Inhibitory regulation of osteoclast bone resorption
by signal regulatory protein α

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ABSTRACT Osteoclasts mediate bone resorption, which is critical for bone development, maintenance, and repair. Proper control of osteoclast development and function is important and deregulation of these processes may lead to bone disease, such as osteoporosis. Previous studies have shown that the cytosolic protein tyrosine phosphatase SHP-1 acts as a suppressor of osteoclast differentiation and function, but putative inhibitory receptors that mediate recruitment and activation of SHP-1 in osteoclasts have remained unknown. In the present study, we identify the SHP-1-recruiting inhibitory immunoreceptor signal regulatory protein (SIRP) α as a negative regulator of osteoclast activity. SIRPα is expressed by osteoclasts, and osteoclasts from mice lacking the SIRPα cytoplasmic tail and signaling capacity display enhanced bone resorption in vitro. Consequently, SIRPα-mutant mice have a significantly reduced cortical bone mass. Furthermore, osteoclasts from SIRPα-mutant mice show an enhanced formation of actin rings, known to be instrumental in bone resorption. SIRPα mutation did not significantly affect osteoclast formation, implying that the role of SIRPα was limited to the regulation of mature osteoclast function. This identifies SIRPα as a bona fide inhibitory receptor that regulates the bone-resorption activity and supports a concept in which osteoclast function is balanced by the signaling activities of activating and inhibitory immunoreceptors.—Van Beek, E. M., de Vries, T. J., Mulder, L., Schoenmaker, T., Hoeben, K. A., Matozaki, T., Langenbach, G. E. J., Kraal, G., Everts, V., van den Berg, T. K. Inhibitory regulation of osteoclast bone resorption by signal regulatory protein α. FASEB J. 23, 000–000 (2009). www.fasebj.org

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Osteoclasts are multinucleated cells from the macrophage lineage that are highly specialized in bone resorption and as such play a pivotal role in normal bone development, turnover, and repair. Deregulation of osteoclast differentiation and function may lead to pathological conditions, such as osteoporosis or osteopetrosis, and therefore both the formation and activity of osteoclasts need to be tightly controlled. The development of osteoclasts involves the differentiation and fusion of myeloid precursor cells into mature bone-resorbing osteoclasts, and this is driven by the action of receptor activator of NF-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) (1–3). The immunoreceptor tyrosine-based activation motif (ITAM) is a highly conserved region in the cytoplasmic domain of signaling chains of adapter proteins and receptors and is a critical mediator of intracellular signals. ITAM signaling is required for the differentiation and function of B and T cells in adaptive immunity and regulates the function of innate immune cells, including natural killer cells, and myeloid cells such as macrophages, neutrophils, and dendritic cells. Recent studies have demonstrated that ITAM adapter proteins are also involved in the formation and function of osteoclasts. Mice deficient in both of the ITAM adapter proteins, DNAX-activating protein (DAP) 12 and Fc receptor γ-chain (FcRγ), are osteopetrotic, owing to impaired osteoclast formation and bone resorption (4–6). In addition, mice deficient in the tyrosine kinases Syk and c-Src, acting downstream of DAP12 and FcRγ, develop severe osteopetrosis, which is apparently due to diminished bone resorption (6–8). Furthermore, triggering of DAP12- and FcRγ-associated activating immunoreceptors, such as triggering receptors expressed

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on myeloid cells-2 (TREM-2), paired immunoglobulin-like receptor-A (PIR-A), osteoclast-associated receptor (OSCAR), and signal regulatory protein β1 (SIRPβ1) enhanced osteoclastogenesis and promoted bone resorption, suggesting that activating immunoreceptor signaling plays a regulatory role in osteoclast formation and function (5, 9).

In the immune system, ITAM receptor signaling is generally counterbalanced by inhibitory receptors that recruit cytosolic Src homology-2-containing protein tyrosine phosphatases (SH2-PTPases), such as SHP-1 and/or SHP-2, via cytoplasmic ITIMs. Interestingly, motheaten mice with a deficiency or loss-of-function mutation in SHP-1, which constitutes the major cytosolic SH2-PTPase in hematopoietic cells, develop severe osteoporosis, which is associated with an increase in osteoclast differentiation and bone resorption, indicating that SHP-1 controls osteoclast formation and activity (10–12). It is therefore anticipated that osteoclasts express inhibitory receptors that, through recruitment and activation of SHP-1, inhibit osteoclast formation and/or activation. However, until now the relevant inhibitory receptors on osteoclasts have not been defined.

