PYK2 in Osteoclasts Is an Adhesion Kinase, Localized in the Sealing Zone, Activated by Ligation of $\alpha_v\beta_3$ Integrin, and Phosphorylated by Src Kinase

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Abstract

Osteoclast activation is initiated by adhesion to the bone surface, followed by cytoskeletal rearrangement, the formation of the sealing zone, and a polarized ruffled membrane. This study shows that PYK2/CAKβ/RAFTK, a cytoplasmic kinase related to the focal adhesion kinase, is highly expressed in rat osteoclasts in vivo. Using murine osteoclast-like cells (OCLs) or their mononuclear precursors (pOCs), generated in a coculture of bone marrow and osteoblastic MB1.8 cells, we show: (*a*) tyrosine phosphorylation of PYK2 upon ligation of β_3 integrins or adhesion of pOCs to serum, vitronectin, osteopontin, or fibronectin but not to laminin or collagen; (*b*) coimmunoprecipitation of PYK2 and c-Src from OCLs; (*c*) PYK2 binding to the SH2 domains of Src; (*d*) marked reduction in tyrosine phosphorylation and kinase activity of PYK2 in OCLs derived from Src (−/−) mice, which do not form actin rings and do not resorb bone; (*e*) PYK2 phosphorylation by exogeneous c-Src; (*f*) translocation of PYK2 to the Triton X-100 insoluble cytoskeletal fraction upon adhesion; (*g*) localization of PYK2 in podosomes and the ring-like structures in OCLs plated on glass and in the sealing zone in OCLs plated on bone; and (*h*) activation of PYK2, in the presence of MB1.8 cells, parallels the formation of sealing zones and pit resorption in vitro and is reduced by echistatin or calcitonin and cytochalasin D. Taken together, these findings suggest that Src-dependent tyrosine phosphorylation of PYK2 is involved in the adhesion-induced formation of the sealing zone, required for osteoclastic bone resorption. (*J. Clin. Invest.* 1998. 102: 881–892.) Key words: tyrosine kinase • podosome • clear zone • bone resorption • vitronectin receptor

Introduction

Integrins are cell surface receptors that mediate cell-extracellular matrix (ECM)[1] and cell–cell interactions and the initiation of signals that control many cell functions (1, 2). The cytoplasmic focal adhesion kinase (FAK) has been implicated in integrin signaling, which includes changes in intracellular Ca^{2+}, lipid turnover, and tyrosine phosphorylation and leads to cytoskeletal rearrangements and gene expression (3–5).

PYK2/CAKβ/RAFTK is a member of the focal adhesion kinase family (3–5). PYK2 and FAK are 65% homologous; both lack a transmembrane region, SH2 or SH3 domains, but have proline-rich regions in the C terminus. PYK2 is expressed in neuronal and hemopoietic cells, including megakaryocytes and various lymphocytic and myeloid cell lines (5, 6), which also express FAK. PYK2 was suggested to play a different role than FAK (7–11). In megakaryocytes and B cells, PYK2 is tyrosine phosphorylated in an integrin-dependent manner and localizes to "focal adhesion-like structures" (12, 13). In vitro, PYK2 binds to the protein tyrosine kinases Src and Fyn, as well as to $p130^{cas}$, paxillin, and Grb-2 (3, 7, 12–15). Recently, PYK2 and Src were reported to play a role in the signaling pathways that link G-protein–coupled receptors with mitogen-activated protein kinase (7).

Osteoclasts are the bone-resorbing cells and play a key role in bone remodeling (16). Osteoclast adhesion to the bone surface induces cytoskeletal reorganization and cell activation (17). The recognition of extracellular matrix components possibly mediated by $\alpha_v\beta_3$ is thought to initiate osteoclast function (18, 19). In vitro integrin-mediated adhesion to vitronectin, fibronectin, or collagen induces osteoclast spreading and actin rearrangement (20). In addition, signaling pathways, which involve c-Src, c-Cbl, or PI3-kinase, were shown to be important for osteoclastic bone resorption (21–24).

The object of this study was to examine a possible role for PYK2 in osteoclast activation. We show that PYK2 is highly expressed in osteoclasts in vivo and in osteoclast-like cells (OCLs) in culture. Tyrosine phosphorylation of PYK2 is induced upon attachment to vitronectin, osteopontin, and fibronectin and is mediated by β_3 integrins. PYK2 is found in the sealing zone, and its phosphorylation correlates with the formation of the sealing zone and with OCL bone resorption. In addition, in OCLs, PYK2 associates with c-Src in an SH2-dependent manner. Furthermore, in the resorption-deficient Src (−/−) OCLs, PYK2 is not tyrosine phosphorylated. These findings suggest that PYK2 may play a role in Src-dependent intracellular pathways that link β_3–integrin mediated osteoclast adhesion to osteoclast activation and the formation of the osteoclast sealing zone on the bone surface.

Methods

Antibodies and other reagents. Anti-FAK (mAb 2A7), anti-p60src (mAb GD11), and antiphosphotyrosine (mAb 4G10) were from Upstate Biotech (Lake Placid, NY); antipaxillin (mAb 349) and anti-PYK2 (mAb 11) were from Transduction Labs (Lexington, KY). Anti-PYK2 polyclonal antibodies were developed using a purified C-terminal fragment of mouse PYK2 (Research Genetics, Huntsville, AL). Goat anti–N-terminal domain of PYK2 was from Santa Cruz

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1. *Abbreviations used in this paper:* D_3, $1\alpha,25(OH)_2D_3$; ECL, enhanced chemiluminescence; ECM, extracellular matrix; FAK, focal adhesion kinase; GST, glutathione-S-transferase; OCL, osteoclast-like cell; pOC, prefusion osteoclast-like cell.

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Biotech (Santa Cruz, CA). Anti-β_2 integrin (mAb 18/2) antibody was from ATCC (Rockville, MD), anti-β1 (mAb 9EG7) and anti-β_3 (mAb 2C9.G2) integrins were from PharMingen (San Diego, CA), and F(ab)′2 of anti–rat or –hamster IgG, FITC- and TRITC-conjugated IgG were from Jackson Labs (West Grove, PA). HRP-secondary antibodies were from Amersham (Arlington Heights, IL); collagenase and dispase were from Wako, Inc. (Dallas, TX). Tissue culture reagents were from GIBCO BRL (Grand Island, NY). 1α,25-$(OH)_2D_3$ (D_3) was a gift from Dr. M. Uskokovich, Hoffmann-LaRoche (Nutley, NJ). Recombinant c-Src was from Upstate Biotech, glutathione-S-transferase (GST) fusion proteins of Fyn, Lyn, and PI3-kinase were from PharMingen and Santa Cruz. GST-fusion proteins of c-Src and PYK2 were generated by PCR using oligonucleotides flanking the indicated domains, cloned into the pGEX-4T plasmid (Pharmacia Biotech, Piscataway, NJ), and expressed according to manufacturer.

Isolation of osteoclast-like cells and of prefusion osteoclast-like cells. Bone marrow–derived OCLs were prepared as previously described (25). Osteoblastic MB1.8 cells were cocultured with murine bone marrow cells in α-MEM containing 10% FBS and 10 nM D_3. After 7 d, the cocultures were washed in PBS and treated with collagenase/dispase (each at 0.01% [wt/vol] in PBS) at 37°C for 20 min. After MB1.8 cells were removed, lysates of attached OCLs were prepared as described below. The prefusion osteoclast-like cells (pOCs) were isolated from the cocultures as described (25) with the use of 10 mM EDTA in Ca^{2+}/Mg^{2+}-free PBS to lift pOCs. Purity of the preparations was 85–95%, and the cells obtained in this manner have the same properties as pOCs isolated using the previous method. Bone resorption activity was determined by plating MB1.8 cells (pretreated with D_3 for 48 h) on bovine cortical bone slices and then adding pOCs for the indicated times. Cells were then either solubilized and immunoprecipitated or immunostained as described below. The resorption pits were determined as described (25).

Heterozygous Src (+/−) mice were obtained from Jackson Labs (Bar Harbor, ME) and mated to generate osteopetrotic Src (−/−) mice, which were identified by the lack of tooth eruption. OCLs were prepared from spleen cells isolated from 3-wk-old Src (−/−) mice and their normal Src (+/?) littermates as described (24).

RNA isolation and Northern blot analysis. Total cellular RNA was isolated as described (26). A specific probe for PYK2 (570 bp) was generated by PCR using the primers: 5′-(AGTGACATTTATCAGATGGAG) and 3′-(GAATGGACTGTGCACCGAGCC), with OCL-derived cDNAs as template. A probe for FAK (700 bp) was generated according to the published sequences, using the primers: 5′-(CAGCACACAATCCTGGAGGAG) and 3′-(GCTGAAGCTTGACACCTCAT) with cDNAs of MB1.8 cells as template (5, 27).

