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SLP-76 Couples Syk to the Osteoclast Cytoskeleton

Jennifer L. Reeve,*† Wei Zou,* Yuli Liu,* Jonathan S. Maltzman,‡ F. Patrick Ross,* and Steven L. Teitelbaum2*

The capacity of the osteoclast (OC) to resorb bone is dictated by cytoskeletal organization, which in turn emanates from signals derived from the αβ3 integrin and c-Fms. Syk is key to these signals and, in other cells, this tyrosine kinase exerts its effects via intermediaries including the SLP adaptors, SLP-76 and BLNK (B cell linker). Thus, we asked whether these two SLP proteins regulate OC function. We find BLNK-deficient OCs are normal, whereas cytoskeletal organization of those lacking SLP-76 is delayed, thus modestly reducing bone resorption in vitro. Cytoskeletal organization and bone resorption are more profoundly arrested in cultured OCs deficient in BLNK and SLP-76 double knockout (DKO) phenotypes. In contrast, stimulated bone resorption in vivo is inhibited ~40% in either SLP-76−/− or DKO mice. This observation, taken with the fact that DKO OCs are rescued by retroviral transduction of only SLP-76, indicates that SLP-76 is the dominant SLP family member in the resorptive process. We also find SLP-76 is phosphorylated in a Syk-dependent manner. Furthermore, in the absence of the adaptor protein, integrin-mediated phosphorylation of Vav3, the OC cytoskeleton-organizing guanine nucleotide exchange factor, is abrogated. In keeping with a central role of SLP-76/Vav3 association in osteoclastic resorption, retroviral transduction of SLP-76, in which the Vav binding site is disrupted (3YF), fails to normalize the cytoskeleton of DKO OCs and the resorptive capacity of the cells. Finally, c-Fms-activated Syk also exerts its OC cytoskeleton-organizing effect in a SLP-76/Vav3-dependent manner. The Journal of Immunology, 2009, 183: 1804–1812.

Osteoclasts (OCs),3 the polykaryons of the myeloid lineage, are the exclusive cells responsible for bone resorption and the targets of antiresorptive therapies used in the treatment of osteoporosis and inflammatory osteolysis. The OC creates a sealing zone at the site of attachment to bone, permitting localized secretion of proteases into an acidic microenvironment needed for skeletal degradation. Formation of the sealing zone requires cytoskeletal rearrangement, which creates an actin ring-like structure (1). The αβ3 integrin plays a central role in the process of actin ring formation and we have focused on delineating the mechanism of αβ3 signaling in OCs.

Because the OC is of hematopoietic lineage, it is not surprising that it shares signaling pathways with other immune cells, such as those initiated by the TCR. Analogous to the TCR, αβ3 integrin occupancy induces rapid phosphorylation of Src and Syk family tyrosine kinases essential for actin ring formation and OC function (2).

SLPs (Src homology region 2 (SH2) domain-containing leukocyte proteins) are expressed throughout the hematopoietic lineage and phosphorylated by Syk family kinases in multiple cell types (3). SLP-76 is the dominant family member expressed in T cells and involved in TCR signaling. SLP-76 also regulates signal transduction in platelets, mast cells, dendritic cells, and neutrophils, whereas SLP-65/BLNK (B cell linker) mediates BCR signaling (3). In both T and B cells, these adaptor proteins are phosphorylated by Syk family kinases after receptor engagement, allowing for assembly of a multimeric complex through three structural domains. The N-terminal acidic-rich region contains three phospho-inducible tyrosines (Y112, Y128, and Y145), which are binding sites for the guanine nucleotide exchange factor (GEF), Vav, the adaptor, Nick, and Tec kinases. The central proline-rich region associates with phospholipase Cγ and the Grb2-related adapter downstream of Shc (Gads) via an R-X-K-X motif. In the C terminus, the adaptor ADAP (adhesion and degranulation-promoting adaptor protein) and HPK1 kinase bind via the SH2 domain (3).

The function of this scaffolding complex was first characterized in the context of T cell development as T cells in SLP-76−/− mice fail to mobilize calcium or induce NFAT and AP1 gene regulation following TCR engagement and are arrested in the double negative three (DN3) stage CD4+ CD8− (4–6).

In addition to its role in T cell development, SLP-76 impacts the cytoskeleton in many cells. It is required, for example, to form the immune synapse in T cells, a process dependent on cytoskeletal remodeling (7). SLP-76 is also involved in platelet spreading on fibrinogen, a dynamic cytoskeletal event regulated by the αιβ3 integrin (8–10).

We find that OCs lacking Syk and Vav3 fail to spread normally and do not form actin rings, reflecting a disorganized cytoskeleton. As a result, Syk- or Vav3-deficient OCs are unable to efficiently resorb bone (2, 11). We demonstrate that SLP-76 is the key SLP family member in the OC, promoting cytoskeletal organization and...
resorptive function by linking Syk to Vav3 in α,β₃, and c-Fms-dependent manner.

