Targeted Inhibition of Phospholipase C γ2 Adaptor Function Blocks Osteoclastogenesis and Protects from Pathological Osteolysis*

Received for publication, April 19, 2013, and in revised form, September 12, 2013. Published, JBC Papers in Press, September 30, 2013, DOI 10.1074/jbc.M113.477281

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Background: Genetic deletion of PLCγ2 prevents osteoclast differentiation and reduces bone resorption in vitro and in vivo. Results: Ectopic expression of the tandem SH2 domains of PLCγ2 impairs osteoclastogenesis and protects from bone loss in wild-type mice.

Conclusions: Targeting PLCγ2 adaptor function is an efficient strategy to block osteoclast differentiation.

Significance: This work provides a framework to design specific PLCγ2 inhibitors.

Phospholipase C γ2 (PLCγ2) is a critical regulator of innate immune cells and osteoclasts (OCs) during inflammatory arthritis. Both the catalytic domain and the adaptor motifs of PLCγ2 are required for OC formation and function. Due to the high homology between the catalytic domains of PLCγ2 and the ubiquitously expressed PLCγ1, molecules encompassing the adaptor motifs of PLCγ2 were designed to test the hypothesis that uncoupling the adaptor and catalytic functions of PLCγ2 could specifically inhibit osteoclastogenesis and bone erosion. Wild-type (WT) bone marrow macrophages (BMM) that overexpress the tandem Src homology 2 (SH2) domains of PLCγ2 (SH2(N+C)) failed to form mature OCs and resorb bone in vitro. Activation of the receptor activator of NF-κB (RANK) signaling pathway, which is critical for OC development, was impaired in cells expressing SH2(N+C). Arrest in OC differentiation was evidenced by a reduction of p38 and 14-3-3 phosphorylation as well as decreased NFATc1 and c-Fos/c-Jun levels. Consistent with our hypothesis, SH2(N+C) abrogated formation of the RANK-Gab2 complex, which mediates NF-κB and AP-1 activation following RANK ligand (RANKL) stimulation. Furthermore, the ability of SH2(N+C) to prevent inflammatory osteolysis was examined in vivo following RANKL or LPS injections over the calvaria. Both models induced osteolysis in the control group, whereas the SH2(N+C)-treated cohort was largely protected from bone erosion. Collectively, these data indicate that inflammatory osteolysis can be abrogated by treatment with a molecule composed of the tandem SH2 domains of PLCγ2.

Pathological bone loss is a debilitating complication associated with prosthetic implants, osteoarthritis, rheumatoid arthritis, and periodontal disease (1) and results from an increase in the number and/or function of the bone-resorbing OCs (2). OCs are differentiated from BMM precursors in the presence of RANKL and macrophage colony-stimulating factor (M-CSF), which bind RANK and c-Fms, respectively (3). Pro-inflammatory cytokines TNF-α, IL-1β, and IL-6, which are produced at inflamed joints, further promote OCG and resorptive activity (4). Such crosstalk between immune cells and OCs highlights the need for a therapeutic treatment capable of reducing inflammation and preventing bone erosion.

PLCγ1 and PLCγ2 molecules are critical regulators of innate and adaptive immune responses and are activated during OC differentiation. These enzymes convert phosphatidylinositol 4,5-bisphosphate into two second messengers, inositol 1,4,5-trisphosphate and diacylglycerol, leading to activation of inositol 1,4,5-trisphosphate/calcium and diacylglycerol-dependent pathways downstream of RANK, integrins, and a variety of immune receptors (5). For example, in vitro studies of T lymphocytes demonstrate that PLCγ1 is a critical modulator of T cell receptor responses (6–8). However, PLCγ2 is ubiquitously expressed, and its global deletion leads to early embryonic lethality in the mouse (9). Thus, an approach to inhibit PLCγ1 function is likely to have broad off-target effects. PLCγ2 expression is confined to cells of hematopoietic lineage, including B lymphocytes, natural killer cells, mast cells, neutrophils, dendritic cells, and OCs (10–14). Plcg2−/− mice have increased bone mass (osteopetrosis) due to reduced differentiation of BMMs into OCs (15). Additionally, Plcg2−/− mice are protected from serum transfer-induced arthritis and antigen-induced arthritis due to functional defects in neutrophils and dendritic cells, respectively (13, 14). Therefore, PLCγ2 is a promising target for therapeutic design in the context of inflammatory osteolysis. However, due to its high homology with the more ubiquitously expressed PLCγ1, it is necessary to find inhibitors that can selectively block PLCγ2 activation.

