PLCγ2 regulates osteoclastogenesis via its interaction with ITAM proteins and GAB2

Dailing Mao,1 Holly Epple,2 Brian Uthgenannt,1 Deborah V. Novack,3 and Roberta Faccio1

1Department of Orthopaedic Surgery and Cell Biology, 2Department of Medicine and Cell Biology, and 3Department of Medicine, Division of Bone and Mineral Diseases, Washington University School of Medicine, St. Louis, Missouri, USA.

Introduction
Bone erosion is a major hallmark of rheumatoid arthritis and is solely executed by the bone-resorbing cells, the osteoclasts (OCs) (1). These cells arise from macrophage precursors and differentiate into the mature polykaryon after stimulation with receptor activator of NF-κB ligand (RANKL) and M-CSF (2). OCs are recruited to sites of inflammation or differentiate at these sites owing to elevated levels of local RANKL secreted by cells in the inflamed tissue. Levels of TNF-α, IL-1, and M-CSF are also elevated during inflammatory responses and they collaborate with RANKL to further increase the rate of OC formation and bone resorption (3–6).

Targeted mutagenesis of the p50/p52 components of NF-κB and NF-κB pathways blocks in vivo osteoclastogenesis (10–12).

Excessive bone loss in arthritic diseases is mostly due to abnormal activation of the immune system leading to stimulation of osteoclasts. While phospholipase Cγ (PLCγ) isoforms are known modulators of T and B lymphocyte–mediated immune responses, we found that blockade of PLCγ enzymatic activity also blocks early osteoclast development and function. Importantly, targeted deletion of Pleg2 in mice led to an osteopetrotic phenotype. PLCγ2, independent of PLCγ1, was required for receptor activator of NF-κB ligand–induced (RANKL-induced) osteoclastogenesis by differentially regulating nuclear factor of activated T cells c1 (NFATc1), activator protein–1 (AP1), and NF-κB. Specifically, we show that NFATc1 upregulation is dependent on RANKL-mediated phosphorylation of PLCγ2 downstream of Dap12/Fc receptor γ (Dap12/FcRγ) receptors and is blocked by the PLCγ inhibitor U73122. In contrast, activation of JNK and NF-κB was not affected by U73122 or Dap12/FcRγ deletion. Interestingly, we found that in osteoclasts, PLCγ2 formed a complex with the regulatory adapter molecule GAB2, was required for GAB2 phosphorylation, and modulated GAB2 recruitment to RANK. Thus, PLCγ2 mediates RANKL-induced osteoclastogenesis and is a potential candidate for antiresorptive therapy.

Research article

The Journal of Clinical Investigation

American Society for Clinical Investigation

http://www.jci.org

Volume 116
Number 11
November 2006

2869

Nonstandard abbreviations used: AP1, activator protein–1; BMM, BM macrophage; FeRγ, Fc receptor γ; IP3, inositol-1,4,5-triphosphate; ITAM, immune receptor tyrosine activation motifs; NFATc1, nuclear factor of activated T cells c1; OB, osteoblast; OC, osteoclast; PLC, phospholipase C; RANK, receptor activator of NF-κB; RANKL, RANK ligand; SFK, Src family kinase; TRAP, tartrate-resistant acid phosphatase.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J Clin. Invest. 116:2869–2879 (2006). doi:10.1172/JCI28775.
The PLCγ family consists of 2 members, PLCγ1 and PLCγ2 (24–26). Both isoforms require phosphorylation on specific tyrosine residues for their catalytic activity. In addition, they contain 2 SH2 and 1 SH3 domains through which they can interact with other signaling proteins, suggesting adapter function. PLCγ1 is widely distributed, while PLCγ2 is primarily limited to cells of hematopoietic lineage (24). Mice deficient in PLCγ1 develop normally to embryonic day 8.5 but die soon after due to undefined defects in growth and development (28). The disruption of PLCγ2 in mice does not result in embryonic lethality, but animals show internal bleeding, decreased B cell number and function, defective platelet aggregation to collagen, and a block in Fc receptor–mediated responses in mast cells and NK cells (29–31).