Signal regulatory protein α (SIRPα) is an inhibitory immunoreceptor that contains ITIMs, which on ligand binding become phosphorylated and mediate the recruitment and activation of the cytosolic tyrosine phosphatases (PTPases) SHP-1 and SHP-2 (13–16). The extracellular region of SIRPα is composed of Ig-like domains, which share a particularly close structural similarity with T-cell receptor and B-cell receptor chains (17, 18). SIRPα is expressed predominantly by myeloid and neuronal cells (19–21). The broadly expressed CD47 surface receptor acts as a cellular ligand for SIRPα and has been proposed to act as a signal of “self” (20, 22–24), which by interacting with SIRPα controls cellular effector functions, including growth, differentiation, adhesion, transendothelial migration of monocytes and granulocytes, and migration of Langhans cells (25, 26). A role for SIRPα in osteoclast differentiation and/or function has not been established. Of relevance, in vitro studies have suggested that CD47-SIRPα interactions may play a role in the fusion of macrophages leading to multinucleated cell formation, as occurs also during osteoclastogenesis (27–29).

In the present study, we have investigated the involvement of SIRPα signaling in osteoclast differentiation and activation, using mice that have a defect in SIRPα signaling (30). Our results show that SIRPα-mutant mice have significantly decreased cortical bone mass, despite apparently normal osteoclast development in vivo and in vitro. Furthermore, osteoclasts generated from these mice show an increased bone-resorption capacity in vitro. This is associated with enhanced formation of actin rings, which are known to be important for proper bone degradation. Thus, SIRPα is identified as an inhibitory receptor that selectively controls osteoclast function.

MATERIALS AND METHODS

Mice

C57BL/6 mice with a targeted deletion of the SIRPα cytoplasmic region have been described previously (30). The mice that were originally generated onto the 129/Sv background had been backcrossed onto the C57BL/6 mice for 10 generations. Wild-type C57BL/6 mice of the same genetic background were maintained together with the SIRPα-mutant mice in the breeding facility of Vrije Universiteit Medical Center. Mice were kept in specifically pathogen-free conditions according to Federation of European Laboratory Animal Science Associations recommendations and used at 6 wk of age. Permission for animal experiments described here was obtained from the Animal Welfare Committee of Vrije Universiteit.

Bone marrow isolation

Bone marrow cells were isolated as described before (31). The cells were resuspended through a 21-gauge needle and filtered over a 100-μm pore size cell strainer filter (Falcon/Becton Dickinson, Franklin Lakes, NJ, USA) and kept on ice in culture medium (α-minimal essential medium; Gibco BRL, Paisley, Scotland) supplemented with 5% FCS (HyClone, Logan UT, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (antibiotic antimiycotic solution; Sigma-Aldrich, St. Louis, MO, USA) until further use.

Bone marrow cell populations

Various hematopoietic cell populations in the bone marrow, including granulocytes, monocytes, immature myeloid cells, lymphoid cells, erythroid cells, and early blasts, were identified, as described previously (32), by double staining with biotinylated ER-MP12 monoclonal antibody (mAb) (against CD31) and fluorescein isothiocyanate-labeled ER-MP20 mAb (against Ly-6C) (generously provided by Dr. P. Leenen, Department of Immunology, Erasmus University Medical Center, Rotterdam, The Netherlands). After a 1-h incubation at 4°C, cells were washed 3 times with PBS-0.1% BSA. ER-MP12 binding was detected using Alexa 633-streptavidin conjugate (Molecular Probes, Eugene, OR, USA) for 30 min at 4°C. After 3 washes with PBS-0.1% BSA, cells were analyzed on a FACSCalibur system (BD Biosciences, San Jose, CA, USA) using Cell Quest software (BD Biosciences).

Osteoclastogenesis

Bone marrow cells were plated in 96-well flat-bottom tissue-culture-treated plates (Costar, Cambridge, MA, USA) at a density of 1.0 × 10^5 cells/well in 150 μl of culture medium containing 30 ng/ml recombinant murine M-CSF (R&D Systems, Minneapolis, MN, USA) and 20 ng/ml recombinant murine RANKL (RANKL-TEC, R&D Systems). Cells were cultured on 650-μm-thick bovine cortical bone slices or on plastic. Culture medium was replaced every 3 d. After either 5 d for tartrate-resistant acid phosphatase (TRACP) staining or 6 d for the analysis of bone resorption, wells were washed with PBS and either fixed in 4% PBS-buffered formaldehyde and stored at 4°C (for TRACP staining) or in water (for bone resorption), respectively. TRACP staining was performed according to the manufacturer’s instruction (Sigma-Aldrich).

