Protein tyrosine phosphatases ε and α perform nonredundant roles in osteoclasts

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INTRODUCTION

The mass of bone and its physical properties are regulated by the opposing activities of osteoblasts, which synthesize bone matrix, and osteoclasts, which degrade it. Osteoclasts are large, multinucleated cells that are formed by fusion of precursor cells from the hematopoietic monocyte-macrophage lineage in response to molecular signals, which include macrophage colony stimulating factor (M-CSF; CSF-1) and receptor activator of nuclear factor κB ligand (RANKL; Boyle et al., 2003; Bruzzaniti and Baron, 2006; Teitelbaum, 2007). Osteoclasts (OCLs) adhere to bone, and from their ventral membrane they secrete acid and proteolytic enzymes onto the bone surface that degrade the organic and mineral components of the matrix (Bruzzaniti and Baron, 2006). OCLs adhere to matrix by use of podosomes—specialized adhesion structures that are centered on an actin-rich core. Integrins and associated molecules that surround the podosomal core transduce the signal generated by physical contact with matrix to the core, resulting in changes in its subcellular organization and stability (Destaing et al., 2003, 2008; Luxenburg et al., 2006b). The organization of podosomes in OCLs is typical of the activation state of the cell. Active osteoclasts (fully polarized cells, referring to the functional difference that develops in mature osteoclasts from their precursors) have podosomes that are arranged as a large array or belt at the cell periphery. In less-active or inactive (nonpolarized) cells, podosomes are organized in discrete rings, in smaller, less-developed clusters, or are spread at random throughout the cell.

Tyrosine phosphorylation of proteins plays central roles in the production of OCLs and in regulating their function. Phosphorylation is critical for signaling processes mediated by RANKL and M-CSF, whose receptor is the tyrosine kinase c-Fms (Ross, 2006; Wada et al., 2006). Moreover, absence of the Src tyrosine kinase reduces

ABSTRACT Female mice lacking protein tyrosine phosphatase ε (PTP ε) are mildly osteopetrotic. Osteoclasts from these mice resorb bone matrix poorly, and the structure, stability, and cellular organization of their podosomal adhesion structures are abnormal. Here we compare the role of PTP ε with that of the closely related PTP α in osteoclasts. We show that bone mass and bone production and resorption, as well as production, structure, function, and podosome organization of osteoclasts, are unchanged in mice lacking PTP α. The varying effects of either PTP on podosome organization in osteoclasts are caused by their distinct N-termini. Osteoclasts express the receptor-type PTP α (RPTPa), which is absent from podosomes, and the nonreceptor form of PTP ε (cyt-PTPe), which is present in these structures. The presence of the unique 12 N-terminal residues of cyt-PTPe is essential for podosome regulation; attaching this sequence to the catalytic domains of PTP α enables them to function in osteoclasts. Serine 2 within this sequence regulates cyt-PTPe activity and its effects on podosomes. We conclude that PTPs α and ε play distinct roles in osteoclasts and that the N-terminus of cyt-PTPe, in particular serine 2, is critical for its function in these cells.

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the ability of OCLs to resorb bone and organize their podosomal adhesion structures properly (Soriano et al., 1991; Luxenburg et al., 2006b; Miyazaki et al., 2006; Destaing et al., 2008). The kinase Pyk2, which functions in close collaboration with Src, also plays important roles in OCLs (Gil-Henn et al., 2007). Physical contact of these cells with matrix activates integrin signaling and increases podosomal protein phosphorylation significantly (Luxenburg et al., 2006a), further supporting the link between OCL adhesion, podosome function, and protein tyrosine phosphorylation. The central roles of tyrosine kinases in regulating OCLs indicate that tyrosine phosphatases, which counter kinase activity, also play critical roles in these processes. Among these, the nonreceptor-type PTP SHP1 inhibits OCL function and formation in vivo (Aoki et al., 1999; Umeda et al., 1999), whereas its close structural relative SHP2 performs opposite roles (Bauler et al., 2011). The receptor-type PTP CD45 inhibits OCL function, leading to reduced OCL activity and increased bone mass in CD45-deficient mice (Shvietel et al., 2008). Loss of the dual-specificity phosphatase MKP1 in mice reduces the amount of OCLs but appears to render them more active (Carlson et al., 2009; Sartori et al., 2009). Studies in cell culture systems established the nonreceptor-type PTP-PEST as a positive regulator of OCL differentiation and adhesion, most likely through its role in dephosphorylating and activating Src and Pyk2 (Chellaiah et al., 2007; Chellaiah and Schaller, 2009; Eleniste et al., 2012). Similar findings were obtained in culture regarding the receptor-type PTPRO (PTP-oc; Amoui et al., 2007; Yang et al., 2007; Sheng et al., 2009).

