblast growth. Thus increased GDE3 expression accelerates GroPIns catabolism, decreasing osteoblast proliferation, and inducing cell differen-

[6] S. Mariggiò 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 $PCCl_3$ cells (13), reinforcing the link between GroPIns and the regulation of cell growth.

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