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Holly Epple
Washington University School of Medicine in St. Louis

Viviana Cremasco
Washington University School of Medicine in St. Louis

Kaihua Zhang
Washington University School of Medicine in St. Louis

Dailing Mao
Washington University School of Medicine in St. Louis

Gregory D. Longmore
Washington University School of Medicine in St. Louis

See next page for additional authors

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Phospholipase Cγ2 Modulates Integrin Signaling in the Osteoclast by Affecting the Localization and Activation of Src Kinase▽†

Holly Epple,[1,2]‡ Viviana Cremasco,[3]‡ Kaihua Zhang,[3] Dailing Mao,[3] Gregory D. Longmore,[1,2]* and Roberta Faccio[2,3]*

Departments of Medicine,[1] Cell Biology,[2] and Orthopedics,[3] Washington University, St. Louis, Missouri

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Integrin engagement induces a cascade of signaling pathways that include tyrosine phosphorylation of numerous proteins that lead to modulation of the actin cytoskeleton. Src is a major intracellular mediator of integrin-dependent functions, but the mechanism(s) by which Src is regulated in response to integrin signals is not fully understood. Here, we demonstrate an important role for phospholipase C gamma 2 (PLCγ2) in Src activation in the osteoclast. Through analysis of primary cells from PLCγ2$^{-/-}$ mice, PLCγ2 was found to be an important regulator of $\alpha_v\beta_3$ integrin-mediated bone osteoclast cell adhesion, migration, and bone resorption. Adhesion-induced PYK2 and Src phosphorylation is decreased in the absence of PLCγ2, and the interaction of Src with β_3 integrin and PYK2 is dramatically reduced. Importantly, PLCγ2 was found to be required for proper localization of Src to the sealing actin ring, and this function required both its catalytic activity and adapter domains. Based on these results, we propose that PLCγ2 influences Src activation by mediating the localization of Src to the integrin complex and thereby regulating integrin-mediated functions in the osteoclast.

Integrins are transmembrane proteins that serve as a link between the extracellular matrix and the cellular cytoskeleton and are a primary means by which the cell interacts with and responds to its environment (16, 19). Integrin engagement induces various intracellular signaling responses leading to reorganization of the actin cytoskeleton, including activation of protein tyrosine kinases (5, 12, 15, 16). By modulating these signaling pathways, integrins can affect cellular processes such as adhesion, migration, proliferation, and apoptosis (18, 33).

Integrins are intimately linked to the function of some specialized cell types, such as bone-derived osteoclasts. Osteoclasts are giant, multinucleated cells that form from hematopoietic bone marrow precursor cells in response to the osteoclastogenic factors receptor activator of NFκB ligand (RANKL) and macrophage colony stimulating factor (M-CSF). Osteoclasts remove mineralized bone matrix during bone modeling and remodeling (4, 36). In order to resorb bone, the osteoclast must adhere tightly to the bone surface and does so by reorganizing the actin cytoskeleton to establish a sealing zone. Next, acids and lysosomal enzymes must be secreted into the resorption lacunae to break down the mineralized bone matrix. Finally, osteoclasts migrate over the bone surface so as to sustain resorptive activity (2, 31).

The bone-resorptive function of osteoclasts is dependent on integrin receptor engagement with the bone extracellular matrix (26, 27, 32). Osteoclasts specifically express high levels of the $\alpha_v\beta_3$ integrin (7, 26). Mice lacking β_3 integrin exhibit late-onset osteopetrosis, and osteoclasts from these mice are defective in adhesion, spreading, actin ring formation, and bone resorption in vitro (9, 22). Since integrins are crucial to the unique biologic function of osteoclasts and primary osteoclasts can be readily isolated and cultured ex vivo, osteoclasts provide a particularly good cellular system in which to elucidate integrin-mediated signaling mechanisms and correlate these mechanisms with relevant biologic function.

Activation of integrin receptor complexes leads to remodeling of the actin cytoskeleton through the formation and activation of a large signaling complex called the "adhesion complex" (7). The adhesion complex contains enzymes and adaptor and scaffolding proteins. Src and PYK2, two enzymes implicated in signaling from the β_3 integrin, have been shown to promote bone resorption (3, 8). Following $\alpha_v\beta_3$ integrin engagement, c-Src is phosphorylated at Tyr416, activating its kinase activity and inducing an interaction between the Src homology 2 (SH2) domain of Src and PYK2 (1, 8). Calcium signals lead to PYK2 autophosphorylation and subsequent further tyrosine phosphorylation by Src (8, 17).

Recent findings have demonstrated the importance of immune receptor-activated motif (ITAM)-containing receptors Dap12 and FcRγ in modulation of integrin signaling events (23). Deletion of Dap12 in osteoclasts leads to dysfunctional cells with aberrant actin organization and impaired bone-resorptive capacity (9). Indeed, c-Src, the $\alpha_v\beta_3$ integrin, and the ITAM motif of Dap12 appear to act in concert to modulate osteoclastic bone resorption (40). However, in these cells ac-

* Corresponding author. Mailing address for Roberta Faccio: Washington University School of Medicine, Department of Orthopedics, 660 South Euclid, Campus Box 8233, St. Louis, MO 63110. Phone: (314) 747-4602. Fax: (314) 362-0334. E-mail: faccior@wudosis.wustl.edu. Mailing address for Gregory Longmore: Washington University School of Medicine, Division of Hematology, 660 S. Euclid Ave., Campus Box 8125, St. Louis, MO 63110. Phone: (314) 362-8834. Fax: (314) 362-8826. E-mail: glongmor@im.wustl.edu.

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‡ These authors contributed equally to the work.

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tivation of Src occurred independently of the ITAM receptor (40). Thus, the means by which c-Src is recruited to the $\alpha_v\beta_3$ receptor, fully activated, and modulates integrin signaling, particularly in osteoclasts, remains to be elucidated.

Phospholipase C gamma (PLCγ) is activated downstream of ITAM-containing receptors in several hematopoietic cells. Two isoforms exist, PLCγ1 and PLCγ2; the former is more widely expressed, while the latter is confined to cells of the hematopoietic compartment. Like other PLC proteins, PLCγ regulates intracellular calcium levels and protein kinase C activation via cleavage of the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP_2) (6). PLCγ is unique among the PLC family members, however, in that in addition to its enzymatic activity, PLCγ can also act as an adaptor protein via two SH2 domains and an SH3 domain (6).

Recent data have implicated PLCγ in integrin-mediated functions. Phosphorylation of PLCγ1 at integrin complexes in fibroblasts contributes to motility regulation (14), while in platelets, PLCγ2 phosphorylation, downstream of the $\alpha_{IIb}\beta_3$ integrin, is essential for cell spreading (38). Engagement of the $\alpha_v\beta_3$ integrin in osteoclasts also induces phosphorylation of PLCγ2 in a Src-dependent manner (25), but the significance of integrin-mediated PLCγ2 signaling to osteoclast function is not known.

PLCγ2$^{-/-}$ mice are osteopetrotic and have decreased numbers of osteoclasts in vivo and in vitro due to defective NFAT2 upregulation (20). NFAT2 is a critical transcription factor activated by RANKL and controls osteoclast differentiation (21). Interestingly, NFAT2 overexpression promotes osteoclastogenesis even in the absence of RANKL signals (21). Here, we show that exogenous expression of NFAT2 in PLCγ2$^{-/-}$ cells is able to restore the abilities of PLCγ2$^{-/-}$ cells to differentiate into mature osteoclasts, but these cells cannot resorb bone. Utilizing primary cells from PLCγ2$^{-/-}$ mice, we show that PLCγ2 is required for the integrin-dependent functions of osteoclast adhesion, migration, and bone resorption. Following integrin engagement, PLCγ2 was found to affect Src activation by modulating the efficient recruitment of Src to the osteoclast actin ring at the plasma membrane and thus influencing the adhesion-induced associations between Src, β_3 integrin, and PYK2. We conclude that PLCγ2 is an important mediator of integrin signaling in the osteoclast.

MATERIALS AND METHODS

Primary cell culture. Wild-type (WT) and PLCγ2$^{-/-}$ mice on a C57BL/6 background have been previously described (37). Bone marrow-derived macrophages (BMDM) were isolated from 6- to 8-week-old mice as previously described (9, 24). Briefly, the marrow spaces of mouse long bones were flushed with serum-free alpha medium (Gibco). Isolated cells were plated at a concentration of 5×10^6 cells per p100 petri dish and grown in the presence of 100 ng/ml M-CSF for 2 to 5 days. To form osteoclasts, macrophages were cultured on tissue culture-treated dishes with 100 ng/ml glutathione *S*-transferase–RANKL and 10 ng/ml M-CSF for 5 days, with fresh medium added every 48 h.