Protein tyrosine phosphatase ε (PTPe) is another positive regulator of OCL function. Two major protein forms of PTPe are known: the receptor type (RPTPa), which is an integral membrane protein, and the nonreceptor type (cyt-PTPe), which is predominantly cytosolic but can also be found in association with the cell membrane and in the nucleus. Both proteins are produced from the single Ptpre gene by use of alternative promoters (Krueger et al., 1990; Elson and Leder, 1995a,b; Nakamura et al., 1996; Tanuma et al., 1999). RPTPa and cyt-PTPe are identical throughout their sequence, with the exception of their N-termini, where the transmembranial and extracellular domains of RPTPa are replaced in cyt-PTPe by a short sequence of 12 hydrophilic amino acids (Elson and Leder, 1995a). Two additional proteins are produced from the Ptpre gene: p67 PTPe, which is produced by initiation of translation at an internal ATG codon present in mRNAs for RPTPa and cyt-PTPe; and p65 PTPe, which is produced by proteolytic cleavage of RPTPa, cyt-PTPe, or p67 PTPe (Gil-Henn et al., 2000, 2001). cyt-PTPe is expressed strongly in OCLs but not in osteoblasts; RPTPa is not expressed significantly in either cell type (Chiusaroli et al., 2004). Young female homozygous PTPe-deficient mice (EKO mice; Peretz et al., 2000), which lack all known forms of PTPe protein, exhibit increased trabecular bone mass, caused primarily by reduced OCL-mediated bone resorption. Accordingly, collagen telopeptide concentrations in serum of EKO mice are reduced, and OCL-like cells produced in vitro from bone marrow of EKO mice resorb mineralized matrix less well (Chiusaroli et al., 2004). Recruitment of hematopoietic precursor cells from the bone marrow to the circulation, which depends on OCL function, is also reduced in female EKO mice (Kollet et al., 2006). Loss of PTPe disrupts the structure, cellular organization, and stability of podosomes in OCLs and is consistent with reduced function of these cells (Chiusaroli et al., 2004). After activation of integrin molecules, cyt-PTPe is phosphorylated at its C-terminal Y638 by partially activated Src; cyt-PTPe then dephosphorylates Src at Y527, thus fully activating the kinase and promoting OCL adhesion and activity (Granot-Attas et al., 2009). Loss of cyt-PTPe disrupts podosomal structure and function at least in part due to reduced Src activation downstream of integrins (Chiusaroli et al., 2004; Granot-Attas et al., 2009).

The receptor-type PTP α (RPTPa) is closely related to RPTPe. Both PTPs are the only known members of the type IV subfamily of receptor-type PTPs, both possess short and heavily glycosylated extracellular domains, and the amino acid sequences of their catalytic domains and adjacent sequences are 72% identical. RPTPa is a ubiquitous protein that has been linked to, among other functions, cytoskeletal reorganization and cell migration (Zeng et al., 2003; Chen et al., 2006) and promotion of neural cell adhesion molecule–dependent neurite outgrowth (Bodrikov et al., 2005). In several cases, RPTPa functions by dephosphorylating and activating Src or one of its related kinases (Zheng et al., 1992; den Hertog et al., 1993; Ponniah et al., 1999; Su et al., 1999), a property shared by PTPe (Berman-Golan and Elson, 2007; Granot-Attas et al., 2009). A shorter form of RPTPa, p66 PTPa, is produced by proteolytic cleavage of RPTPa and is thus analogous to p65 PTPe (Gil-Henn et al., 2001). No form of PTP α that is similar to cyt-PTPe is known. In this study we show that RPTPa does not play a unique role in OCLs. Building on this result, we analyze the contributions of various domains of RPTPa and cyt-PTPe, the form of PTPe that is present in OCLs, to the ability of either PTP to function in these cells. We find that the 12 N-terminal amino acid residues of cyt-PTPe, which are unique to this form of PTPe, and in particular serine 2 within this region, are critical for enabling cyt-PTPe to function in OCLs.

RESULTS
Mice lacking RPTPa exhibit normal bone structure
To examine whether RPTPa-deficient (AKO) mice exhibit abnormalities in bone structure, we first examined the expression of RPTPa in osteoclasts. In agreement with previous studies, protein blotting studies indicate that OCLs express both RPTPa and cyt-PTPe (Figure 1A; Chiusaroli et al., 2004). As expected, RPTPa and cyt-PTPe were each absent from OCLs prepared from AKO or EKO mice, respectively (Figure 1A). Previous studies indicate that RPTPa, but not cyt-PTPe, is expressed in osteoblasts (Chiusaroli et al., 2004).

Histomorphometric analysis of cancellous bone from tibiae of wild-type (WT) and AKO female mice did not reveal any abnormalities in bone structure, we first examined the expression of RPTPa in osteoclasts. In agreement with previous studies, protein blotting studies indicate that OCLs express both RPTPa and cyt-PTPe (Figure 1A; Chiusaroli et al., 2004). As expected, RPTPa and cyt-PTPe were each absent from OCLs prepared from AKO or EKO mice, respectively (Figure 1A). Previous studies indicate that RPTPa, but not cyt-PTPe, is expressed in osteoblasts (Chiusaroli et al., 2004).

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OCLs, and the fraction of bone surface that was in contact with OCLs in these preparations was similar in AKO and WT mice (Figure 1C and Table 1). In agreement, levels of collagen telopeptides in serum, an indication of OCL activity in vivo, were unchanged in AKO mice, indicating that bone resorption is unchanged in AKO mice (Table 2). We conclude that the absence of RPTPa does not disrupt normal bone structure or its formation or degradation in vivo. In these respects RPTPa differs considerably from cyt-PTPe, whose absence results in increased bone mass in vivo due to reduced OCL-mediated bone resorption (Chiusaroli et al., 2004; Granot-Attas et al., 2009; Table 1). RPTPa cannot compensate for loss of cyt-PTPe, indicating that their roles in this cell type are distinct.

**RPTPa-deficient osteoclasts are produced and function normally in vitro**

To examine the possible role of RPTPa in OCLs in greater detail, we isolated nucleated bone marrow cells from tibiae and femora of WT, AKO, EKO, and DKO mice. Cells were grown in culture in the presence of M-CSF and RANKL, which promote differentiation of precursor cells into OCL-like cells. Cells from mice of all four genotypes were produced similarly in vitro; the ability of AKO OCLs to resorb bone in vitro was similar to that of WT cells (Figure 2, A and B). Because the manner in which podosomes are arranged in OCLs correlates with their activity, we also scored the fraction of OCLs in culture in which podosomes were arranged as belts at the cell periphery, in small rings, or as clusters/ata random (Figure 2C). OCL cultures prepared from WT and AKO mice contained similar fractions of cells in each of these three structural categories. In contrast, EKO OCLs exhibited a marked shift in podosomal organization: the fraction of OCLs containing a well-formed sealing zone–like structure was decreased significantly, whereas more cells displayed podosomes arranged at random (Figure 2D; Chiusaroli et al., 2004; Granot-Attas et al., 2009). Podosomal organization in DKO OCLs was similar to that in EKO OCLs (Figure 2D). We conclude that RPTPa does not perform a unique role in podosomal organization in OCLs in culture: its absence does not affect normal podosomal organization in WT OCLs, whereas the disruption observed in podosomal organization when PTP ε is absent is not affected by the presence or absence of RPTPa.