Attachment of pOCs to ECM. Polystyrene dishes (Becton Dickinson, Lincoln Park, NJ) were coated at 4°C for 15 h with either FBS or 25 μg/ml human fibronectin (NY Blood Center, New York) or 10 μg/ml human vitronectin or 50 μg/ml recombinant osteopontin or 25 μg/ml mouse laminin (GIBCO BRL) or 25 μg/ml collagen type I or IV (Collaborative Biomed, Bedford, MA). Before cell attachment, pOCs (3×10^5 cells/ml) were washed (2×) with serum-free α-MEM medium and 0.1% BSA and then either kept in suspension or allowed to attach to ECM-coated plates for the indicated times at 37°C. Cells were solubilized in modified RIPA buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.2% sodium deoxycholate, 1 mM EDTA, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin) on ice for 20 min and prepared for immunoprecipitation.

Integrin clustering Antibody-induced integrin clustering was performed as reported (28). Cell suspensions (3×10^5 cells/ml) were incubated with anti-β_1, anti-β_2 or anti-β_3 antibodies at 25 μg/ml, 4°C for 1 h. Cells were washed with ice-cold serum-free medium (2×) with 100 mM vanadate and resuspended in medium containing 50 μg/ml of goat F(ab)′2 anti–rat IgG or anti–hamster IgG, followed by incubation at 37°C for 30 min. Cells were lysed in modified RIPA buffer and subjected to immunoprecipitation and -blotting as described below.

Translocation into the cytoskeletal fraction. Translocation of PYK2 into the cytoskeletal fraction was measured as described (29). pOCs were cultured with or without MB1.8 cells at equal cell density (3.0×10^5 cells) on plastic tissue culture plates or bone for 2 h at 37°C. Cells were washed gently with ice-cold PBS with 1 mM vanadate, then extracted in 0.5% Triton X-100, 10 mM Tris-HCl, pH 7.4, including phosphatase and protease inhibitors, for 5 min on ice. The soluble fractions were collected, and the insoluble fractions were then solubilized in RIPA buffer. Both were immunoblotted using anti-PYK2 antibodies. The levels of PYK2 were quantitated using an Imaging Densitometer (Model GS-700; BioRad, Hercules, CA) and expressed as percentage of total PYK2 protein in both fractions combined.

Immunoprecipitation. Cell lysates in RIPA buffer were immunoprecipitated using anti-PYK2 antibodies (2 μg) for 2 h, followed by protein G-sepharose (Pharmacia) for 1 h at 4°C. After washing with RIPA (4×), proteins were separated on SDS-PAGE (12% gels; Novex, San Diego, CA) and blotted onto Immobilon-P transfer membranes (Millipore, Bedford, MA). Phosphotyrosine and PYK2 protein were estimated by immunoblotting either with HRP-conjugated antiphosphotyrosine mAb 4G10 or with anti-PYK2 antibodies, followed by HRP-anti–rabbit IgG. Blots were developed by enhanced chemiluminescence (ECL; Amersham) at different exposure times for quantitation. Imaging densitometry was used to estimate the specific activity of tyrosine phosphorylated PYK2, which was quantitated as the ratio of phosphorylated PYK2 to its protein content, and expressed relative to controls (cells in suspension).

In vitro protein association assays. OCL lysates (1 mg/ml) were in TNE buffer and incubated with 10 μg of GST-fusion protein coupled with glutathione-sepharose beads for 2 h at 4°C. The beads were washed with lysis buffer (4×) and with PBS (1×), then subjected to immunoblot analysis using anti-PYK2 antibodies. For coimmunoprecipitation, pOCs (2.5×10^6 cells) were solubilized in TNE lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 10% glycerol, with phosphatase and protease inhibitors). Clarified cell lysates were incubated with anti-PYK2 N-terminal domain or anti-Src antibodies and sequentially blotted with anti-Src and anti-PYK2 antibodies, as described above.

Kinase assay. pOCs were plated on plastic or bone surfaces with or without MB1.8 cells (3×10^5 cells/ml). Cells were washed in PBS and solubilized in TNE buffer at the indicated times. In assays using inhibitors, cocultures were plated on bone slices for 4 h, then treated with cytochalasin D (5 μM) or calcitonin (100 nM) for 20 min or with echistatin (1 μM) for 1 h. PYK2 was immunoprecipitated from the clarified lysates. Half of the immunoprecipitates were subjected to blotting with antiphosphotyrosine or anti-PYK2 antibodies, and the other half was washed with kinase assay buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM $MnCl_2$ and 1 mM dithiothreitol), followed by the addition of 5 μCi [γ-^{32}P] ATP (3,000 Ci/mmol, Amersham) and 1 μM cold ATP, incubated for 10 min at 30°C. The reaction was subjected to SDS-PAGE and autoradiography. Specific activity of ^{32}P-labeled PYK2 was quantitated as described above.

Phosphorylation of PYK2 by the recombinant c-Src was performed as described by the manufacturer (Upstate Biotech). Lysates of Src (+/?) and Src (−/−) OCLs (800 μg/ml) were immunoprecipitated with anti–N-PYK2 antibodies and washed with RIPA buffer (5×) and kinase buffer (1×). Each precipitate was equally divided and incubated with [γ-^{32}P] ATP (1 μCi/μl) with and without recombinant c-Src (20 U per sample) for 10 min at 30°C.

Immunofluorescence staining and confocal microscopy. Immunofluorescent labeling of podosomes and sealing zone structures in OCLs was performed as described (17). PYK2 was visualized using anti-PYK2 antibodies, followed by TRITC anti–rabbit IgG. Actin was stained with 500 mU/ml FITC-phalloidin (Molecular Probes, Inc., Eugene, OR). Cells on glass coverslips were photographed using a Zeiss Axiophot epifluorescence microscope, and cells on bone slices were viewed with a confocal laser scanning microscope consisting of a Wild Aristophan fluorescent microscope and Leica Lasertechnik GmbH 1.05 software (Heidelberg, Germany) (19).

Immunoperoxidase staining of tissue sections. Tibiae were dissected from Sprague Dawley female rats (6 mo old) and fixed in 4% paraformaldehyde (in PBS) for 24 h, then demineralized in 20% EDTA, 10% formalin for 6 wk, and embedded in paraffin. Deparaffinized sections (10 μm) were permeabilized with 0.1% Triton X-100, PBS, and incubated with 2% BSA and then with 0.5% hydrogen peroxide (30 min each at 25°C). The slides were washed in 0.1% Triton X-100, PBS (3×, 10 min each). This was repeated after each subsequent step. Sections were incubated with anti-PYK2 polyclonal antibodies (10 μg/ml), incubated with HRP-conjugated second antibody, and developed with diaminobenzidine. All sections were counterstained with methyl green.

Results

Expression of PYK2 in osteoclasts in vitro and in vivo. PYK2 expression was not detected in MB1.8 cells but was readily detected in the coculture with bone marrow, even in the absence of D_3 treatment, probably due to the presence of PYK2 in hemopoietic cells (Fig. 1 *A*). With D_3 treatment, PYK2 expression increased approximately twofold and was further enriched (~ 16-fold) in the purified OCL fraction (Fig. 1 *A*). In contrast, the osteoblastic MB1.8 cells appear to be the major source of FAK in the coculture. In OCLs, FAK expression was barely detected. These findings suggest that in the coculture system PYK2 expression is abundant in osteoclasts.

PYK2 protein expression examined by Western blot analysis (Fig. 1 *B*) was not detected in MB1.8 cells. Similar levels of PYK2 were found in purified pOCs, cultured with or without MB1.8 cells, indicating that PYK2 is primarily derived from OCLs in this system. The size of PYK2 is ~ 110 kD, but several lower molecular weight bands (75 kD, 80 kD, and 97 kD) were seen, possibly due to proteolytic degradation. Similar calpain-dependent degradation products of PYK2 were reported in platelets stimulated with thrombin (10).

Abundant expression of PYK2 was also observed in vivo in osteoclasts attached to bone using immunohistochemistry (Fig. 2). PYK2 was also detected in megakaryocytes and small cells in the bone marrow, which could be lymphocytes or platelets (Fig. 2). PYK2 was not detected in other cells, including osteoblasts. In control sections, when the primary antibody was omitted, no staining was observed (data not shown).

Ligation of β_3 integrin increases PYK2 tyrosine phosphorylation in prefusion osteoclast-like cells. Since mature osteoclasts are isolated based on tight adherence to tissue culture plates and cannot be detached undamaged by conventional methods, pOCs provide the opportunity to examine the effect of adhesion on PYK2 tyrosine phosphorylation. These cells are isolated in high purity (85–95%) and express the cellular markers found in multinucleated OCLs, such as tartrate resistant acid phosphatase, calcitonin receptors, cathepsin K, and most importantly $\alpha_v\beta_3$ integrin (25). When pOCs maintained in suspension are allowed to attach for 1 h to plates coated either with serum or with various ECM proteins, there is a marked increase in PYK2 tyrosine phosphorylation (Fig. 3 *A*). Largest effects were seen with vitronectin or osteopontin, less with serum, fibronectin, or denatured type I collagen but not type IV collagen or laminin. Phosphorylation increased during 1 h following plating on vitronectin (Fig. 3 *B*), a time course that parallels OCLs spreading and formation of actin-rich adhesion structures on bone surfaces (25, 30).