Although NF-κB, AP1, and NFATc1 are all critical for RANKL-induced osteoclastogenesis (9, 18, 32), due to their ubiquitous expression, selective therapies targeting these signaling pathways in OCs are not yet in clinical use. In this study, we show that targeted deletion of Plcg2 in mice results in an in vivo osteopetrotic phenotype, independent of PLCγ1. In vitro, Plcg2-null cells fail to form multinucleated OCs and exhibit decreased RANKL-mediated NFATc1 expression; impaired JNK, c-Jun, and IκBα phosphorylation; and thus defective AP1 and NF-κB activation. Our data reveal a role for PLCγ2 in bone homeostasis apart from its contribution to B cell development. The combined effect of PLCγ2 on lymphocyte and OC differentiation could represent a novel target for the treatment of inflammatory osteolytic diseases.

Results

Inhibition of PLCγ enzymatic activity blocks OC differentiation and disrupts actin ring formation. PLCγ isoforms are known activators of the NFAT family of transcription factors in lymphoid cells, downstream of immune receptor signaling via ITAMs (33). Since NFAT and ITAMs are both critical regulators of OC differentiation and cells lacking the ITAM-containing adapters Dap12/FcRγ display severe osteopetrosis (20), we asked whether PLCγ family members were also required for efficient OC differentiation and function. We therefore tested the capacity of the PLCγ inhibitor U73122 to block OC formation and bone resorption. Addition of U73122 to the culture media blocked OC development, as demonstrated by the absence of tartrate-resistant acid phosphatase–positive (TRAP-positive) cells, compared with the numerous multinucleated OCs obtained in wells cultured only with RANKL and M-CSF (Figure 1A). Replacing the media with fresh osteoclastogenic media lacking the inhibitor restored the ability of the cells to differentiate, indicating that the compound was not toxic to the cells (Figure 1A). Culture of OCs on dentin in the presence of U73122 blocked bone resorption (Figure 1B). Furthermore, addition of U73122 to mature resorbing cells rapidly disrupted the organization of the actin rings, indicating that this compound can block bone resorption independent of its capacity to affect differentiation (Figure 1C).

PLCγ2 regulates bone mass in vivo. To determine whether PLCγ1, PLCγ2, or both are required for OC formation, we turned to the analysis of mice lacking these proteins. Plcg1−/− mice die in utero by day 9, rendering the analysis of their bone phenotype difficult. Plcg2−/− mice, however, are viable. Thus, femurs from 4-month-old sex-matched WT and Plcg2−/− mice were subjected to histological examination (Figure 2, A–E) and μCT analysis (Figure 2, F–I). Plcg2−/− mice had a more than 3-fold increase in the percentage of trabecular bone volume versus total bone volume compared with their WT counterparts, as determined by histological examination (Figure 2B) and as shown in μCT reconstructed 3D images (Figure 2F). Histomorphometric analysis also indicated that the number of osteoblasts (ObS) per bone perimeter was similar in WT and Plcg2−/− mice (Figure 2C), while the number of OCs per bone perimeter and the OC surface were significantly decreased in the null animals (Figure 2, D and E). The number of trabeculae (Figure 2G) and their thickness (Figure 2H) were significantly higher in the Plcg2−/− compared with WT mice, while the space between the trabeculae was smaller (Figure 2I). Thus, PLCγ2 regulates bone mass in vivo.

PLCγ2 is required for OC differentiation. To determine whether PLCγ2 modulates the capacity of ObS to form bone or to promote OC differentiation, primary ObS were isolated from newborn mice, and their capacity to mineralize extracellular matrix or induce osteo-