**Materials and Methods**

**Mice**

BLNK−/−, SLP-76−/−, Syk−/−, and linker of activated T cells (LAT)− and Gads-deficient (LAT−/− and Gads−/−) mice were previously described (4, 12–15). BLNK mice on a C57BL/6 background were purchased from The Jackson Laboratory; Syk−/− mice were a gift from Dr. V. Tybulewicz (National Institute for Medical Research, London, U.K.) and are on a 129.sv background; C57BL/6 LAT−/− mice were a gift from Dr. L. Samelson (National Institutes of Health, Bethesda, MD); C57BL/6 Gads mice were provided by A. Chan (Genentech, San Francisco, CA). SLP-76−/− were backcrossed to C57BL6 background for over 10 generations. All mice were housed in the animal facility at Washington University School of Medicine. To overcome the perinatal lethality of SLP-76−/−, DKO, and Syk−/− mice, we used a bone marrow transplantation strategy to generate radiation chimera. Embryos were collected from heterozygous Syk−/+; SLP-76−/+, or Syk−/+;BLNK−/+ breeding pairs at embryonic day E16–20; fetal liver cells were isolated from and injected into the tail vein of lethally irradiated (1000 Gy) wild-type (WT) C57BL/6 recipient mice. Chimeras were used for in vivo experiments or as a source of bone marrow-derived macrophages (BMM), following 4 wk of marrow reconstitution.

In vivo studies used radiation chimera generated from SLP-76−/− and SLP-76−/−;BLNK−/− littermates or SLP-76−/−;BLNK−/− (control) and SLP-76−/−;BLNK−/−;DKO (DKO) chimeras (1–5 in each group). A total of 10 μg of parathyroid hormone (PTH)–34 (Bachem) in 25 μl of buffer (0.1% BSA, 0.1 mM HCl) or buffer alone was injected supracaudally every 6 h for 4 days. Mice were sacrificed, and calvaria were isolated for histology. Fasting serum was collected and analyzed for collagen fragments using the RatLaps ELISA in duplicate (Immunodiagnostic Systems). Three calvarial sections from each mouse were stained for tetrarose-resistant acid phosphatase (TRAP) and analyzed histomorphometrically at ×16 using the Osteomeasure software.

**Macrophage isolation and OC culture**

Following sacrifice by CO₂ inhalation, the long bones from mice were isolated and flushed with α-MEM. Bone marrow was cultured on bacterial plastic, which promotes growth of monocyte cells and prevents admixture of mesenchymal cell. The cells were cultured with α-10 medium supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin supplemented with 1:10 CMG (conditioned medium supernatant containing recombinant M-CSF (16)). On day 4 of culture, the remaining cells, which have been selected as a pure population of macrophages, were lifted using 1X trypsin/EDTA and replated at 5 × 10⁵ cells in a tissue culture-treated plastic 96-well plate in α-10 medium supplemented with 100 ng/ml GST-RANKL and 1:50 CMG. The cells were cultured over time (3–6 days) and stained for TRAP (Sigma-Aldrich). As shown (see supplemental Fig. S2), 50 ng/ml purified RANKL (receptor activator of NF-κB ligand; PeproTech) and 10 ng/ml M-CSF (R&D Systems) were used as previously described (17). For actin ring and pit reorative assays, cells were cultured as described on bovine bone slices. For actin ring and pit reative assays, cells were cultured as described on bovine bone slices.

**Western blotting and immunoprecipitation**

Following sacrifice by CO₂ inhalation, the long bones from mice were isolated and flushed with α-MEM. Bone marrow was cultured on bacterial plastic, which promotes growth of monocyte cells and prevents admixture of mesenchymal cell. The cells were cultured with α-10 medium supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin supplemented with 1:10 CMG (conditioned medium supernatant containing recombinant M-CSF (16)). On day 4 of culture, the remaining cells, which have been selected as a pure population of macrophages, were lifted using 1X trypsin/EDTA and replated at 5 × 10⁵ cells in a tissue culture-treated plastic 96-well plate in α-10 medium supplemented with 100 ng/ml GST-RANKL and 1:50 CMG. The cells were cultured over time (3–6 days) and stained for TRAP (Sigma-Aldrich). As shown (see supplemental Fig. S2), 50 ng/ml purified RANKL (receptor activator of NF-κB ligand; PeproTech) and 10 ng/ml M-CSF (R&D Systems) were used as previously described (17). For actin ring and pit reorative assays, cells were cultured as described on bovine bone slices. For actin ring and pit reative assays, cells were cultured as described on bovine bone slices.

**Plasmid and retroviral transduction**

The SLP-76-pMX-IRES-BSR was subcloned from a SLP-76 plasmid, previously described (18), into pMX-IRES-BSR by introducing the BamHI 5’ and XhoI 3’ restriction sites with a hemagglutinin (HA)-tag at the 3’ end.

The 3YF and R443K mutations were generated by mutating SLP-76-pMX vector using site-directed mutagenesis (Stratagene). The Δ157–222 construct was created by standard molecular biology techniques. A total of 8 μg of the pMX retrovector plasmid was transfected into Plat-E packaging cell line (19) using the Transfectol reagent (GeneChoice) according to the manufacturer’s instructions. The viral supernatant was collected 48 h posttransfection and added to BMMs in α-10 medium with 1:10 CMG and 4 μg/ml polybrevine (Sigma-Aldrich). After 24 h of incubation with the virus, transduced cells were selected using 1 μg/ml blasticidin (Calbiochem) for 3 days and plated for OC precursors as described. Using the described constructs, GFP was tagged to the N terminus of WT SLP-76 and mutated and inserted into the pMX retrovector.