* This work was supported, in whole or in part, by National Institutes of Health Grants RO1 AR52921 AR53628 (to R.F.). This work was also supported by Shriners Hospital Grant 85120 (to R.F.) and an Arthritis Foundation grant (to R.F.).
1 Both authors contributed equally to this work.
2 Supported in part by the Institutional Metabolic Skeletal Disorders Training Program Grant T32 AR060719-01 from the National Institutes of Health.
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4 The abbreviations used are: OC, osteoclast; OCG, osteoclastogenesis; BMM, bone marrow macrophage; PLCγ2, phospholipase C γ2; RANK, receptor activator of NF-κB; RANKL, RANK ligand; SH2, Src homology; TRAP, tartrate-resistant acid phosphatase; Ad, adenovirus.
PLCy1 and PLCy2 are unique from other PLC molecules because they contain two tandem SH2 motifs and an SH3 adaptor motif. Initial studies suggested that these adaptor motifs harbor an intrinsic regulatory function of the catalytic activity of the protein (16), exposing the catalytic domain upon PLCy1/2 phosphorylation (17). However, it is now established that these SH2 and SH3 motifs can also mediate protein-protein binding via homeodomain interactions (17). Considering that the catalytic domains of PLCy1 and PLCy2 are over 90% homologous, whereas their adaptor motifs are less than 60% homologous, the latter regions are a potential target for specific inhibition.

The scaffolding function of PLCy2, in addition to its catalytic activity, is required for OC formation. Cells with a point mutation in the SH2 domain of PLCy2 are incapable of in vitro OCG despite intact catalytic function (18). Thus, we hypothesized that the scaffolding function of endogenous PLCy2 could be disrupted through a dominant-negative effect by a molecule encompassing the adaptor domains of PLCy2. We report that a molecule composed of the tandem SH2 motifs of PLCy2 is able to abrogate OCG in vitro and in vivo by disrupting protein interactions between RANK and Gab2. This approach may represent a novel method of targeting PLCy2 to prevent inflammatory bone loss.

EXPERIMENTAL PROCEDURES

Plasmids and Retrovirus Generation—The SH2 or SH3 domains of PLCy2 were cloned into the blasticidin-resistant pMX retroviral vector and fused with HA. To generate retrovirus, PLAT-E cells were transfected with expression vector by using a TransIT transfection reagent (Mirus Bio). Viral supernatants were collected on days 2 and 3 after transfection and immediately used to transduce freshly isolated BMMs. After 24 h, medium containing 1 μg/ml blasticidin was added to cells for 48 h to select for expressing cells.

Primary Cell Culture—Bone marrow was isolated from long bones of 6–8-week-old C57BL/6 mice and cultured in α-minimum Eagle's medium containing 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin and glucose (α-10 medium), with 0.1% H2O2) for 15 min. Bone resorption pits were visualized with a light microscope and quantified using Image J software (National Institutes of Health; rsbweb.nih.gov/ij).

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—Analysis of bone resorption was completed as described previously (18). Briefly, BMMs were plated on bovine bone slices and cultured with 0.01 CMG14-12 and 100 ng/ml GST-RANKL for 10 days. Fresh medium was added every 2 days. Cells were removed from the bone surface by using mechanical force and 2 N NaOH. Bone slices were stained with 20 μg/ml peroxidase-conjugated wheat germ agglutinin for 30 min (Sigma) followed by 3,3′-diaminobenzidine (0.52 mg/ml in PBS containing 0.1% H2O2) for 15 min. Bone resorption pits were visualized with a light microscope and quantified using Image J software.

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Real-time PCR—BMMs were cultured with 100 ng/ml RANKL and 0.01 volume of CMG14-12 for 4 h in α-minimum Eagle's medium containing 2% FBS and then stimulated with RANKL (100 ng/ml) or M-CSF (100 ng/ml) minimum Eagle's medium for the indicated times. Cells were lysed in radioimmunoprecipitation assay lysis buffer supplemented with HALT protease and phosphatase inhibitor cocktail (Pierce).