Figure 1

PLCγ inhibition blocks osteoclastogenesis and actin ring formation. (A) WT OCs were generated with RANKL (100 ng/ml) and M-CSF (10 ng/ml) in the presence of the PLC inhibitor U73122 (5 μM) for 4 days on plastic. In some wells, the media with the inhibitor was replaced with fresh osteoclastogenic media, and cells were allowed to differentiate for 4 more days (Withdrawal U73122). (B) WT OCs were grown on dentin with or without U73122 for 10 days. Cells were then removed and pits stained with hematoxylin red (Magnification, ×200). CTR, control. (C) WT OCs were generated on dentin in the absence of the inhibitor, then treated with vehicle or U73122 for 1 hour. Cells were fixed and actin stained using FITC-phalloidin (Magnification, ×200).
Osteopetrosis in mice lacking PLCγ2. (A) TRAP staining of decalcified histological sections of WT and Plcg2−/− proximal femurs. Magnification, ×40. (B–E) Quantitative analysis of bone parameters from histological sections of WT and Plcg2−/− bone femurs (n = 8) showing: percentage of bone volume versus total bone volume (BV/TV) (B); number of OBs (nOB) per bone perimeter (C); number of OCs (nOC) per bone perimeter (D); fraction of trabecular surface covered by OCs (E). (F) 3D reconstitution of μCT scans of WT and Plcg2−/− femurs. (G–I) 3D trabecular quantitative parameters of bone structure (n = 6). Graphs show mean ± SEM, with significant differences compared with WT indicated.
to modulate T cell and B cell receptor–mediated NF-κB activation, we hypothesized that this pathway could also be regulated by PLCγ2 in OCs. Nuclear extracts from WT and Plcg2−/− BMMs stimulated with RANKL were subjected to EMSA analysis, and data show that in the null cells, activation of NF-κB was virtually absent (Figure 5D, bottom panel). In agreement with this result, IkBα phosphorylation and nuclear translocation of p65 were also decreased in Plcg2−/− BMMs (Figure 5, B and C). Collectively, these data suggest that PLCγ2 plays a central role in RANKL-mediated osteoclastogenesis by controlling activation of AP1 and NF-κB and upregulation of NFATc1.

PLCγ2 is phosphorylated by RANKL via Dap12-mediated costimulatory signals in a Src family kinase–dependent manner. Having shown that PLCγ2 is required for the activation of osteoclastogenic signaling pathways downstream of RANKL, we turned to the mechanism by which PLCγ2 itself is activated in OCs. In immune cells, PLCγ isoforms are tyrosine phosphorylated in a Src family kinase–dependent (SFK-dependent) manner (24). Incubation of WT cells with the SFK inhibitor PP2 (5 μM) completely blocked RANKL-mediated PLCγ2 phosphorylation and NFATc1 upregulation (Figure 6A), demonstrating a concordant dependence on SFK in OC lineage cells.

To determine whether lack of PLCγ2 was abrogating the activation of PLCγ1 in response to RANKL and M-CSF, WT and Plcg2−/− OC precursors (BMMs grown in RANKL-containing media for 2 days) were stimulated with RANKL or M-CSF. Interestingly, RANKL-mediated PLCγ1 phosphorylation was barely measurable in both WT cells and cells lacking PLCγ2 (Figure 6B) despite detectable levels of PLCγ1 protein in both cell types. In contrast, a strong PLCγ1 phosphorylation signal was observed after M-CSF stimulation in WT and Plcg2−/− cells (Figure 6C).

Since costimulatory signals are required for RANKL-mediated calcium oscillation and NFATc1 expression, we determined whether PLCγ2 phosphorylation was dependent on the RANKL costimulatory receptors Dap12 and FcRγ. We found that PLCγ2 phosphorylation and NFATc1 nuclear localization were decreased in Plcg2−/− FcRγ−/− cells treated with RANKL (Figure 6D, E and F). Reexpression of Dap12, but not empty vector, in the double null OCs (Figure 6D) completely restored PLCγ2 phosphorylation (Figure 6E) and expression of NFATc1 in the nucleus (Figure 6F). Thus, PLCγ2 is phosphorylated downstream of Dap12 in an SFK-dependent manner, following RANKL stimulation.