The vitronectin receptor $\alpha_v\beta_3$ is the most abundant integrin in osteoclasts (31), while the fibronectin receptor $\alpha_v\beta_1$ and the laminin/collagen receptor $\alpha_2\beta_1$ are present at a lower level (32, 33). The role of these integrins in adhesion-induced tyrosine phosphorylation of PYK2 was therefore examined. pOCs were incubated with either anti-β_1, anti-β_2, or anti-β_3 monoclonal antibodies followed by secondary antibodies, to enhance clustering. Ligation of the β_3 integrin increases PYK2 tyrosine phosphorylation, while ligation of the β_1 or β_2 integrins does not (Fig. 3 *C*).

c-Src binds to PYK2 in osteoclast-like cells via the SH2 domain. Since integrin-mediated tyrosine phosphorylation of FAK recruits c-Src (34, 35) and c-Src plays a role in the organization of the osteoclast cytoskeleton (24), we examined a possible role for c-Src in PYK2 phosphorylation in osteoclasts. In lysates prepared from adherent OCLs, PYK2 is constitutively

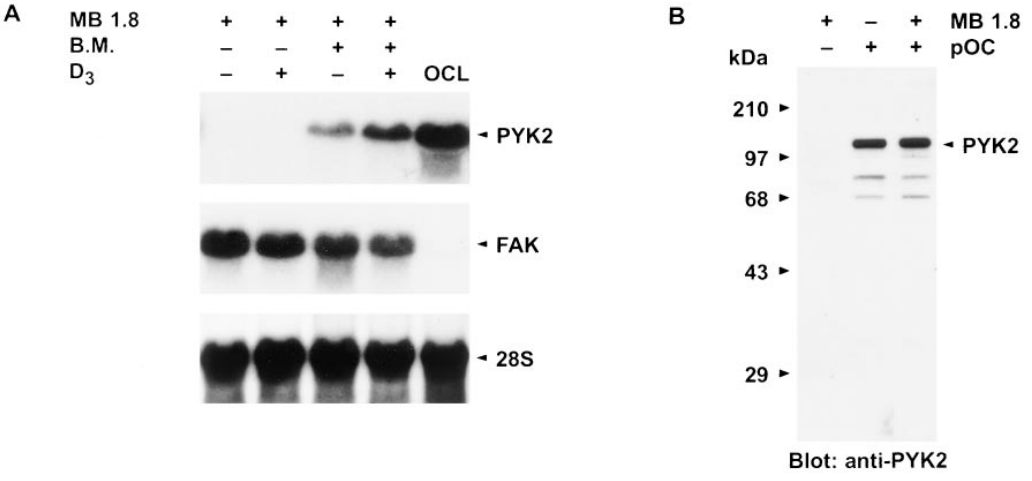

Figure 1. Specific expression of PYK2 in osteoclast-like cells in the coculture system. (*A*) Bone marrow cells (*B.M.*) were cocultured with MB 1.8 cells in the absence or presence of $1\alpha,25(OH)_2D_3$ (D_3). Total RNA (20 μg per lane) from OCLs and MB1.8 cells was subjected to Northern blot analysis and first hybridized with cDNA probe for PYK2 and then with probe for FAK. The same filter was rehybridized with a probe for 28S ribosomal RNA. (*B*) Equal amounts of protein (20 μg per lane) isolated from pOCs and MB1.8 cells either alone or in combination were separated on SDS-PAGE, immunoblotted with rabbit anti-PYK2 antibodies, and developed by ECL.

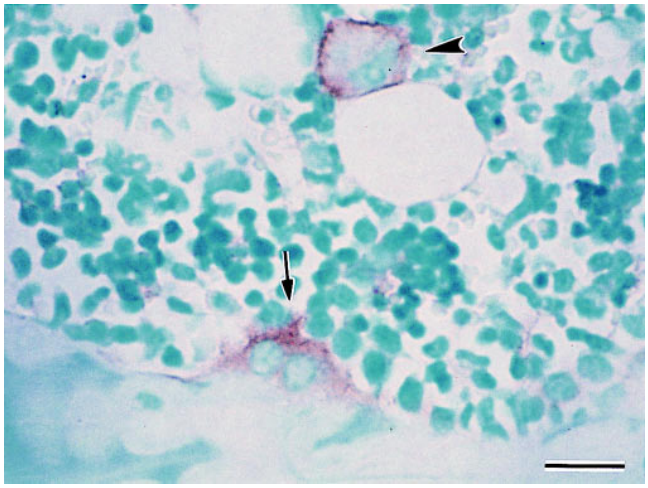

Figure 2. Expression of PYK2 in osteoclasts in vivo. Tissue sections from rat tibiae were incubated with anti-PYK2 antibodies (10 μg/ml) followed by HRP-conjugated anti–rabbit antibodies and developed with diaminobenzidine. Sections were counterstained with methyl green. Expression of PYK2 is detected at high level in multinucleated osteoclasts (*arrow*) along the bone surfaces and in megakaryocytes (*arrowhead*) in bone marrow. No significant PYK2 expression was found in other cells. Bar, 25 μm.

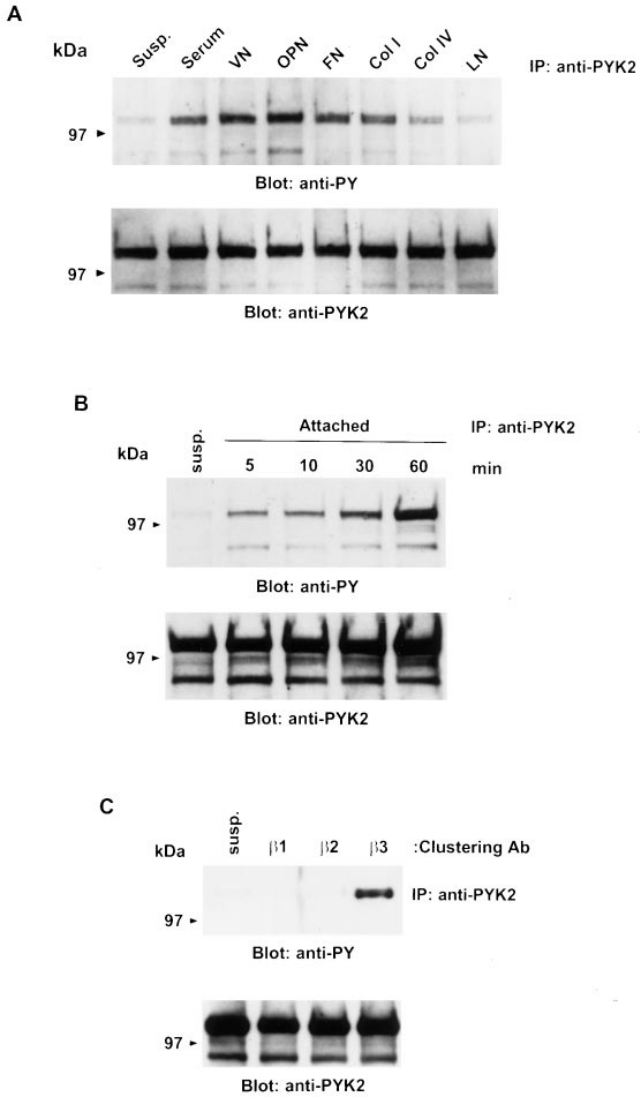

Figure 3. Cell adhesion or clustering of β_3 integrin induces tyrosine phosphorylation of PYK2 in prefusion osteoclast-like cells. (*A*) In the absence of serum, prefusion OCLs (pOCs, 3×10^5 cells/ml) were either kept in suspension (*Susp.*) or allowed to adhere for 1 h to dishes coated with either serum, vitronectin (*VN*, 10 μg/ml), osteopontin (*OPN*, 50 μg/ml), fibronectin (*FN*, 25 μg/ml), collagen type I (*Col I*, 25 μg/ml), collagen type IV (*Col IV*, 25 μg/ml), or laminin (*LN*, 25 μg/ml). (*B*) pOCs were allowed to attach to dishes coated with VN for the indicated times at 37°C. (*C*) pOCs were in suspension (*susp.*) or incubated with anti-β_1, anti-β_2, or anti-β_3 integrin at 25 μg/ml for 1 h at 4°C, followed by secondary antibodies (50 μg/ml) for 30 min at 37°C. Cell lysates were immunoprecipitated and blotted as described in Methods.

tyrosine phosphorylated (see Fig. 5 *B*) and coimmunoprecipitates with c-Src, using either antibodies against the N-terminal domain of PYK2 or anti–c-Src (Fig. 4 *A*). In addition, using GST-fusion proteins, we found that PYK2 binds to Src-SH2 domain but not Src-SH3 (Fig. 4 *B*). Interestingly, PYK2 isolated from adherent OCLs did not bind to the SH2 domains of other Src family kinases (Fyn and Lyn) or of PI3 kinase, suggesting selective interaction of PYK2 with the Src-SH2 domain.

