Syk Tyrosine 317 Negatively Regulates Osteoclast Function via the Ubiquitin-Protein Isopeptide Ligase Activity of Cbl*

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Cytoskeletal organization of the osteoclast (OC), which is central to the capacity of the cell to resorb bone, is induced by occupancy of the αvβ3 integrin or the macrophage colony-stimulating factor (M-CSF) receptor c-Fms. In both circumstances, the tyrosine kinase Syk is an essential signaling intermediary. We demonstrate that Cbl negatively regulates OC function by interacting with SykY317. Expression of nonphosphorylatable SykY317F in primary Syk−/− OCs enhances M-CSF- and αvβ3-induced phosphorylation of the cytoskeleton-organizing molecules, SLP76, Vav3, and PLCγ2, to levels greater than wild type, thereby accelerating the resorptive capacity of the cell. SykY317 suppresses cytoskeletal organization and function while binding the ubiquitin-protein isopeptide ligase Cbl. Consequently, SykY317F abolishes M-CSF- and integrin-stimulated Syk ubiquitination. Thus, Cbl/SykY317 association negatively regulates OC function and therefore is essential for maintenance of skeletal homeostasis.

OCs2 are multinucleated cells generated by fusion of mononuclear progenitors of the monocyte/macrophage family under the aegis of M-CSF and receptor activator of nuclear factor κB ligand (RANKL) (1). Upon mineralized matrix recognition, the OC polarizes its fibrillar actin, eventuating in the formation of an acidified extracellular microenvironment that degrades bone. Failure to undergo this polarization results in OC hypo-function and consequently in varying degrees of osteopenia and consequently in normal skeletal mass.

1 The abbreviations used are: OC, osteoclast; RANKL, receptor activator of nuclear factor κB ligand; BMMs, bone marrow macrophages; GST, glutathione S-transferase; TRAP, tartrate-resistant acid phosphatase; CTx, C-terminal cross-linking telopeptide of bone collagen; M-CSF, macrophage colony-stimulating factor; WT, wild type; E3, ubiquitin-protein isopeptide ligase; PBS, phosphate-buffered saline; PLCγ2, phospholipase Cγ2; ERK, extracellular signal-regulated kinase.

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Integrins are transmembrane α/β heterodimers that mediate cell-cell and cell-matrix interactions and generate intracellular signals when occupied by ligands (3). The integrin, αvβ3, is expressed by OCs, and binding of this complex to bone is pivotal to the resorptive process (4).

M-CSF recognizes its transmembrane receptor tyrosine kinase, c-Fms, and induces receptor autophosphorylation at seven tyrosine residues within the cytoplasmic domain (5). Several Src homology-2 domain-containing molecules are recruited to the phosphorytous residues upon M-CSF binding and initiate signaling cascades that lead to cytoskeletal organization, survival, and proliferation of OC lineage cells (5–7). Both the αvβ3 integrin and M-CSF are important regulators of OC actin remodeling (4, 6, 8).

Syk is a 72-kDa nonreceptor tyrosine kinase, which mediates αvβ3- and c-Fms-induced OC cytoskeletal organization and function in a phosphorylation-dependent manner via a process involving activation of associated adaptor proteins, such as SLP-76 and Vav3 (9, 10). A number of Syk tyrosine residues undergo phosphorylation following engagement of the integrin and Fcγ receptor in immune (11) and mast cells (12). Three conserved tyrosine residues in the Syk linker region, namely Tyr317, Tyr342, and Tyr346, lie within consensus sequences for recognition by Src homology 2 domains, suggesting they transduce signals. Although phospho-SykY342 and phospho-SykY346 may serve as positive signaling regulators (12–14), phosphorylation of SykY317 creates a binding site for c-Cbl, an E3 ubiquitin ligase proposed to prompt ubiquitination and subsequent degradation of Syk (15, 16). Hence, SykY317 is a candidate negative regulatory site, but its role in OC function and/or differentiation is unknown.

Cbl is a 120-kDa protein that is tyrosine-phosphorylated following activation by growth factors, cytokines, and integrins. It has two distinct but related activities, serving both as an adaptor protein (17, 18) and E3 ubiquitin ligase (19, 20). Cbl functions principally as an adaptor in OCs by participating in signaling complexes that are important in the assembly and remodeling of the actin cytoskeleton (18, 21). In other cell types, Cbl is also a negative regulator of receptor and nonreceptor tyrosine kinases, as it promotes their degradation (22). OCs and their precursors express c-Cbl and another family member Cbl-b that compensates for the absence of c-Cbl (23, 24). As combined deletion of both isoforms eventuates in early embryonic lethality (24), it is not clear if c-Cbl functions as an E3 ubiquitin ligase in OCs. We establish that Cbl, recognizing SykY317, prompts the ubiquitination of the kinases thereby arresting activation of cytoskeleton-organizing molecules and thus OC function. The Cbl-SykY317 complex is therefore important in maintenance of normal skeletal mass.