PLCγ2 catalytic activity is required for NFATc1 upregulation but not for JNK and IkBα phosphorylation. Our data indicate that PLCγ2 regulates NFATc1 and activates JNK and IkBα. In contrast, Dap12/FcRγ modulates NFATc1 (Figure 6F) but not JNK (Figure 6E) or IkBα (20). Thus, we hypothesized that PLCγ2 regulates JNK and IkBα independent of Dap12/FcRγ. To determine whether PLCγ2 catalytic activity is required for JNK and IkBα phosphorylation, WT OC precursors, cultured in the presence of the PLC inhibitor U73122 (5 μM), were stimulated with RANKL for 0 to 60 minutes (Figure 7A). While dampening NFATc1 upregulation, the inhibitor had no effect on p-JNK and p-IkBα, suggesting that the catalytic activity of PLCγ2 is not required for phosphorylation of JNK and IkBα.

Mutations of 2 histidine residues, conserved among all PLC family members, have been shown to inhibit the enzymatic activity of PLCγ1 by 90%–95% in vitro (35). Thus, we generated a PLCγ2 catalytic inactive construct carrying the double histidine 327/372 to phenylalanine mutation (PLCγ2 H/F). This mutant was proven to be lipase inactive compared with WT full-length PLCγ2 in 293 cells (data not shown). Plcg2−/− cells transduced with PLCγ2 H/F were incapable of generating mature, TRAP-positive OCs compared with cells expressing WT PLCγ2 (Figure 7B) or of promoting NFATc1 upregulation (Figure 7C). In contrast, the ability of PLCγ2 H/F to mediate JNK and IkBα phosphorylation was...
indistinguishable from that of WT PLCγ2 (Figure 7D). These data indicate that PLCγ2 can modulate RANK-mediated signaling independent of its catalytic activity by acting as an adapter molecule. One potential interacting protein is GAB2, which associates with RANK and mediates RANK-induced activation of NF-κB and JNK but not NFATc1 (16). Furthermore, GAB2 has been shown to bind PLCγ2 in mast cells (36). To determine whether PLCγ2 binds to GAB2 in OCs, we reciprocally immunoprecipitated GAB2 and PLCγ2 and found that the 2 molecules associated (Figure 7E). Immunoprecipitation with IgG control antibodies revealed the absence of non-specific bands (data not shown). To further determine whether PLCγ2 modulates GAB2 activation or its recruitment to RANK, WT and Plcg2−/− BMMs, treated with RANKL, were immunoprecipitated with an anti-GAB2 Ab (Figure 7F) or with rabbit IgG control Ab (data not shown), followed by immunoblotting with phosphotyrosine (4G10) and RANK. Results showed that PLCγ2 modulates GAB2 phosphorylation and its association with RANK, suggesting that this may be the mechanism by which PLCγ2 controls AP1 and NF-κB activation.

TNF-α cannot reverse the arrested capacity of PLCγ2-null cells to became OCs. The data presented above show that PLCγ2 is central in RANKL signaling. In OCs, TNF-α appears to be the dominant cytokine mediating inflammatory osteolysis and augments the...
osteoclastogenic potential of RANKL-exposed OC precursors (3). Due to the capacity of TNF-α to phosphorylate JNK and IkBz in BMMs and OCs (37), we tested its effect in modulating the above-mentioned signaling pathways in cells lacking PLCγ2. TNF-α-mediated phosphorylation of JNK and IkBz occurred similarly in WT and null cells (Figure 8A). Furthermore, TNF-α was not capable of inducing PLCγ2 phosphorylation (Figure 8A), suggesting that the mechanism by which TNF-α regulates JNK and IkBz is PLCγ2 independent. This result prompted us to determine whether TNF-α could rescue the osteoclastogenic defect of Plcg2−/− cells. Interestingly, while TNF-α augmented RANKL-mediated osteoclastogenesis of WT cells (WT OCs without TNF-α, 19.3 ± 1.5/well; WT OCs with TNF-α, 45.3 ± 1.99/well), the cytokine did not have any effect on the null cells, which remained blocked at the early stage of OC development (Plcg2−/− OCs, not detected) (Figure 8B). Consistent with this finding, TNF-α, compared with RANKL, was not capable of promoting NFATc1 upregulation in WT or in PLCγ2-null cells (Figure 8C).