**RT-PCR**

Total RNA was isolated using RNaseasy kits (Qiagen). First strand cDNA was generated from 1 μg of total RNA using the SuperScript First Strand Synthesis system for RT-PCR (Invitrogen) as instructed by the manufacturer. PCR for DC-STAMP (dendritic cell–specific transmembrane protein) levels was performed with 1 μl of diluted cDNA reaction mixture using Taq polymerase and primers 5’-CCA CGA CCT AGC TGG CT-3’ and 5’-CCA GTG CCA GCC GCA ATC AA-3’ in a volume of 50 μl for 30 amplification cycles (94, 58, and 72°C).

**Western blotting and immunoprecipitation**

Cells were washed with ice-cold PBS and lysed in RIPPA buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and 1X Protease Inhibitor Cocktail (Roche). After 10 min of incubation on ice, lysates were cleared of debris by 10 min of centrifugation at 14,000 rpm. Then 30–40 μg were subjected to 8% SDS-PAGE and transferred onto a PVDF membrane. Following blocking with 0.1% casein in PBS, primary Abs were incubated overnight at 4° and probed with fluorescent-labeled secondary Abs (The Jackson Laboratory) and detected on the Odyssey Infrared Imaging System (LI-COR Biosciences). For immunoprecipitation studies, 750 μg of cleared lysates were incubated with 2 μg of primary Ab for overnight at 4°, followed by 3-h incubation with protein A- or protein G-Sepharose beads. Washed beads were resuspended in 2X SDS loading buffer, boiled for 5 min, centrifuged, and subjected to SDS-PAGE.

**Primary Abs** include rabbit anti-Vav3 and mouse anti-phosphotyrosine mAb 4G10 (Millipore); rabbit anti-SLP-76, rabbit anti-β₂ integrin, and rabbit anti-BLNK (Cell Signaling Technology); rabbit anti-Syk (N-19), mouse anti-Vav, and mouse anti-NFAT (Santa Cruz Biotechnology); mAb 327, mouse anti-Src provided by Dr. A. Shaw (Washington University School of Medicine, St. Louis, MO), and sheep anti-SLP-76 previously described (20).

**Rac assays**

Rac pull-down assay was performed from Pierce. Cell lysates were prepared according to the manufacturer’s instructions. A total of 500 μg of protein was subjected to GST pull-down followed by multistep washes, the beads were resuspended in 2X SDS loading buffer and analyzed on a 12% SDS-PAGE. After transfer, the membrane was immunoblotted for Rac1. Rac G-LISA was purchased from Cytoskeleton. Cell lysates from suspended and adhered cells were prepared using the manufacturer’s lysis buffer. A total of 12.5 μg of protein per well was used in the assay, which was performed according to instructions. Each sample was tested in triplicate.

**Statistics**

For in vivo studies comparing histological analysis and serum collagen fragment values of four genotypes, a one-way ANOVA was performed with Tukey post hoc in group comparisons. All significant values (p < 0.05) are reported. The remaining statistics used a Student t test, comparing mutant/deficient cells to the WT/control group. Cell culture and biochemical data are representative of experiments performed in at least duplicate or triplicate. Data are presented as mean ± SD.

**Results**

SLP-76 and BLNK are phosphorylated in OCs by integrin occupancy in a Syk-dependent manner.

To determine the impact of OC differentiation on expression of SLP, we cultured WT BMMs with M-CSF and RANKL. Immunoblot of cell lysates prepared daily, for 5 days, reveals that...
SLP-76 and BLNK are present in all stages of OC differentiation (Fig. 1A).

In T cells, the Syk family kinase, Zap70, phosphorylates SLP-76 after TCR engagement. Similarly, in B cells, Syk phosphorylates BLNK following BCR ligation (21, 22). Given that Syk is activated by occupied α,β3 ligation (2), we asked whether SLP-76 is also phosphorylated by the integrin in OCs. To this end, control BMMs, containing at least one WT Syk allele (Syk+/−), were cultured with M-CSF and RANKL for 4 days to generate pre-fusion OCs. The cells were serum and cytokine starved, lifted with 0.5% EDTA, and either replated on vitronectin-coated plates or maintained in suspension. Cleared cell lysates were immunoblotted for phosphotyrosine (p-Y) and SLP-76 or BLNK. SLP-76 and BLNK are present in all stages of OC differentiation (Fig. 1A).

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**SLP-76 is required for OC function but not differentiation**

In vivo, OCs and bone mass of BLNK−/− mice are indistinguishable from their heterozygous and WT littermates (see supplemental Fig. S1).4 Similarly, BLNK-deficient OCs spread normally, form actin rings, and are capable of normal bone degradation in vitro.