EXPERIMENTAL PROCEDURES

Mice—Syk−/− (129/SV background) mice were described previously (25). Because of perinatal lethality of Syk−/− mice, we generated bone marrow chimeras by transplanting Syk−/−
fetal liver cells into lethally irradiated WT recipients (9). Chimeras were used as a source of bone marrow macrophages (BMMs) 4–8 weeks after bone marrow transplantation.

All mice used in these experiments were 6–8 weeks old and housed in the animal care unit of Washington University School of Medicine, where they were maintained according to the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care. All animal experimentation was approved by the Animal Studies Committee of Washington University School of Medicine.

Reagents—Recombinant murine M-CSF was obtained from R & D Systems (Minneapolis, MN). Glutathione S-transferase (GST)-RANKL was expressed in our laboratory as described (26). The source of antibodies is as follows: mouse anti-Syk monoclonal antibody from Abcam (Cambridge, MA); anti-phosphotyrosine monoclonal antibody 4G10 and rabbit anti-Vav3 from Upstate (Charlottesville VA); monoclonal antibody 327, directed against the c-Src protein, were gifts of Dr. A. Shaw (Department of Pathology, Washington University School of Medicine, St. Louis, MO); rabbit anti-Src p-Y416 antibody, rabbit anti-phospho-Cbl (Y774) antibody, rabbit anti-SLP-76 antibody, and rabbit anti-phospho-PLCγ2 antibody from Cell Signaling (Beverly MA); goat anti-SLP-76 as described previously (27); rabbit anti-Syk (N-19), mouse anti-ubiquitin, rabbit anti-Cbl, and mouse anti-PLCγ2 antibody from Santa Cruz Biotechnology (Santa Cruz, CA). The plasmid transfection reagent FuGENE 6 was purchased from Roche Applied Science. All other chemicals were obtained from Sigma.

Macrophage Isolation and OC Culture—Primary BMMs were prepared as described previously (28) with slight modification. Marrow was extracted from femora and tibiae of 6–8-week-old mice with minimum Eagle’s α-medium and cultured in minimum Eagle’s α-medium containing 10% inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin (α-10 medium) with 1:10 CMG condition media (29) on bacterial plastic dishes. Cells were incubated at 37 °C in 6% CO2 for 3 days and then washed with PBS and labeled with 1× trypsin/EDTA (Invitrogen) in PBS. A total of 5×105 cells were cultured in 200 μl of minimum Eagle’s α-medium containing 10% heat-inactivated fetal bovine serum with 100 ng/ml GST-RANKL and 30 ng/ml mouse recombinant M-CSF in 96-well tissue culture plates, some containing sterile bone slices. Cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) activity after 6 days in culture, using a commercial kit (387-A, Sigma). For pre-OC generation, 1.5×106 BMMs were plated per 10-cm tissue culture dish and cultured in 30 ng/ml M-CSF and 100 ng/ml GST-RANKL for 3 days.

Staining of Actin Ring and Bone Resorptive Pits—For actin ring staining, cells were cultured on bovine bone slice in the presence of M-CSF and RANKL for 6 days at which time cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, rinsed in PBS, and immunostained with Alexa 488 phalloidin (Molecule Probes). To quantitate resorption lacunae, cells were removed from bone slices with mechanical agitation. Bone slices were incubated with peroxidase-conjugated wheat germ agglutinin (Sigma) for 1 h and stained with 3,3′-diaminobenzidine (Sigma).

Media C-terminal Cross-linking Telopeptide of Bone Collagen (CTX) Assay—BMMs were cultured on bovine bone slice in 96-well plates with RANKL and M-CSF for 6 days. α-10 medium was changed 1 day before harvesting. Medium CTx concentration was determined using a CrossLaps for Culture ELISA kit (Nordic Bioscience Diagnostics A/S, Herlev, Denmark).

Syk Kinase Assay—BMMs were cultured on tissue culture plates with RANKL and M-CSF for 3 days. Cells were lifted with 0.1% EDTA and then replated on vitronectin-coated plates for 30 min. Cells were lysed, and Syk kinase activity was tested using Omnia plate-based assay kit (Invitrogen).