Plasmids, retrovirus generation, and antibodies. Full-length PLCγ2 was obtained from M. Katan (Cancer Research UK Centre for Cell and Molecular Biology, Chester Beatty Laboratories) and cloned into the blasticidin-resistant pMX retroviral vector. PLCγ2 mutants were made using a QuikChange XL site-directed mutagenesis kit (catalog no. 200517; Stratagene), following the manufacturer's instructions. NFAT2 was obtained from A. Rao (Harvard Medical School, Boston, MA) and cloned into the puromycin-resistant pMX retroviral vector. To generate retrovirus, PLAT-E cells, which stably express retroviral packaging genes (24), were transfected with expression vector by using Transit transfection reagent and the manufacturer's protocols (Mirus, Madison, WI). Viral supernatants were collected on day 2 and day 3 posttransfection and immediately used to infect freshly isolated BMDM. After 24 h, medium containing the appropriate selection (2 μg/ml puromycin or 1 μg/ml blasticidin) was added to cells for 48 h to select for expressing cells. For Western blotting and immunofluorescence analysis, the following antibodies were used: anti-PLCγ2, PLCγ1, NFAT2, polyclonal Src, actin (Santa Cruz), β_3 integrin, PYK2 (BD), p402 PYK2 (Biosource), p416 Src (Cell Signaling), and monoclonal Src (generous gift from Steve Teitelbaum).

Cell adhesion, migration, and bone resorption. BMDM were treated with differentiation medium for 2 days to produce prefusion osteoclasts (preOCs). Cells were starved for 6 h, lifted from the dish by using 0.5 mM EDTA in phosphate-buffered saline (PBS) and a cell lifter (Corning Inc.), and plated on glass coverslips coated with 5 μg/ml vitronectin (BD Biosciences). After various times, cells were fixed in 4% paraformaldehyde for 10 min. The number of adherent cells per field was enumerated using a light microscope (Olympus).

The bottoms of 8-μm-pore Boyden chamber membranes (Corning Inc.) were coated with 5 μg/ml vitronectin and blocked in 2% heat-inactivated bovine serum albumin for 30 min. Serum-free medium with or without 100 ng/ml M-CSF was added to the bottom chamber, and 100,000 day 2 preOCs were added to the upper Boyden chamber membrane in serum-free medium. Cells were allowed to migrate for 6 to 8 h at 37°C. Cells remaining on the upper membrane were removed by mechanical force while cells on the bottom membrane were fixed in 100% methanol for 15 min. Cells were stained with modified Giemsa stain (Sigma), and the number of cells per field was enumerated using an inverted light microscope (Olympus).

Bone resorption analysis was completed as described previously (39). Briefly, macrophage cells were plated on dentin slices and cultured with 10 ng/ml M-CSF and 100 ng/ml RANKL for 10 days, with fresh medium 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). For development, 3,3′-diaminobenzidine (0.52 mg/ml in PBS containing 0.1% H_2O_2) was added onto the bone slices for 15 min. Bone resorption pits were analyzed using a light microscope (Olympus) and quantified using Image J software.

Immunoprecipitation and kinase assays. For immunoprecipitation, cells were harvested in lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 10% glycerol) supplemented with protease inhibitors and clarified by centrifugation. The protein concentration of each sample was determined using bicinchoninic acid protein assay (Pierce) and normalized. Samples were incubated with primary antibody overnight at 4°C and with protein G agarose beads (Amersham) for 1 h. Beads were washed three times in lysis buffer and immunoprecipitates subjected to Western blotting.

For Src kinase assays, preOC cells were starved for 6 h and then stimulated for 45 min on a vitronectin-coated surface. Whole-cell lysates were collected in lysis buffer (see above), normalized, and immunoprecipitated with 2 μg monoclonal Src antibody for 1 h at 4°C. Beads were washed two times in lysis buffer and one time in reaction buffer (Upstate). The Src kinase assay was completed following the manufacturer's instructions (Upstate Biotechnology).

Lipase assay. Infected cells were harvested in lysis buffer, and WT PLCγ2 or PLCγ2 mutants were immunoprecipitated using a mouse anti-FLAG M2-agarose beads (Sigma). After 2 h, beads were washed and resuspended in lipase buffer (50 mM NaH_2PO_4, 100 mM KCl, 0.8 mM EGTA, 0.8 mM $CaCl_2$) plus 0.15% OG (*N*-octyl-β-D-glucopyranoside). Tritium-labeled PIP_2 was added to the sample for 15 min, and then inorganic phase was recovered using a mixture of HCl, KCl, and chloroform. Scintillation fluid was added to the inorganic phase, and radioactive counts were determined.

Immunofluorescence and microscopy. Cells were fixed with 4% paraformaldehyde for 10 min and permeabilized in blocking buffer (0.015% Triton X-100 [Sigma], 3% normal goat serum, and 1% immunoglobulin G-free bovine serum albumin [Jackson Immunological Research] in PBS) for 30 min. Primary antibody was added for 12 h at 4°C and then rinsed three times with PBS. Secondary antibody was added for 1 to 2 h at room temperature and then washed three times with PBS. Coverslips were incubated with fluorescein isothiocyanate- or CY3-phalloidin (Molecular Probes) and DAPI (4′,6′-diamidino-2-phenylindole) (to identify nuclei) for 10 min at room temperature, washed, and mounted on glass slides. Slides were analyzed using an Olympus IX70 inverted microscope with either a 10× APO, 0.4-numerical-aperture lens or a 60× APO, 1.4-numerical-aperture lens. Images were captured with a CoolSnap camera (Roper Scientific) and analyzed using Metamorph software (Universal Imaging Corporation). All images from the same set of experiments were taken without changing the camera settings.

RESULTS

Overexpression of NFAT2 rescues osteoclast differentiation defect of PLCγ2$^{-/-}$ BMDM but not bone resorption. Loss of PLCγ2 in mice results in osteopetrotic bones (20). While osteoclasts are present in the bones of PLCγ2$^{-/-}$ mice, their numbers are significantly reduced, and primary BMDM from PLCγ2$^{-/-}$ mice fail to became mature, multinucleated osteoclasts when treated with RANKL and M-CSF in ex vivo cultures (20). This defect in differentiation is due to a decreased upregulation of NFAT2 (20). Since overexpression of NFAT2 in BMDM has been shown to increase the RANKL-induced differentiation capacities of these cells (21), we reasoned that overexpression of NFAT2 in the PLCγ2$^{-/-}$ BMDM could rescue the defect in osteoclast differentiation. BMDM were isolated from the long bones of WT and PLCγ2$^{-/-}$ mice and retrovirally transduced with empty vector, NFAT2, or PLCγ2. After selection, cells were treated with M-CSF and RANKL for 5 to 7 days to induce osteoclast differentiation. Cells were then fixed and stained to identify tartrate-resistant acid phosphatase-positive (TRAP$^+$), multinucleated osteoclasts. WT cells expressing both empty vector and NFAT2 formed large, TRAP$^+$ osteoclasts (Fig. 1A and B). Expression of NFAT2 in WT cells increased the number of osteoclasts formed compared to expression of empty vector alone (Fig. 1F), as previously observed (21). PLCγ2$^{-/-}$ cells expressing empty vector did not form osteoclasts (Fig. 1C); however, expression of either NFAT2 or PLCγ2 in PLCγ2$^{-/-}$ cells was able to rescue the defect in osteoclast differentiation (Fig. 1D and E). Notably, expression of NFAT2 in PLCγ2$^{-/-}$ cells did not promote differentiation to the same extent as that seen with WT cells overexpressing NFAT2 (Fig. 1F). Importantly, we did not detect any difference in RANK (the receptor for RANKL) expression levels in the two cells types either with or without NFAT2 expression (Fig. 1G). These results confirmed that upregulation of NFAT2 is an essential function of PLCγ2 in promoting osteoclast differentiation and that osteoclast differentiation of PLCγ2$^{-/-}$ cells could be rescued by simply overexpressing NFAT2.

Since the ability to remodel and resorb the bone is a unique feature of the osteoclast, we asked whether overexpression of NFAT2 could rescue the capacities of PLCγ2$^{-/-}$ osteoclasts to resorb bone. WT and PLCγ2$^{-/-}$ BMDM transduced with either NFAT2 or PLCγ2 were plated on a dentine bone surface in the presence of M-CSF and RANKL. WT osteoclasts expressing either empty vector or NFAT2 displayed numerous actin rings (Fig. 1H and inserts). Although PLCγ2$^{-/-}$ cells expressing NFAT2 formed mature osteoclasts, the actin rings appeared abnormal. Compared to WT or PLCγ2$^{-/-}$ cells rescued with full-length PLCγ2, they were small and contained central dots of actin condensed in the middle (Fig. 1H, phalloidin-stained images). Mirroring the abnormal actin ring, PLCγ2$^{-/-}$ osteoclasts expressing NFAT2 exhibited decreased bone-resorptive capacity compared to PLCγ2-expressing cells (Fig. 1H [quantified in Fig. 1I]). In sum, although expression of NFAT2 rescued the PLCγ2$^{-/-}$ defect in osteoclast differentiation, PLCγ2 was critical for proper osteoclast actin ring formation and bone resorption.

PLCγ2 is expressed in mature osteoclasts and localizes to the actin ring. To determine the role of PLCγ2 in osteoclast function, we first examined the expression of PLCγ isoforms in response to RANKL-induced osteoclast differentiation. BMDM from WT and PLCγ2$^{-/-}$ mice were treated with M-CSF and RANKL to induce osteoclast differentiation. Total cell lysates were collected every day for 5 days and Western blotted to determine the levels of PLCγ2 and PLCγ1 protein. RANKL-induced osteoclast differentiation did not alter the levels of either isoform (Fig. 2A). Importantly, PLCγ1 levels were not altered in PLCγ2$^{-/-}$ cells compared to those in WT cells, suggesting that PLCγ1 did not compensate for the loss of PLCγ2 (Fig. 2A).