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RNA analysis and real-time quantitative PCR

RNA isolation and real-time quantitative PCR were performed as described in detail previously (33, 34). Real-time PCR primers were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA) (Supplemental Table S1). To avoid amplification of genomic DNA, each amplicon spanned ≥1 intron. To test for PCR efficiencies, one of the osteoclast samples was used to generate a standard curve for all of the genes studied. The PCR reactions of the different amplicons had equal efficiencies. Porphobiligenon deaminase (PBGD) was used as the housekeeping gene (35). Expression of this gene was not affected by any of the experimental conditions. Samples were normalized for the expression of PBGD by calculating the ΔΔCt (Ctgene of interest − Ctgene of reference). Expression of each gene was calculated relative to the mean of the PBGD Ct value.

Bone resorption

Bone resorption was visualized and quantified as described previously (36), with minor adaptations. Cells were removed with 0.25 M NH4OH for 30 min. The slices were washed in distilled water, incubated in a saturated alum [KAl(SO4)2]12H2O solution for 10 min, washed in distilled water, and stained with Coomassie Brilliant Blue. The surface areas of individual resorption pits were measured using Image-Pro Plus software (MediaCybernetics, Silver Spring, MD, USA).

Immunoprecipitation and Western blot analysis

For immunoprecipitation of SIRPα, bone marrow cells were plated in 48-well flat-bottom tissue culture treated plates (Costar) at a density of 2.5 × 103 cells/well in 400 μl of culture medium containing 30 ng/ml M-CSF and 20 ng/ml RANKL-TEC. Culture medium was replaced every 3 d. After 5.5 d, 1 mM sodium orthovanadate (Na3VO4; New England Biolabs, Leusden, The Netherlands) was added to culture medium supplemented with 1 mM Na3VO4. Subsequently, cells were lysed with Igepal lysis buffer (Sigma-Aldrich) containing 1 mM Na3VO4 and protease inhibitor cocktail (Roche Diagnostics, Almere, The Netherlands) for 1 h. SIRPα was immunoprecipitated by antibody (Molecular Probes). Rat anti-mouse CD25 (clone P84, IgG1; BD Pharmingen, San Diego, CA, USA) or isotype control antibody (37) bound to protein G-Sepharose beads for 1 h at 4°C. Beads were washed with Igepal lysis buffer containing 1 mM Na3VO4 and protease inhibitor cocktail and resuspended in SDS sample buffer. Western blot analysis was performed with anti-SHP-1 (SH-PTP1, rabbit polyclonal; Santa Cruz Biotechnology, Heidelberg, Germany) and anti-SIRPα (Ab8120, rabbit polyclonal; Abcam, Cambridge, UK) antibodies, followed by horseradish peroxidase-linked secondary antibodies. Proteins were detected with SuperSignal West Dura (Pierce Biotechnology, Rockford, IL, USA) and a gel imager (Epi Chemi II Darkroom combined with a 12-bit SensiCam charged-coupled device camera driven by Labworks 4.0 (UVP, Inc., Upland, CA, USA)).

Micro-computed tomography (micro-CT) imaging

Three-dimensional reconstructions of trabecular and cortical bone of the tibiae were generated with a high-resolution micro-CT system (μCT 40; Scanco Medical AG, Brüttisellen, Switzerland). Tibiae of 6 male wild-type and 6 male SIRPα-mutant mice, all of which were 42 d old, were mounted in a cylindrical specimen holder (polyetherimide; 20.5 mm outer diameter; 0.7 mm wall thickness) to be captured in a single scan. They were secured with synthetic foam and were completely submerged in deionized water. Scans with an isotropic resolution of 10 μm were made using a 70-kV peak-voltage X-ray beam. The computed linear attenuation coefficient of the X-ray beam in each volume element (voxel) was stored in an attenuation map and represented by a gray value in the reconstruction. Specific volumes of interest (VOIs) were selected. To analyze trabecular bone, a region of 5% of the tibiae length above and under the metaphysis was evaluated. Cortical bone analysis was performed in the region between 45 and 55% along the length of the tibiae. To discriminate between bone and background, the reconstructions were segmented using an adaptive threshold determination procedure, as described previously (38). All bone regions were segmented using the same threshold for comparison purposes. Multiple cortical and trabecular bone parameters were determined using morphometric software supplied by the manufacturer [for trabecular bone: bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and connectivity density (Conn.D); for cortical bone: cortical thickness (Ct.Th) and second moment of inertia (MOI)].