Plasmids and Retroviral Transduction—Wild type human Syk cDNAs, a gift from Dr. Sanford Shattil (University of California, San Diego), were subcloned into the BamHI and Xhol sites of a pMX retroviral vector in which the puromycin resistance sequence was replaced with one coding for blastocidin resistance. Y317F mutant was generated using the QuickChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA). WT and Y317F mutant Syk cDNA was transected transiently into Plat-E packaging cells using FuGENE 6 transfection reagent (Roche Applied Science). Virus was collected 48 h after transfection. BMMs were infected with virus for 24 h in the presence of 100 ng/ml M-CSF and 4 μg/ml Polybrene (Sigma). Cells were selected in the presence of M-CSF and 1 μg/ml blastocidin (Calbiochem) for 3 days prior to use as OC precursors.

Western Blotting and Immunoprecipitation—Cultured cells were washed twice 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 Na3VO4, 1 mM NaF, and 1× protease inhibitor mixture (Roche Applied Science). After incubation on ice for 10 min, cell lysates were clarified by centrifugation at 15,000 rpm for 10 min. Forty micrograms of total lysates were subjected to 8% SDSPAGE and transferred onto polyvinylidene difluoride membranes. Filters were blocked in 0.1% casein in PBS for 1 h and incubated with primary antibodies at 4 °C overnight followed by probing with fluorescence-labeled secondary antibodies (The Jackson Laboratory). Proteins were detected with the Odyssey infrared imaging system (LI-COR Biosciences).

RESULTS

SykY317F Enhances OC Spreading—SykY317F is phosphorylated in the OC, in response to αvβ3 integrin engagement (9) or M-CSF stimulation (10). To assess the role of SykY317 in OC function, we retrovirally transduced hemagglutinin-tagged SykWT and SykY317F into Syk–/– BMMs, which after 3 days of culture in M-CSF express equal amounts of the native and mutated protein (Fig. 1A).

Syk–/– BMMs differentiate normally into OCs, but the mutant polypektions are dysfunctional as they fail to organize their cytoskeleton and optimally resorb bone (9). To assess the role of SykY317 in OC function, we treated SykWT and SykY317F BMMs with increasing doses of RANKL and M-CSF for 6 days (Fig. 1, B and C). Mirroring our previous data, WT Syk rescues the cytoskeletal abnormalities of Syk–/– OCs (9) and, in keeping with unaltered OC number in Syk–/– mice, does not affect
osteoclastogenesis (Fig. 1B). Surprisingly, SykY317F not only rescues the cytoskeletal abnormalities of Syk−/− OCs (Fig. 1, B–D), but cells expressing the nonphosphorylatable mutant actually spread more effectively than WT (Fig. 1, B and E). The enhancement of size and spreading is particularly evident at lower concentrations of RANKL, indicating that SykY317F sensitizes OCs to the cytokine.

To ensure that these morphological abnormalities of SykY317F OCs do not reflect accelerated differentiation, we measured a series of markers of osteoclastogenesis in BMMs exposed to M-CSF and RANKL with time. Expression of characteristic osteoclastogenic proteins is not enhanced in SykY317F cells (Fig. 1F) nor are changes in specific intracellular signaling events that mediate OC differentiation (Fig. 1, G and H), namely RANKL-induced NF-κB, assessed by IκB-α phosphorylation and degradation, as well as c-Jun N-terminal kinase, ERK1/2, and p-38 phosphorylation. M-CSF-driven ERK1/2 and AKT phosphorylation are also normal in SykY317F OCs.

SykY317F Enhances OC Function—To further explore the cytoskeletal features of SykY317F-expressing OCs, we maintained BMMs on bone slices in M-CSF and RANKL for 6 days, in parallel with experiments depicted in Fig. 1. The actin cytoskeleton was visualized with fluorescein isothiocyanate-phalloidin. In correlation with their size, SykY317F OCs on the resorptive substrate have enlarged actin rings (Fig. 2A), a critical hallmark of cytoskeletal organization. Establishing that the unusual cytoskeletal morphology of SykY317F-bearing OCs translates into the capacity of the cells to degrade bone, their ability to form resorptive pits is significantly increased, relative to WT, particularly at low dose RANKL (Fig. 2, B and C). Most importantly, medium CTx of SykY317F OCs on bone is increased 3-fold relative to WT (Fig. 2D). The fact that mutant and WT OC numbers are indistinguishable (Fig. 1D) establishes that the enhanced bone degradation reflects accelerated resorptive activity per cell and not stimulated osteoclastogenesis.