To obtain nuclear extracts from RANKL-treated cells, tissue culture plates were washed with H2O2, and the adherent cells were lysed with hypotonic buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 10% glycerol) supplemented with protease inhibitors and clarified by centrifugation. The protein concentration of each sample was determined using bichinchoninic acid protein assay (Bio-Rad), and 1 mg of protein from each sample was used for immunoprecipitation. Samples were incubated with anti-PLCy2 (Santa Cruz Biotechnology) or anti-Gab2 antibody (Millipore) overnight at 4 °C and with protein G-agarose beads (Amersham Biosciences) for 3 h at 4 °C. Beads were washed three times in lysis buffer, and immunoprecipitates were used for Western blotting.

RANKL, M-CSF, and Vitronectin Stimulation—For RANKL and M-CSF stimulation, pre-OCs were starved for 4 h in α-minimum Eagle's medium containing 2% FBS and then stimulated with RANKL (100 ng/ml) or M-CSF (100 ng/ml) minimum Eagle's medium for the indicated times. Cells were lysed in radioimmunoprecipitation assay lysis buffer supplemented with HALT protease and phosphatase inhibitor cocktail (Pierce).

To obtain nuclear extracts from RANKL-treated cells, tissue culture plates were washed with H2O2, and the adherent cells were lysed with hypotonic buffer (10 mM HEPES, 1.5 mM MgCl2, 1 mM KCl, 1 mM DTT, and protease and phosphatase inhibitors) followed by the addition of 0.1% Nonidet P-40. After centrifugation, the supernatants were collected (cytosolic fraction), whereas the pellets (nuclear fraction) were suspended in high salt buffer (hypotonic buffer plus 400 mM NaCl).

For vitronectin stimulation, pre-OCs were washed with PBS and lifted with 10% trypsin/EDTA in PBS. Cells were replated on tissue culture plates coated with vitronectin for the indicated length of time and lysed in radioimmunoprecipitation assay lysis buffer supplemented with HALT protease and phosphatase inhibitor cocktail.

Western Blot Analysis and Antibodies—The protein concentration of cell lysates or nuclear extracts was determined using bichinchoninic acid protein assay (Bio-Rad). An equivalent amount of protein for each sample was mixed with 5× loading
buffer (312.5 mM Tris, pH 6.8, 10% SDS, 50% glycerol, 0.05% bromphenol blue, 10% β-mercaptoethanol), separated by SDS-PAGE, and transferred to PVDF membranes. Membranes were probed with antibodies as indicated. Antibodies specific to the phosphorylated forms of PLCγ1, PLCγ2, ERK, JNK, c-Jun, IκBα, and Src (Tyr-416) were purchased from Cell Signaling Technology. Antibodies against p65, NFATc1, PLCγ2, TRAF6, RANK, lamin B, and Sp1 were purchased from Santa Cruz Biotechnology. The monoclonal antibody against β-actin was purchased from Sigma-Aldrich. Polyclonal Gab2 antibody was purchased from Millipore.

**In Vivo Osteolysis Models**—WT C57Bl/6 mice were treated with 100 μg of RANKL and 10⁷ PFU of adenovirus containing LacZ (Ad-LacZ control) or SH2(N+C) (Ad-SH2(N+C)) by subcutaneous injections over the calvaria daily for 5 days. Mice were sacrificed on day 6, and the calvaria were collected for histological analysis. The calvaria were preserved in 10% buffered formalin overnight (16 h) and decalcified with 14% EDTA for 4 days. Paraffin-embedded sections were stained for TRAP to visualize OCs (Sigma).

All mice used in these experiments were housed in the animal care unit of the Washington University School of Medicine, where they were maintained according to guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care. All animal experimentation was approved by the Animal Studies Committee of the Washington University School of Medicine.