Phosphorylation of the C-terminal tyrosine of PTPs ε and ε is often critical to allow these PTPs to fulfill their physiological roles (e.g., Zheng et al., 2000; Berman-Golan and Elson, 2007; Sines et al., 2007; Rousso-Noori et al., 2011). In OCLs, cyt-PTPe is phosphorylated at Y638 after integrin-mediated contact with matrix, thus increasing the ability of cyt-PTPe to activate Src. In agreement, Src activity is reduced in EKO OCLs, and increasing Src activity can correct the abnormal stability of podosomes in EKO OCLs (Granot-Attas et al., 2009). The C-terminal sequences of cyt-PTPe and RPTPa proteins are almost identical (Figure 3A), and phosphorylation of RPTPa at its C-terminal Y789 was shown to affect the physiological role of RPTPa in a number of nonbone systems. Examination of RPTPa in OCLs differentiated from primary bone marrow cells of WT mice indicated that this PTP is phosphorylated at Y789 in adherent OCLs. Phosphorylation of Y789 is not detected in OCLs held in suspension but is detected when these cells are allowed to reattach to a surface coated with fibronectin, a ligand of the αvβ3 integrin present in OCLs (Figure 3B). Physical contact of OCLs with matrix therefore induces C-terminal phosphorylation of RPTPa, much as it does for cyt-PTPe (Granot-Attas et al., 2009). However, autophosphorylation of Src at Y416, an indicator of Src activity that is reduced in adherent EKO OCLs (Granot-Attas et al., 2009), is unchanged in adherent AKO OCLs (Figure 3C), indicating that RPTPa is not
Critical role for the N-terminus of cyt-PTPe in osteoclasts

The high degree of similarity between the catalytic domains of RPTPα and cyt-PTPe led us to examine the molecular basis for their nonredundant roles in OCLs. We note that in addition to each being the product of a distinct gene and displaying a slightly different sequence, the isoform of PTP ε that is expressed in OCLs is the nonreceptor, predominantly cytosolic cyt-PTPe, whereas RPTPα is a receptor-type integral membrane protein (Figure 4, A and B). To determine whether either of these distinctions affects the function of these PTPs in OCLs, we examined the abilities of various forms of PTPα and ε to function in OCLs, using rescue of the podosomal disorganization observed in EKO OCLs as readout.

Stability and proper organization of podosomes in OCLs have been linked repeatedly with the ability of these cells to resorb matrix (e.g., Chiusaroli et al., 2004; Miyazaki et al., 2004; Gil-Henn et al., 2007; Destaing et al., 2008; Granot-Attas et al., 2009). Podosomes of EKO OCLs are abnormally stable and disorganized, with relatively few EKO OCLs displaying a well-organized podosomal belt at their periphery. Expression of cyt-PTPe in EKO OCLs rescues their abnormal podosomal stability (Chiusaroli et al., 2004; Granot-Attas et al., 2009). To examine whether cyt-PTPe could also rescue podosomal organization in EKO OCLs, we infected these cells with adenoviruses expressing cyt-PTPe. OCLs in which podosomes were arranged as a podosomal belt at the cell periphery, in small rings, or in clusters (Figure 2C) were scored and compared with those seen in WT and in mock-infected EKO OCLs. As seen in Figure 4C, expression of WT cyt-PTPe in EKO OCLs rescued their abnormal podosome organization pattern and made it similar to that of WT OCLs, thus validating use of podosomal organization as a readout for the EKO OCL phenotype in this study.

**TABLE 1:** Histomorphometric analysis of the cancellous region of the tibial metaphysis of 7-wk-old female mice.

| Parameter                        | WT (n = 7)     | AKO (n = 7)    | EKO (n = 7)    | DKO (n = 8)    |
|----------------------------------|----------------|---------------|---------------|---------------|
| BV/TV (%)                        | 3.44 ± 0.53    | 3.24 ± 1.01   | 9.61 ± 1.41*  | 11.49 ± 2.84* |
| Tb.Th (μm)                       | 22.45 ± 1.85   | 22.62 ± 2.43  | 32.02 ± 1.26* | 31.68 ± 2.54* |
| Tb.N (/mm)                       | 1.51 ± 0.17    | 1.31 ± 0.22   | 2.94 ± 0.35*  | 3.31 ± 0.63*  |
| Tb.Sp (μm)                       | 700 ± 95       | 843 ± 102     | 341 ± 47**    | 365 ± 81*     |
| MS/BS (%)                        | 22.36 ± 2.42   | 22.45 ± 2.51  | 32.63 ± 3.79* | 36.03 ± 1.32* |
| MAR (μm/d)                       | 2.71 ± 0.28    | 2.83 ± 0.35   | 2.50 ± 0.19   | 3.14 ± 0.19   |
| BFR/BS (μm²/μm²/yr)             | 215 ± 23       | 219 ± 23      | 287 ± 32      | 410 ± 20*     |
| BFR/BV (%)                       | 2024 ± 303     | 1976 ± 184    | 1834 ± 244    | 2670 ± 181    |
| Ob.S/BS (%)                      | 15.50 ± 2.79   | 17.16 ± 2.14  | 13.07 ± 2.54  | 24.54 ± 3.63* |
| N.Ob/BS (/mm)                    | 11.51 ± 2.43   | 14.66 ± 1.60  | 11.14 ± 2.62  | 21.53 ± 2.95* |
| OS/BS (%)                        | 10.95 ± 2.61   | 15.82 ± 2.83  | 13.21 ± 2.47  | 16.91 ± 2.72  |
| O.Th (μm)                        | 3.88 ± 0.42    | 3.83 ± 0.42   | 4.64 ± 0.38   | 4.86 ± 0.38   |
| Oc.S/BS (%)                      | 11.36 ± 1.40   | 10.86 ± 2.08  | 7.39 ± 1.91   | 8.58 ± 2.04   |
| N.Oc/BS (/mm)                    | 5.39 ± 0.52    | 4.93 ± 1.11   | 3.42 ± 0.88   | 4.02 ± 0.95   |