Kinase Activity of SykY317F Is Not Increased—The capacity of Syk to organize the OC cytoskeleton depends upon its phosphorylation, mediated in the context of integrin activation by c-Src, and in response to M-CSF by autophosphorylation (9, 10). Thus, the stimulated function of SykY317F-bearing OCs might reflect enhanced c-Src and/or Syk activation.
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mine whether integrin-induced activity of the two kinases is enhanced in mutant osteoclastic cells, we cultured WT- and Syk<sup>Y317F</sup>-expressing BMMs in RANKL and M-CSF. After 3 days, the cells were lifted and replated on the αβ3 ligand, vitronectin, or maintained in suspension. As seen in Fig. 3A, integrin-induced Syk phosphorylation is unaltered by the Y317F mutation. Similarly, αβ3-stimulated c-Src activity, as manifest by its Tyr<sup>416</sup> phosphorylation and total protein phosphorylation in Syk<sup>Y317F</sup>-expressing OCs is indistinguishable from WT (Fig. 3B). Like αβ3 occupancy, c-Fms activation prompts similar Syk (Fig. 3C) and total protein (Fig. 3D) phosphorylation in WT and mutant Syk-expressing cells. Furthermore, the Y317F mutant does not alter Syk kinase activity induced by αβ3 occupancy (Fig. 3E).

**Syk<sup>Y317F</sup> Super-activates Cytoskeleton-organizing Signaling Molecules**—Syk is an intermediary in the αβ3- and c-Fms-activated cytoskeletal organizing pathway in the OC, which involves target signaling molecules, including the guanine nucleotide exchange factor Vav3 (9). We therefore reasoned that the enhanced cytoskeleton-organizing capacity of Syk<sup>Y317F</sup> should be reflected by increased activity of Vav3. In fact, whether stimulated by integrin or c-Fms occupancy, Vav3 phosphorylation is enhanced in mutant osteoclastic cells (Fig. 4, A and B). PLCγ2 (30, 31) and SLP-76 (32) are also integrin- and M-CSF-induced OC cytoskeleton-organizing signaling molecules, which in other cells are activated by Syk (33, 34). Like Vav3, phosphorylation of both molecules, whether induced by αβ3 or c-Fms occupancy, is increased in Syk<sup>Y317F</sup> OCs (Fig. 4, C–F). Thus, augmented OC function in a circumstance wherein Syk<sup>Y317F</sup> is not phosphorylated reflects activation of cytoskeleton-organizing molecules.

**Syk<sup>Y317F</sup> Inhibits Cbl-mediated Ubiquitination**—The unaltered kinase capacity of Syk<sup>Y317F</sup> suggests its enhanced net activity, at least in part, reflects increased abundance. In other cell types, phosphorylated Syk<sup>Y317F</sup> binds c-Cbl, which functions as E3 ligase, ultimately promoting Syk ubiquitination and degradation. Thus, failure of Syk<sup>Y317F</sup> to undergo phosphorylation may prolong the half-life of the protein. To address this hypothesis, in OCs, we first established that αβ3 engagement phosphorylates Syk<sup>Y317F</sup> (Fig. 5A). Furthermore, whereas WT Syk/Cbl association is enhanced by αβ3 occupancy, the same is not true in the context of Syk<sup>Y317F</sup>, a circumstance in which other Syk tyrosines are phosphorylated (Fig. 5B). We then determined whether the unaltered abundance of Syk<sup>Y317F</sup> is due to decreased c-Cbl association. Indeed, the Y317F mutant, but not WT, Syk/Cbl association was impaired following αβ3 occupancy (Fig. 5C). Moreover, the Y317F mutation uniquely prevented Syk ubiquitination (Fig. 5D). Thus, Syk<sup>Y317F</sup> failure to undergo phosphorylation prolongs the half-life of the protein, implicating an increase in protein abundance and concomitantly increased kinase activity.