Next, we set out to identify the subcellular localization of PLCγ2 in primary osteoclasts. WT and PLCγ2$^{-/-}$ BMDM were retrovirally transduced with empty vector or NFAT2 and grown in the presence of RANKL to form mature osteoclasts. PLCγ2 was found to localize to the edge of the WT osteoclast, colocalizing with the actin ring (Fig. 2B, merge in yellow). PLCγ2 localization was not altered by overexpression of NFAT2, since the immunostaining pattern was the same in WT cells expressing either empty vector or NFAT2 (Fig. 2B, top and middle rows). As an antibody specificity control, PLCγ2$^{-/-}$ osteoclasts expressing NFAT2 were probed with the PLCγ2 antibody (Fig. 2B, bottom row). That PLCγ2 localized to the actin rings of mature osteoclasts, combined with the observations that PLCγ2-null cells formed abnormal actin rings and failed to resorb bone, strongly suggested that PLCγ2 governed critical integrin-dependent signaling events important for osteoclast function.

PLCγ2 promotes preOC adhesion, spreading, and haptotactic migration. $\alpha_v\beta_3$ integrin function is critical for osteoclast adhesion, migration, and ultimately bone resorption (9, 22). Since PLCγ2 has been biochemically implicated as a potential component of the integrin adhesive complex (25), we asked whether PLCγ2 contributed to $\alpha_v\beta_3$ integrin function in the osteoclast. Although PLCγ2$^{-/-}$ BMDM fail to undergo terminal osteoclast differentiation, they could form mononuclear preOCs when exposed to RANKL for 2 days, as demonstrated by the fact that the levels of expression of β_3 integrin and Src, early markers of osteoclast commitment, were similar to those in WT cells (Fig. 3A). Thus, we tested the adhesion capacities of WT and PLCγ2$^{-/-}$ preOCs plated on vitronectin-coated plates for the indicated times. There were decreased numbers of adherent PLCγ2$^{-/-}$ preOCs at all time points examined compared to WT control levels (Fig. 3B), and importantly, all null cells had round morphologies, suggesting a spreading defect (Fig. 3C). Expression of PLCγ2 in PLCγ2$^{-/-}$ cells rescued the defects in adhesion and spreading (Fig. 3B and C). NFAT2 overexpression in PLCγ2$^{-/-}$ preOCs did not rescue the adhesive defect of these cells, indicating that NFAT2 expression, per se, did not affect integrin-mediated adhesion (data not shown).

Next, the abilities of WT and PLCγ2$^{-/-}$ preOCs to migrate were determined using a Boyden chamber assay. Haptotactic-stimulated migration toward the vitronectin matrix (−M-CSF) and M-CSF-induced chemotactic migration (+M-CSF) were determined by counting the number of cells that migrated to the bottom of the Boyden chamber membrane. PLCγ2$^{-/-}$ preOCs exhibited decreased haptotaxis toward vitronectin relative to WT preOCs (Fig. 3D). In both WT and PLCγ2$^{-/-}$ preOCs, the addition of M-CSF induced twofold increases in

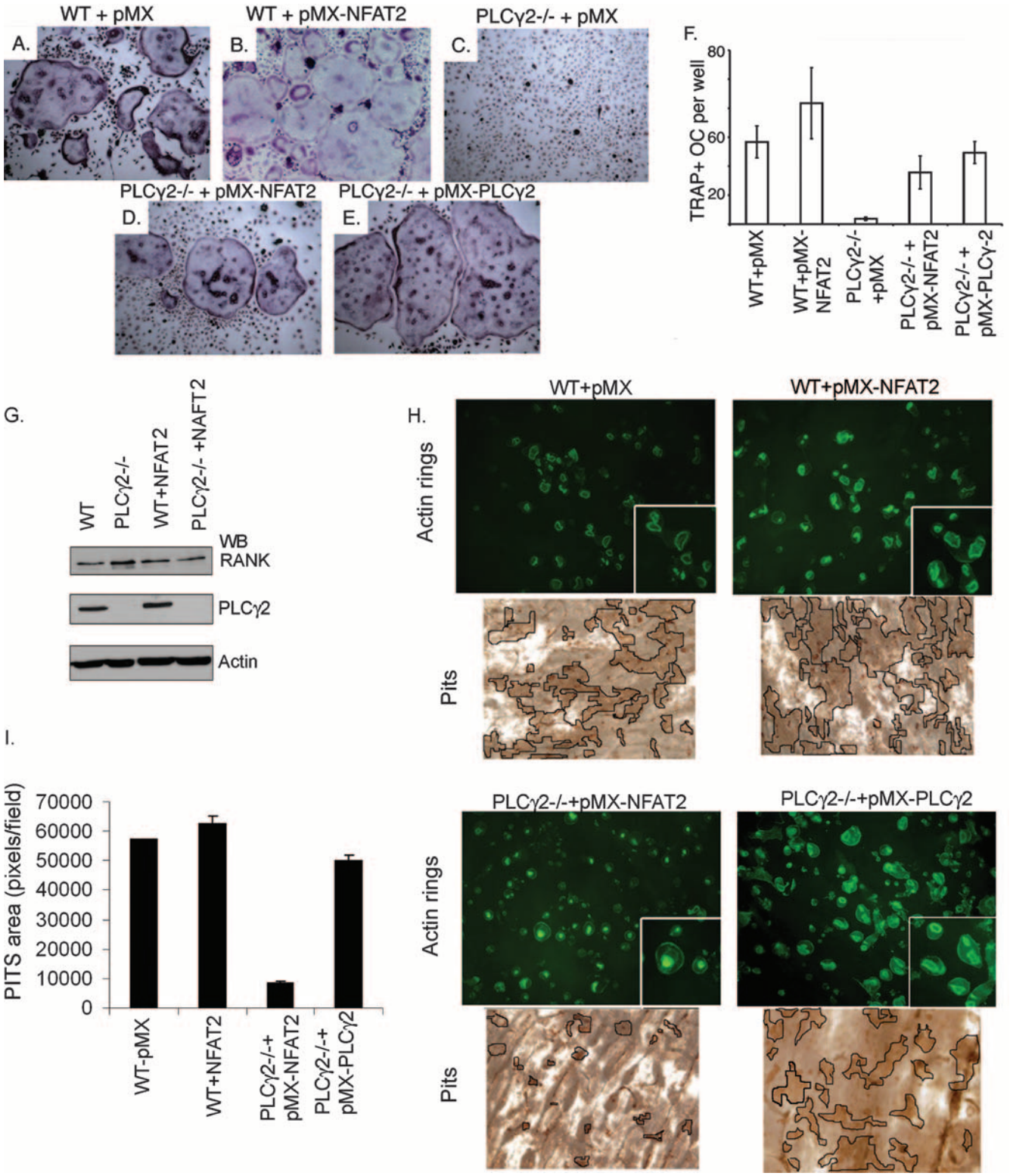

FIG. 1. Overexpression of NFAT2 rescues the defect in RANKL-dependent osteoclast differentiation of PLCγ2$^{-/-}$ cells but not bone resorption. (A to E) WT and PLCγ2$^{-/-}$ BMDM were retrovirally infected with pMX (empty vector) (A, C), pMX-NFAT2 (B, D), or pMX-PLCγ2 (E). Cells were grown in the presence of 10 ng/ml M-CSF and 100 ng/ml RANKL for 5 to 7 days, fixed, and TRAP stained. TRAP^{+} osteoclasts were observed using a light microscope with a 10× objective. (F) The number of TRAP^{+} osteoclasts per well was determined and graphed for each sample. (G) WT and PLCγ2$^{-/-}$ BMDM were retrovirally infected with empty vector (pMX) or NFAT2 and grown in the presence of 10 ng/ml M-CSF and 100 ng/ml RANKL. After 3 days, cells were lysed and Western blotted (WB) for the indicated protein. (H) WT and PLCγ2$^{-/-}$ BMDM retrovirally infected with empty vector (pMX), NFAT2 (pMX-NFAT2), or PLCγ2 (pMX-PLCγ2) were cultured on dentin for 10 days with RANKL and M-CSF, fixed, and stained with fluorescein isothiocyanate-phalloidin to detect actin rings (top row; 10× objective). Inserts were enlarged fourfold. Cells were removed with 2 N NaOH and resorption pits visualized by staining with peroxidase-conjugated wheat germ agglutinin. Pits were analyzed by light microscopy with a 10× objective. Black lines delineate the resorbed areas (bottom row). (I) Area of bone resorption per field was determined using Image J software and graphed for each sample.