Parameters are trabecular bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular spacing (Tb.Sp), mineralizing surface (MS/BS), mineral apposition rate (MAR), bone formation rate per unit bone surface area (BFR/BS), bone formation rate per bone volume (BFR/BV), percentage bone surface in contact with osteoblasts (Ob.S/BS); osteoblast number per unit of bone surface length (N.Ob/BS); osteoid surface (OS/BS); osteoid thickness (O.Th); percentage bone surface in contact with osteoclasts (Oc.S/BS), and osteoclast number per unit bone surface length (N.Oc/BS). Similar values were obtained for BV/TV, Tb.Sp, Tb.Th, and Tb.N in 7-wk-old WT and AKO female mice by micro-CT (unpublished data).

*p < 0.05 compared with WT.

*p < 0.05 compared with AKO.

*p < 0.001 compared with EKO.

| Serum CTX (ng/ml) | WT  | AKO  | EKO  | DKO  |
|-------------------|-----|------|------|------|
|                   | 108.1 ± 7.7 | 121.3 ± 10.8 | 72.9 ± 3.8* | 95.1 ± 5.8 |

Shown are mean ± SE, n = 16–18 mice/genotype. *p = 0.00004 vs. WT by Student’s t test.

**TABLE 2:** Collagen telopeptide concentrations in serum of 7-wk-old female mice.

Phosphorylation of Src at Y416 is reduced in DKO OCLs to levels observed in adherent EKO OCLs (Figure 3C), further indicating that RPTPα does not play a unique role in OCLs in this respect as well. pY416 Src, which is detected in adherent OCLs, is reduced significantly in OCLs held in suspension and is restored in an integrin-mediated manner when OCLs are allowed to readhere to a surface coated with fibronectin (Figure 3D). Despite their initial differences in pY416 Src levels (Figure 3C), OCLs from WT, EKO, AKO, and DKO mice were all capable of reattaching to fibronectin-coated surface and restoring initial levels of pY416 Src (Figure 3D). This indicates that cyt-PTPe is not the exclusive regulator of integrin-mediated activation of Src, which can occur to some extent in the absence of this phosphatase, possibly by other PTPs or directly by the β-integrin chain (Arias-Salgado et al., 2003). In all, the role of RPTPα in production or function of OCLs in vivo or in vitro is either minimal or is redundant with the roles of other, non-PTP ε, PTPs.
To compare the effect of various forms of PTP α and ε on podosomal organization in EKO OCLs, we constructed a series of adenoviral expression vectors for expressing proteins shown in Figure 4A. Each adenoviral vector was able direct expression of the relevant protein product (Supplemental Figure S1), and its effect on podosomal organization was scored. As seen in Figure 4D, the fraction of

FIGURE 2: Properties of PTP α-deficient osteoclasts. (A) Bone marrow cells from WT, AKO, EKO, and DKO female mice were cultured with M-CSF and RANKL for 6 d and then stained for TRAP (red). Bar, 200 μm. (B) Bone marrow cells from WT and AKO female mice were seeded on fragments of bovine bone and grown for 8 d in the presence of M-CSF and RANKL. Cells were then removed and the bone fragments stained with Coomassie brilliant blue to highlight resorption pits. Bar, 400 μm. (C) WT OCLs prepared from 7-wk-old female mice were grown on glass coverslips, fixed, stained with phalloidin–Alexa 488, and examined by cell imaging. Shown are examples of the three podosomal arrangement types: sealing zone–like structure (SZL, large single belt at the cell periphery), rings (mixture of small rings and individual, scattered podosomes), and clusters (individual or grouped podosomes, no rings). Bar, 10 μm. Dashed line marks outer perimeter of the cell shown. (D) Percentages (mean ± SD) of OCLs of the four genotypes in which the actin-rich podosomal cores were arranged in SZL-like structures (SZL), rings (R), or clusters (C). n = 317–638 OCLs/genotype. **p < 0.05 vs. WT by Student’s t test.

FIGURE 3: Phosphorylation of RPTPa and Src in OCLs. (A) C-terminal sequences of cyt-PTPα and RPTPa. The phosphorylatable tyrosine (Y789 in RPTPa, Y638 in cyt-PTPα) is highlighted. (B) Primary OCLs prepared from bone marrow of WT mice were grown on plastic plates (Ad), serum-starved, lifted, maintained in suspension for 30 min (Sus), and then seeded on plates coated with fibronectin (FN). Cells were lysed, and pY789 RPTPa was detected by protein blotting. (C) Phosphorylation of Src at its activating Y416 in adherent OCLs from WT, EKO, AKO, and DKO female mice. Top, representative protein blot. Bottom, bar diagram summarizing two to seven independent repeats per genotype (each compared with pY416 Src levels in WT OCLs processed in parallel; mean ± SE). (D) Src undergoes integrin-dependent phosphorylation in the absence of PTP α and/or ε. Adherent, suspended, and readherent OCLs from the four genotypes were analyzed for pY416 Src by protein blotting. Note that in all cases, Src is hypophosphorylated in suspended cells (Sus) and rephosphorylated when cells reattach to fibronectin-covered surface (FN). Intensity of pY416 Src phosphorylation varies among genotypes as in C; exposure of pY416 Src images was adjusted for each genotype to allow visualization of phosphorylation.