**Syk Tyrosine 317 Promotes OC Function**—To determine whether the enhanced kinase activity of Syk<sup>Y317F</sup> translates to increased OC function, we cultured WT and Syk<sup>Y317F</sup>-expressing BMMs in RANKL and M-CSF. After 3 days, the cells were lifted and replated on vitronectin (Fig. 5E). Phosphorylated tyrosine (p-Y) in Syk immunoprecipitates (IP) was determined by immunoblot. B cells were cultured with or without M-CSF (100 ng/ml) for 5 min. Phosphorylated tyrosine in Syk immunoprecipitates was determined by immunoblot. D, cells were cultured as in C. Total tyrosine-phosphorylated proteins and phosphorylated Src(Y416) were determined by immunoblot. C, cells were cultured with or without M-CSF (100 ng/ml) for 5 min. Phosphorylated tyrosine in Syk immunoprecipitates was determined by immunoblot. B, cells were cultured with RANKL and M-CSF for 3 days. A, cells were then lifted and either maintained in suspension (S) or plated on vitronectin (A) for 30 min. Phosphorylated tyrosine (p-Y) in Syk immunoprecipitates (IP) was determined by immunoblot.

**Syk<sup>Y317F</sup> Enhances OC Function**—The unaltered kinase capacity of Syk<sup>Y317F</sup> suggests its enhanced net activity, at least in part, reflects increased abundance. In other cell types, phosphorylated Syk<sup>Y317F</sup> binds c-Cbl, which functions as E3 ligase, ultimately promoting Syk ubiquitination and degradation. Thus, failure of Syk<sup>Y317F</sup> to undergo phosphorylation may prolong the half-life of the protein. To address this hypothesis, in OCs, we first established that αβ3 engagement phosphorylates Syk<sup>Y317F</sup> (Fig. 5A). Furthermore, whereas WT Syk/Cbl association is enhanced by αβ3 occupancy, the same is not true in the context of Syk<sup>Y317F</sup>, a circumstance in which other Syk tyrosines are phosphorylated (Fig. 5B, and data not

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**FIGURE 2.** **Syk<sup>Y317F</sup> Enhances OC Function.** In parallel with the experiments depicted in Fig. 1, Syk<sup>Y317F</sup> BMMs, transduced with either WT Syk or Syk<sup>Y317F</sup>, were cultured with M-CSF (30 ng/ml) and increasing amounts of RANKL for 6 days on bone slices. A, actin ring formation was determined by immunofluorescence following fluorescein isothiocyanate-phalloidin staining. B, after 6 days, OCs were removed and the bone slices stained with horseradish peroxidase-labeled wheat germ agglutinin to visualize resorption lacunae. C, histomorphometric analysis of pit area/field. D, medium was collected after 6 days in M-CSF and RANKL (100 ng/ml) and assayed for CTx concentration. *, p < 0.01; **, p < 0.001.

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**FIGURE 3.** **Syk<sup>Y317F</sup> Does Not Alter Syk Kinase Activity.** Syk<sup>Y317F</sup> BMMs, transduced with either WT Syk or Syk<sup>Y317F</sup>, were cultured with RANKL and M-CSF for 3 days. A, cells were then lifted and either maintained in suspension (S) or plated on vitronectin (A) for 30 min. Phosphorylated tyrosine (p-Y) in Syk immunoprecipitates (IP) was determined by immunoblot. B, cells were treated as in A. Total tyrosine-phosphorylated proteins and phosphorylated Src(Y416) were determined by immunoblot. C, cells were treated with or without M-CSF (100 ng/ml) for 5 min. Phosphorylated tyrosine in Syk immunoprecipitates was determined by immunoblot. D, cells were treated as in C. Total tyrosine-phosphorylated proteins were determined by immunoblot. (Numbers represent densitometric analysis relative to suspension, or 0 min of M-CSF, in WT cells.) E, cells were then lifted and replated on vitronectin for 30 min. Cells were lysed and incubated for reaction for 30, 60, and 90 min. The kinase activity was tested using fluorescence enzyme-linked immunosorbent assay reader. Numbers represent average of three time points. RFU, relative fluorescent units.
Expression of phosphorylated Syk Tyr317 and total Syk was determined by immunoblot. Actin serves as loading control.

FIGURE 4. Syk Tyr317 super-activates cytoskeleton-organizing signaling molecules. Syk-/- BMMs, transduced with either WT Syk or Syk Tyr317F, were cultured with RANKL and M-CSF for 3 days. A, cells were lifted and either maintained in suspension (S) or plated on vitronectin (A) for 30 min. Phosphorylated tyrosine (p-Y) in Vav3 immunoprecipitates (IP) was determined by immunoblot. B, cells were serum- and cytokine-starved and treated with M-CSF (100 ng/ml) for 5 min. Phosphorylated tyrosine in Vav3 immunoprecipitates was determined by immunoblot. C, cells were treated as in A. Phosphorylated tyrosine in PLCγ2 immunoprecipitates was determined by immunoblot. D, cells were serum- and cytokine-starved and treated with M-CSF (100 ng/ml) for 5 min. Phosphorylated tyrosine in PLCγ2 immunoprecipitates was determined by immunoblot. E, cells were treated as in A. Phosphorylated tyrosine in SLP76-PLCγ2 immunoprecipitates was determined by immunoblot. (Numbers represent densitometric analysis relative to suspension or 0 min of M-CSF in WT cells.)