Tyrosine phosphorylation and kinase activity of PYK2 are reduced in Src (−/−) OCLs, and PYK2 is a substrate of exogeneous c-Src. Since in Src (−/−) mice, osteoclast function is severely compromised, we examined the state of tyrosine phosphorylation and activation of PYK2 in OCLs from Src (−/−) mice. Tyrosine phosphorylation of several proteins ~ 100–120 kD and 50–60 kD is markedly reduced in Src (−/−) OCLs compared with Src (+/?) (Fig. 5 *A*). In the same blots, PYK2 levels in Src (−/−) OCLs were comparable with those in Src (+/?). The absence of c-Src in Src (−/−) OCLs is also documented (Fig. 5, *A* and *C*). Most importantly, the level of tyrosine phosphorylation of PYK2 in Src (−/−) OCLs is markedly reduced (Fig. 5 *B*) compared with OCLs from normal littermates. Similarly, the level of ^{32}P-phosphorylation associated with PYK2 immunoprecipitated from Src (−/−) OCLs is also significantly reduced (Fig. 5 *C*). In the same preparation, the level of tyrosine phosphorylation of paxillin (Fig. 5 *B*), an adhesion-dependent cytoskeletal protein, was not altered.

Furthermore, PYK2 is directly phosphorylated by exogeneous c-Src (Fig. 5 *D*). To reduce the level of associated endogeneous proteins, PYK2 immunoprecipitated from OCL cell lysates, were extensively washed with RIPA buffer before addition of exogeneous c-Src. In PYK2 immunoprecipitated from Src (+/?) OCLs, there is some ^{32}P-incorporation possibly due to autophosphorylation or to a small amount of associated endogeneous kinases. There is no detectable ^{32}P-incorporated into PYK2 immunoprecipitated from Src (−/−) OCLs. However, in the presence of exogeneous Src, PYK2 is highly phosphorylated, whether derived from control cells or Src (−/−) OCLs, indicating that PYK2 can be a direct substrate of c-Src in OCLs.

Adhesion-induced translocation of PYK2 into the cytoskeletal fraction. Since the sealing zone formed upon osteoclast adhesion is very rich in actin filaments, we examined the partition of PYK2 between the cytoskeletal (Triton insoluble) and cytoplasmic (Triton soluble) compartments in pOCs adhering

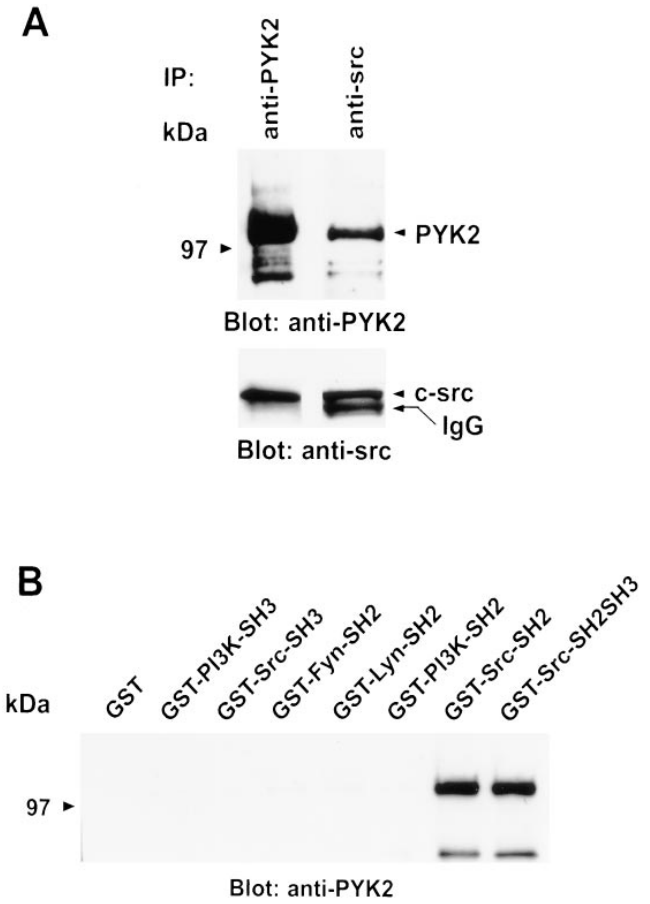

Figure 4. c-Src binds to PYK2 via the SH2 domain. (*A*) Co-immunoprecipitation of PYK2 and c-Src from OCLs. Lysates (1 mg/ml) prepared from purified OCLs were immunoprecipitated either with anti–N-PYK2 (2 μg/ml) or with anti-Src antibodies (2 μg/ml). Precipitates were washed and separated on 12% SDS-PAGE and blotted with either anti-PYK2 or anti-Src antibodies. ECL film development for the coprecipitated proteins was 10 min and for the immuno-precipitated proteins was 0.5–1 min. (*B*) OCL lysates (1 mg/ml) were incubated with 10 μg of GST or of GST fusion proteins containing SH2 and SH3 domains of the indicated kinases, for 3 h at 4°C, washed and separated on SDS-PAGE, and then blotted with anti-PYK2 antibodies.

to plastic or bone surfaces in the absence or presence of MB1.8 cells. In the absence of MB1.8 cells, PYK2 was in the soluble (noncytoskeletal) fraction and attachment of pOCs did not cause PYK2 translocation into the Triton-insoluble fraction (Fig. 6, *top*). In suspended pOCs in the presence of MB1.8 cells, a small portion of PYK2 (10%) was found in the cytoskeletal Triton-insoluble fraction (Fig. 6, lane *2*). However, on plastic or bone surfaces, in the presence of MB1.8 cells about a third of PYK2 redistributed to the Triton-insoluble fraction, 24% (lane *4*) and 34% (lane *6*) of total PYK2, respectively (Fig. 6, *bottom*). Tyrosine phosphorylation of PYK2 in the Triton-soluble and insoluble fractions were not significantly different, as determined by Western blotting with antiphosphotyrosine antibodies (data not shown). In vitro kinase assays of PYK2 could not be performed in the Triton-insoluble fractions because of the presence of SDS in the extraction buffer.

Adhesion-dependent activation of PYK2 kinase in OCLs is enhanced by the osteoblastic MB1.8 cells. We and others reported that osteoblastic cells support the fusion of pOCs and are necessary for the resorption of mineralized matrix by OCLs (25). As shown in Fig. 7 *A*, MB1.8 cells had no significant effect on PYK2 tyrosine phosphorylation in pOCs seeded either on plastic (relative specific activities 3.9 with MB1.8 cells vs. 4.1 without) or bone (4.3 vs. 3.2). However, in vitro kinase activities measured by ^{32}P-incorporation into PYK2 (immunoprecipitated in the presence of orthovanadate), were approximately twofold higher in pOCs plated with MB1.8 cells (Fig. 7 *B*), suggesting that the interaction of pOCs with MB1.8 cells, while not detectably affecting the level of tyrosine phosphorylation in PYK2, might increase its kinase activity. The kinase activities measured under these conditions include PYK2 autophosphorylation as well as the activity of other kinases coimmunoprecipitated with PYK2.

Activation of PYK2 kinase parallels the formation of the sealing zone and bone resorption activity. Findings reported above suggest the involvement of PYK2 in the cytoskeletal reorganization associated with OCL fusion and bone resorption. We therefore examined kinase activity in PYK2 immunoprecipitated from lysates of pOCs cocultured with MB1.8 cells plated on bone and actively resorbing bone for 1, 2, 4, 8 or 21 h (Fig. 8 *A*). Resorption pits were counted in the same bone slices (Fig. 8 *B*). In parallel cocultures stained with phalloidin, F-actin containing sealing zone structures were also counted (Fig. 8 *B*). As shown, there was a sustained increase in the specific activity of in vitro kinase activity associated with the PYK2 immunocomplexes (Fig. 8 *A*) that paralleled the formation of sealing zones and of resorption pits (Fig. 8 *B*). In addition, a number of phosphorylated proteins were coimmunoprecipitated with PYK2, including detectable bands of ~ 250 kD, 68–70 kD, and 40–50 kD. The total level of PYK2 protein was significantly reduced at 21 h, along with reduction in total protein in cell lysates at that time. The same observation was made in three repeated experiments. Although the reason for this apparent reduction in extracted protein is not known, it may be due to degradation of PYK2 in activated osteoclasts, as observed in thrombin-activated platelets (10) or tighter association of cytoskeletal proteins with the bone surface and reduced extractibility under the mild solubilization conditions required for the immunoprecipitation and in vitro kinase assays.