EKO OCLs that displayed a well-organized podosomal belt was significantly lower than in WT OCLs. Expressing in EKO OCLs either cyt-PTPα or the artificial protein cyt-PTPα, in which the 12 N-terminal
residues of cyt-PTPe replaced the membrane-spanning and extra-cellular domain of RPTPa (Figure 4B), increased the fraction of cells displaying well-organized podosomal belt structures to that found in WT OCLs. In contrast, RPTPe and RPTPa did not do so. We conclude that despite the sequence differences that exist between them, the catalytic domains of both PTPs can function in OCLs to...

**FIGURE 4:** Nonreceptor forms of PTPs ε and α can rescue the podosomal organization phenotype of EKO osteoclasts. (A) Schematic representation of various constructs of PTP ε and PTP α used in this study. Dashed horizontal lines: cell membrane; oval, unique N-terminus from cyt-PTPe; small rectangle, Lck myristoylation motif; large vertical rectangles, PTP domains of PTP α (black) or PTP ε (white). (B) N-terminal sequences of RPTPa/e, cyt-PTPa/e, and p67 PTPe. Dashed underline marks membrane-spanning regions of RPTPe and RPTPa. Solid underline marks the 12 N-terminal residues of cyt-PTPe, which are unique to this isoform (and were included as the N-terminus of the artificial cyt-PTPa protein).

(C) Expression of cyt-PTPe in OCLs from PTPe-deficient mice rescues their podosomal organization phenotype. Cultures of OCLs prepared from bone marrow of WT or EKO mice, some expressing exogenous cyt-PTPe as indicated, were examined as described in Figure 2D. *p ≤ 0.03, **p ≤ 0.006 vs. WT by Student’s t test. *n = 199–918 osteoclasts analyzed per treatment.

(D) Rescue of the EKO OCL podosome organization phenotype by various PTP ε and PTP α molecules. WT and EKO OCLs prepared from 7-wk-old female mice infected with adenoviruses expressing the indicated constructs and then processed as in Figure 2D. Percentage of cells (mean ± SD) in which podosomes are arranged as podosomal belts (SZL) for clarity of presentation. The complete distributions of SZL, R, and C cells in this experiment are shown in Supplemental Figure S2. *p ≤ 0.04, **p ≤ 0.008 vs. WT.
ensure proper podosomal organization. We also conclude that the
distinction between the N-terminal sequences of both PTPs affects
their ability to function in OCLs.

Lck-PTPe and Lck-PTPa, forms of cyt-PTPe or cyt-PTPa whose
N-termini include an Lck myristoylation motif, did not rescue the
podosomal organization phenotype of EKO OCLs (Figure 4D).
Lck-tagged PTPs localize to the cell membrane (e.g., Andersen
et al., 2001) but lack the membrane-spanning and extracellular
regions of RPTPe/RPTPa. This result indicates that interaction with
extracellular ligands, such as dimerization-induced inhibition of
RPTPa (Jiang et al., 1999, 2000), is not the cause of the inability of
both molecules to rescue the EKO OCL podosomal phenotype.
Finally, p67 PTPe, which lacks the 27 N-terminal amino acid residues
of cyt-PTPe and is entirely cytosolic (Figure 4B; Gil-Henn et al., 2000),
did not rescue the podosomal organization phenotype of EKO
OCLs (Figure 4D). In all, the only forms of PTPs α and ε that rescued
podosomal organization in EKO OCLs were cyt-PTPe and cyt-PTPa.
Both proteins include the 12 N-terminal amino acids of cyt-PTPe,
which are absent from all other proteins examined in this study.
We conclude therefore that these 12 residues are required to allow cyt-
PTPe to support proper organization of podosomes in OCLs.

We showed previously that cyt-PTPe is found also in podosomes
of OCLs (Granot-Attas et al., 2009). To determine whether p67 PTPe
and cyt-PTPa proteins can be associated with podosomes, we ex-
pressed both proteins in EKO OCLs, isolated podosome-enriched
fractions from the cells, and examined whether either protein is
present in them. In these experiments fractionations of actin and
Src, which are found throughout the cell and in podosomes, and of
tubulin, which is not found in podosomes, were followed as controls
for the quality of the fractionation process. This study revealed that
cyt-PTPe and cyt-PTPa, which possess the N-terminus of cyt-PTPe,
and p67, which does not, are all present in podosome-rich fractions
of OCLs (Figure 5). We conclude therefore that despite its role in
ensuring proper podosomal organization in OCLs, the N-terminus
of cyt-PTPe is not required for actual localization of cyt-PTPe in
podosomes. Of note, similar fractionation experiments revealed that
RPTPa was not present in OCL podosome–enriched fractions ir-
respective of whether endogenous or exogenous RPTPa was exam-
ined (Figures 5 and 6E). It is therefore possible that at least part of
the functional differences between cyt-PTPe and RPTPa in OCLs
arise also from the absence of RPTPa from podosomes.