Discussion

The interaction between the immune and skeletal systems is a significant cause of pathological bone loss in rheumatoid arthritis, periodontal diseases, and tumor-associated bone metastasis (38). This exaggerated bone loss is most frequently due to abnormal activation of the immune system, leading to hyperstimulation and differentiation of the OCs (2, 39). The identification of signaling pathways or molecules common to the immune and bone systems could help in designing therapies for the variety of diseases that affect both bone and immunity. Recently, an activating point mutation in the murine PLCγ2 gene has been shown to lead to severe spontaneous inflammation and autoimmunity, mediated by B cells and non-B and non-T hematopoietic cells (40). The chronic inflammation resulted in severe arthritis of the small joints, leading to phalangeal erosion (40). This observation suggested that the activating mutation in PLCγ2 gene could also be responsible for the hyperactivation and recruitment of the OCs at the inflammatory sites.

In this study we have identified what we believe to be a novel role for the immunomodulatory protein PLCγ2 as a key factor in the regulation of bone homeostasis. Targeted deletion of Plcg2 led to increased bone mass due to blockade of OC differentiation in unmanipulated mice, with decreased RANKL-mediated signaling to API and NF-κB and defective upregulation of NFATc1, independent of PLCγ1. Despite the presence of normal levels of PLCγ1 in the Plcg2-null cells, PLCγ1 could not compensate for the lack of its cognate isomorph. Furthermore, we found that PLCγ2, and not PLCγ1, was
phosphorylated by RANKL. This result suggests that the 2 PLCγ isoforms exert different, nonoverlapping functions in OCs.

Differential regulation of the 2 PLCγ isoforms within the same cell type has been previously demonstrated in platelets, where activation of glycoprotein VI by collagen resulted in a substantial increase in tyrosine phosphorylation of PLCγ2 but not PLCγ1 (41, 42). Since activation of PLCγ isoforms is tightly regulated by the action of a number of receptor tyrosine kinases (RTKs) (24–26), we asked whether M-CSF, via its receptor c-Fms, could lead to phosphorylation of PLCγ1, PLCγ2, or both. We found that PLCγ1, and to a lesser extent PLCγ2, was phosphorylated by M-CSF. In support of this finding, analysis of Plcg1−/− ES cells generated few hematopoietic cells with a reduced level of monocyte/macrophage differentiation (29). Furthermore, we did not detect an M-CSF–dependent proliferative defect in BMMs and OCs lacking PLCγ2, and we observed that M-CSF–mediated activation of ERK and JNK occurred normally. These results indicate that PLCγ2 is not involved in M-CSF signaling and suggest that PLCγ1 may regulate this pathway in OC lineage cells.

The phosphorylation of PLCγ2 in response to RANKL was mediated in an SFK-dependent manner. PP2, a well-known Src kinase inhibitor, completely blocked RANKL-mediated PLCγ2 activation. Moreover, Dap12 was required for phosphorylation of PLCγ2 in response to RANKL stimulation. It is possible that SFKs phosphorylate PLCγ2 directly. However, it is more likely that SFKs phosphorylate the tyrosine residues in the ITAM motif of Dap12, leading to recruitment and activation of Syk, which in turn can activate PLCγ1, as occurs in other cell types (B cells and mast cells) (43, 44). We did not determine which Src family member is involved in PLCγ2 activation, since the inhibitor used blocked various SFKs. These results indicate that PLCγ isoforms are differentially phosphorylated in OCs, PLCγ2 primarily by RANKL and PLCγ1 by M-CSF.