Treatment with echistatin, cytochalasin D, or calcitonin inhibits PYK2 kinase activity. The above observations suggested a relationship between PYK2 kinase activity and the organization of the osteoclastic cytoskeleton. We therefore examined the effect of agents known to disrupt osteoclastic cytoskeletal organization, such as cytochalasin D, echistatin, or calcitonin on PYK2 activity in OCLs (36, 37). pOCs were cocultured with MB1.8 cells on bone slices for 4 h, the time required for the formation of actin ring structures in OCLs on glass coverslips and the formation of sealing zones on bone (Fig. 8 *B*). Subsequent treatment of the cocultures with cytochalasin D for 20 min inhibited PYK2 kinase activity by 61%, suggesting that cytoskeletal association played a role in kinase activity. Treatment with inhibitors of bone resorption, echistatin for 1 h or calcitonin for 20 min, resulted in 38 and 27% inhibition in PYK2 kinase activity, respectively (Fig. 8 *C*).

Localization of PYK2 in the actin-rich ring-like adhesion structures in osteoclast-like cells. To complement above biochemical findings, we examined microscopically the presence of PYK2 in the actin rings of multinucleated OCLs, the formation of which is promoted in pOCs by coculture with D_3-treated MB1.8 cells. In pOCs plated on glass coverslips for 1 h

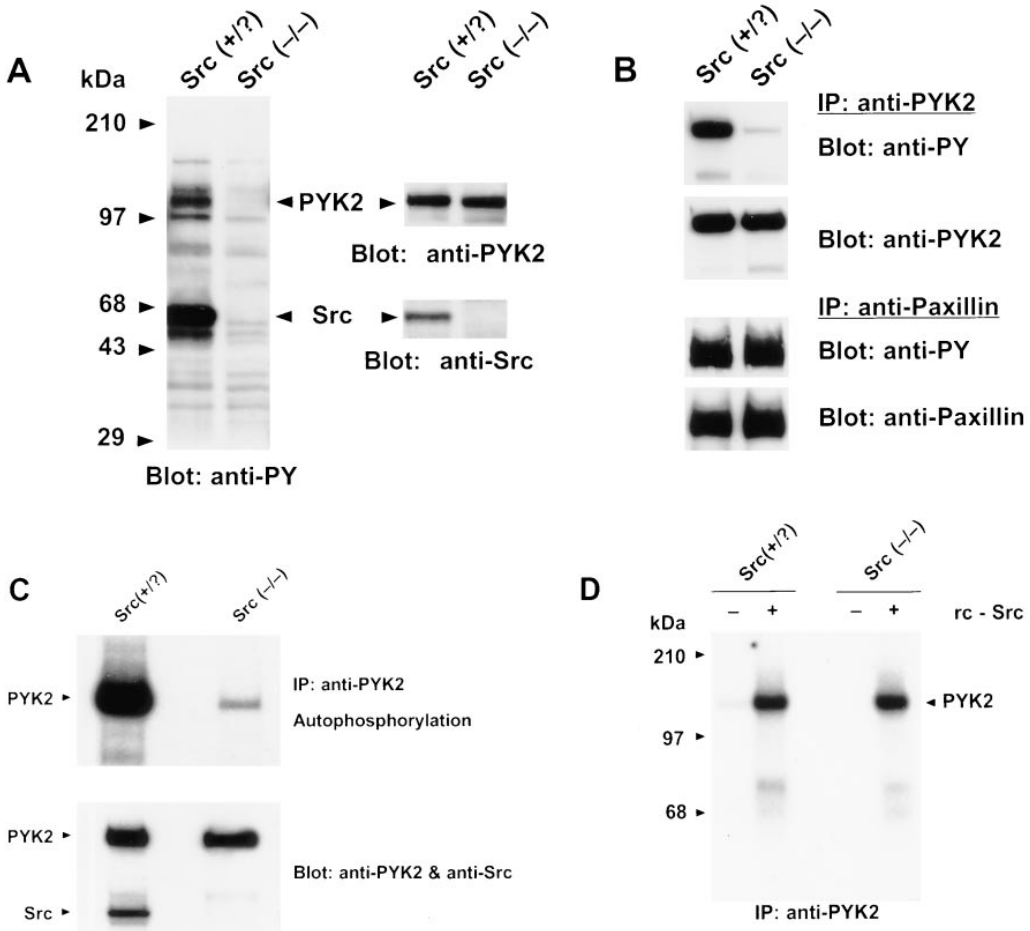

Figure 5. Tyrosine phosphorylation and kinase activity of PYK2 in Src (−/−) osteoclast-like cells. (*A*) Src (+/?) and Src (−/−) OCL lysates (20 μg/lane) were subjected to immunoblotting with HRP-conjugated anti-phosphotyrosine (anti-PY), then reblotted with anti-PYK2 and anti-Src, followed by HRP-conjugated secondary antibodies. (*B*) The same lysates were then immunoprecipitated with anti-PYK2, followed by immunoblotting with anti-PY and anti-PYK2 antibodies. Paxillin was used as control. (*C*) From Src (+/?) and Src (−/−) OCL lysates, immunoprecipitated PYK2 was subjected to in vitro kinase assay (*top*), and the levels of PYK2 and Src (*bottom*) were detected using Western blot analysis. (*D*) Immunoprecipitated PYK2 was assayed with or without c-Src (20 U) as described in Methods.

in the presence of MB1.8 cells, F-actin is localized in discrete dots or podosomes and in the forming rings of a spreading osteoclast (Fig. 9 *b*). This contrasts with the localization of F-actin in stress fibers, observed in the neighboring MB1.8 cells (in *green*, Fig. 9 *b*). PYK2 localization shown in red is similar to that of actin in podosomes and in the cell body (compare Fig. 9, *a* and *b*). Colocalization of the two proteins appears in yellow (Fig. 9 *c*). Note the complete absence of PYK2 in MB1.8 cells. After plating (4 h), pOCs fuse to form large, spread, multinucleated cells (OCLs) with F-actin organized in ring-like structures around the cellular periphery, shown in green (Fig. 9 *e*). In these cells, PYK2 also reorganizes and localizes in these rings (in *red*, Fig. 9 *d*). Perinuclear localization of PYK2 is also detected. Colocalization of PYK2 and F-actin is seen in yellow

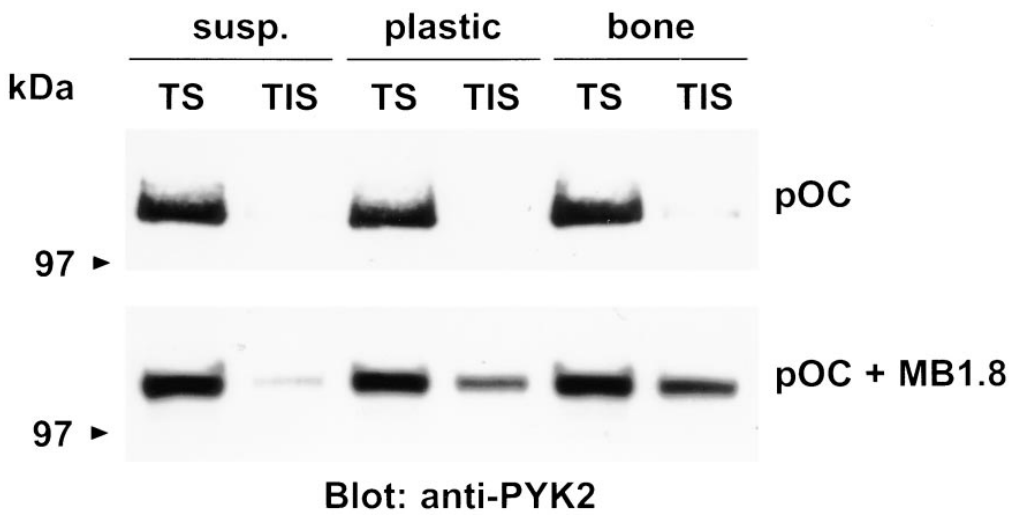

Figure 6. Adhesion-dependent translocation of PYK2 to the Triton-insoluble cytoskeletal fraction in the prefusion osteoclast-like cells. pOCs were incubated alone (*top*) or with D_3-pretreated MB1.8 cells (*bottom*) at 3×10^5 cells each. Cells were in suspension (*susp.*) or plated on plastic or bone slices for 2 h at 37°C, then fractionated as described in Methods. Triton-soluble (*TS*) and insoluble fractions (*TIS*) were blotted with anti-PYK2 antibodies. In cocultures of pOCs and MB1.8, PYK2 in the TIS fraction relative to the PYK2 protein in both fractions, was 10% in susp., 24% on plastic, and 34% on bone.