Serine 2 affects cyt-PTPe activity and is required for
cyt-PTPe to regulate podosome organization in OCLs

We next examined the 12 N-terminal residues of cyt-PTPe in further
detail. As seen in Figure 6A, this sequence contains three positively
charged residues (R4, K5, R9) and four potential phosphorylation
sites (S2, S3, S8, T11). R4, K5, and R9 participate in a functional
nuclear localization signal (Kraut et al., 2002), making their involve-
ment in podosomal regulation possibly less likely. We therefore
turned our attention to serine 2, the first of the four serine/threonine
residues. Expression of S2D cyt-PTPe, which mimics phosphoryla-
tion at this site, rescued the podosomal arrangement phenotype of
EKO OCLs. In contrast, S2A cyt-PTPe, which mimics dephosphoryla-
tion at this site, did not do so. In fact, expression of this latter con-
struct in EKO OCLs exacerbated podosomal disorganization, sug-
gesting that it may function in a dominant-negative role (Figure 6B).
We note that the catalytic activities of both S2A and S2D cyt-PTPe
are higher than that of WT cyt-PTPe (Figure 6C). This result indicates
that presence of serine 2 inhibits cyt-PTPe catalytic activity and sug-
gests a regulatory role for this residue. However, the effect of serine
2 of cyt-PTPe on podosomal organization in OCLs is most likely not
mediated by changes in cyt-PTPe activity, since S2A and S2D cyt-
PTPe exert opposite effects on podosomal organization despite
both being active and activating Src throughout the cell to similar
extents in OCLs (Figure 6D).

Fractionation studies indicated that S2D cyt-PTPe and S2A cyt-
PTPe are found in podosome-enriched fractions of OCLs to similar
extents (Figure 6E), in agreement with our previous conclusion that
the N-terminus of cyt-PTPe does not control podosomal localization
of cyt-PTPe (Figure 5). The S2A and S2D mutations did not affect
nuclear localization of cyt-PTPe (unpublished data), indicating that
serine 2 does not interact functionally with the adjacent nuclear lo-
calization signal. These results strongly suggest that serine 2, possi-
bly through its phosphorylation, is required for cyt-PTPe to regulate
podosomal organization in OCLs.

DISCUSSION

Results presented here indicate that lack of RPTPa does not affect
the mass or structure of trabecular bone. AKO mice also exhibit
unaltered rates of bone synthesis and degradation, and their OCLs
are produced, function, and signal normally. In parallel, we confirm
that loss of the related cyt-PTPe disrupts the structure, stability, and
organization of podosomes in OCLs and results in increased trabe-
cular bone mass. Moreover, in all experiments performed in culture,
OCLs from EKO mice performed identically to OCLs from DKO
mice that lack both RPTPa and cyt-PTPe. Loss of RPTPa therefore
did not affect the phenotypes induced by loss of cyt-PTPe. We
increased leptin sensitivity and resistance to diet-induced obesity in mice. Yadav and others (2004) have shown that alteration of leptin receptor signaling in the hypothalamus, leading to leptin resistance, can affect osteoblasts via the sympathetic nervous system (SNS).

Hypothalamic leptin signaling can affect osteoblasts in an indirect manner. Hypothalamic leptin signaling in mice can affect osteoblasts via the sympathetic nervous system (SNS), which supports OCL-mediated bone resorption in part by activating Src downstream of integrin receptors (Granot-Attas et al., 2009). Although both PTPs are closely related and perform several of their physiological functions by dephosphorylating and activating Src, loss of cyt-PTPe, but not RPTPa, reduces Src activity in cultured OCLs (Figure 3C); this suggests that these PTPs differ in their ability to activate Src in OCLs. Because proper Src activity is essential for OCL function, this may explain in part the functional differences between both PTPs. This functional distinction most likely arises from a more basic difference between the isoforms of these PTPs that are expressed in OCLs—the receptor-type RPTPa versus the nonreceptor-type cyt-PTPe. RPTPa and its receptor-type PTP ε counterpart RPTPa cannot rescue the podosomal organization phenotype of EKO OCLs. In contrast, cyt-PTPe and the similarly structured artificial protein cyt-PTPa do rescue this phenotype; this indicates that despite some sequence differences between both PTPs, the catalytic domains of PTP α can function in OCLs when presented in the proper structural context.

Functional differences in the phenotypes of osteoclasts lacking one of two closely related PTPs provide an opportunity to identify domains within cyt-PTPe that are essential for its function in this cell type. The main structural feature that sets apart RPTPa from cyt-PTPe is the presence of transmembrane and extracellular domains in RPTPa, which are replaced by a unique sequence of 12 N-terminal amino acids in RPTPa, thereby creating a unique N-terminus for both PTPs. The latter active model is not likely, since the sequences of the extracellular domains of RPTPa and RPTPe differ significantly from one another and from the N-termini of Lck-PTPe, Lck-PTPa, and p67, which also cannot regulate podosomal organization. The active model would rely on the unlikely assumption that the widely divergent N-termini of these five molecules share the ability to actively prevent rescue of the podosomal organization phenotype of EKO OCLs. These results also argue against the possibility that RPTPa and RPTPe are inhibited from functioning in OCLs by binding of an extracellular ligand(s). The alternative model, by which presence of the 12 N-terminal amino acids of cyt-PTPe is required for affecting podosomal organization in OCLs, is strengthened significantly by the finding that the S2A mutation in this sequence might cause the absence of the 12 N-terminal residues of cyt-PTPe from both PTPs or by a particular function that is fulfilled actively by the transmembrane and extracellular domains of either PTP.