Histology

Tibiae of 42-d-old wild-type and SIRPα-mutant mice were fixed for 24 h at room temperature in 4% formaldehyde and 1% glutaraldehyde, buffered in 0.1 M sodium cacodylate buffer (pH 7.4). Next, tibiae were decalcified for 2 wk in a solution containing 10% EDTA, 1% formaldehyde, and 1% glutaraldehyde, which was replaced 2×/wk. Fixed specimens were dehydrated through a series of ethanol solutions and embedded in Epon 812 (TAAB Laboratories, Aldermaston, UK). Semithin sections (1–2 μm) were cut and stained with methylene blue. The number of osteoclasts per millimeter of trabecular surface area was determined using QWin software (Leica, Wetzlar, Germany).

Confocal microscopy

Bone marrow cells were seeded on cortical bone slices and allowed to mature for 5.5 d in the presence of M-CSF and RANKL, as described above. Slices were washed in PBS, fixed in acetone at −20°C for 4 min, and subsequently dried at room temperature. Nonspecific binding to cells was blocked for 30 min with 10% normal goat serum, followed by an overnight incubation at 4°C with rat anti-mouse SIRPα (clone P84, IgG1) (37). After 3 washes with PBS, the slides were incubated with an Alexa 488-conjugated goat anti-rat IgG antibody (Molecular Probes), Rat anti-mouse CD25 (clone PG61.5.3, IgG1) (39) served as an isotype-matched negative control. F-actin was stained according to a method described previously using Alexa 488-phalloidin (Molecular Probes) (40). Nuclei were stained with propidium iodide (Sigma-Aldrich). Image stacks were generated using confocal laser scanning microscopy (Leica) using an argon laser (Alexa 488 and propidium iodide).

RESULTS

Osteoclastogenesis is independent of osteoclast SIRPα signaling

The inhibitory receptor-associating PTPase SHP-1 has been demonstrated to play a nonredundant negative
role in osteoclast differentiation and activation (10, 12), but the putative inhibitory immune receptors that mediate SHP-1 recruitment and activation and induce downstream signaling events in osteoclasts have not been identified. Confocal microscopy was used to study the cellular expression and localization of SIRPα in osteoclasts that were generated from murine bone marrow cells and cultured on cortical bone slides in the presence of M-CSF and RANKL. Consistent with the generalized expression of SIRPα among cells of the myeloid lineage, a plasma membrane and diffuse cytoplasmic staining were observed for SIRPα in both multinucleated osteoclasts and mononuclear osteoclast precursors (Fig. 1A). The SIRPα cytoplasmic tail contains ITIM motifs that are responsible for the recruitment of the cytosolic tyrosine phosphatase SHP-1 (15). To evaluate the binding of SHP-1 to SIRPα in osteoclasts we performed an immunoprecipitation experiment. As shown by SIRPα immunoprecipitation and Western blotting with a SHP-1-specific antibody (Fig. 1B), SHP-1 can associate constitutively with SIRPα in osteoclasts.

To investigate a direct role for SIRPα in osteoclast differentiation and activity, we performed studies in SIRPα-mutant mice. These mice, which were described previously (30), lack most of the cytoplasmic region of SIRPα, including all of the ITIMs implicated in signaling. First, we studied osteoclast formation in vitro by culturing bone marrow cells from wild-type and SIRPα-mutant mice in the presence of M-CSF and RANKL. In