**RESULTS**

**SH2(N+C) Blocks OCG in Vitro**—To design an efficient strategy to specifically oppose endogenous PLCγ2 activity in the context of pathological bone loss, we focused on the protein modular domains as the catalytic motif is highly homologous to PLCγ1. Retroviral constructs harboring the N- and C-terminal SH2 domains alone or in combination (N+C), the SH3 domain, or encompassing both SH2 and SH3 regions were generated (Fig. 1A). These constructs were transduced into WT BMMs that also express endogenous PLCγ2. Expression of the indicated domains was confirmed by Western blot (Fig. 1B). BMMs...
were treated with RANKL and M-CSF, and differentiation to mature OCs was evaluated by TRAP staining. RANKL and M-CSF induced differentiation of precursor cells into OCs in all groups, except in the presence of the SH2(N/H11001C) and partially in SH2(N/H11001C)/H11001SH3-expressing cells. Ectopic expression of SH2(N/H11001C) significantly impaired OC development (number of TRAP cells in SH2(N/H11001C), 1.3 ± 0.88 versus empty vector, 153.3 ± 8.99, p < 0.0001). A 52% inhibition of OCG was observed in WT cells expressing SH2(N+C)+SH3, whereas the differentiation of cells expressing a single SH2 (either SH2-N or SH2-C) or the SH3 motif alone was similar to cells infected with the empty vector (pMX; Fig. 1, C and D). Consistent with its inhibitory effect on OC formation, BMMs that were transduced with SH2(N+C) and grown on cow bone slices in the presence of RANKL and M-CSF for 7 days formed fewer resorptive pits when compared with pMX-transduced controls (Fig. 2).

To further understand which stage of OC differentiation was blocked by the expression of SH2(N+C), real-time PCR was used to quantify expression of the early osteoclastogenic marker TRAP and the master transcription regulator of OCG NFATc1, the functional OC protease cathepsin K, and the calcitonin receptor expressed by mature OCs (Fig. 3). WT cells transduced with empty vector alone showed an up-regulation for all conventional markers of OCG during 4 days of culture in the presence of RANKL and M-CSF. In contrast, expression of OC differentiation markers was dramatically reduced in WT cells expressing SH2(N+C), indicating that this molecule targets signaling pathways involved early in the OC differentiation process.

**RANK Signaling Cascade Is Inhibited by SH2(N+C) in Vitro—**
To understand how SH2(N+C) inhibits OCG in vitro, we analyzed activation of signaling pathways downstream of RANK/
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RANKL stimulation, M-CSF/c-Fms stimulation, or vitronectin/α5β1 integrin ligation. WT day 2 pre-OCs expressing SH2(N+C) or vector control were stimulated with 100 ng/ml RANKL for 0–60 min, as indicated. Pre-OCs expressing SH2(N+C) failed to activate downstream RANK signals, as assayed by Western blot for phosphorylation of PLCγ2, Iκ-βα, and p38 and up-regulation of c-Fos and NFATc1 (Fig. 4A). Activation of JNK was unaffected (not shown). Furthermore, nuclear translocation of NFATc1 and phosphorylated c-Jun was inhibited by SH2(N+C) expression (Fig. 4B). In contrast, when pre-OCs were stimulated with 100 ng/ml M-CSF over the indicated time course, cells expressing SH2(N+C) induced phosphorylation of the downstream molecules AKT and ERK equivalent to cells expressing the vector alone (Fig. 4C). Phosphorylation of Pyk2, c-Src, and Iκ-βα was decreased in SH2(N+C)-expressing pre-OCs plated on vitronectin (Fig. 4D). All together, these results indicate that SH2(N+C) primarily inhibits the RANKL- and integrin-mediated signaling pathways, which are integral to OC differentiation.

SH2(N+C) Blocks Association of Gab2 with PLCγ2 and RANK—To understand the mechanism by which SH2(N+C) interferes with the RANKL signaling cascade, a co-immunoprecipitation assay of cell lysates from RANKL-stimulated pre-OCs was performed. Following RANKL binding to its receptor RANK, the signaling adaptor Gab2 is recruited to the RANK complex (20). Gab2 associates with PLCγ2 to stimulate phosphorylation of Iκ-βα leading to OCG (15, 20). SH2(N+C) dampened the association of PLCγ2 with Gab2 when compared with pre-OCs expressing vector only (Fig. 5A). Furthermore, SH2(N+C) also impaired the association of RANK with Gab2 but did not affect recruitment of TRAF6 to Gab2 (Fig. 5B). Thus, SH2(N+C) inhibits RANK signaling cascades by impeding protein scaffolding at the receptor.