In contrast to cyt-PTPe, cyt-e and the similarly closely related PTPs provide an opportunity to identify domains within cyt-PTPe that are essential for its function in this cell type. The main structural feature that sets apart RPTPa from cyt-PTPe is the presence of transmembrane and extracellular domains in RPTPa, which are replaced by a unique sequence of 12 N-terminal amino acids in RPTPa, thereby creating a unique N-terminus for both PTPs. The latter active model is not likely, since the sequences of the extracellular domains of RPTPa and RPTPe differ significantly from one another and from the N-termini of Lck-PTPe, Lck-PTPa, and p67, which also cannot regulate podosomal organization. The active model would rely on the unlikely assumption that the widely divergent N-termini of these five molecules share the ability to actively prevent rescue of the podosomal organization phenotype of EKO OCLs. These results also argue against the possibility that RPTPa and RPTPe are inhibited from functioning in OCLs by binding of an extracellular ligand(s). The alternative model, by which presence of the 12 N-terminal amino acids of cyt-PTPe is required for affecting podosomal organization in OCLs, is strengthened significantly by the finding that the S2A mutation in this sequence might cause the absence of the 12 N-terminal residues of cyt-PTPe from both PTPs or by a particular function that is fulfilled actively by the transmembrane and extracellular domains of either PTP. The latter active model is not likely, since the sequences of the extracellular domains of RPTPa and RPTPe differ significantly from one another and from the N-termini of Lck-PTPe, Lck-PTPa, and p67, which also cannot regulate podosomal organization. The active model would rely on the unlikely assumption that the widely divergent N-termini of these five molecules share the ability to actively prevent rescue of the podosomal organization phenotype of EKO OCLs. These results also argue against the possibility that RPTPa and RPTPe are inhibited from functioning in OCLs by binding of an extracellular ligand(s). The alternative model, by which presence of the 12 N-terminal amino acids of cyt-PTPe is required for affecting podosomal organization in OCLs, is strengthened significantly by the finding that the S2A mutation in this sequence might cause the absence of the 12 N-terminal residues of cyt-PTPe from both PTPs or by a particular function that is fulfilled actively by the transmembrane and extracellular domains of either PTP. The latter active model is not likely, since the sequences of the extracellular domains of RPTPa and RPTPe differ significantly from one another and from the N-termini of Lck-PTPe, Lck-PTPa, and p67, which also cannot regulate podosomal organization. The active model would rely on the unlikely assumption that the widely divergent N-termini of these five molecules share the ability to actively prevent rescue of the podosomal organization phenotype of EKO OCLs. These results also argue against the possibility that RPTPa and RPTPe are inhibited from functioning in OCLs by binding of an extracellular ligand(s). The alternative model, by which presence of the 12 N-terminal amino acids of cyt-PTPe is required for affecting podosomal organization in OCLs, is strengthened significantly by the finding that the S2A mutation in this sequence.
abrogates its ability to do so. The latter results highlight the importance of serine 2 in regulating cyt-PTPε in OCLs and raise the possibility that cyt-PTPε function in OCLs is regulated by phosphorylation at this residue.

The mechanism by which serine 2 affects cyt-PTPε function in osteoclasts is not clear. The 12 N-terminal residues of cyt-PTPε do not control physical access of cyt-PTPε to podosomes, since cyt-PTPε and its mutants S2A and S2D, as well as p67, which lacks this sequence, are all detected in podosome-enriched fractions of OCLs. Although serine 2 down-regulates the catalytic activity of cyt-PTPε, activity alone does not explain the ability of cyt-PTPε to regulate podosomal organization, since S2A and S2D cyt-PTPε, which affect podosomal organization in opposite ways, are similarly active. It is possible that the N-terminus of cyt-PTPε allows it to engage in protein–protein interactions or regulates protein stability in a way that is essential for its function in osteoclasts; further studies are needed to address this issue.

**MATERIALS AND METHODS**

**Antibodies**
Polyclonal antibodies used included anti-PTPε (Elson and Leder, 1995b), which cross-reacts with RPTPα; anti–phospho-PTPε, which reacts specifically with PTPε phosphorylated at its C-terminal tyrosine residue (Y638 in cyt-PTPε = Y695 in RPTPε; Berman-Golan and Elson, 2007) and cross-reacts with RPTPα phosphorylated on its C-terminal Y789; and anti-pY416 Src (Cell Signaling Technology, Danvers, MA). Monoclonal antibodies used included anti–v-Src (clone 327; Calbiochem, San Diego, CA), anti-α-tubulin (clone DM1A; Sigma-Aldrich, St. Louis, MO), and anti-actin (clone AC-40; Sigma-Aldrich). Horseradish peroxidase (HRP)–conjugated secondary antibodies for protein blotting were from Jackson ImmunoResearch Laboratories (West Grove, PA). Enhanced chemiluminescence reagents were from Biological Industries (Beit Haemek, Israel).

**Mice**
Gene-targeted mice lacking PTPε (Peretz et al., 2000) or RPTPα (Bodrikov et al., 2005), as well as DKO mice lacking both PTPs and WT controls, were used in a mixed 50% 129 SvEv, 50% C57Black/6 genetic background. In some studies EKO and WT mice were in the pure 129 SvEv background. All mice were handled in accordance with Israeli law and Weizmann Institute regulations, and studies were approved by the Weizmann Institute’s Animal Ethics Committee.

**Micro-CT and bone histomorphometry**
Micro-CT was performed on a volume of 1.8 mm³ of cancellous bone starting 0.3 mm distal to the proximal tibial growth plate, using an eXplore Locus SP system (GE Healthcare, London, Canada). Histomorphometric measurements were performed on 5-μm sagittal sections of tibiae embedded in methyl methacrylate resin as described (Aoki et al., 1999). Histomorphometric parameters were measured in a 1.84-mm² area of secondary spongiosa starting 0.3 mm from the proximal growth plate, using the Osteomeasure analysis system (Osteometrics, Atlanta, GA). Data were analyzed for statistical significance by Student’s t-test.