![Figure 1. SIRPα is expressed in osteoclasts, but SIRPα signaling is not required for osteoclastogenesis. A) Osteoclasts generated from bone marrow cells of wild-type mice by culturing for 3.5 d in the presence of M-CSF and RANKL and seeded on cortical bone slides express SIRPα. Abundant SIRPα was seen both in mononuclear (arrowheads) and multinucleated (arrows) cells. Cells were fixed and stained with the anti-SIRPα monoclonal antibody (clone p84, rat IgG) and Alexa 488 (green)-conjugated goat anti-rat IgG antibody and evaluated by confocal microscopy. Nuclei were visualized by propidium iodide (red) staining. B) Osteoclasts derived from bone marrow cells of the wild-type mice and cultured on plastic in the presence of M-CSF and RANKL. After 30 min of incubation with culture medium supplemented with Na3VO4, cells were lysed and cellular lysate was subjected to immunoprecipitation (IP) with anti-SIRPα (SIRPα, clone P84, rat IgG) coupled to G-Sepharose beads or isotype control (−). Western blot (WB) analysis was performed with antibodies against SHP-1 (SH-PTP1, rabbit polyclonal antibody) and anti-SIRPα (Ab8120, Rabbit polyclonal). Note that SHP-1 associates with SIRPα in osteoclasts. C) Bone marrow cells of 6-wk-old male control and SIRPα-mutant mice were subjected to flow cytometric analysis with antibodies against CD31 (ER-MP12) and Ly-6C (ER-MP20). Flow cytometric dot-plot of ER-MP12/20-labeled bone marrow cells. D) Six different cell populations, including granulocytes, monocytes, immature myeloid cells, lymphoid cells, erythroid cells, and early blasts, could be distinguished. No difference was found in bone marrow composition between wild-type and SIRPα-mutant mice based on the percentage of the variant subpopulations. Data are presented as means ± se. E) Osteoclasts, generated from bone marrow cells of wild-type and SIRPα-mutant mice and cultured on cortical bone slides in the presence of M-CSF and RANKL for 5 d, were stained for TRACP, a specific enzyme marker of osteoclasts. Nuclei were stained with DAPI. Specimens were photographed with a blue filter, visualizing TRACP staining in black. Note that mononuclear, binuclear, and multinucleated cells (arrows) are present in both cultures. F) Number of multinucleated (>3 nuclei) cells per square centimeter per 105 bone marrow cells originally seeded. Data are expressed as means ± se of two independent experiments (both wild-type and SIRPα-mutant mice, n=5). No differences were seen in the number of osteoclasts generated from the bone marrow of both wild-type and SIRPα-mutant mice. G) Cultured osteoclasts were analyzed by quantitative PCR for the osteoclast markers DC-STAMP, calcitonin receptor, cathepsin K, and TRACP. Abundance of mRNA relative to that of PBGD is shown. No differences were found between wild-type and SIRPα-mutant mice. Data are presented as means ± se. Scale bars = 100 μm.
these experiments, suboptimal concentrations of M-CSF and RANKL were used to optimize the conditions for detecting any effect of the SIRPα mutation on osteoclast formation and differentiation. Of note, no difference in bone marrow composition was observed between wild-type and SIRPα-mutant animals with respect to the relative numbers of granulocytes, monocytes, immature myeloid cells, lymphoid cells, erythroid cells, and early blasts that were identified on the basis of CD31 (ER-MP12) and Ly-6C (ER-MP20) expression (32) (Fig. 1C, D). After differentiation for 6 d in the presence of M-CSF and RANKL, multinucleated osteoclasts were identified after staining for TRACP activity and 4,6-diamidino-2-phenylindole (DAPI) (Fig. 1F). No significant differences in the formation and differentiation of mature osteoclasts were found between the cultures from SIRPα-mutant and wild-type mice, regardless of whether the cells had been cultured on cortical bone slices (Fig. 1E–G) or on plastic (not shown). In particular, there were no differences in the average numbers of nuclei in multinucleated osteoclasts (Fig. 1F), indicating that SIRPα signaling is dispensable for cellular fusion during osteoclastogenesis. This finding is perhaps somewhat surprising in light of previous studies in which a role for CD47-SIRPα interactions in macrophage fusion was suggested (27–29). Furthermore, the expression levels of typical osteoclast markers dendritic cell-specific transmembrane protein (DC-STAMP), calciitonin receptor, cathepsin K, and TRACP were determined by real-time quantitative PCR, and these were unaltered in osteoclasts from SIRPα-mutant mice compared with the wild-type cells (Fig. 1G and Supplemental Fig. S1C), strongly suggesting a similar degree of osteoclast differentiation. Taken together, these data show that SIRPα is present on osteoclasts, but its signaling does not play a significant role in osteoclast formation and differentiation.