SH2(N+C) Blocks OC Formation in Vivo—Finally, the potential efficacy of targeting PLCγ2 adaptor motifs to inhibit bone erosion was examined in vivo, using two established models of OCG. To facilitate these studies, adenoviral vectors were generated to express SH2(N+C) or β-galactosidase (LacZ) as a control. LacZ expression after one intra-articular injection of 10⁷ PFU was confirmed by 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal) staining (data not shown). In the first model, 100 μg of RANKL and 10⁷ PFU of Ad-SH2(N+C) or Ad-LacZ were administered to WT mice by subcutaneous injections over the calvaria daily for 5 days. Mice were sacrificed on day 6, and calvaria were prepared for histological examination to assess the number of TRAP⁺ OCs. WT mice receiving SH2(N+C) adenovirus displayed significantly
fewer OCs when compared with LacZ-expressing controls (Fig. 6A). Furthermore, the effect of SH2(N+C) in the context of inflammatory bone loss was examined using an established model of OCG initiated by a single LPS injection over the calvaria. 30 μg of LPS and 10⁷ PFU of SH2(N+C) or control LacZ adenovirus were injected over the calvaria at day 0 followed by 10⁷ PFU of SH2(N+C) or control LacZ adenovirus every other day for 5 days. Mice were sacrificed on day 6, and the calvaria were collected for histological analysis. Representative images of histological sections stained to detect TRAP⁺ OCs (red cells) and quantification of the percentage of quantification of OC surface per bone surface (Oc.S/B.S.) and OC number per bone surface (Oc.N/B.S.) are shown. Error bars indicate mean ± S.D. B, WT C57Bl/6 mice were treated with 30 μg of LPS and 10⁷ PFU of Ad-LacZ or Ad-SH2(N+C) by supracalvarial injection at day 0 followed by Ad-LacZ or Ad-SH2(N+C) every other day. Mice were sacrificed on day 6, and the calvaria were collected for histological analysis. Representative images of histological sections stained to detect TRAP⁺ OCs and quantification of OC surface per bone surface (Oc.S/B.S.) and OC number per bone surface (Oc.N/B.S.) are shown. Error bars indicate mean ± S.D.

**DISCUSSION**

Inhibitors that block the catalytic activity of PLC proteins, such as ET-18-OCH₃ and U73122, have been used to establish the function of PLCs and the importance of phosphatidylinositol 4,5-bisphosphate hydrolysis into inositol 1,4,5-trisphosphate and diacylglycerol in various cellular contexts. However, the major limitation of these inhibitors is lack of specificity. Using a genetic model consisting of Plcγ₂-deficient mice, we previously showed that PLCγ₂ is required for the maintenance of basal bone mass and for protection from inflammatory arthritis (13–15). Thus, the goal of the current study was to identify a specific approach to target endogenous PLCγ₂ function to negate pathological bone loss. PLCγ₂ is important for both OC differentiation and bone resorption. We previously proposed that in the context of OCG, PLCγ₂ serves a dual purpose both as an active lipase, leading to expression of the master osteoclastogenic transcription factor NFATc1, and as a critical scaffolding molecule facilitating NF-κB and AP-1 induction. Point mutations in the SH2
domains of PLC-γ2 (R564K/R672K) render BMMs unable to differentiate to OCs despite intact catalytic activity (18). Here, we show that the overall effect of the SH2(N+C) was inhibition of the RANK signaling cascade and suppression of OCG. Our observations support an independent scaffolding function of PLC-γ2 in which the SH2 motifs of PLC-γ2 mediate the association between RANK and Gab2. Our results suggest that ectopic expression of SH2(N+C) exerts a dominant-negative effect on formation of this complex by sequestering Gab2 and thus impairing its binding to the receptor RANK. These observations are consistent with modeling of intracellular RANK receptor interactions (21, 22). During the first hour of RANKL stimulation (early activation phase), PLC-γ2 is recruited to RANK, and Gab2 binds weakly to the recruited PLC-γ2 without interacting directly with RANK (21). Following prolonged RANKL stimulation (beyond 24 h), Gab2 may bind to the hepatic control region (HCR) of RANK and recruit TRAF6 (21, 22). Thus, it is hypothesized that the scaffolding function of PLC-γ2 is critical in the early phase, whereas its catalytic activity may mediate signaling during the early and late phases of RANKL stimulation (21, 22). Importantly, concurrent exposure to SH2(N+C) and RANKL can limit OCG in vitro and in vivo.

