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J Immunol 2009; 183:1804-1812; Prepublished online 10 July 2009;
doi: 10.4049/jimmunol.0804206
http://www.jimmunol.org/content/183/3/1804

Supplementary Material

http://www.jimmunol.org/content/suppl/2009/07/13/jimmunol.0804206.DC1

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SLP-76 Couples Syk to the Osteoclast Cytoskeleton

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The capacity of the osteoclast (OC) to resorb bone is dictated by cytoskeletal organization, which in turn emanates from signals derived from the $\alpha_\beta_3$ 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 $\sim 40\%$ 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), 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 $\alpha_\beta_3$ integrin plays a central role in the process of actin ring formation and we have focused on delineating the mechanism of $\alpha_\beta_3$ 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, $\alpha_\beta_3$ 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 cytoproteins) 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 She (Gads) via an R-X-X-K motif. In the C terminus, the adaptor ADAP (adhesion and degranulation-promoting adapter 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 API 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 $\alpha_{\text{IIb}}\beta_3$ 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 Syp to Vav3 in a αβγδ
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. Tyubulewicz (National Institute for Medical Research, London, U.K.) and are on a 129sv 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 C57BL/6 background for over 10 generations. All mice were housed in the animal facility at Washington University School of Medicine, St. Louis, MO, and were maintained in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care. All animal studies were approved by the Animal Studies Committee of 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 chimeras. 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 chimeras generated from SLP-76−/− and BLNK−/− littermates or SLP-76−/−/BLNK−/− (control) and SLP-76−/−/BLNK−/− (DKO) littermates (α = 4–5 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 calvariae were isolated for histology. Fasting serum was collected and analyzed for collagen type I.

Macrophage isolation and OC culture

Cells were washed with ice-cold PBS and lysed in RIPA 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 5% SDS-PAGE and transferred onto a PVDF membrane. Following blocking with 0.1% casein in PBS, primary Abs were incubated overnight at 4°C and probed with fluorescent-labeled secondary Abs (The Jackson Laboratory) and detected on the Odyssey Infrared Imager System (LI-COR Biosciences). For immunoprecipitation studies, 750 μl of cleared lysates were incubated with 2 μg of primary Ab for overnight at 4°C, followed by 3× 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-BLNK 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 using FTC membranes. Lysates were prepared according to the manufacturer’s instructions. A total of 500 μg of protein was subjected to Sigma-G-LISA pull-down. Following multiple 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. 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

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 from 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 αβ1 integrin ligation (22), 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<sup>+/−</sup>), 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 from adhered (A) or suspended (S) cells were immunoprecipitated for either SLP-76 (B) or BLNK (C), and the immunoprecipitates were immunoblotted for phosphotyrosine (p-Y) and SLP-76 or BLNK.

**FIGURE 1.** SLP-76 and BLNK are phosphorylated, in OCs, by integrin occupancy in a Syk-dependent manner. A, WT BMMs, cultured with M-CSF and RANKL until time were lysed, and the lysate immunoblotted for SLP-76 and BLNK. B, α1 integrin subunit (arrow) serves as a marker of OC differentiation. B and C: Syk<sup>−/−</sup> and Syk<sup>+/−</sup> BMMs 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 from adhered (A) or suspended (S) cells were immunoprecipitated for either SLP-76 (B) or BLNK (C), and the immunoprecipitates were immunoblotted for phosphotyrosine (p-Y) and SLP-76 or BLNK.

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

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

In contrast to BLNK<sup>−/−</sup> 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<sup>−/−</sup> or SLP-76<sup>−/+</sup> (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<sup>−/−</sup> and control precursors form TRAP-expressing multinucleated cells by day 3 (Fig. 2A). Confirming normal OC differentiation, SLP-76<sup>−/−</sup> 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<sup>+/−</sup> OCs are indistinguishable from those derived from naïve WT mice, the majority of those lacking SLP-76 fail to spread, and exhibit a morphology similar to Syk<sup>−/−</sup> 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<sup>−/−</sup> counterparts, however, the phenotype of SLP-76<sup>−/−</sup> 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 resorbed 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<sup>−/−</sup>/BLNK<sup>−/−</sup> (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),<sup>7</sup> we used SLP-76<sup>−/−</sup>/BLNK<sup>−/−</sup> 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 multinucleated, TRAP-positive cells that express osteoclastogenic markers normally, but form smaller OCs with a “crenated” appearance (Fig. 3, A and B). Unlike SLP-76<sup>−/−</sup> cells (Fig. 2A), however, prolonged exposure to M-CSF and RANKL does not rescue “crenated” DKO OCs, indicating that BLNK compensates partially for the loss of SLP-76.

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. Unlike SLP-76<sup>−/−</sup> OCs, SLP-76<sup>−/−</sup>/BLNK<sup>−/−</sup> cells possess a reduced 43% without SLP-76 (Fig. 2F). Hence, SLP-76 does not mediate OC maturation, but its absence impairs OC function.