AP1, NF-κB, and NFATc1 are members of well-described osteoclastogenic pathways activated by RANKL. We found that PLCγ2

Figure 7
PLCγ2 forms a complex with GAB2 and modulates its activation. (A) WT preOCs cultured with or without the PLC inhibitor U73122 (5 μM) for 3 days were stimulated with RANKL and subjected to Western blot analysis for phospho-JNK, phospho-IκBα, and NFATc1. β-Actin served as control. (B) WT and Plcg2−/− BMMs retrovirally transduced with empty vector (pMX), WT PLCγ2, or catalytically inactive PLCγ2 (PLCγ2 H/F) were cultured with RANKL (100 ng/ml) and M-CSF (10 ng/ml) for 7 days, and multinucleated OCs were detected by TRAP staining. Objective, ×10. (C) The same cells as shown in B were subjected to RANKL stimulation and Western blot analysis to detect NFATc1 expression. (D) Plcg2−/− BMMs retrovirally transduced with pMX, WT PLCγ2, or PLCγ2 H/F were treated with RANKL, and phosphorylation of IκBα and JNK was determined by Western blot analysis. PLCγ2 expression levels are shown. β-Actin served as loading control in C and D. (E) PLCγ2 and GAB2 were reciprocally immunoprecipitated in WT and Plcg2−/− BMMs treated with RANKL and subjected to Western blot analysis using anti-PLCγ2 and anti-GAB2 Abs, respectively. TCL, total cell lysate. (F) GAB2 was immunoprecipitated in WT and Plcg2−/− BMMs and subjected to Western blot analysis using anti-phosphotyrosine Ab (clone 4G10), anti-RANK, and anti-PLCγ2.
Based on the observation that PLCγ2 for activation of NF-κB and JNK, but not NFATc1, pathways (16). Based on the observation that PLCγ2 forms a complex with Gab2 in OCs and in other cell types (36), we hypothesized that PLCγ2 could regulate some RANKL signaling pathways by acting as an adapter molecule. One possibility is that PLCγ2 modulates the Gab2/RANK complex. In support of this hypothesis, we found that PLCγ2 is required for Gab2 phosphorylation and mediates Gab2 recruitment to RANK, suggesting that API1 and NF-κB activation might be dependent on the capacity of PLCγ2 to form a complex with Gab2. In addition, the finding that the PLC inhibitor U73122, which blocks IP3-mediated calcium influx (45), and a catalytically inactive form of PLCγ2 completely abrogate osteoclastogenesis by affecting NFATc1 upregulation suggest that both the catalytic activity and the adapter function of PLCγ2 are required for osteoclastogenesis. Thus, overall our data support the model (Figure 9) in which the catalytic activity of PLCγ2 is required for NFATc1 upregulation downstream of the ITAM motif of Dap12, while PLCγ2, acting as an adapter molecule, mediates Gab2 phosphorylation and its recruitment to RANK, leading to API1 and NF-κB activation.

The specific requirement of PLCγ2 for activation of NF-κB, API1, and NFATc1 signaling pathways is manifested in a basal in vivo osteopetrotic phenotype characterized by defective OC recruitment. Although the number of OCs in vivo was significantly reduced in Plcg2-null compared with WT animals, the presence of a few TRAP-positive cells could reflect the presence of a milieu of cytokines circulating in vivo that partially rescue the differentiation of PLCγ2-null OCs. TNF-α potently stimulates osteoclastogenesis by augmenting RANKL-mediated signaling pathways (46). TNF-α can also induce NF-κB and IkBα phosphorylation (37). Thus, we sought to determine whether TNF-α could reverse the ability of the PLCγ2-null cells to become OCs. Interestingly, while TNF-α could activate NF-κB and IkBα in Plcg2-null BMMs, it could not reverse their differentiation defect, most likely due to the inability of TNF-α to induce NFATc1 upregulation. In conclusion, our data indicate that PLCγ2 is required for the maintenance of bone homeostasis and RANKL-mediated osteoclastogenesis both in vitro and in vivo.