FIGURE 5. Syk Tyr317 abolishes Syk-Cbl association in OCs. A, WT BMMs were cultured with RANKL and M-CSF for 3 days. The cells were then lifted and either maintained in suspension (S) or plated on vitronectin (A) for 30 min. Expression of phosphorylated Syk Tyr317 and total Syk was determined by immunoblot. Actin serves as loading control. B–D, Syk-/- BMMs, transduced with either WT Syk or Syk Tyr317F, were cultured with RANKL and M-CSF for 3 days. B, cells were then lifted and either maintained in suspension (S) or plated on vitronectin (A) for 30 min. Cbl immunoprecipitates (IP) were probed by immunoblot for Syk. IgG immunoprecipitate serves as negative control. C, cells were treated as in B. Phosphorylated tyrosine (p-Y) in Cbl immunoprecipitates (IP) was determined by immunoblot. D, cells were treated with M-CSF (100 ng/ml) for 5 min. Phosphorylated tyrosine in Cbl immunoprecipitates was determined by immunoblot. E, cells were treated as in B. Phosphorylated Cbl in total cell lysates was determined by immunoblot using a specific phospho-Cbl Tyr744 antibody.

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Because Cbl is an E3 ligase, its interaction with Syk may yield ubiquitination and consequent degradation of the tyrosine kinase. We therefore asked if αvβ3 or c-Fms ligation induces Syk ubiquitination and if so is the event altered by Syk Tyr317F. To this end, we pretreated WT and Syk Tyr317F pre-OCs with the proteasome inhibitor, MG-132, for 1 h before exposure to M-CSF. As shown in Fig. 6A, the cytokine induces ubiquitination of WT Syk (and/or associated proteins), which is totally arrested in the presence of Syk Tyr317F. Syk Tyr317F also dampens αvβ3-induced Syk ubiquitination (Fig. 6B). To determine the potential impact of the cytokine on Syk degradation, we treated WT and Syk Tyr317F marrow macrophages with M-CSF and RANKL for 3 days to commit them to the OC phenotype. The cells were exposed to cycloheximide in the absence of cytokines for 1 h to arrest protein synthesis. The cells were treated with M-CSF, and the quantity of WT and mutated Syk was determined by immunoblot during the next 4 h. Consistent with its activity being unaltered in nonstimulated cells (Fig. 4), basal amounts of the tyrosine kinase are similar in those bearing WT and Tyr317F mutated Syk (Fig. 6C). On the other hand, M-CSF promotes progressive degradation of WT Syk but not Syk Tyr317F. Interestingly, degradation of Syk is apparent prior to detectable ubiquitination.

DISCUSSION

All forms of pathological bone loss, including those attending autoimmune osteolysis, represent enhanced resorption relative to formation. In most circumstances, accelerated skeletal degradation reflects a combination of increased OC number and activity, the latter typically associated with cytoskeletal reorganization. In this circumstance, the cell polarizes to form actin rings and a ruffled membrane, both participating in delivery of matrix-degrading molecules into the resorptive microenvironment between bone and the juxtaposed plasma membrane (35).

Although the general morphological features of OC polarization have been long appreciated, insights into the relevant molecular mechanisms are recent. Matrix-derived signals, mediated via the αvβ3 integrin and M-CSF, are particularly important in organizing the cytoskeleton of the resorptive cell. In fact, the integrin and cytokine share many components of a canonical signaling pathway eventuating in OC polarization and bone degradation. This signaling complex includes the shown). Despite the fact that Syk Tyr317 fails to recognize Cbl, the mutated kinase does not alter total Cbl tyrosine phosphorylation or that of Cbl Tyr744, one of the major sites of tyrosine phosphorylation (Fig. 5, C–E). Thus, Cbl phosphorylation does not depend on its recognition of Syk.
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Syk-deficient OCs fail to spread, lack actin rings, and have attenuated bone resorptive activity. As expected, all three defects are rescued by expressing WT Syk in cells lacking the tyrosine kinase. The novel feature of this study is that, as in B-cells and mast cells, SykY317 also blunts the OC. Alternatively, inhibition of phosphorylation of the residue, which occurs upon αvβ3 occupancy, “super-rescues” the cytoskeletal and resorptive dysfunction of Syk−/− OCs as manifest by spreading, actin ring formation, and bone degradation. These cells are reminiscent of those generated in the absence of SHP1, another molecule that physiologically restrains OC function (42). The fact that the stimulatory effect SykY317F exerts on resorptive activity reflects accelerated activity and not increased OC number is consonant with normal expression and activation of differentiation and immediate signaling molecules, respectively.