Osteoclast bone resorption is inhibited by SIRPα signaling

To determine whether SIRPα is a critical regulator of osteoclast function, we compared the bone-resorption activity of in vitro generated bone marrow-derived osteoclasts from wild-type and SIRPα-mutant mice cultured on bovine cortical bone slices. Interestingly, osteoclasts from the SIRPα-mutant mice proved to resorb considerably more bone compared with those from wild-type mice (1.0±0.3 and 3.2±0.5%, respectively) (Fig. 2A, B). This difference in osteoclast activity was not only reflected in the total amount of bone resorbed but also by a difference in the average pit size (1.0±0.1 and 1.9±0.2 mm², respectively), suggesting that the bone-resorption capacity of individual osteoclasts was increased (Fig. 2C). These data show that SIRPα-derived signals play a regulatory role in osteoclast function.

Reduced cortical bone mass in SIRPα-mutant mice

To examine whether the enhanced bone-resorption capacity of osteoclasts generated by cells isolated from SIRPα-mutant mice is reflected by reduced bone mass in vivo, the tibiae of SIRPα-mutant mice and wild-type mice were analyzed for a variety of bone parameters using micro-CT. This analysis demonstrated that the Ct.Th of the bone, measured at the midshaft of the tibiae, was significantly less in the SIRPα-mutant mice (0.21±0.016 and 0.16±0.005 mm, respectively) (Fig. 3A, B). This reduction occurred despite normal length of the tibiae (Fig. 3C). Other parameters for trabecular and cortical bone, including BV/TV, Tb.N, Tb.Th, Tb.Sp, Conn.D, and second MOI, did not differ significantly between the wild-type and SIRPα-mutant mice. Furthermore, the SIRPα-mutant mice displayed a small (i.e., 13%) but significant reduction in their body weight (Fig. 3D), a finding that was also reported previously (22), which may in fact be explained by the reduction in volume of cortical bone, that represents ~75% of the total bone in the body (41).

To confirm in vivo that the reduced Ct.Th observed in the SIRPα-mutant mice was a result of an enhanced bone resorption and was not caused by a difference in osteoclast number, the tibiae of the wild-type and SIRPα-mutant mice were analyzed by histology for the presence and number of osteoclasts (Fig. 3E). Apparently, the defect in SIRPα signaling of the mutant mice did not affect the number of osteoclasts per millimeter of trabecular bone surface (Fig. 3F). Also, there was no difference in the average number and distribution of nuclei per osteoclast between the two genotypes (Fig. 3G). Finally, as suggested by histological analyses, there were no indications for prominent differences in osteoblast activity and the amount of osteoid at the shaft of the tibiae (Supplemental Fig. S1), suggesting roughly
comparable bone formation capacity in SIRPα-mutant and wild-type animals.

Collectively, these in vivo results suggest that SIRPα-mutant mice have significantly reduced cortical bone mass, despite apparently normal osteoclast numbers and apparently normal osteoblast function. This finding provides further support for the idea that SIRPα signaling plays inhibitory role in osteoclast activity.

Enhanced actin ring formation in the SIRPα-mutant mice

To gain initial insight into the mechanism underlying the increased bone resorption by the SIRPα-mutant mice, we focused on actin ring formation in osteoclasts. In bone-resorbing osteoclasts, these actin rings are an intrinsic part of the adhesive structure, the so-called sealing zone, that isolates the resorptive microenvironment from the general extracellular space and is a prerequisite for efficient bone resorption (3, 42). Of relevance, signaling by activating immunoreceptors, which is mediated via c-Src and Syk, promotes actin ring formation (8). We therefore anticipated that SIRPα signaling might affect actin ring formation. Osteoclasts were generated from bone marrow cells on cortical bone slices and stained with fluorescence-labeled phalloidin to visualize filamentous actin. We therefore anticipated that SIRPα signaling might affect actin ring formation.