Previous studies reported that Plcg2-null mice have defective B cell maturation and mast cell activation (29). The inability of Plcg2-null cells to respond to both basal and inflammation-mediated...
osteoclastogenesis in vitro clearly supports the important role of PLCγ2 in osteoclast differentiation. Thus, the intrinsic defect of Pleg2−/− OCs together with the deficient B cell–mediated immune response suggest that targeting PLCγ2 could be a novel approach for antiresorptive therapies. Antirheumatic therapies directed to selectively deplete human CD20-positive B cells (e.g., Rituximab) or to directly target OC activation and function (RANKL inhibitors and bisphosphonates) have been very useful approaches in recent clinical trials (47). However, in most cases, only a combination of both treatments will resolve both the inflammatory and the ongoing osteolytic responses. Thus, PLCγ2 becomes an interesting target for the cure of rheumatoid diseases due to its dual role in the activation of a particular subset of immune cells, B lymphocytes, and for its capacity to control OC development in vivo. Therefore, a combined therapy affecting both the inflammatory and the osteolytic components of the rheumatoid disease might increase the hope that disease modification and remission in rheumatoid arthritis is a realistic goal.

Methods

Mice. Pleg2−/−, Dap12−/−, FeRγ−/−, and WT mice were on a C57BL/6 background and have been previously described (20, 29). All mice used in these experiments were 6−16 weeks old and bred and maintained with sterilized food, water, and bedding at the Animal Facility of the Washington University School of Medicine. All experiments were approved by the Animal Studies Committee of Washington University.

Histochemistry and μCT. Five-micron sections of fixed, decalcified, paraffin-embedded long bones were stained with either H&E or embedded in plastic for μCT. 3D images from intact mouse femurs were obtained on a μCT40 scanner (Scanco Medical).

Mouse OCs. BM Ms were isolated from long bones of 6- to 8-week-old mice as described previously (48). We obtained OCs by culturing BM Ms with glutathione-S-transferase–RANKL (GST-RANKL) (100 ng/ml) and M-CSF (10 ng/ml) for 5 days. For coculture experiments, OBs and BM Ms were mixed and cultured for 10–14 days in the presence of 1,25 Vit D3 (10−8 M). For rescue experiments, macrophages were obtained as described above; transduced with Flag-Dap12, Flag-PLCγ2, or Flag-PLCγ2 H/F; selected in puromycin- (for Dap12 constructs) or blastocystin-containing media (for PLCγ2 constructs) for 3 days; and cultured with M-CSF and RANKL to obtain OCs, as previously described (49). The PLCγ2 H/F mutation was made using a QuikChange XL Site-Directed Mutagenesis Kit (catalog 200517; Stratagene) following the manufacturer’s instructions.

Mouse OBs. We isolated OB precursors from calvariae of mice that were 4 days old, expanded them in media alone until confluent, and then cultured them with β-glycerophosphate and ascorbic acid to induce OB differentiation until bone nodules formed.

Western blot analysis and antibodies. BM Ms or OCs starved for 6 hours in serum-free medium were stimulated with RANKL (100 ng/ml) or M-CSF (100 ng/ml) and lysed at the times indicated in the Results and Figures 4–8 in RIPA lysis buffer supplemented with protease inhibitors and sodium orthovanadate. In some experiments, OCs were cultured in the presence of the Src inhibitor PP2 (5 μM; Calbiochem) or PLC inhibitor U73122 (5 μM; BIOMOL), starved, and then stimulated with RANKL. For Western blot analysis of phospho-PLCγ1 and -PLCγ2, phospho-ERK, phospho-JNK, phospho–c-Jun, phospho-ικB, and phospho-Src (Y416), we used polyclonal antibodies from Cell Signaling Technology. Polyclonal antisem against p65, NFATc1, and PLCγ2 were purchased from Santa Cruz Biotechnology Inc. The monoclonal antibody against β-actin was obtained from Sigma-Aldrich. Polyclonal GAB2 Ab was purchased from Upstate USA Inc. to obtain nuclear extract from RANKL-treated cells, plates were washed with H2O and cells lysed with hypotonic buffer (10 mM HEPES, 1.5 mM MgCl2, 1 mM KCl, 1 mM DTT, and protease and phosphatase inhibitors), followed by addition of 0.1% NP40. After centrifugation, the supernatants were collected (cytosolic fraction), while the pellets (nuclear fraction) were suspended in high-salt buffer (hypotonic buffer plus 400 mM NaCl). Extracts were quantified by a modified Coomassie method (Pierce). Nonradioactive EMSA was performed following the manufacturer’s instructions (Pierce) using AP1 and NF-κB biotinylated oligonucleotides (for AP1: 5′-GGGGAAAGTCCCCTCAACTT-3′ and 5′-CCACAAGATTCTGGGGACTC-3′; and cathepsin K using 5′-GAATGGCAGGGGACTTTCCCAGGCT-3′ and 5′-GGAAATGCCCTCCCTCAACTT-3′). An excess (200×) of nonbiotinylated oligonucleotides was used for competition control.