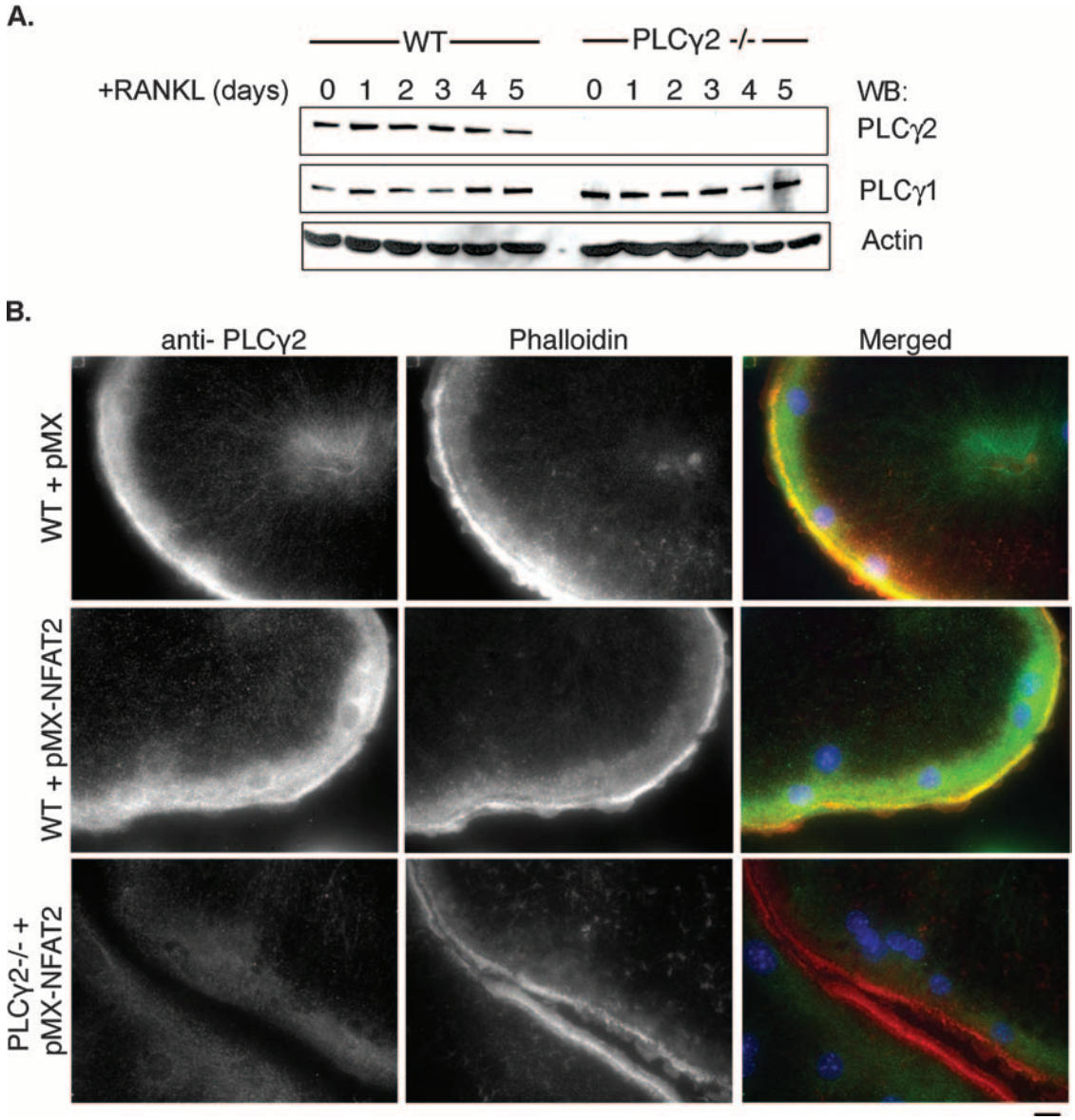

FIG. 2. PLCγ2 expression is stable during osteoclast differentiation, and PLCγ2 is localized to the actin ring in the osteoclast. (A) WT and PLCγ2$^{-/-}$ BMDM were grown in the presence of 10 ng/ml M-CSF and 100 ng/ml RANKL for 5 days. Whole-cell lysates were collected every 24 h and Western blotted (WB) for the indicated proteins. (B) WT and PLCγ2$^{-/-}$ BMDM were retrovirally infected with pMX (empty vector) (top row) or pMX-NFAT2 (bottom two rows). Osteoclasts were probed with PLCγ2 polyclonal antibody and costained with Cy3-phalloidin and DAPI. Images were obtained with an Olympus IX70 inverted fluorescence microscope using a 60× APO, 1.4-numerical-aperture lens. Scale bar, 5 μm.

migration compared to the level for vitronectin alone (Fig. 3D). This indicated that the response of PLCγ2$^{-/-}$ preOCs to M-CSF-stimulated chemotaxis was not altered; rather, the migration defect resulted primarily from an impaired haptotactic response to vitronectin, an integrin-dependent function. These data indicated that PLCγ2 played an important role in osteoclast adhesion, spreading, and migration, all integrin-dependent processes important for the bone-resorptive function of the osteoclast.

PLCγ2 promotes activation of Src and PYK2 in response to integrin engagement. How, then, does PLCγ2 influence integrin function in the osteoclast? To determine whether the absence of PLCγ2 influenced integrin complex-associated kinase activity, we compared the integrin-induced tyrosine phosphorylation (i.e., activation) levels of Src and PYK2 in WT and PLCγ2$^{-/-}$ preOCs following adhesion to vitronectin. PLCγ2$^{-/-}$ cells showed decreased phosphorylation of tyrosine 416 of Src and tyrosine 402 of PYK2, which was normalized following retroviral reintroduction of PLCγ2 into PLCγ2$^{-/-}$ cells (Fig. 4A). Since PLCγ2$^{-/-}$ cells expressed normal levels of PLCγ1 (Fig. 2A), it was unlikely that PLCγ1 was compensating for the absence of PLCγ2. To confirm the nonredundant role of these two isoforms, their activation following adhesion was determined. Only PLCγ2, and not PLCγ1, became phosphorylated following attachment to vitronectin (see Fig. S1 in the supplemental material).

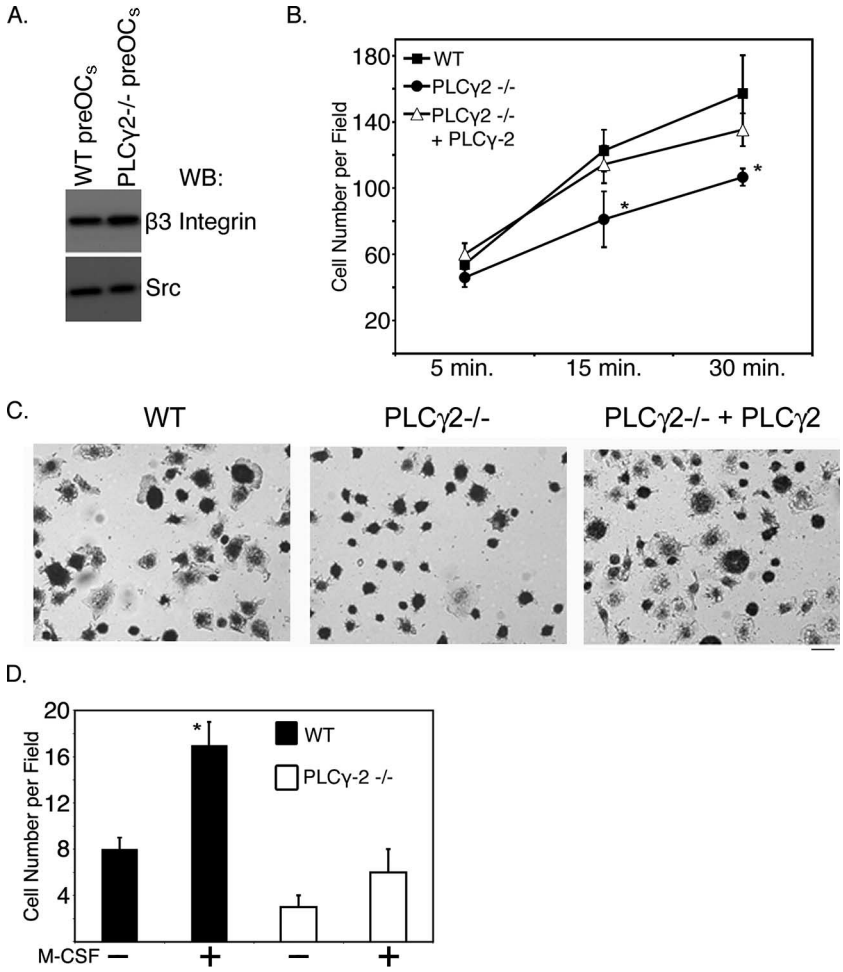

FIG. 3. PLCγ2$^{-/-}$ preOCs exhibit defective adhesion and spreading on vitronectin and a decreased haptotactic migration response. (A) WT and PLCγ2$^{-/-}$ BMDM were allowed to differentiate for 2 to 3 days in osteoclastogenic medium to form preOCs. Cell lysates were Western blotted (WB) for the indicated proteins. (B, C) WT (filled squares), PLCγ2$^{-/-}$ (filled circles), and PLCγ2$^{-/-}$ plus pMX-PLCγ2 (open triangles) preOCs were serum and cytokine starved for 6 h, plated on vitronectin (5 μg/ml), washed, and fixed after various times. Cells were imaged with a light microscope to observe spreading (C) and number of adherent cells per field enumerated (B). Each data point represents an average of eight independent fields, and standard deviations were calculated. An asterisk represents a P value of <0.001. Scale bar, 10 μm. (D) WT and PLCγ2$^{-/-}$ preOCs were allowed to migrate toward a filter coated with vitronectin in the absence (−) or presence (+) of 100 ng/ml M-CSF. Migrated cells were stained with Geimsa and counted under a light microscope. At least three fields were analyzed per data point. The increase observed in WT cells after addition of M-CSF was considered significant, with a P value of <0.001.