In contrast to BLNK−/− mice, which are born in Mendelian ratio, global deletion of SLP-76 generates few viable pups. In fact, we recovered one knockout animal in 250 mice produced from heterozygous breeding pairs, a yield precluding meaningful morphological or biochemical evaluation of the skeleton. To circumvent this problem we i.v. injected E16–20 SLP-76−/− or SLP-76+/− (control) fetal liver cells into lethally irradiated WT mice. Following marrow reconstitution, BMMs were isolated from the radiation chimeras and differentiated into OCs in M-CSF and RANKL. Both SLP-76−/− and control precursors form TRAP-expressing multinucleated cells by day 3 (Fig. 2A). Confirming normal OC differentiation, SLP-76−/− and control cells express c-Src and the β3 integrin subunit equally during osteoclastogenic culture (Fig. 2B) and do not generate a reduced number of multinucleated TRAP-positive cells (Fig. 2C). However, whereas day 3 SLP-76+/− OCs are indistinguishable from those derived from native WT mice, the majority of those lacking SLP-76 fail to spread, and exhibit a morphology similar to Syk−/− cells that are smaller and have a crenated appearance (2) (Fig. 2A). In fact, the modestly increased number of SLP-76-deficient OCs at this juncture (Fig. 2C) may reflect available space in light of attenuated spreading. Unlike their Syk−/− counterparts, however, the phenotype of SLP-76−/− OCs resolves with time, mirroring WT by day 6, as manifest by TRAP staining (Fig. 2A), number (Fig. 2C), spreading (Fig. 2D) and capacity to form actin rings (Fig. 2E). In fact, the numbers of mutant and control OCs are similarly reduced by day 6, likely reflecting equivalent apoptosis of terminally differentiated cells (23) (Fig. 2, A, C, and D). Despite unimpeded differentiation of the mutant cells and their normalization of spreading with time, the quantity of bone resolved during 6 days of osteoclastogenesis is reduced 43% without SLP-76 (Fig. 2F). Hence, SLP-76 does not mediate OC maturation, but its absence impairs OC function.

**BLNK compensates for absence of SLP-76 in vitro**

SLP-76 is a Syk target, but the spreading defect of OCs lacking the tyrosine kinase is more prolonged than of those deficient in the adaptor protein (2). This observation raised the possibility that BLNK partially compensates for the loss of SLP-76 in linking Syk to the OC cytoskeleton. We tested this hypothesis by generating SLP-76−/−/BLNK−/− (DKO) embryos and injecting their liver cells into irradiated WT hosts. Chimerism was confirmed by Western blot analysis and quantitative RT-PCR demonstrating less than 2% SLP-76 remaining in WT mice transplanted with SLP-76-deficient bone marrow (data not shown). Based on the fact that BLNK deficiency, alone, does not impact the OC (see supplemental Fig. S1), we used SLP-76+/−/BLNK−/− litters as control (Figs. 3, 4, and shown in Fig. 6). Similar to those deleted of only SLP-76, BMMs lacking both SLP proteins differentiate into OCs, however, the phenotype of SLP-76−/− and control cells express c-Src and the β3 integrin subunit equally during osteoclastogenic culture (Fig. 2B) and do not generate a reduced number of multinucleated TRAP-positive cells (Fig. 2C). However, whereas day 3 SLP-76+/− OCs are indistinguishable from those derived from native WT mice, the majority of those lacking SLP-76 fail to spread, and exhibit a morphology similar to Syk−/− cells that are smaller and have a crenated appearance (2) (Fig. 2A). In fact, the modestly increased number of SLP-76-deficient OCs at this juncture (Fig. 2C) may reflect available space in light of attenuated spreading. Unlike their Syk−/− counterparts, however, the phenotype of SLP-76−/− OCs resolves with time, mirroring WT by day 6, as manifest by TRAP staining (Fig. 2A), number (Fig. 2C), spreading (Fig. 2D) and capacity to form actin rings (Fig. 2E). In fact, the numbers of mutant and control OCs are similarly reduced by day 6, likely reflecting equivalent apoptosis of terminally differentiated cells (23) (Fig. 2, A, C, and D). Despite unimpeded differentiation of the mutant cells and their normalization of spreading with time, the quantity of bone resolved during 6 days of osteoclastogenesis is reduced 43% without SLP-76 (Fig. 2F). Hence, SLP-76 does not mediate OC maturation, but its absence impairs OC function.

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OCs enlarge by fusing with their counterparts raising the possibility that the relatively small size of the DKO cells represents attenuated fusion. In this circumstance, one would expect a relative paucity of nuclei in mutant OCs. We find, however, such is not the
case (Fig. 3C). Furthermore, expression of DC-STAMP mRNA, whose product is involved in cell fusion, is temporally indistinguishable in DKO and control BMMs undergoing osteoclastogenesis (Fig. 3D). Hence, the DKO OC phenotype does not represent attenuated fusion but likely reflects dysfunctional cytoskeletal organization. Consistent with this posture, DKO OC actin ring

FIGURE 2. SLP-76-deficient BMMs differentiate into OCs normally but exhibit delayed spreading and reduced resorptive capacity. A, SLP-76+/+ and SLP-76−/− BMMs, isolated from radiation chimera marrow, were cultured in M-CSF and RANKL for 3 or 6 days. The cells were stained for TRAP activity at a magnification ×40 (upper and lower) and a magnification ×200 (middle). B, SLP-76+/+ and SLP-76−/− BMMs were cultured in M-CSF and RANKL over time. Lysates were immunoblotted for the OC differentiation markers, β3 integrin subunit and c-Src; β-actin serves as a loading control. C and D, Quantification of total (C) and spread (D) OCs in A. E and F, SLP-76+/+ and SLP-76−/− BMMs were cultured on bone slices, with M-CSF and RANKL, for 6 days. The resultant OCs were labeled with FITC-phalloidin to visualize actin rings at ×200 magnification (E). They were then removed and resorptive pits stained with peroxidase-conjugated wheat germ agglutinin followed by 3,3′-diaminobenzidine and magnified at ×200 and quantified (F). *** p < 0.001.