SLP-76, an adaptor protein lacking intrinsic enzyme activity, plays a key role in T-cell receptor-derived signals, including those that regulate the actin cytoskeleton (43). SLP-76 is phosphorylated by Syk in a number of cells, and we find the same holds following αvβ3 engagement or M-CSF treatment of OCs. In keeping with the negative regulatory function of SykY317, SLP-76 is hyper-phosphorylated in SykY317F-bearing cells. In consequence, Vav3, which is a component of the cytoskeleton-organizing complex recruited by activated SLP-76, also undergoes enhanced phosphorylation in the mutant OCs. Thus, SykY317 restrains OC cytoskeletal organization by dampening activation of effector molecules.

Cbl family proteins are evolutionarily conserved negative regulators that associate with protein-tyrosine kinases upon their activation. Cbl recognizes, ubiquitinates, and negatively regulates Syk in T-cells and mast cells and does so by binding to phosphorylated SykY317 (16, 22, 44, 45). Having defined the role of WT Syk in OCs (9), these observations prompted us to determine whether the bone resorptive function of the kinase is altered by preventing Tyr317 phosphorylation. Similar to its association with other αvβ3-associated proteins in OCs, Cbl recognizes Syk upon integrin activation (21) in this circumstance by binding Tyr317. Although the nonphosphorylatable Y317F mutant blocks Syk/Cbl association, it does not alter activation of either molecule. This observation, taken with the normal Cbl phosphorylation extant in Syk−/− OCs (data not shown), indicates Syk and Cbl activation are independent events.

On the other hand, the Y317F mutant profoundly reduces Syk ubiquitination, particularly in the context of M-CSF.

FIGURE 6. SykY317F inhibits Syk ubiquitination and degradation. Syk−/− BMMs, transduced with either WT Syk or SykY317F, were cultured with RANKL and M-CSF for 3 days. A, cells were pretreated with proteasome inhibitor MG-132 (10 μM) for 1 h and then stimulated with M-CSF with time. Syk immunoprecipitates (IP) were immunoblotted for ubiquitin (Ub). B, cells, lifted and maintained in suspension in the presence MG-132 (10 μM) for 1 h, were retained in suspension (S) or plated to vitronectin (A) for 3 h. Syk immunoprecipitates (IP) were immunoblotted for ubiquitin. C, cytokine- and serum-starved cells were pretreated with the protein synthesis inhibitor cycloheximide (20 μg/ml). After 20 min, M-CSF was added and Syk immunoblotted with time. Actin serves as loading control. Numbers represent densitometric analysis related to 0 h in each group. p-Y, phosphorylated tyrosine.

ITAM proteins, Dap12 and Fcγ, Vav3, the SLP adaptor proteins, and the small GTPase, Rac (9, 10, 36). Interestingly, inactivation of any of these complex-residing proteins yields “created-appearing” OCs that fail to spread.

c-Src is also a key component of both adhesion- and cytokine-stimulated cytoskeleton organization associating with the β3 integrin subunit cytoplasmic domain as well as that of c-Fms (8, 37). In fact, until the recent discovery of the role of Syk, c-Src was the only nonreceptor tyrosine kinase with an established role in the OC (38). Like c-Src, Syk is phosphorylated downstream of the integrin and c-Fms, but the mechanism of activation differs in each circumstance. Whereas Syk is phosphorylated by c-Src in the context of αvβ3 integrin occupancy, “super-rescues” the cytoskeletal and resorptive dysfunction of Syk−/− OCs as manifest by spreading, actin ring formation, and bone degradation. These cells are reminiscent of those generated in the absence of SHP1, another molecule that physiologically restrains OC function (42). The fact that the stimulatory effect SykY317F exerts on resorptive activity reflects accelerated activity and not increased OC number is consonant with normal expression and activation of differentiation and immediate signaling molecules, respectively.