The phosphorylation of Src at tyrosine 416 correlates with its kinase activity (28). We also directly measured the kinase activities of Src in WT and PLCγ2$^{-/-}$ preOCs following integrin activation in an in vitro kinase assay. Src activity from PLCγ2$^{-/-}$ cells stimulated by adhesion to vitronectin was decreased 50% compared to that from WT cells (Fig. 4B). Together, these data indicated that PLCγ2 was critical for the activation of Src and PYK2 kinases following integrin activation.

PLCγ2 promotes the formation of a β_3 integrin/Src/PYK2 complex. In addition to regulating enzyme activity, phosphorylation of components of the adhesion complex can influence protein-protein interactions within the complex (8). Therefore, we asked whether the absence of PLCγ2 influenced the formation of the integrin adhesive complex in response to integrin engagement. WT and PLCγ2$^{-/-}$ preOC cells were plated on a vitronectin-coated surface, and at various times postadhesion, Src or PYK2 was immunoprecipitated from cell lysates and bound products Western blotted for components of the integrin adhesive complex. In WT cells, an adhesion-induced interaction of Src with β_3 integrin and PYK2 was detected; however, these same associations were not detected in the PLCγ2$^{-/-}$ cells (Fig. 5A). Furthermore, PYK2 and PLCγ2 also associated following integrin-dependent engagement, as detected specifically in WT cells (Fig. 5B). When PYK2 was immunoprecipitated, an adhesion-induced interaction with Src was detected in WT lysates; however, this interaction was barely detectable in PLCγ2$^{-/-}$ lysates (Fig. 5B). To determine whether PLCγ catalytic activity was required for the formation of the Src/PYK2/β_3 complex, WT adherent cells were treated with the PLCγ inhibitor U73122 for 3 h. As expected, untreated WT cells fully supported the association of Src with PYK2 and β_3; however, this complex was not detected in cells treated with the PLCγ inhibitor (Fig. 5D). These data indi-

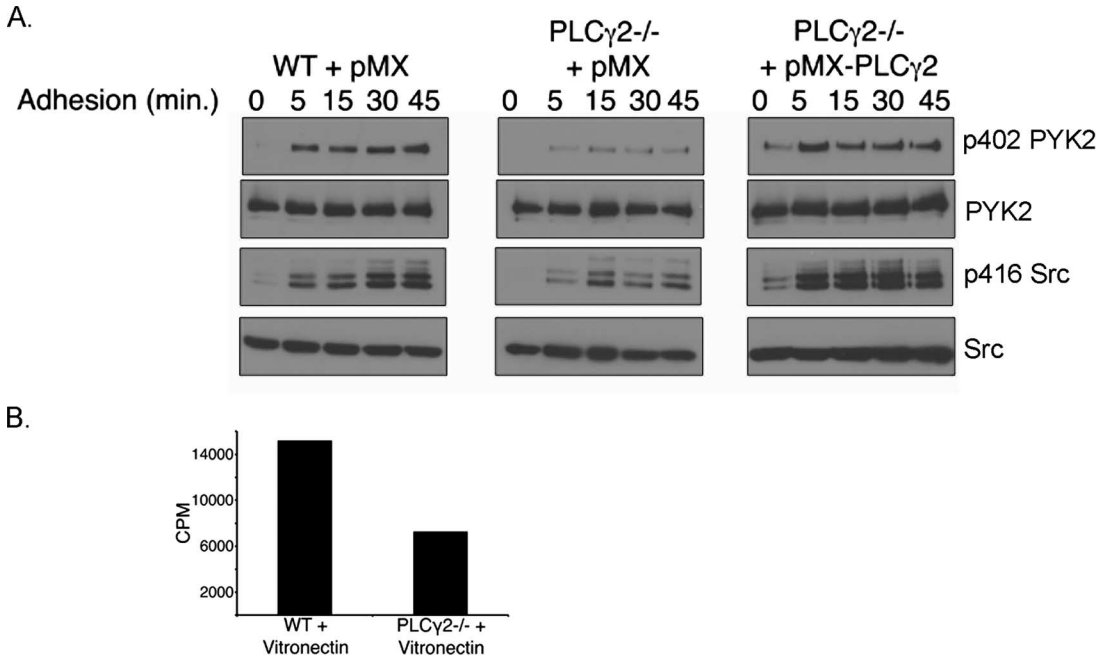

FIG. 4. PLCγ2$^{-/-}$ cells exhibit a defect in Src and PYK2 phosphorylation and decreased Src kinase activity. (A) WT and PLCγ2$^{-/-}$ preOCs infected with empty vector (pMX) or PLCγ2$^{-/-}$ cells infected with pMX-PLCγ2 were starved for 6 h and stimulated by adhesion to vitronectin for the indicated times. Whole-cell lysates were Western blotted for the indicated proteins. (B) WT and PLCγ2$^{-/-}$ preOCs were starved and stimulated by adhesion to vitronectin for 45 min. Whole-cell lysates were collected and normalized. From equal amounts of protein, endogenous Src was immunoprecipitated and kinase activity determined in vitro, using an exogenous substrate. Counts per minute (CPM) of ^{32}P-labeled substrate were determined using a scintillation counter.

cated that PLCγ2 contributed to the association of Src and PYK2 with the integrin adhesion complex following integrin activation.

PLCγ2 controls the subcellular localization of Src. One possible mechanism by which PLCγ2 could influence Src and PYK2 activation at the adhesive complex is by facilitating localization of these proteins to the integrin adhesive complex, as suggested by the coimmunoprecipitation experiments presented above. To determine whether PLCγ2 affected the localization of Src and PYK2 to integrins in the osteoclast, WT or PLCγ2$^{-/-}$ BMDM were transduced with empty vector, NFAT2, or full-length PLCγ2 and differentiated to form mature osteoclasts. Phalloidin staining, to visualize the actin cytoskeleton, revealed that equivalent actin rings formed in both WT and PLCγ2$^{-/-}$ mature osteoclasts expressing either NFAT2 or PLCγ2 when plated on glass (Fig. 6A to C), in contrast to what was observed when PLCγ2$^{-/-}$ cells were plated on a bone surface (Fig. 1G). The staining patterns for total PYK2 were similar in WT and PLCγ2$^{-/-}$ cells, localizing at the actin ring (Fig. 6D to F). However, PYK2 phosphorylated at Y402 was found at the peripheral membrane juxtaposed to the actin ring only in cells expressing PLCγ2 (Fig. 6G to I; also see Fig. S2 in the supplemental material). Both total and activated forms of Src were also mislocalized in PLCγ2$^{-/-}$ cells (Fig. 6J to O). In WT cells, Src localized exclusively to the outer edge of the cell in close proximity to the phalloidin-stained actin ring, while in PLCγ2$^{-/-}$ cells, Src staining was present diffusely throughout the cell and did not specifically localize to the actin ring (Fig. 6J to L). Staining with a phosphospecific antibody to tyrosine 416 of Src (active Src) revealed the presence of p416-Src in WT cells predominantly at the edge of the cell juxtaposed to the actin ring, while p416-Src staining was dramatically reduced in PLCγ2$^{-/-}$ osteoclasts (Fig. 6M and O; also see Fig. S2 in the supplemental material). Importantly, reintroduction of full-length PLCγ2 in PLCγ2$^{-/-}$ cells resulted in Src and p416-Src staining patterns similar to those in WT osteoclasts (Fig. 6L and O). The faint staining of activated Src and PYK2 observed in PLCγ2$^{-/-}$ osteoclasts correlated with decreased phosphorylated levels of the two proteins following attachment to vitronectin, as determined by Western blotting (Fig. 4A), indicating that PLCγ2 controlled both activation and localization of p-Src and p-PYK2 in osteoclastic cells.