Osteoclasts were generated from bone marrow cells on cortical bone slices and stained with fluorescence-labeled phalloidin to visualize filamentous actin. In osteoclasts of both mouse strains, actin rings were identified (Fig. 4A), but not all osteoclasts displayed actin rings. In the SIRPα-mutant mice the proportion of osteoclasts with actin rings was significantly higher (Fig. 4B). Nearly all mutant osteoclasts, but only 75% of wild-type osteoclasts, contained actin ring. Moreover, the average number of actin rings per osteoclast was higher in mutant than in wild-type cells (Fig. 4B). Again, in these experiments, the average number of nuclei of the osteoclasts analyzed did not differ between the two mouse strains (data not shown). Taken together, these findings indicate that SIRPα inhibits actin ring formation, and this may explain, at least in part, the elevated bone-resorption activity of SIRPα-mutant osteoclasts.
DISCUSSION

In the present study, we have investigated the involvement of SIRPα/H9251, as a prototypic inhibitory receptor, in osteoclast formation and bone resorption by using SIRPα-mutant mice that lack the cytoplasmic domain of the receptor and therefore carry an intrinsic defect in signaling. Our results demonstrate that SIRPα/H9251 is expressed by osteoclasts and that SIRPα signaling plays a nonredundant regulatory role in the bone-resorption activity of osteoclasts. This conclusion is based on the following in vitro and in vivo observations.

In vitro-generated osteoclasts from the SIRPα-mutant mice showed a strongly enhanced bone-resorption activity in comparison with the cells generated from wild-type mice. In vivo, this enhancement of osteoclast activity was also reflected in a decrease in Ct.Th of the tibiae. These findings are in agreement with studies performed in motheaten mice that lack the cytosolic tyrosine phosphatase SHP-1, which is considered to be a major cytosolic PTPase in hematopoietic cells and is known to transduce at least some of the intracellular signals generated by SIRPα in macrophages (10, 12). In fact, to our knowledge, SIRPα, which does indeed associate with SHP-1 in osteoclasts, as shown in the present study, represents the first reported example of a bona fide ITIM-containing inhibitory immunoreceptor that inhibits osteoclast activity.

In contrast to the reported observations in SHP-1-deficient mice, which display both enhanced osteoclast formation and bone-resorption activity, we did not observe any detectable differences in the formation and differentiation of osteoclasts, evaluated either in vitro or in vivo, between wild-type and SIRPα-mutant mice. This observation indicates that the SIRPα-mutant reproduces only the part of the SHP-1 phenotype that relates to the regulation of bone resorption and suggests that other inhibitory immune receptors that signal via SHP-1 may also be relevant, particularly with respect to osteoclast formation. To our knowledge, only one candidate SHP-1-binding inhibitory receptor, i.e., the CMRF-35-like molecule 1 (CML-1), with such an activity has been reported (43). However, whereas overexpression of CML-1 inhibits osteoclastogenesis in an in vitro model using RAW264.7 cells, CML-1 is actually down-regulated during normal osteoclastogenesis. Although this finding strongly suggests that inhibitory receptors can indeed have inhibitory activity with respect to osteoclastogenesis, the relevant inhibitory receptors controlling osteoclast formation in vivo remain to be identified.

The observation that SIRPα-mutant mice do not show any detectable defects in osteoclast formation in general and in osteoclast multinucleation in vitro (Fig. 1E) and in vivo (Fig. 3G) may be somewhat unexpected.

Figure 4. Enhanced actin ring formation in SIRPα-mutant mice. Osteoclasts derived from bone marrow cells of the wild-type and SIRPα-mutant mice were cultured on cortical slides and stained with Alexa Fluor 488 phalloidin. A) Images were obtained by confocal laser scanning microscopy. Actin rings are green, nuclei (propidium iodide staining) are red. Scale bars = 100 μm. B) Number of actin rings per osteoclast was determined. In the SIRPα-mutant mice, almost all osteoclasts contain an actin ring, and the percentage of osteoclasts with >1 actin ring is increased. On average, 35 ± 5.4 osteoclasts/mouse (n=5/genotype) were analyzed. Data are expressed as means ± se. *P < 0.05.