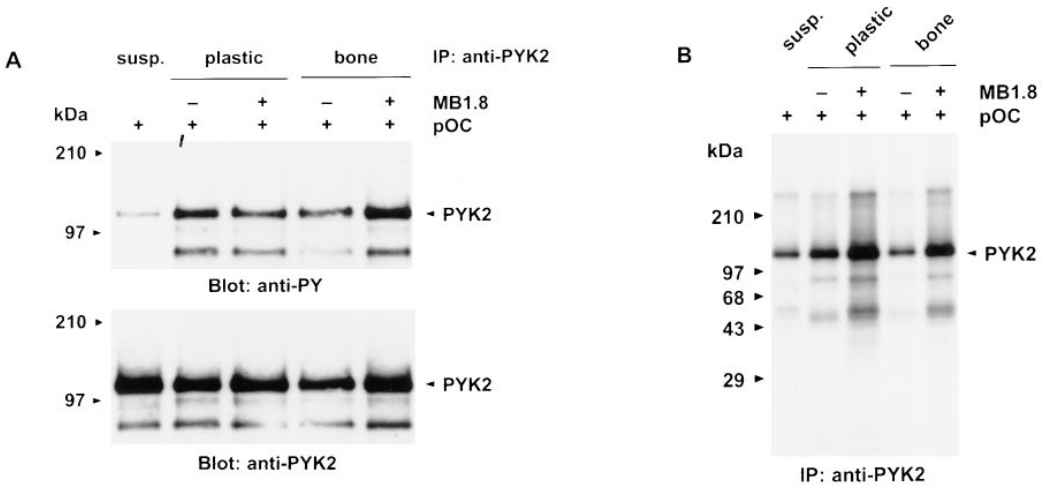

Figure 7. PYK2 kinase activity in osteoclast-like cells is enhanced by the osteoblastic MB 1.8 cells. pOCs were in suspension (*susp.*) or plated on plastic or bone without or with D_3-pretreated MB1.8 cells (3×10^5 cells each) for 1 h at 37°C. Cells were solubilized in TNE lysis buffer as described in Methods, followed by immunoprecipitation with anti-PYK2 antibodies (2 μg/ml). (*A*) Half of PYK2 immunoprecipitates were blotted with antiphosphotyrosine (*anti-PY*), then with anti-PYK2 antibodies. (*B*) Half of PYK2 immunoprecipitates were subjected to in vitro kinase assay to determine total [^{32}P] incorporation into PYK2 immunocomplexes. Specific activity of PY or [^{32}P] incorporated into PYK2 were calculated as described in Methods. In *A*, the fold increase of PY in PYK2 when pOCs were plated on plastic alone or with MB 1.8 cells was (4.1 and 3.9) and on bone (3.2 and 4.3), respectively. In *B*, the fold increase in [^{32}P]-PYK2 when pOCs were plated alone or with MB1.8 cells on plastic was (2.3 and 3.9) and on bone (1.8 and 3.8), respectively.

in the overlapping image (Fig. 9 *f*). In these OCL ring structures, PYK2 also colocalizes with the cytoskeletal proteins vinculin and paxillin (data not shown).

PYK2 localization in the sealing zone of osteoclast-like cells on bone surfaces. The actin-rich ring-like structures formed in OCLs in vitro are thought to be related to the tight sealing zones formed by osteoclasts on bone matrix during polarization before bone resorption. In resorbing OCLs on bone (Fig. 10), PYK2 (in *red*) was concentrated in the sealing zone and colocalized with F-actin (in *green*) in the ring-like structure and possibly in the ruffled border within the ring (Fig. 10, *a–c*). PYK2 was found in the double circle structure of the mature sealing zone, which colocalizes with vinculin and paxillin (data not shown). In addition, confocal microscopic optical sections

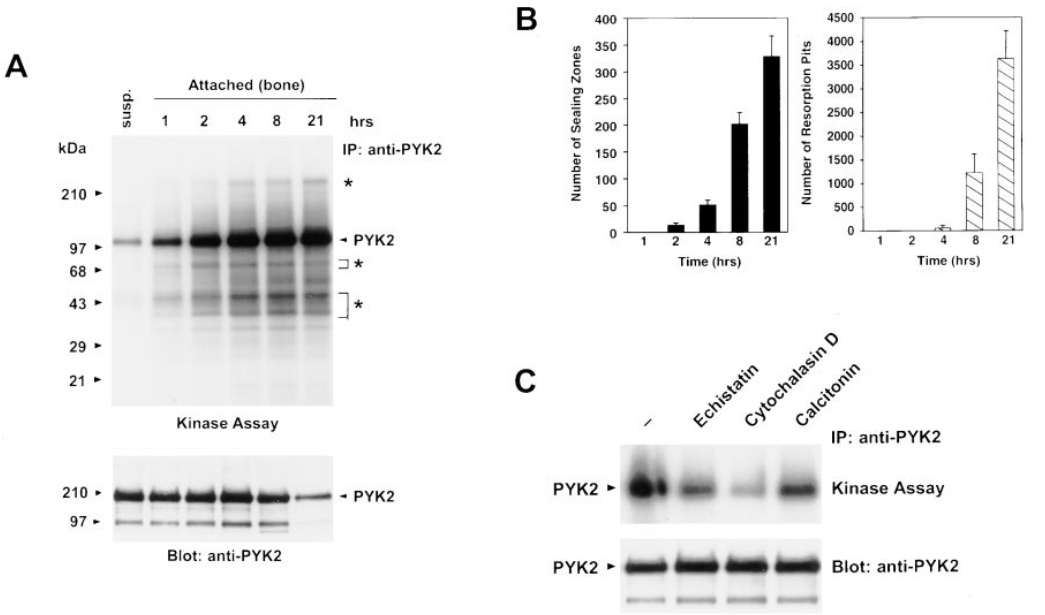

Figure 8. PYK2 kinase activity correlates with actin ring formation and bone resorption; treatment with echistatin, cytochalasin D or calcitonin inhibits PYK2 kinase activity in osteoclast-like cells. pOCs and D_3-pretreated MB1.8 cells (3×10^5 cells each) were coincubated in suspension (*susp.*) for 1 h or plated on bone slices for 1, 2, 4, 8, and 21 h at 37°C. At the indicated times, cells were solubilized in TNE lysis buffer and analyzed as followed: (*A*) [^{32}P] incorporation into PYK2 and associated proteins during the in vitro kinase assay. Note that the number of detected phosphorylated proteins in the PYK2 immunocomplexes (asterisk) increased with time. (*B*) Parallel cocultures of pOCs and MB 1.8 cells plated on bone slices were fixed and stained with phalloidin at the indicated time points to quantitate the number of sealing zones and resorption pits (per bone slice). Data are means of triplicated samples, ±SD. Relative specific activity of [^{32}P] incorporated into PYK2 was quantitated as described in Methods. Fold increases in [^{32}P]-PYK2 were 4.9, 5.9, 6.3, 8.4, and 19.3 at 1, 2, 4, 8, and 21 h, respectively. (*C*) pOCs with D_3-pretreated MB1.8 cells were plated on bone slices for 4 h at 37°C, followed by treatment without (control) or with either echistatin (1 μM) for 1 h, cytochalasin D (5 μM), or calcitonin (100 nM) for 20 min. Immunoprecipitated PYK2 was subjected to kinase assay as described above. Percent reduction of PYK2 phosphorylation determined in cocultures treated with echistatin was 38% with cytochalasin D 61% and with calcitonin 27%, relative to untreated control.