PLCγ2 catalytic activity and adapter function are required for Src localization. PLCγ2 contains a catalytic domain that converts PIP_2 into IP_3 (inositol-1,4,5-trisphosphate) and DAG (1,2-*sn*-diacylglycerol) and two tandem SH2 motifs and one SH3 domain involved in protein-protein interaction. To determine the mechanism by which PLCγ2 modulated Src localization and activation, we infected PLCγ2-null BMDM with a catalytic inactive PLCγ2 construct (PLCγ2 H/F carrying the mutations H327F/H372F) and with mutants carrying point mutations in either the two tandem SH2 motifs (PLCγ2 SH2 with point mutations R564K/R672K) or the SH3 domain (PLCγ2 SH3 carrying the mutation P820L). We first analyzed the capacities of these mutated forms of PLCγ2 to support osteoclast differentiation and bone resorption. As previously shown (20), PLCγ2 catalytic activity was required for proper formation of multinucleated spread osteoclasts (Fig. 7A). In addition, mutations in the SH2 motifs, but not in the SH3 domain, also

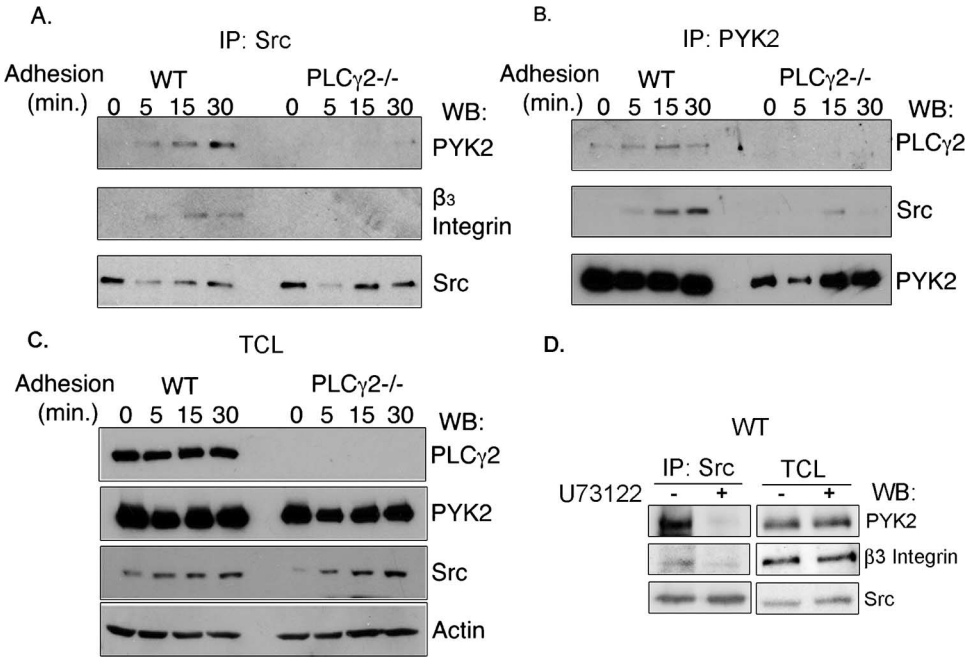

FIG. 5. Protein-protein interactions between components of the integrin adhesion complex are decreased in PLCγ2$^{-/-}$ cells. (A, B) WT and PLCγ2$^{-/-}$ preOCs were starved for 6 h and stimulated by adhesion to vitronectin for various times. Lysates were collected, normalized, and immunoprecipitated with a Src monoclonal antibody (A) or a PYK2 monoclonal antibody (B). Immunoprecipitates were Western blotted (WB) for the indicated proteins. (C) Whole-total cell input lysates (TCL) were Western blotted for the indicated proteins. Equal amounts of proteins were loaded in each lane. (D) WT adherent osteoclasts were treated with U73122 (5 μM) for 3 h or left untreated. Lysates were immunoprecipitated with a Src monoclonal antibody and Western blotted for the indicated proteins. The input lysates (TCL) were Western blotted for the same proteins. Equal amounts of proteins were loaded in each lane.

blocked osteoclast differentiation compared to cells expressing WT PLCγ2 (Fig. 7A and data not shown). Control experiments demonstrated that these mutants were expressed at similar levels following retroviral transduction (Fig. 7B). Furthermore, we also confirmed that PLCγ2 H/F exhibited 70% decreased lipase activity in vitro compared to cells expressing WT PLCγ2, while the catalytic activity in cells expressing the PLCγ2 SH2 mutant was intact (Fig. 7C). Next, we tested the abilities of these two PLCγ2 mutants to confer bone resorption capacity and to promote Src localization in PLCγ2$^{-/-}$ cells. NFAT2 was cotransfected with PLCγ2 H/F or SH2 mutants so as to ensure proper osteoclast differentiation. Mutation of the catalytic domain or disruption of the adapter function affected the capacities of the osteoclasts to resorb bone (Fig. 7D and E), while not altering the localization of PLCγ2 at the cell periphery (Fig. 7F). Importantly, Src localization remained altered in cells expressing PLCγ2 H/F and PLCγ2-SH2 mutants compared to that in WT osteoclasts or osteoclasts transduced with WT PLCγ2 (Fig. 8). In fact, while Src was primarily found at the cell periphery, localizing at the actin ring, in cells expressing full-length PLCγ2 (Fig. 8A to C and G to I), the kinase clustered in different areas of the cell, sometimes assuming a perinuclear distribution, in osteoclasts expressing PLCγ2 H/F or PLCγ2-SH2 mutants.

These data indicated that the catalytic activity and the adapter function of PLCγ2, conferred by the two SH2 motifs, were both independently required for proper localization of Src to the membranes of osteoclasts as well as efficient bone resorption. Overall, these findings suggested that appropriate localization of Src could be a critical mechanism by which PLCγ2 affects integrin function in osteoclasts.

DISCUSSION

Considering the role of integrins in essential cellular processes, it is important to elucidate the precise molecular events that occur in response to integrin engagement. The lipase PLCγ is phosphorylated and enzymatically activated upon integrin engagement (11, 25, 30). We now show in the osteoclast that PLCγ2 is critical for the integrin-mediated processes of adhesion, migration, and bone resorption (Fig. 1 and 3). PLCγ2 is also required for integrin-mediated phosphorylation of the kinases Src and PYK2 (Fig. 4). Importantly, in the absence of PLCγ2, the adhesion-induced interaction of Src with β_3 integrin and PYK2 is dramatically reduced (Fig. 5) and Src is not properly localized to the actin ring (Fig. 6). Finally, we show that both the catalytic activity and the adapter domains of PLCγ2 are required for proper Src localization (Fig. 8). These results implicate PLCγ2 as an important transducer of integrin signals in the osteoclast.

The mechanism by which Src is regulated in response to integrin signals is not fully understood. Phosphorylation at Tyr527 by C-terminal Src kinase induces an intramolecular interaction with the SH2 domains of Src that inhibits Src activity, while autophosphorylation at Tyr416 is required for activation of the kinase (28). Protein-protein interactions with the SH2 and SH3 domains of Src can also relieve the intramolecular inhibitory state and serve to activate Src. For instance,