SLP-76, an adaptor protein lacking intrinsic enzyme activity, plays a key role in T-cell receptor-derived signals, including those that regulate the actin cytoskeleton (43). SLP-76 is phosphorylated by Syk in a number of cells, and we find the same holds following αvβ3 engagement or M-CSF treatment of OCs. In keeping with the negative regulatory function of SykY317, SLP-76 is hyper-phosphorylated in SykY317F-bearing cells. In consequence, Vav3, which is a component of the cytoskeleton-organizing complex recruited by activated SLP-76, also undergoes enhanced phosphorylation in the mutant OCs. Thus, SykY317 restrains OC cytoskeletal organization by dampening activation of effector molecules.

Cbl family proteins are evolutionarily conserved negative regulators that associate with protein-tyrosine kinases upon their activation. Cbl recognizes, ubiquitinates, and negatively regulates Syk in T-cells and mast cells and does so by binding to phosphorylated SykY317 (16, 22, 44, 45). Having defined the role of WT Syk in OCs (9), these observations prompted us to determine whether the bone resorptive function of the kinase is altered by preventing Tyr317 phosphorylation. Similar to its association with other αvβ3-associated proteins in OCs, Cbl recognizes Syk upon integrin activation (21) in this circumstance by binding Tyr317. Although the nonphosphorylatable Y317F mutant blocks Syk/Cbl association, it does not alter activation of either molecule. This observation, taken with the normal Cbl phosphorylation extant in Syk−/− OCs (data not shown), indicates Syk and Cbl activation are independent events.

On the other hand, the Y317F mutant profoundly reduces Syk ubiquitination, particularly in the context of M-CSF.
Because Syk degradation is arrested in cells bearing the mutated tyrosine kinase, a reasonable conclusion would hold that the “super-spread” and hyper-resorptive phenotype of SykY317F OCs reflects protection of the tyrosine kinase from Cbl-mediated proteosomal degradation. This thesis, however, is challenged by an apparent temporal inconsistency between suppressed ubiquitination of SykY317F and its increased abundance in M-CSF-treated cells (Fig. 6). Specifically, degradation of WT Syk is detected with 1 h of exposure to the cytokine, although SykY317F is protected. In contrast, ubiquitination of WT Syk is apparent only after 3 h, a discrepancy that may represent distinct sensitivities of the two assays. This postulate is in keeping with our observation that coinfect with Cbl phosphorylation, c-Fms-mediated Bim degradation, in OCs, is evident within 5 min of ligand occupancy (not shown), while ubiquitination of c-Fms-mediated Bim degradation, in OCs, is evident within 5 min of ligand occupancy (not shown), while ubiquitination of c-Fms-mediated Bim degradation, in OCs, is evident within 5 min of ligand occupancy (not shown), while ubiquitination of c-Fms-mediated Bim degradation, in OCs, is evident within 5 min of ligand occupancy (not shown), while ubiquitination of c-Fms-mediated Bim degradation, in OCs, is evident within 5 min of ligand occupancy (not shown), while ubiquitination of c-Fms-mediated Bim degradation, in OCs, is evident within 5 min of ligand occupancy (not shown), while ubiquitination of c-Fms-mediated Bim degradation, in OCs, is evident within 5 min of ligand occupancy (not shown), while ubiquitination of c-Fms-mediated Bim degradation, in OCs, is evident within 5 min of ligand occupancy (not shown), while ubiquitination of c-Fms-mediated Bim degradation, in OCs, is evident within 5 min of ligand occupancy (not shown), while ubiquitination of c-Fms-mediated Bim degradation, in OCs, is evident within 5 min of ligand occupancy (not shown), while ubiquitination of c-Fms-mediated Bim degradation, in OCs, is evident within 5 min of ligand occupancy (not shown), while ubiquitination of c-Fms-mediated Bim degradation, in OCs, is evident within 5 min of ligand occupancy (not shown), while ubiquitination of c-Fms-mediated Bim degradation, in OCs, is evident within 5 min of ligand occupancy (not shown), while ubiquitination of c-Fms-mediated Bim degradation, in OCs, is evident within 5 min of ligand occupancy (not shown), while ubiquitination of c-Fms-mediated Bim degradation, in OCs, is evident within 5 min of ligand occupancy (not shown), while ubiquitination of c-Fms-mediated Bim degradation, in OCs, is evident within 5 min of ligand occupancy (not shown), while ubiquitination of c-Fms-mediated Bim degradation, in OCs, is evident within 5 min of ligand occupancy (not shown).