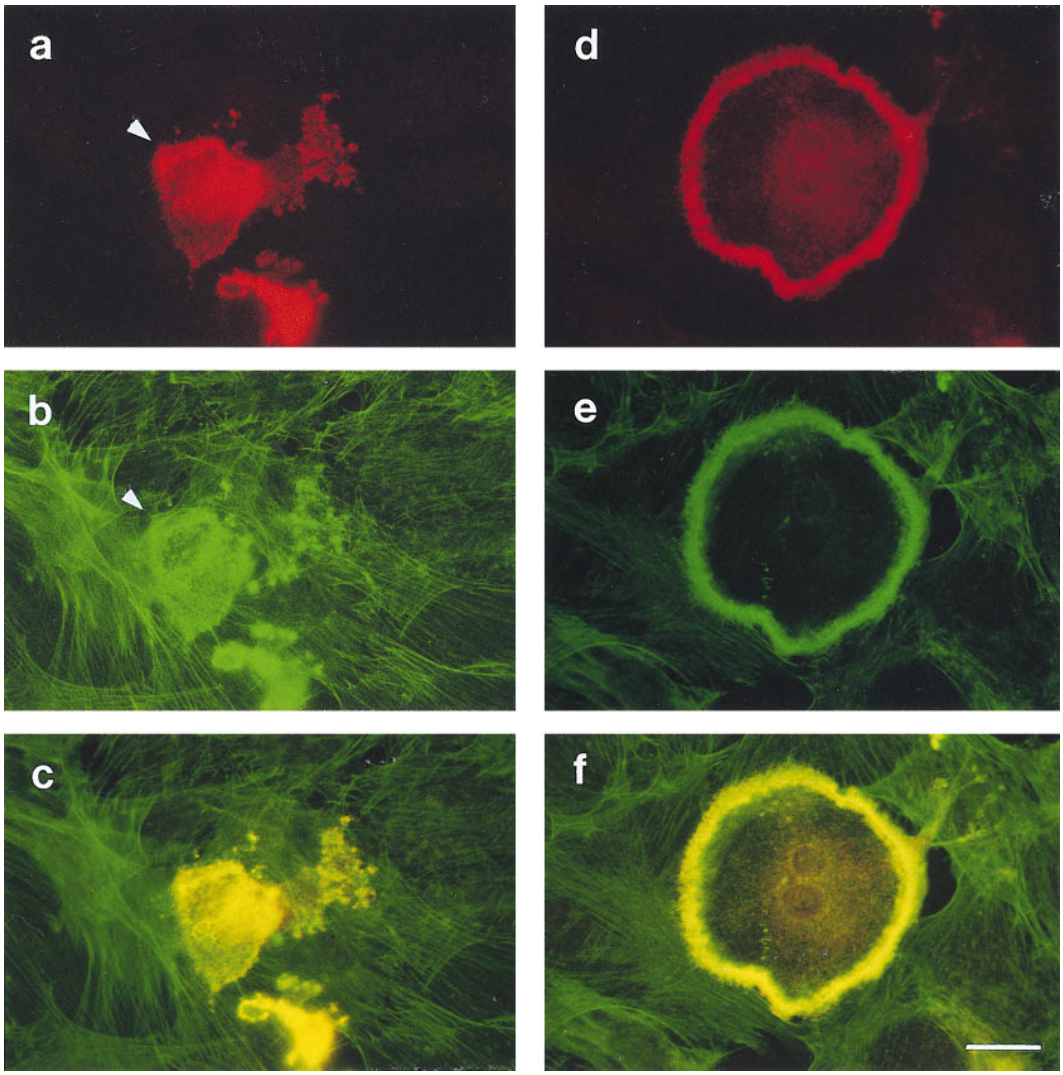

Figure 9. Localization of PYK2 in the ring-like adhesion structures in osteoclast-like cells. pOCs were cocultured with D_3-pretreated MB1.8 cells on glass coverslips, then fixed at 1 h (*a–c*) or 4 h (*d–f*) and immunostained with anti-PYK2 antibodies, followed with TRITC-conjugated anti–rabbit IgG (*red*) and FITC- phalloidin (*green*). PYK2 localizes to podosomes (*arrowheads*) and ring-like adhesion structures in OCLs (*red*, *a* and *d*). F-actin localizes to the same adhesion structures in OCLs and in stress fibers in MB1.8 cells (*green*, *b* and *e*). Colocalization of PYK2 and F-actin is seen in yellow (*c* and *f*). Bar, 10 μm.

through the sealing zone showed PYK2 staining in the whole sealing zone and also distributed in the cytoplasm where colocalization with F-actin is not observed (*red*, Fig. 10, *d–f*).

In Src (−/−) OCLs, PYK2 is diffusely localized in the cytoplasm and focal-adhesion–like contacts. Since PYK2 was not tyrosine phosphorylated in Src (−/−) OCLs, we examined the localization of PYK2 in these cells. In contrast to the typical ring-like adhesion contacts found in OCLs derived from the normal mice (Fig. 11, *d–f*), podosomes and ring-like structures were completely absent in Src (−/−) OCLs (Fig. 11, *a–c*). Instead, there were small focal adhesion-like structures underneath the nucleus and around the cell periphery, visualized by F-actin localization shown in green (Fig. 11 *b*). In Src (−/−) OCLs, PYK2 in red was distributed throughout the cytoplasm and was also localized to these small focal adhesion contacts (Fig. 11 *a*). Overlapped localization of PYK2 and F-actin is shown in yellow (Fig. 11 *c*).

Discussion

The main findings of this study are that in osteoclasts, PYK2 is an adhesion kinase that is tyrosine phosphorylated in a c-Src–dependent manner upon ligand engagement or ligation of the $\alpha_v\beta_3$ integrin and is activated by osteoclast interaction with osteoblastic (MB1.8) cells. Furthermore, PYK2 localizes to the sealing zone of resorbing osteoclasts, along with F-actin, vinculin, and paxillin, suggesting that it plays a role in the attachment-dependent cytoskeletal reorganization required for osteoclast activity.

PYK2 is highly expressed in osteoclasts in vivo and osteoclast-like cells generated in cocultures of murine bone marrow and osteoblastic MB1.8 cells. The adhesion-dependent kinase FAK was previously reported to be present in osteoclasts (38). However, in this study, we found that FAK is highly expressed in osteoblastic MB1.8 cells and in primary calvaria osteoblasts (data not shown) but was only barely detectable in osteoclasts. In contrast, PYK2 appears to be the predominant adhesion-dependent kinase present in osteoclasts, as demonstrated by Northern analysis, Western blots, and immunostaining methods. With the same methods, expression of PYK2 was undetectable in the osteoblastic MB1.8 cells. We could therefore conclude that PYK2 phosphorylation and activation in the cocultures of MB1.8 cells and purified pOCs reflected PYK2 changes in osteoclasts.

Osteoclastic bone resorption is a multistep process that starts with matrix recognition, osteoclast attachment, polariza-

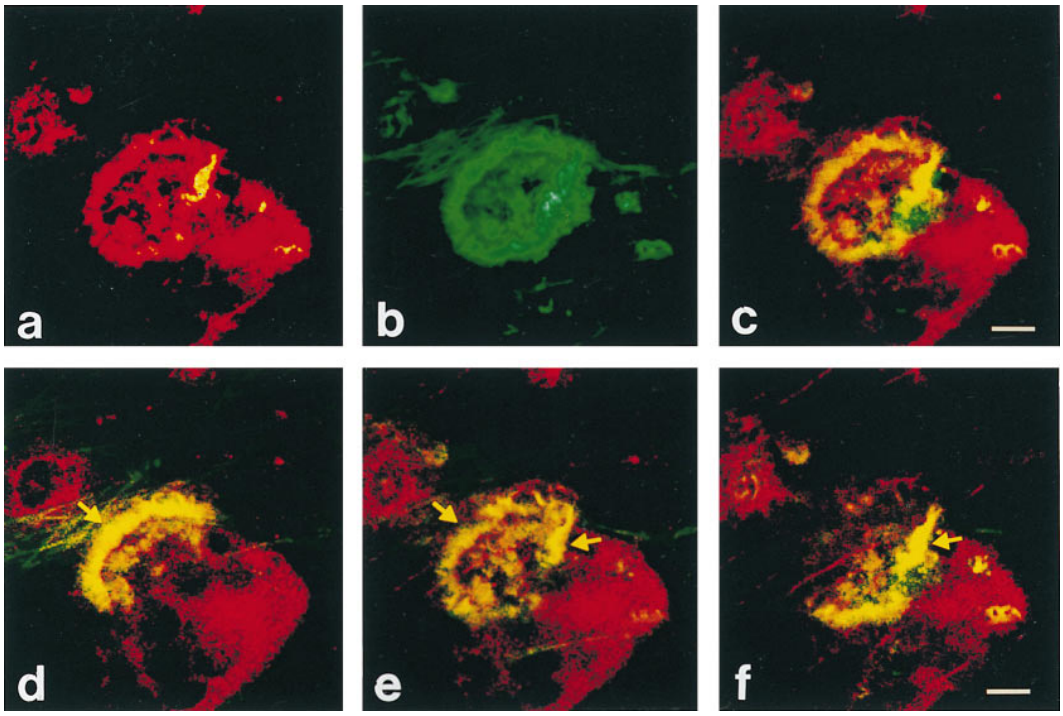

Figure 10. Pseudocolor confocal images of PYK2 colocalization with F-actin in resorbing osteoclast-like cells on bone. Co-localization of PYK2 (*a*, *red*) with F-actin (*b*, *green*) in the sealing zone, is shown in yellow (*c–f*). Extended images merged from 12 optical sections with 0.5-μm intervals close to the bone surface (5.5-μm thickness) (*a–c*). Optical sections with 1.5-μm intervals showing the colocalization through the sealing zone (*d–f*, *arrows*). Bars, 10 μm.

tion, and formation of the actin-rich sealing zone on the bone surface. Osteoclasts express at least three integrins: the vitronectin receptor $\alpha_v\beta_3$, the collagen receptor $\alpha_2\beta_1$, and the fibronectin receptor $\alpha_v\beta_1$ (39). $\alpha_v\beta_3$ ligands were shown to inhibit bone resorption, suggesting that its function is rate limiting for osteoclast activity (19, 37). The tyrosine phosphorylation of PYK2 in pOCs upon adhesion to known substrates of $\alpha_v\beta_3$ or the ligation of β_3 integrins suggests that PYK2 may play a role in the function of $\alpha_v\beta_3$ in osteoclasts. The increase in tyrosine phosphorylation of PYK2 in pOCs correlated with the spreading of OCLs and the formation of actin rings on substrate-coated surfaces, which normally required at least 1 h (25, 30). This is in contrast to the faster (20 min) tyrosine phosphorylation of PYK2 in macrophages (our unpublished observation) or of FAK in fibroblasts (27). Tyrosine phosphorylation of PYK2 also has been reported in megakaryocytes and T-lymphocytes upon attachment to fibronectin and upon ligation of β_1 and β_3 integrins, respectively (5, 40).