FIGURE 3. DKO OCs normally differentiate but are dysfunctional. Control (SLP-76+/+/BLNK−/−) and DKO BMMs were cultured in M-CSF and RANKL over time, fixed, and stained (A) for TRAP activity at a magnification of ×40 (upper and lower) or magnification of ×200 (middle) or immunoblotted (B) for c-Src, NFATc1, and cleaved cathepsin K (Cat K) as markers of OC differentiation. β-actin serves as loading control. C, The number of nuclei per OC was quantified in day 5 TRAP-stained cultures. D, DKO and control BMMs were cultured in M-CSF and RANKL over time. DC-STAMP mRNA was assessed by RT-PCR. Actin served as loading control. E and F, Control and DKO BMMs were cultured on bone in RANKL and M-CSF for 5 days. The cells were fixed and labeled with FITC-phalloidin to visualize actin rings (E) or removed and the resorptive pits stained with peroxidase-conjugated wheat germ agglutinin lectin (F) at a magnification of ×200. Resorptive area per field was histomorphometrically determined. G and H, DKO BMMs were transduced with WT SLP-76 or empty vector. Following selection with blasticidin, the transduced cells were plated on plastic (G) or bone (H) in M-CSF and RANKL. Five days later, the number of spread OCs (G) and the percentage of OCs exhibiting actin rings (H) were determined. * p < 0.05 and *** p < 0.001.
formation is compromised (Fig. 3E) and resorption pit excavation is reduced more than what occurs with deletion of only SLP-76 (Fig. 3F). Confirming that their phenotypic abnormalities reflect absence of SLP-76, DKO OCs retrovirally reconstituted with WT-SLP-76 spread normally and generate abundant actin rings (Fig. 3, G and H). Hence, BLNK partially compensates for the absence of SLP-76 in vitro and SLP-76 fully normalizes BLNK-deficient OCs.

Our capacity to generate DKO OCs stands in contrast to the failure of Shinohara et al. (17) to do so. Because the M-CSF and RANKL used in this study are generated in our laboratory, we determined whether the discrepancy reflects reagent differences. Thus, we cultured DKO and control BMMs with commercial, recombinant RANKL and M-CSF in concentrations used by Shinohara et al. (17). DKO OCs are generated in these conditions (see supplemental Fig. S2) and are indistinguishable from those illustrated in Fig. 3A.

Absence of SLP-76 compromises OC function in vivo

To determine the impact of the adaptor protein on bone resorption in vivo, we supracalvarially injected PTH (1–34), four times daily for 4 days, into radiation chimera mice with SLP-76+/+ or SLP-76−/− OC genotypes. Similar to our in vitro observations, OCs are recruited in the absence of SLP-76 in vivo. In contrast, SLP-76−/− OCs are generally smaller and more elongated than normal cells (Fig. 4A). We repeated the experiment using DKO radiation chimeras and littermate controls, observing similar results. Confirming impaired bone resorption, PTH-enhancement of serum collagen fragments is blunted in SLP-76−/− and DKO mice (Fig. 4B). In contrast to the in vitro compensatory properties of BLNK, however, reduction of PTH-induced bone resorption is equivalent in SLP-76−/− and DKO mice. Conversely, the total number of OCs bearing SLP-76−/− or DKO mutations is not reduced, indicating suppressed resorption in these animals reflects OC dysfunction rather than recruitment (Fig. 4C). Butressing this conclusion, the proportion SLP-76−/− or DKO OCs juxtaposed to bone or the percentage of bone surface in contact with such mutant OCs is reduced in PTH-treated animals (Fig. 4, D and E). Interestingly, the percentage of SLP-76−/−/BLNK−/− (control) OCs on bone is slightly, but significantly, reduced relative to SLP-76−/− OCs with intact BLNK. Although the significance of this observation is obscure, it raises the possibility that deletion of three SLP family alleles impacts OC bone attachment.

Gads and LAT are dispensable for OC spreading and function

In TCR-mediated signaling, SLP-76 is a component of a plasma membrane-residing quaternary complex including LAT, phospholipase Cγ1, and Gads, a Grb2 family member containing SH2 and SH3 domains. Membrane-associated LAT is phosphorylated by Syk following receptor engagement and recruits SLP-76 through its interaction with Gads (14, 24). Gads is constitutively bound to SLP-76 through its SH2 domain and recognizes LAT via the Gads SH2 domain only after phosphorylation (3, 25). In contrast to the TCR, however, αιβ3 integrin-stimulated platelet cytoskeletal organization, activated via SLP-76 phosphorylation, is unimpeded in the absence of LAT or Gads (8–10).

Like αιβ3 in the platelet, SLP-76-mediated OC activation is independent of LAT and Gads. Specifically, LAT−/− and Gads−/− OCs spread normally and resorb bone as effectively as WT cells (see supplemental Fig. S3, A, C, D, and F). Moreover, integrin-mediated SLP-76 phosphorylation is intact in LAT−/− and Gads−/− OCs (see supplemental Fig. S3, B and E). Lastly, a SLP-76 mutant (R237A) specifically disrupting its association with Gads and family member, Grb2 (26), rescues the DKO phenotype as effectively as WT SLP-76 (see supplemental Fig. S3, G). Thus, it is unlikely that the normal phenotype of Gads null
cells reflects compensation by related proteins. Activation of SLP-76 in response to integrin activation in OCs is, therefore, LAT- and Gads-independent and mirrors signaling in the platelet rather than the TCR.