<sup>4</sup>The online version of this article contains supplemental material.
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 irradiation chimera mice with SLP-76 mutant OCs. 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. 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.

**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 SH3 domain and recognizes LAT via the Gads SH2 domain. In contrast to the TCR, however, SLP-76 phosphorylation is intact in LAT−/− or Gads−/− cells. Moreover, integrin-stimulated platelet cytoskeletal organization, activated via SLP-76 phosphorylation, is unimpeded in the absence of LAT or Gads.

Like α_{in}β_{3}, integrin-stimulated platelet cytoskeletal organization, activated via SLP-76 phosphorylation, is unimpeded in the absence of LAT or Gads.
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 phosphotyrosine (3YF) and proline-rich regions (Δ157-222), and the SH2 point mutation (R448K). DKO BMMs were transduced with the four SLP-76 constructs or empty vector. Following selection, the cells were plated with M-CSF and RANKL on plastic. After 5 days, OCs were stained for TRAP activity. 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 localize to the OC surface (Fig. 5H).

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 dipeptide repeats eliminates integrin-injured 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.

FIGURE 6. SLP-76 links Syk to Vav3 following integrin engagement in the OC. BMMs were differentiated into pre-fusion OCs by 3 days culture with M-CSF and RANKL. They were serum and cytokine starved, lifted, and either maintained in suspension (S) or adhered to vitronectin (A). A, HA immunoprecipitates of DKO pre-fusion OCs transduced with HA-tagged SLP-76, immunoblotted for Vav and SLP-76, as loading control. B and C. Lysates of DKO and control (SLP-76+/BLNK−) pre-fusion OCs were immunoprecipitated with an anti-Vav3 (B) or anti-Syk (C) Ab. The immunoprecipitates were immunoblotted for phosphotyrosine (p-Y) and Vav3 (B) or Syk (C) as loading controls. DKO BMMs were transduced with WT or 3YF 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 Vav and SLP-76. E, Lysates of transduced DKO pre-fusion OCs were immunoprecipitated with an anti-Vav3. Immunoprecipitates were immunoblotted for phosphotyrosine 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.

FIGURE 7. SLP-76 activates cytoskeleton-organizing proteins in an M-CSF-dependent manner in OCs. WT pre-fusion OCs were serum and cytokine starved and exposed to M-CSF or vehicle for 5 min. Lysates were immunoprecipitated using an anti-Syk (A) or anti-BLNK (B) Ab. Immunoprecipitates were immunoblotted for phosphotyrosine (p-Y). C, Cytokine-starved Syk+/− or Syk−/− pre-fusion OCs were exposed to M-CSF for 5 min. Lysates were immunoprecipitated with an anti-SLP-76 Ab and immunoprecipitates immunoblotted for phosphotyrosine and SLP-76. D, SLP-76+/− and SLP-76−/− pre-fusion OCs were treated with M-CSF for 5 min. Lysates were immunoprecipitated using an anti-Syk Ab and the immunoprecipitates were immunoblotted for phosphotyrosine and Syk. Vav3 was immunoprecipitated from Syk+/− or Syk−/− (E) or SLP-76+/− and SLP-76−/− (F) cytokine-starved pre-fusion OCs treated with M-CSF for 5 min. The immunoprecipitates were immunoblotted for phosphotyrosine and Vav3.
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 \( \beta_1 \) integrin-null OCs through a Syk-dependent mechanism (30). Thus, there is commonality in the means by which activated c-Fms and \( \alpha_\beta_1 \) rearrange the OC cytoskeleton. To determine whether like the integrin, the cytoskeleton-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 \( \alpha_\beta_1 \) 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 \( \alpha_\beta_1 \)-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 FcR 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 \( \alpha_\beta_1 \) 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 as Gads\(^{-/-}\) OCs are normal. Additionally, integrin-mediated SLP-76 phosphorylation occurs in the absence of Gads. Because a homologous Grb2 family member may compensate for the loss of Gads, we examined LAT-deficient cells, which also spread normally and are functional. These observations are reminiscent of platelets in which adhesion-dependent signals also do not require the Gads/LAT complex (8, 10, 34). Our data indicate that SLP-76 recruitment to the OC plasma membrane is via the tyrosine or the proline region, as mutations within each affects GFP-tagged SLP-76 localization.

Despite the differences in SLP-76 activation in platelets and T cells, both \( \alpha_{\text{Itb}} \beta_1 \) 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 DOC 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).
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 α5β1- 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 integrin, phosphorylates SLP-76 (2, 29). Phosphorylated SLP-76, via its tyrosine-region, recognizes Vav3 and forms a complex that includes small GTPases at the cell surface. SLP-76 is, therefore, yet another member of the emerging signaling pathway for which the OC regulates its cytoskeleton and, thus, its unique capacity to resorb bone.

Disclosures
The authors have no financial conflict of interest.

References