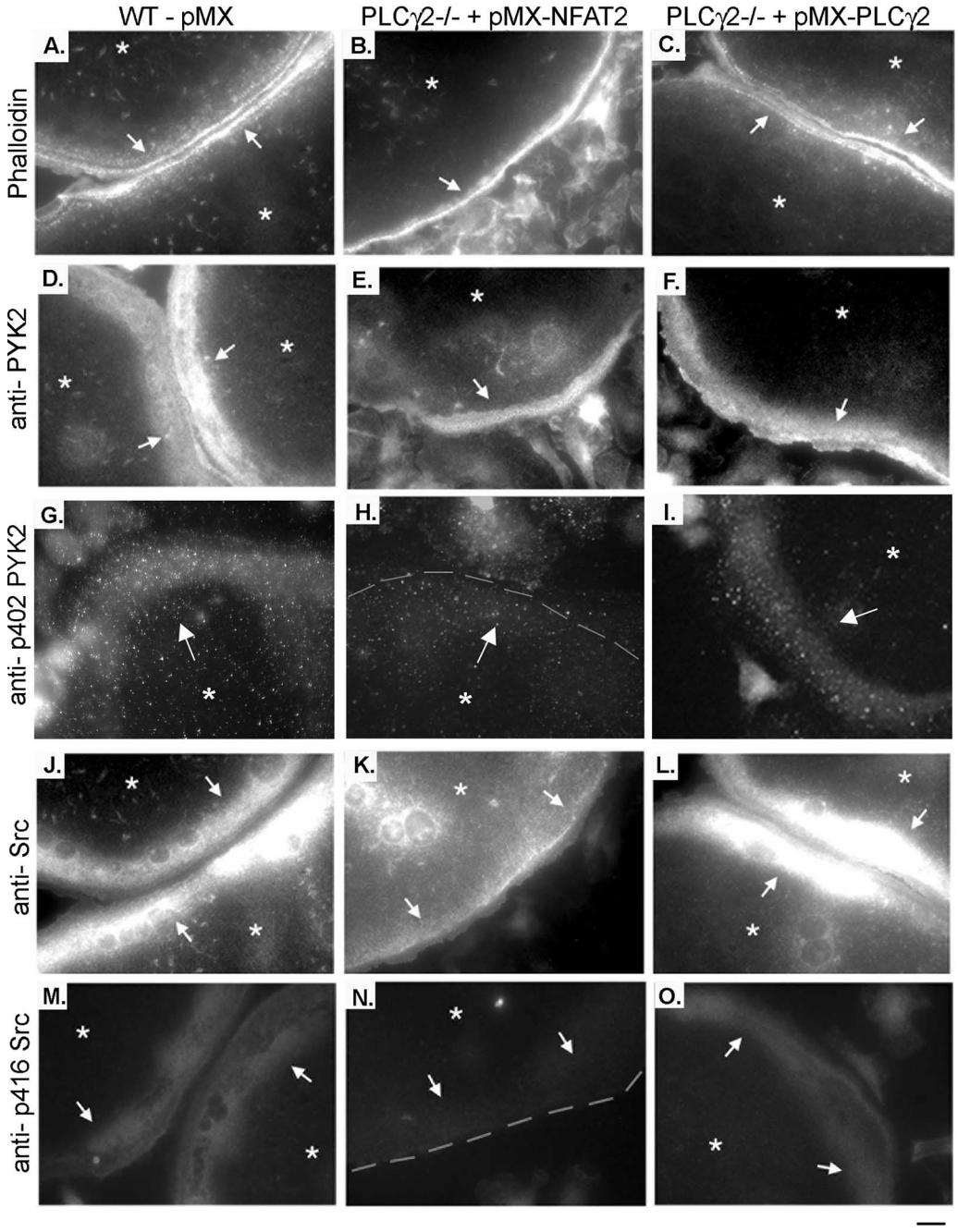

FIG. 6. Total Src and activated Src and PYK2 are mislocalized in PLC$\gamma 2^{-/-}$ osteoclasts. WT BMDM, retrovirally transduced with pMX empty vector (A, D, G, J, and M), and PLC$\gamma 2^{-/-}$ BMDM, retrovirally transduced with pMX-NFAT2 (B, E, H, K, and N) or pMX-PLC$\gamma 2$ (C, F, I, L, and O), were grown in differentiation medium for 5 to 7 days to allow osteoclast differentiation. Cells were then fixed and stained with phalloidin (A to C) or incubated with antibodies to PYK2 (D to F), p402 PYK2 (G to I), Src (J to L), or p416 Src (M to O). An asterisk marks each mature osteoclast present in a field. All other cells are immature preOCs, macrophages, or stromal cells. Arrows mark the area at or near the actin sealing ring in mature osteoclasts. In panels H and N, a dotted line delineates the edge of the mature osteoclast. Images were captured using an Olympus IX70 inverted microscope (60× APO, 1.4-numerical-aperture lens), with a CoolSnap camera, and analyzed using Metamorph software. Scale bar, 5 μm.

when the SH3 domain of Src interacts with the tail of the β_3 integrin, following integrin clustering, phosphorylation of Src at Tyr416 is induced (1). A less appreciated aspect of Src regulation is the localization of Src to sites where it is activated or the localization of activated Src near substrates, such as its recruitment to clustered integrins.

Based on our results, we propose that PLC$\gamma 2$ modulates $\alpha_v\beta_3$ integrin signaling by affecting the formation and/or stability of the integrin adhesion complex as well as the subcellular localization of Src. In support of this model, adhesion-induced interactions of Src with β_3 integrin and PYK2 are significantly reduced in PLC$\gamma 2^{-/-}$ osteoclasts (Fig. 5A). This

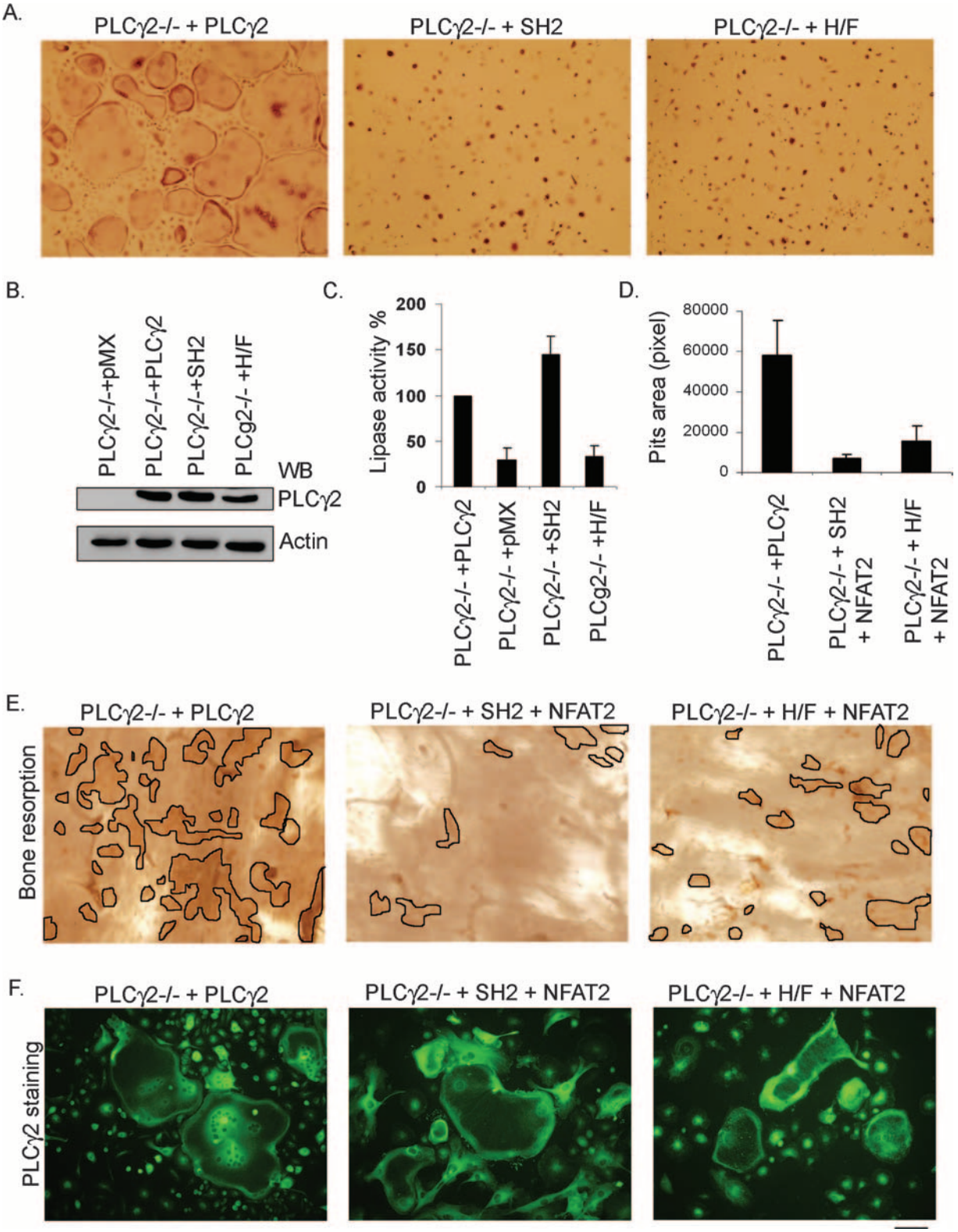

FIG. 7. Both catalytic activity and adapter function of PLCγ2 are required for bone resorption in osteoclasts. (A) PLCγ2$^{-/-}$ BMDM were retrovirally transduced with pMX-PLCγ2, PLCγ2-SH2 (construct carrying the mutations R564K/R672K), or the PLCγ H/F mutant (catalytically inactive construct with mutated H327F/H372F). Cells were grown in the presence of 10 ng/ml M-CSF and 100 ng/ml RANKL for 5 to 8 days, fixed, and TRAP stained. TRAP^{+} osteoclasts were observed using a light microscope with a 10× objective. (B) Infected BMDM were lysed and Western blotted (WB) for PLCγ2 expression. Actin expression served as a loading control. (C) Transduced proteins were immunoprecipitated using a FLAG antibody, and production of IP_3 was determined using a radioactive probe. Lipase activity is expressed as percentage of the WT PLCγ2 level, which was given an arbitrary value of 100. (D, E) BMDM infected with pMX-PLCγ2 or coinfected with indicated PLCγ2 mutants plus NFAT2 were plated on dentin and grown in differentiation medium for 10 days. After cells were removed, pits were visualized by staining with peroxidase-conjugated wheat germ agglutinin (E). Area of resorbed bone was determined using Image J software (D). Scale bar, 20 μm. (F) Plcγ2$^{-/-}$ BMDM were retrovirally infected with Plcγ2 or PLCγ2 mutants SH2 and H/F plus NFAT2 and grown in differentiation medium for 5 to 8 days. Osteoclasts were fixed and immunostained with PLCγ2 antibody.