SLP-76 N-terminal tyrosine and proline-rich regions organize the OC cytoskeleton

To identify associated proteins mediating SLP-76-stimulated cytoskeletal organization, we mutated its N-terminal phospho-tyrosine (3YF) and proline-rich regions (Δ157-222), and SH2 point mutation (R448K). DKO BMMs were transduced with the four SLP-76 constructs or empty vector. B, Following selection, the cells were plated with M-CSF and RANKL on plastic. After 5 days, OCs were stained for TRAP activity. C, The number of spread cells in B was quantified on days 4 and 5. D, Actin ring formation by transduced cells, generated on bone, visualized by FITC-phalloidin staining. E, The percentage of cells exhibiting actin rings on day 5 was quantified. F, After removal of OCs generated on bone, resorptive lacunae were stained with peroxidase-conjugated wheat germ agglutinin and 3,3'-diaminobenzidine. G, Resorptive pit area was quantified histomorphometrically. H, DKO BMMs were transduced with GFP alone (empty vector), GFP WT SLP-76, or GFP-tagged mutant constructs. Cells were cultured on glass coverslips with M-CSF and RANKL for 5 days to generate OCs. The cells were examined by fluorescent microscopy to determine GFP distribution. *, p < 0.05; **, p < 0.01; and ***, p < 0.001 vs WT. All images were set at a magnification of ×200.

SLP-76 are unable to normalize spreading after prolonged culture with M-CSF and RANKL (Fig. 5, B and C). Interestingly, R448K SLP-76, which specifically disrupts the ability of the SH2 domain to bind phosphotyrosines, partially rescues the DKO cytoskeleton (Fig. 5B). Whereas their spreading on day 4 is compromised, DKO OCs expressing R448K SLP-76 are more akin to WT OCs on day 5, although morphological differences remain (Fig. 5, B and C). Confirming partial rescue, the SH2 domain, but not 3YF or Δ157-222 SLP-76 mutant, yields OCs with actin ring formation similar to WT (Fig. 5, D and E). DKO cells expressing mutations in the N-terminal and the proline-rich regions also fail to effectively degrade bone, whereas those transduced with R448K SLP-76 are normal in this regard (Fig. 5, F and G).
SLP-76 activation requires its proximity to the plasma membrane. We therefore asked whether the failure of 3YF and Δ157–222 SLP-76 to rescue the OC cytoskeleton is attended by defective cell surface localization. To this end, DKO BMMs were transduced with GFP-tagged WT, 3YF, Δ157–222, and R448K SLP-76 constructs. The cells were differentiated into OCs on glass coverslips and fixed on day 5. Mirroring their capacity to rescue the DKO cytoskeleton, WT and R448K SLP-76 constructs or empty vector before 3-day culture with M-CSF and RANKL. D, Lysates were immunoprecipitated with an anti-HA Ab. Immunoprecipitates were immunoblotted for phosphotyrosine (p-Y) and Vav3. F and G, Lysates of transduced DKO pre-fusion OCs were subjected to GST-PAK pull-down, followed by Rac1 immunoblot (F) or used to quantitate GTP-Rac1/2 by ELISA (G). Rac1 serves as loading control in F.

SLP-76 links Syk to Vav3 following integrin engagement in the OC

Having established that the tyrosine region of SLP-76, which contains its putative Vav binding site, regulates cytoskeletal remodeling, we asked whether SLP-76 and Vav3 associate following integrin engagement. Thus, DKO BMMs transduced with HA-tagged SLP-76 were treated with M-CSF and RANKL for 3 days, lifted and maintained in suspension or replated on vitronectin to activate αvβ3. In fact, SLP-76 and Vav3 associate more robustly in vitronectin-adherent cells than with those in suspension (Fig. 6A). To determine whether SLP-76 is proximal to Vav activation in αvβ3 signaling, we immunoblotted Vav3 immunoprecipitates, derived from adherent or suspended DKO and control pre-fusion OCs, with a pan-phosphotyrosine Ab. Absence of the SLP diminishes integrin-induced Vav3 phosphorylation (Fig. 6B), whereas Syk activation is intact (Fig. 6C), establishing that SLP-76 and BLNK are downstream of Syk and proximal to Vav3 in the integrin signaling cascade. Furthermore, adhesion-stimulated Vav3 phosphorylation is reduced in DKO pre-fusion OCs retrovirally expressing 3YF SLP-76, which attenuates SLP-76/Vav3 association (Fig. 6, D and E).