Immunofluorescence. WT or Dap12−/−, FeRγ−/− OCs retrovirally transduced with either the empty vector or full-length Dap12 were cultured on glass, fixed, and stained with anti-NFATc1 Ab (Santa Cruz Biotechnology Inc.), DAPI to detect the nuclei, and FITC-phalloidin to detect actin (48).

Real-time PCR. Total RNA was isolated from cell cultures at various times using the RNaseasy Mini Kit (QIAGEN) and was reverse transcribed to cDNAs using SuperScript II according to the manufacturer’s instructions (Invitrogen). Primers specific for murine NFATc1, TRAP, GAPDH, cathepsin K, and calcitonin receptor were used. For quantitative real-time PCR, NFATc1 was amplified using 5′-CCCGTCATGACTCTTGGCAGATTTCCAGACGG-3′ and 5′-CAATGAAACGTGTTGACCTAC-3′. TRAP was amplified using 5′-AGGTTGAGGGGAGCTTCCGATTTTCTGGCAGAT-3′ and 5′-ggggaaagttcccctcaacctt-3′. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control for normalization of the input RNA amount. The primers used were 5′-GTCTGACTATTGCTCAGCCGGATGATTCATGTCCCTTTCATGCT-3′ and 5′-CCATGAAACGTGTTGACCTAC-3′. TRAP was amplified using 5′-AGGTTGAGGGGAGCTTCCGATTTTCTGGCAGAT-3′ and 5′-GGGAAGTCCCTCCCTCAACTT-3′. An excess (200×) of nonbiotinylated oligonucleotides was used for competition control.

Figure 9

PLCγ2 in RANKL signaling. PLCγ2 is phosphorylated by RANKL in an SFK-dependent manner downstream of Dap12/FeRγ. Activation of PLCγ2 and PLCγ2 catalytic activity are required for NFATc1 upregulation (right arm). PLCγ2 can also bind GAB2 and modulate its activation and recruitment to the RANK-signaling complex (left arm). This interaction might be required for activation of the IkBα/NF-κB and JNK/AP1 pathways independent of Dap12/FeRγ.
Acknowledgments

We gratefully acknowledge Yousef Abu-Amer (Department of Orthopaedic Surgery, Washington University) and Marco Colonna (Department of Pathology and Immunology, Washington University) for helpful suggestions and discussion; James N. Ihle for Pgl2−/− mice (St. Jude Children’s Research Hospital, Memphis, Tennessee); and Matilda Katan for the full-length PLCγ2 construct (Cancer Research UK Centre for Cell and Molecular Biology, Chester Beatty Laboratories). This work was supported by departmental funding from the Orthopaedic Surgery Department, Washington University School of Medicine; by the Arthritis Foundation (R. Faccio); and by an American Society for Bone and Mineral Research career award (to R. Faccio); and by NIH grants (AR47846 and AR48335 to D.V. Novack and AR52911 to R. Faccio).

Received for publication April 7, 2006, and accepted in revised form August 29, 2006.

Address correspondence to: Roberta Faccio, Washington University School of Medicine, Department of Orthopaedic Surgery, Campus Box 8233, 1 Barnes Jewish Hospital Plaza, Suite 11300, St. Louis, Missouri 63110, USA. Phone: (314) 747–6602; Fax: (314) 362-0334; E-mail: faccior@wustl.edu.
signaling: integration of protein tyrosine kinase functions. 