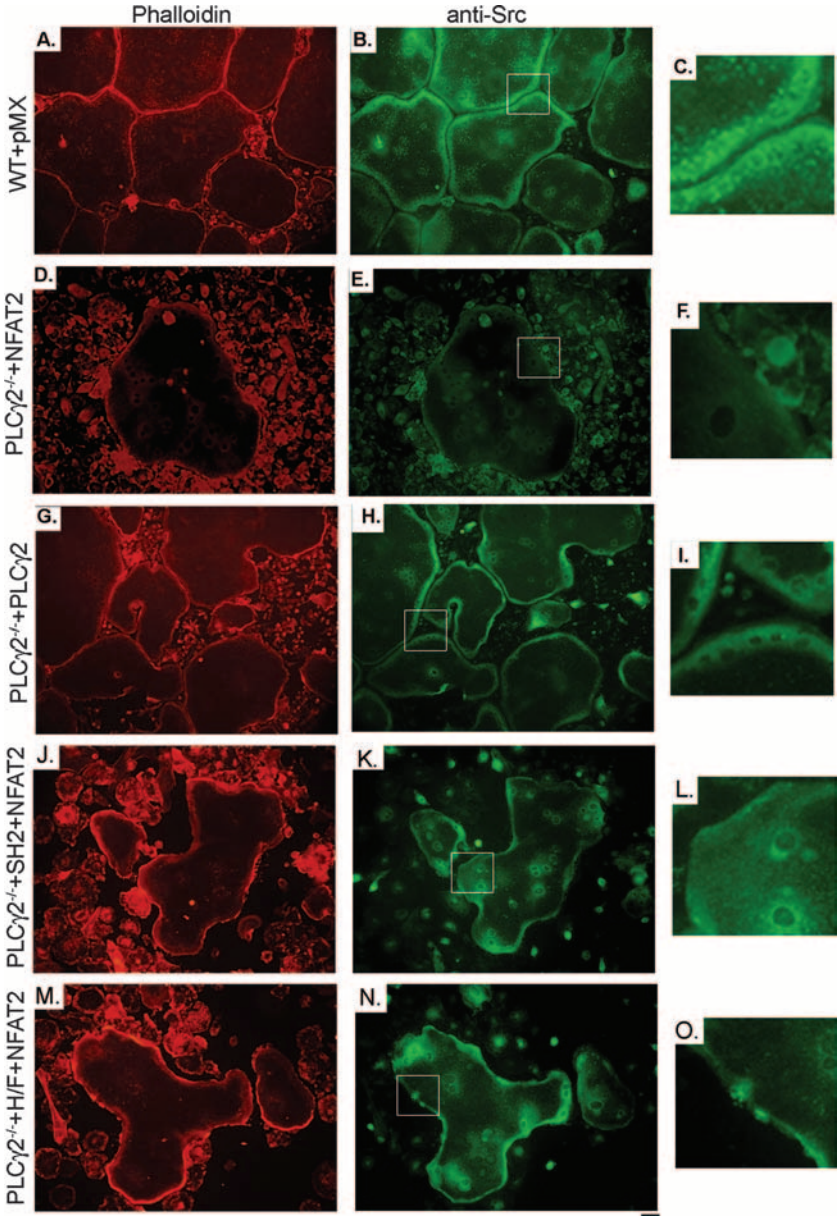

FIG. 8. Both catalytic activity and adaptor function of PLCγ2 are required for Src localization in osteoclasts. WT or PLCγ2$^{-/-}$ BMDM, retrovirally transduced with empty vector (pMX), NFAT2 alone, full-length PLCγ2, or PLCγ2 mutants SH2 and H/F plus NFAT2, were grown on glass in the presence of 10 ng/ml M-CSF and 100 ng/ml RANKL for 5 to 8 days. Upon osteoclast formation, cells were fixed and stained for actin (A, D, G, J, and M) or Src (B, E, H, K, and N). Images were obtained with an Olympus IX70 inverted fluorescence microscope using a 20× APO lens. Panels C, F, I, L, and O each represent a field of Src-stained images enlarged fourfold. Scale bar, 10 μm.

indicates that PLCγ2, which itself has been shown to interact with PYK2 (25) (Fig. 5B), can modulate either the formation or the stability of the integrin adhesion complex. Second, the aberrant localization of Src in the absence of PLCγ2 may result from decreased recruitment of Src to the integrin complex or instability of the complex. It is important to note that low levels of Src are localized to the area of the actin ring in PLCγ2$^{-/-}$ osteoclasts (Fig. 6K); however, in WT cells the majority of total cellular Src is localized to this site of adhesion (Fig. 6J). Decreased recruitment of Src to the cell periphery was not a result of lower expression levels of total Src in PLCγ2-null cells (Fig. 3A).

Genetic ablation of Src in the osteoclast results in a dramatic phenotype characterized by a complete loss of the actin ring (3). The presence of low levels of Src at the membrane in PLCγ2$^{-/-}$ cells may explain the presence of actin rings in these cells when they are plated on glass (Fig. 6B). In contrast, PLCγ2$^{-/-}$ osteoclasts plated on bone (more physiologic) do not exhibit normal actin rings but display disorganized actin structures in the middle of the cell (Fig. 1H). Unlike osteoclasts plated on glass, osteoclasts on a bone surface polarize to form a distinct three-dimensional structure; therefore, it is possible that while PLCγ2 is not required for the formation of the actin ring on glass, per se, it is essential for osteoclast

polarization and formation of tight adhesions on the physiological bone substrate. In support of this hypothesis, PLCγ2$^{-/-}$ cells have adhesion and spreading defects as well as decreased abilities to resorb bone, an indication of aberrant functionality.

PLCγ2 becomes phosphorylated in response to integrin engagement (see Fig. S1 in the supplemental material) (25), and this phosphorylation event is mediated by Src (10, 30). Here, we provide evidence that PLCγ2 is important for regulating the subcellular localization and integrin-mediated activation of Src. How, then, could PLCγ2 be working both downstream and upstream of Src? In some cases, such as the activation of Vav3, Src is important; however, Vav3$^{-/-}$ osteoclasts show decreased Src phosphorylation following attachment (10). Here, one possibility is that following integrin engagement Src can promote PLCγ2 phosphorylation and thus helps to activate its lipase activity, whereas during the spreading process, PLCγ2, via its SH2 and SH3 domains, may serve a scaffolding function independent of its catalytic activity to recruit Src to the adhesion complex. This is supported by the following findings: (i) inhibition of PLCγ catalytic activity by U73122 fails to induce Src/PYK2/β_3 association following integrin engagement (Fig. 5D) and (ii) the catalytic activity and the SH2 adapter motifs of PLCγ2 are independently required for proper Src localization at the actin rings of mature osteoclasts (Fig. 8). As a consequence of these integrin dysfunctions, both PLCγ2 mutants, as in PLCγ2$^{-/-}$ cells, fail to support proper osteoclast function (i.e., bone resorption).

Another possibility is that the catalytic activity of PLCγ2 may be required for PYK2 activation and consequently Src localization. PYK2 autophosphorylation at Tyr402 is a calcium-dependent event that occurs independent of Src activity (34), and autophosphorylation of the PYK2 homologue focal adhesion kinase has been shown to be critical for recruitment of Src to focal adhesions in fibroblast cells (35). Here, we find that PLCγ2-null cells have decreased levels of Tyr402 PYK2 in response to integrin engagement (Fig. 4A and 6). This may contribute to the abnormal Src localization in these cells. Furthermore, treatment with the PLCγ inhibitor U73122, which may influence tyr402 phosphorylation of PYK2, disrupted the Src/PYK2/β_3 adhesive complex in adherent WT osteoclasts. In further support of a role for the lipase activity of PLCγ2, osteoclasts carrying the PLCγ2 catalytic domain mutant displayed improper Src localization (Fig. 8). Finally, it is also possible that PLCγ2, via its SH2 motif, can bind to PYK2, thereby recruiting both PYK2 and Src to the integrin complex. Adhesion-dependent association between PLCγ SH2 motifs and PYK2 in osteoclasts has been previously shown (25). Importantly, our data for PLCγ2-deficient osteoclasts indicate that total PYK2 is properly localized at the actin ring, while its activated form, pPYK2 Y402, is barely detectable. This observation would suggest that PLCγ2 is required for both PYK2 phosphorylation and the recruitment/stability of the activated protein at the sealing zone of the osteoclast, where it can interact with Src and $\alpha_v\beta_3$. This could explain the decreased formation of the Src/PYK2/β_3 complex that we observe in PLCγ2-null osteoclasts in response to integrin engagement and, more importantly, the abnormal localization of Src in cells carrying the PLCγ2 SH2 mutations.

ITAM-containing receptors Dap12 and FcRγ have been involved in integrin signaling in several cell types, including the osteoclasts. Interestingly, however, Src is normally phosphorylated in Dap12/FcRγ$^{-/-}$ osteoclasts (reference 40 and data not shown), suggesting that the mechanism by which PLCγ2 controls Src activation downstream of the integrin receptor is ITAM independent. Both PLCγ1 and PLCγ2 are suggested to participate in integrin and ITAM signaling. Our studies from PLCγ2-null mice clearly suggest a nonredundant role for these two isoforms during both osteoclast differentiation and activity. A differential and nonredundant role for PLCγ1 and -2 in NK cells has been recently reported (29). Here, we show that PLCγ2 is specifically important for the integrin-mediated processes in the osteoclast since the phenotype persists in PLCγ2$^{-/-}$ cells despite the presence of normal PLCγ1 expression. Furthermore, only PLCγ2, and not PLCγ1, is phosphorylated upon integrin-mediated adhesion. We cannot, however, rule out that PLCγ1 may still be playing a role in activating integrin signaling in the osteoclast. For example, we have found that PLCγ1, and to a lesser extent PLCγ2, is primarily activated by M-CSF (20). It is possible that PLCγ1 could contribute to integrin activation via an M-CSF-dependent mechanism. Since deletion of PLCγ1 in the mouse results in embryonic lethality (13), further experiments are required.

In conclusion, our data suggest that PLCγ2 acts as a modulator of integrin signaling in the osteoclast by regulating the activation and protein-protein interactions of adhesion complex kinases, through localization and/or stabilization of Src at integrin adhesion complexes.

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