As Vav3 is a GEF for Rac, we asked whether activity of the small GTPase is also reduced by diminished Vav3 phosphorylation. In fact, Rac activation is equally induced by integrin engagement in DKO cells expressing empty vector, WT, or 3YF SLP-76. Therefore, although Vav3 phosphorylation is decreased in DKO OCs, Rac is activated by a parallel mechanism, independent of SLP-76.
SLP-76 regulates the OC cytoskeleton in a c-Fms-dependent manner

In addition to its capacity to stimulate BMM proliferation, M-CSF promotes organization of the OC cytoskeleton (27–29). Moreover, high-dose M-CSF normalizes the crenated phenotype of β3 integrin-null OCs through a Syk-dependent mechanism (30). Thus, there is commonality in the means by which activated c-Fms and αβ rearrange the OC cytoskeleton. To determine whether like the integrin, the cytokinesis-organizing capacity of c-Fms involves SLP-76, we exposed cytokine-starved pre-fusion OCs to M-CSF for 5 mins. Similar to integrin occupancy M-CSF induces Syk (29), SLP-76, and BLNK phosphorylation (Fig. 7, A and B). Also mirroring αβ signaling, M-CSF-stimulated SLP-76 phosphorylation requires Syk (Fig. 7C), whereas activation of the kinase is independent of SLP-76 (Fig. 7D). Finally, previously described M-CSF-mediated Vav3 phosphorylation is abrogated in the absence of either Syk or SLP-76 (11) (Fig. 7, E and F).

Discussion

The αβ-mediated cytoskeletal remodeling requires Syk and Vav3 (2, 11). Because it is activated by Syk in other cells, we posited that SLP-76, which is required for cytoskeletal rearrangement during immune synapse formation and platelet adhesion (7, 9), mediates actin organization in the OC. After confirming that integrin engagement rapidly phosphorylates SLP-76 and BLNK in a Syk-dependent manner, we established modest deficiencies in the spreading and resorptive capacity of SLP-76−/− OCs. Despite the fact that BLNK-deficient OCs are normal, absence of both SLP adaptors (DKO) prompts a more severe OC phenotype than does deletion of only SLP-76, indicating that BLNK partially compensates for the loss of SLP-76 in vitro.

In other cells, expression of the two SLP adaptors is often mutually exclusive. For example, SLP-76, alone, is present in T cells, and BLNK in B cells (3). The macrophage, however, is unique in that both proteins are substantially expressed but their combined deletion does not affect activation of the cell or FcRγ signaling (31). Given that SLP-76 and BLNK are present throughout osteoclastogenesis, we hypothesized that despite SLP adaptors not functioning in macrophages, they may in OCs. While containing normal osteoclastogenic markers and numbers of nuclei, DKO OCs, in fact, have defective cytoskeletons. Our finding that SLP adaptor proteins do not regulate differentiation or fusion of OCs, but rather their function in response to αβ, integrin or c-Fms activation, differs from that of Shinohara et al. (17) who failed to generate DKO OCs in vitro. Although we cannot account for this discrepancy, we establish it cannot be attributed to osteoclastogenic reagents. Furthermore, the phenotype of DKO OCs is similar to that attending deletion of other OC integrin-activated, cytoskeleton-organizing proteins. Interestingly, differences in DKO osteoclastogenesis mirrors conflicting observations regarding RANKL-mediated generation of DAP12-deficient OCs, which occurs in our hands (29) but not in that of Koga et al. (32).

Supporting our in vitro observations, PTH-treated SLP-76−/− and DKO radiation chimeras fail to efficiently resorb bone in vivo. Interestingly, whereas BLNK partially compensates for absence of SLP-76 in vitro (compare Figs. 2F and 3F), the same does not apply in vivo (Fig. 4B), at least in the context of PTH stimulation. In this regard, the role of cytokine-dependent hematopoietic cell linker CLINK, the third SLP family member, is yet to be determined in the OC (3). As CLINK is expressed only in cytokine-simulated cells, its impact in the complexity of the bone microenvironment may differ from that in cultured OC lineage cells.

Probably reflecting impaired remodeling, radiation chimeras with dysfunctional OCs, such as those lacking Syk, RANK, or Hck/Src, often have normal bone mass (2, 33 and J. Lorenzo, unpublished observations), and we find the same occurs in the context of SLP-76 deficiency. However, the response of such chimeras to stimulated resorption is typically blunted, as we demonstrate in the absence of SLP-76, with or without BLNK. As expected, PTH-treated control animals display a marked increase in OC recruitment and total resorptive activity. DKO and SLP-76−/− radiation chimeras recruit comparable numbers of OCs in response to the hormone. Each fails, however, to increase bone resorption to WT equivalence, indicating that SLP-76, though not involved in OC differentiation, regulates the OC cytoskeleton and, thus, function.

Gads constitutively binds SLP-76 in the TCR. When Syk phosphorylates LAT, it recognizes Gads, and the complex brings SLP-76 to the membrane (3). Gads, however, is not required for SLP-76 recruitment to the OC plasma membrane is via the tyrosine or the proline region, as formations within each affects GFP-tagged SLP-76 localization.