**Osteoclast culture**
Bone marrow from femora and tibiae of 6- to 8-wk-old mice was depleted of erythrocytes by hypotonic lysis and cultured in complete OCL medium (α-MEM [Sigma-Aldrich] containing 10% fetal calf serum [Invitrogen-Life Technologies, Carlsbad, CA], 2 mM glutamine, 50 U/ml penicillin, and 50 g/ml streptomycin and supplemented with 20 ng/ml M-CSF [Peprotech, Rocky Hill, NJ] and 20 ng/ml RANKL [R&D Systems, Minneapolis, MN]). Cells were plated at 5 × 10⁶ or 1 × 10⁶ cells per well of a six- or 24-well plate, respectively, and incubated at 37°C in 5% CO2 for 5 d with daily changes of medium. Cells were fixed and stained for TRAP activity using a commercial kit (Sigma-Aldrich).

**Pit resorption assay**
Bone marrow cells were cultured on bovine cortical bone slices for 7–8 d with M-CSF and RANKL. Cells were removed from bone by treatment with 0.25M NH₂OH. The slices were washed in distilled water, incubated in a saturated alum (KAl(SO₄)₂) solution, washed in distilled water, and stained with Coomassie brilliant blue (0.2% in a solution of 40%H₂O/60% methanol).

**Collagen telopeptide assay**
Serum was prepared from blood collected by retro-orbital bleeding from 7-wk-old female mice that had been fasted overnight. Concentrations of C-telopeptide degradation products of type I collagen were determined using the Ratlaps ELISA system (Immunodiagnostics Systems, Scottsdale, AZ) according to the manufacturer’s instructions.

**Replating experiments**
Primary bone marrow preosteoclasts were starved on their fourth day of differentiation for 4 h in OCL medium containing 1% serum and no cytokines. Cells were detached by a short treatment with 10 mM EDTA, suspended in DMEM containing 20 mM HEPES and 1 mg/ml bovine serum albumin, and incubated at 37°C for 1 h with gentle rotation. Cells were replated on plates precoated with 20 μg/ml fibronectin (Sigma-Aldrich) and analyzed 30 min later.

**Adenoviral infection of OCLs**
Bone marrow from mice was cultured in OCL medium as described. Two days after seeding, medium was replaced with complete OCL medium containing adenoviruses. After overnight incubation the medium was changed and the cells were fed daily with fresh OCL medium (containing cytokines). Adenoviruses were produced with AdEasy XL adenoviral vector system (Stratagene, Agilent Technologies, Santa Clara, CA).

**Fluorescence microscopy**
BM cells were seeded on glass coverslips (Menzel-Glaser, Braunschweig, Germany). On day 2 of differentiation, cells were infected with adenoviruses; after 4 d of differentiation, OCLs were fixed in 3% paraformaldehyde (PFA; Merck, Darmstadt, Germany) in phosphate-buffered saline (PBS) for 20 min and then for an additional 2 min in warm 3% PFA containing 0.5% Triton X-100 (Sigma-Aldrich). After staining with phalloidin conjugated to Alexa 488, cells were mounted with Fluoromount-G solution (Southern Biotech, Birmingham, AL). Images were collected on a deconvolution DeltaVision microscope system equipped with Resolve3D software (Applied Precision, Issaquah, WA). Cells were analyzed visually and scored as displaying a complete podosomal belt structure (fully polarized, FP), partial podosomal belt and rings (partly polarized, PP), or podosomes arranged small clusters or at random (nonpolarized, NP). Analyses were performed in a manner blinded to the genotypes of the cells.

**Differential cell lysis**
Primary OCLs were cultured in six-well plates and in some samples infected with adenoviruses as described. Cells were lysed in

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200 μl/well lysis buffer (20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1% Triton, and 1 mM sodium vanadate with protease inhibitors [1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 40 μM bestatin, 15 μM E-64, 20 μM leupeptin, 15 μM pepstatin; Sigma-Aldrich]) on ice for 10 min with gentle shaking. Cell bodies and cytoplasm were removed and saved. The remaining adherent podosomal structures were washed gently three times with 200 μl of lysis buffer. Lysis buffer was completely removed, and the remaining cell structures were solubilized in 100 μl/well of 20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 0.1% SDS, and 1% sodium deoxycholate with protease inhibitors.

**Protein blotting**

Cells were washed with ice-cold PBS and lysed in NP-40 buffer (50 mM Tris-Cl, pH 8; 150 mM NaCl; 1% NP-40) and protease inhibitors. Sodium pervanadate, 0.5 mM, was included when tyrosine phosphorylation of proteins was evaluated. A 25-μg amount of total lysates was subjected to 7% SDS–PAGE and transferred onto a nitrocellulose membrane (Protran; Whatman GE Healthcare, Maidstone, United Kingdom). The filters were blocked in 5% milk/PBS/Tween for 1 h and incubated with primary antibody at 4°C overnight, followed by probing with secondary antibodies coupled to HRP.

**Activity assays**

For the PTP ε activity assay, the various forms of PTP ε were expressed at similar levels in 293 cells. At 48 h after transfection, cells were lysed in NP-40 buffer supplemented with protease inhibitors. Total phosphatase activity in lysates was assayed in duplicate at 30°C in 96-well plates in reactions containing 100 μl of cell lysate (25 μg of total protein) and 200 μl of assay buffer (50 mM 2-(N-morpholino)ethanesulfonic acid, pH 7.0, 0.5 mM dithiothreitol, 0.5 mg/ml bovine serum albumin, and 10 mM p-nitrophenyl phosphate). Each sample was assayed with or without addition of 0.5 mM PTP inhibitor sodium pervanadate. Activity was measured by following the increase in absorbance at 405 nm for 45 min, during which absorbance was linear with time. Net activity of each form of PTP ε was normalized to its expression level as determined by protein blotting. Src activity was determined by measuring phosphorylation of enolase by Src immunoprecipitated from the cells analyzed, as described in Gil-Henn and Elson (2003).

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