Figure 5. Hypothetical mechanism of the regulation of osteoclast function by activating and inhibitory receptors. ITAM-bearing proteins FcRγ and DAP12 that associated with the activating immunoreceptors such as TREM2 become phosphorylated by Src on ligand binding. This leads to recruitment of Syk, which in turn induces actin ring formation and thereby activates bone resorption. To control the resorption activity, osteoclasts also express inhibitory receptors such as SIRPα, which recruit the tyrosine phosphatases SHP-1 on ligand binding. SHP-1, in turn, inhibits actin ring formation and thereby the resorption activity of osteoclasts.
in light of the previously suggested role of CD47-SIRPα interactions in macrophage fusion in vitro (27–29). One possible explanation for this apparent discrepancy is that osteoclast fusion is indeed mediated by CD47-SIRPα interactions but does not involve concomitant SIRPα signaling. However, an alternative explanation is that CD47-SIRPα interactions, although relevant for fusion of some of the tested macrophage populations, do not play a role during osteoclast fusion at all. It was recently reported by Lundberg et al. (44) that osteoclasts differentiated from CD47-deficient bone marrow cells in the presence of M-CSF and RANKL do not differ in size or number of nuclei per osteoclast, suggesting that normal fusion occurs. Interestingly, although the results of the latter study strongly argue against a role for CD47-SIRPα interactions in osteoclast fusion, they did provide evidence for a role of CD47-SIRPα interactions in osteoclast formation, as reduced numbers of TRACP-positive osteoclasts were observed in the absence of CD47 in vitro as well as in vivo and also by adding blocking antibodies against CD47 or SIRPα in vitro. Unfortunately, neither the bone-resorption activity of CD47-deficient osteoclasts nor the bone mass was evaluated by Lundberg et al. (44), and it therefore remains to be established whether the inhibition of osteoclast function by SIRPα signaling depends on CD47-SIRPα interactions or involves SIRPα signaling that is independent from CD47. Taken together, these results seem to indicate that osteoclast formation is supported by CD47-SIRPα interactions but is independent of SIRPα signaling; cell fusion of osteoclast precursors is independent of both CD47-SIRPα interactions and SIRPα signaling; and osteoclast function is inhibited by SIRPα signaling.

Although our results provide evidence for an inhibitory role of SIRPα signaling in osteoclast bone resorption, it seems possible that not all osteoclasts are equally dependent on SIRPα regulation. For instance, the decrease in Ct.Th observed in SIRPα-mutant mice in vivo was not paralleled by a detectable effect on trabecular bone mass, suggesting that SIRPα primarily regulates the activity of osteoclasts located at the shaft of long bones. Previously, we and others have demonstrated functional heterogeneity between osteoclasts at distinct anatomical locations, including those from cortical and trabecular bone (36, 45). Zenger et al. (46) showed that the intracellular localization of monomeric TRACP was altered in distal metaphyseal osteoclasts in cathepsin K-deficient mice, which indicates functional differences between bone-resorbing osteoclasts within the trabecular metaphyseal bone. Taken together these findings suggest differences in the regulation of differentiation and activation depending on the anatomical localization of the osteoclast. It remains to be established whether these relate to local differences in SIRPα expression and/or signaling.

Our results also provide preliminary insight into the mechanism of regulation by SIRPα of osteoclastic bone resorption. In particular, we observed that the enhanced bone resorption by SIRPα-mutant osteoclasts was associated with increased actin ring formation. The formation of actin rings, which form the foundation of the so-called sealing zone, is a prerequisite for osteoclastic bone resorption, essentially because it allows the generation of an isolated acidic compartment in which bone matrix is degraded by, e.g., proteases and other activities. Actin ring formation is controlled by α3β3 integrins, which on ligand binding trigger autophosphorylation and activation of the tyrosine kinase c-Src. Activated c-Src phosphorylates ITAM motifs on activating immunoreceptor-associated adaptor proteins, in particular DAP12 or FcRγ, which in turn leads to the recruitment and activation of Syk (8, 47, 48). Syk phosphorylates a variety of substrates, including vinculin and paxillin. These proteins promote actin polymerization and the resultant formation of the osteoclast sealing zone. Of relevance in this context, mice deficient in c-Src or Syk develop osteopetrosis, which is due to decreased osteoclast activity (8, 49, 50). Osteopetrosis is also observed in DAP12 and FcRγ double-knockout mice (6). We propose that SIRPα and perhaps other myeloid inhibitory receptors as well function as the inhibitory counterparts of activating immunoreceptors in this pathway. Figure 5 presents a hypothetical mechanism of the regulation of osteoclast function by activating and inhibitory immune receptors. It will be interesting to establish at which levels the proposed SIRPα-SHP-1 signal integrates with the α3β3 integrin/c-Src/activating immunoreceptor/adaptor/Syk/actin pathway.

CONCLUSIONS

We demonstrate that SIRPα signaling controls osteoclast bone resorption. In particular, SIRPα inhibits the generation of actin rings and the formation of the osteoclast sealing zone. More knowledge on the regulation of osteoclast activity by SIRPα may lead to novel methods for treatment of osteoporosis and other bone-remodeling diseases.

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REFERENCES


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