Despite the differences in SLP-76 activation in platelets and T cells, both αβ and the TCR regulate Vav3 and cytoskeletal rearrangement (7, 35). Given the common phenotype of OCs lacking SLP-76, Vav3, or Syk, we propose that the adaptor protein links the two others. This hypothesis is supported by the fact that Vav3 contains an SH2 domain that associates with the phosphorylated tyrosine of the adaptor (3). In fact, like Syk, SLP-76 is necessary for optimal Vav3 activation following integrin engagement. Confirming that at least one of the three phosphorylated tyrosines of SLP-76 recognizes Vav3, their mutation to phenylalanine blocks OC spreading and function, as well as phosphorylation of the GEF. Because the 3YF SLP-76 mutation disrupts other binding partners, such as Tec kinases and Nck, their inactivity possibly contributes to failure of DKO OC rescue. However, the reduction of Vav3 phosphorylation in the DKO cells, coupled with the similar phenotype of Vav3−/− OCs (11), suggests that SLP-76 is functioning, at least, in part through Vav3. Moreover, Tec kinase deficiency impairs osteoclastogenesis via the RANKL signaling pathway, with reduced numbers of TRAP-positive multinucleated cells in culture (17). Because our results are consistent with a defect in cytoskeletal organization and not osteoclastogenesis, it is likely that Tec kinases are not the principal mediators of SLP-76 cytoskeletal effector proteins.

Although DKO cells have a disorganized cytoskeleton and reduced Vav3 phosphorylation, their Rac activity is not impaired. As Vav3 was initially identified as a GEF for RhoA, RhoG, and to a lesser extent Rac (36), it is possible that, in the OC, Vav3 is also a GEF for other small GTPases. Because M-CSF-induced RhoA activation is normal in Vav1/Vav3-deficient OCs (11), it is unlikely that RhoA is a target of integrin-activated Vav3. Alternatively, RhoG may play a role in OC cytoskeletal organization as it shares greater amino acid sequence similarity with Rac family members than with Rho proteins and promotes T cell spreading on fibronectin (36, 37).

c-Fms and αβ activate a number of common cytoskeleton-organizing events in the OC. It is not unexpected, therefore, that the crenated phenotype of β3 integrin-deficient OCs is normalized by culturing cells with high dose M-CSF (30). Consistent with this
observation, M-CSF induces Vav3 phosphorylation and GTPase activation (28, 38), which prompted us to examine SLP-76 in the context of the cytokine. M-CSF induces Syk phosphorylation and in consequence, that of SLP-76 and BLNK. Consistent with the integrin pathway, M-CSF-induced Vav3 phosphorylation is abrogated in the absence of SLP-76 while Syk phosphorylation is normal, confirming once again that SLP-76 is distal to Syk and proximal to Vav3.

Our data suggest a model of αβ2- and c-Fms-mediated organization of the OC cytoskeleton whereby engagement of either receptor rapidly stimulates c-Src and DAP12, the latter recruiting Syk to its ITAM domain. Syk, which is activated by autophosphorylation in the case of c-Fms, or by c-Src in the context of the cytokine. M-CSF induces Syk phosphorylation and, in the context of the cytokine. M-CSF induces Syk phosphorylation and, in consequence, that of SLP-76 and BLNK. Consistent with the integrin pathway, M-CSF-induced Vav3 phosphorylation is abrogated in the absence of SLP-76 while Syk phosphorylation is normal, confirming once again that SLP-76 is distal to Syk and proximal to Vav3.

References

The authors have no financial conflict of interest.

Disclosures

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References

Supplemental Figure 1. BLNK deficient OCs resorb bone normally, in vitro and in vivo. (A) WT, BLNK$^{+/−}$ and BLNK$^{−/−}$ BMMs were cultured on plastic (top panel, 40x) and bone (bottom panel, 200x) with M-CSF and RANKL for 5 days and stained for TRAP activity (top) or labeled with FITC-phalloidin (bottom). (B) On the sixth day of culture on bone, the media was collected and assayed for CTx by ELISA. (C-D) Bones were collected from 6-8 week old mice. (C) Histological sections of the tibiae were stained for TRAP activity (200x). (D) The amount of trabecular bone (BV) was quantified relative to tissue volume (TV).
Supplemental Figure 2. Generation of DKO osteoclasts with commercial RANKL and M-CSF. Following the methods of Shinohara et al (17), DKO and control BMMs were cultured for 3-5 days with 50ng/ml purified RANKL (Peprotech) and 10ng/ml purified M-CSF (R&D Biosystems). The OCs were fixed and stained for TRAP activity.
Supplemental Figure 3. SLP-76 functions independently of LAT and Gads in OCs.

(A) Gads−/− and Gads+/− BMMs were cultured with M-CSF and RANKL for three to five days, fixed, and stained for TRAP. (B) Gads−/− and WT pre-OCs were serum- and cytokine-starved, lifted with EDTA, and either re-plated on vitronectin-coated plates (A) or maintained in suspension (S) for 30 minutes. The lysates were immunoprecipitated with an anti-SLP-76 antibody and immunoblotted for phosphotyrosine (P-Y) and SLP-76. (C) The culture medium from Gads-deficient and control OCs plated on bone was collected on day 6 and analyzed by ELISA for CTx content. (D) WT and LAT−/− BMMs
were cultured, on plastic, with M-CSF and RANKL for three to five days, fixed, and stained for TRAP activity. (E) Lysates of suspended (S) and adherent (A) WT and LAT−/− pre-OCs were immunoprecipitated for SLP-76 and the immunoprecipitates were immunoblotted for phosphotyrosine or SLP-76. (F) CTx content of culture medium of bone-residing LAT−/− and WT OCs. (G) DKO (SLP-76−/− BLNK−/−) BMMs transduced with either WT-SLP-76 or R237A-SLP-76 were cultured with M-CSF and RANKL for five days, fixed, and stained for TRAP. All images were acquired at 40x.