Gab2 belongs to a family of adaptor molecules that mediate signals downstream of a diverse set of receptors for molecules such as growth factors, antigens, cytokines, Toll-like receptor (TLR) agonists, and the RANK receptor. Gab2-deficient mice exhibit impaired OCG and a net increase in bone mass. Gab2 is also necessary for the differentiation of human progenitor cells into OCs (20). In vitro, BMMs from Gab2-deficient mice do not form mature OCs due to an inherent defect that correlates with reduced RANKL-mediated activation of NF-κB, Akt, and JNK. Similarly, ectopic expression of SH2(N+C) primarily affects RANKL signaling in OCs, without altering downstream signals from the M-CSF receptor, thus implying a good level of specificity for this type of targeted approach. Because Gab2-PLCγ2 complex is likely to form in response to various stimuli, inhibitory effects of SH2(N+C) in other contexts are possible and warrant further investigation.

In contrast to SH2(N+C), the anti-osteoclastic effects of molecules encompassing each single SH2 domain or the entire adaptor motif of PLC-γ2, consisting of the two SH2 domains along with the SH3 motif, were very minimal. It is likely that targeting only one SH2 domain is not sufficient to sequester adaptor molecules from forming a complex with RANK. However, it was surprising to find that a larger fragment encompassing the entire adaptor region of PLC-γ2 had less effect than SH2(N+C) molecule. Initial models of PLC-γ1, and perhaps PLC-γ2, function suggested that, at the three-dimensional structural level, the entire adaptor region (SH2(N+C)+SH3) forms a cap that regulates exposure of the catalytic domain to its substrate (17). Indeed, overexpression of SH2(N+C)+SH3 was shown to block phospholipase activity of cell lysates in vitro (16). Similarly, this region exerted a dominant-negative effect to reduce PLC-γ1 enzyme activity in prostate tumor cells and resulted in reduced tumor invasiveness (23). Although these previous studies clearly demonstrate that the entire adaptor region modulates catalytic activity, they do not clarify whether or not this region also mediates protein-protein binding. In OCs, the incomplete inhibition of OCG by SH2(N+C)+SH3 supports the hypothesis that SH2(N+C)+SH3 is not fully capable of blocking PLCγ2 catalytic activity.

SH2(N+C) anti-osteoclastogenic effects are also observed in vivo using two models of bone loss, consisting of RANKL or LPS supracalvarial injections. Although RANKL directly stimulates the differentiation of monocytes into OCs, LPS-induced bone loss is secondary to immune activation. In vivo immune phenotypes caused by genetic alterations of PLCγ2 have been described. We and others (12–14) have shown that PLCγ2 regulates inflammation and autoimmunity, specifically within the bone microenvironment. PLCγ2−/− mice are protected from immune activation in serum transfer arthritis and antigen-induced arthritis (13, 14). In humans, a dominantly inherited disease called PLAID (PLCγ2-associated antibody deficiency and immune dysregulation) is caused by mutant alleles of PLCγ2 in which small, in-frame genomic deletions eliminate part or all of the C-terminal SH2 domain of PLCγ2. Immune cells from these patients displayed functional deregulation, with increased activation in response to some stimuli and decreased activation in other circumstances (12). Although we did not directly test the effects of SH2(N+C) on immune cell activation or function, it is possible that SH2(N+C) may also modulate inflammatory responses involved in LPS-mediated bone loss in addition to directly targeting the OCs. Further studies are needed to examine potential anti-inflammatory effects of SH2(N+C). In conclusion, the results reported here provide strong evidence that PLC-γ2 adaptor function can be targeted in vitro and in vivo to suppress OCG and bone erosion.

Acknowledgment—The Washington University Center for Musculoskeletal Research was supported by National Institutes of Health Grant P30 AR057235.

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