To further examine the possible role of PYK2 in osteoclast function, we used OCLs obtained from cocultures of MB1.8 cells and Src (−/−)–derived spleen cells. Targeted disruption of c-Src in mice causes osteopetrosis. These mice have a normal number of osteoclasts, which attach to bone, but lack ruffled borders (21, 22). Src (−/−) OCLs obtained in cocultures have focal adhesion–like contacts underneath the nucleus and sometimes around the periphery of the cell but do not display the normal adhesion contacts seen in the Src (+/?) OCLs. Even in the presence of normal primary osteoblasts or MB1.8 cells, Src (−/−) OCLs never organize the dot-like adhesion contacts into ring-like structures, which are thought to preceed the formation of sealing zones on bone surfaces (17). In normal adhering OCLs, c-Src is phosphorylated and stably associated with PYK2. In Src (−/−) OCLs, PYK2 tyrosine phosphorylation and its kinase activity was markedly reduced. In addition, PYK2 immunoprecipitated from Src (−/−) OCLs was shown to be a direct substrate of c-Src in vitro, suggesting that the adhesion-dependent tyrosine phosphorylation of PYK2 is mediated by c-Src in osteoclasts in vivo. Furthermore, in Src (−/−) OCLs, PYK2 does not localize to actin-rich adhesion structures, suggesting that Src-kinase activity and PYK2 phosphorylation may be needed for the recruitment of other molecules involved in the organization of the sealing zone in osteoclasts.

Other evidence that supports PYK2 interaction with Src is that overexpression of PYK2 in human embryonic kidney 293T cells stimulates the endogenous Src activity in these cells (10). In addition, PYK2 tyrosine phosphorylation depends, in part, on Src kinase activity and is stimulated by Src binding to the autophosphorylated site Tyr402 on PYK2 (7). Our observation that PYK2 binds GST-Src-SH2 domain is consistent with these reports. Notably, no binding to the SH2 domain in Fyn, Lyn, or PI3 kinase was seen. Interestingly, overexpression of activated Src (Y527F) in PC12 cells induced tyrosine phosphorylation of PYK2 but did not stimulate PYK2 kinase activity (7). Consistent with the findings reported here, PYK2 phosphorylation mediated by Src may generate docking sites for additional signaling proteins that are recruited by PYK2 (7).

Integrin stimulation in fibroblasts induces the activation and redistribution of c-Src to focal contact structures (41). Furthermore, enhanced FAK tyrosine phosphorylation and stable association between Src and FAK were detected in Src-transformed cells (42), whereas FAK tyrosine phosphorylation is reduced in fibroblasts isolated from Src (−/−) mice (43), pointing to several similarities between FAK and PYK2 in fibroblasts and osteoclasts, respectively. The protooncogene product c-Cbl was also found to be tyrosine phosphorylated in a Src-dependent manner in osteoclasts and was suggested to act downstream of c-Src in a signaling pathway that is required for bone resorption (24). The relationship between PYK2 and c-Cbl in osteoclasts requires further study.

Additional insights into the role of PYK2 in the cytoskeletal organization important for osteoclast function is provided

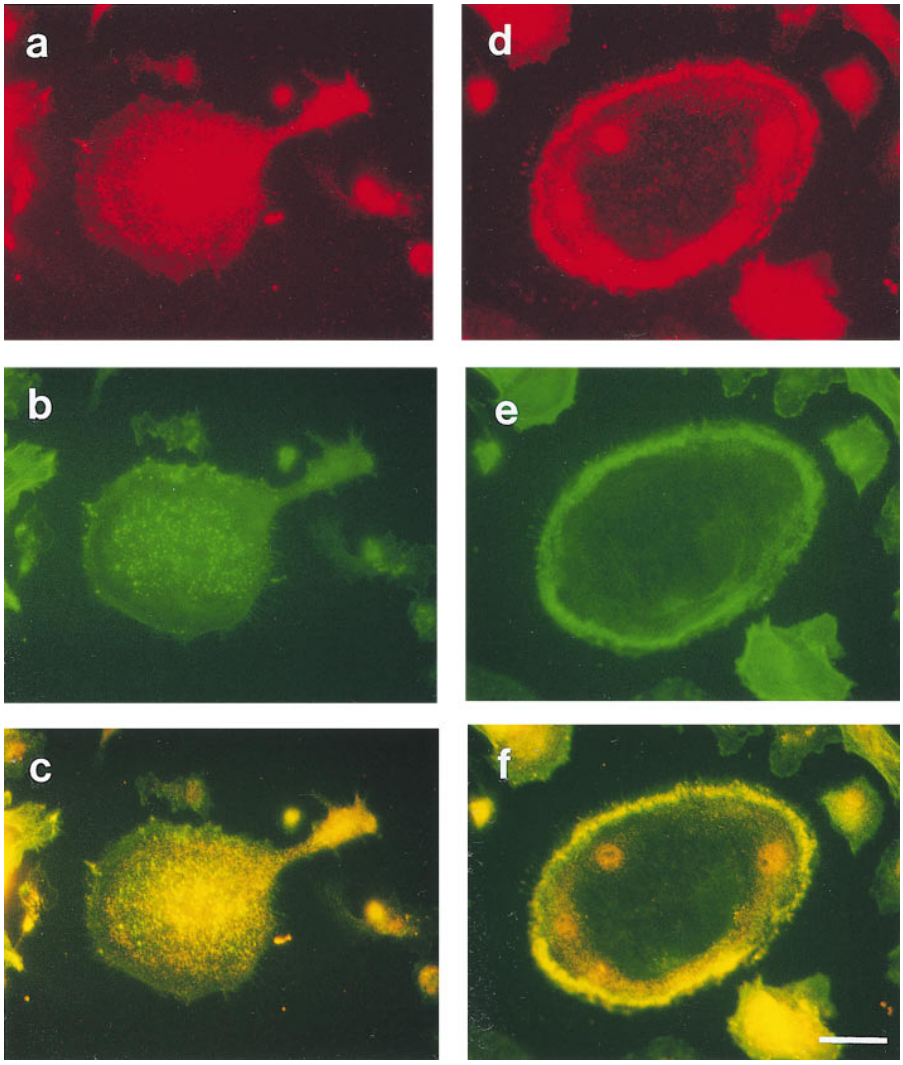

Figure 11. Localization of PYK2 and F-actin in adhesion structures of Src (−/−) osteoclast-like cells. Src (−/−) (*a–c*) and Src (+/?) OCLs (*d–f*) were plated on glass coverslips and coimmunostained with FITC-phalloidin and anti-PYK2 antibodies, followed by TRITC-anti–rabbit IgG. Localization of PYK2 is shown in red (*a* and *d*) and F-actin is shown in green (*b* and *e*) and their colocalization is shown in yellow (*c* and *f*). Bar, 10 μm.

by the effects of osteoblasts. The presence of osteoblasts promotes survival, fusion, and formation of multinucleated OCLs that resorb bone (25, 44). In this system, pOCs do not survive and do not resorb bone without vitamin D_3–treated MB1.8 cells. Indeed, on bone, the presence of these cells was necessary for the activation of OCLs including the formation of F-actin rings and of resorption pits (25). In addition, in the absence of MB1.8 cells, pOCs adhere via podosomes but cannot organize sealing zones on bone surfaces (data not shown). In this study, interaction with MB1.8 cells increased the PYK2 kinase activity, which rose in parallel with the formation of actin rings, associated with osteoclast activation, and with the excavation of resorption pits. In the presence of MB1.8 cells, podosomal adhesion structures in pOCs organize into sealing zones, suggesting a role for PYK2 in the dynamic organization of the actin-rich sealing zone structures. This is supported by the prominent localization of PYK2 in the actin ring of OCLs on glass and in the sealing zone of osteoclasts on bone. Furthermore, disruption of microfilament organization by treatment of OCLs with cytochalasin D rapidly reduced PYK2 kinase activity. We also examined the effects of echistatin and calcitonin, two known inhibitors of osteoclast function. Both, echistatin, a RGD-containing ligand of the $\alpha_v\beta_3$ integrin, and calcitonin, a physiological ligand of a G-protein–linked receptor (36, 37), reduced PYK2 kinase activity to some extent. These findings support a relationship between PYK2 activity, cytoskeletal organization, and osteoclast function.

During bone resorption, several other phosphorylated proteins in addition to c-Src could associate with PYK2 in OCLs. The C-terminal domain of PYK2 contains several functional sites that are conserved in FAK, including two different proline-rich binding regions for $p130^{cas}$ (crk associated substrate) and Graf (GTPase regulator associated with FAK), a binding region for paxillin and an overlapping sequence for focal targeting (45). An association of PYK2 and $p130^{cas}$ was demonstrated in B lymphocytes (13), and $p130^{cas}$ was reported to localize to the actin-rich ring-like structures in OCLs (46). In megakaryocytes, phorbol myristate acetate treatment resulted in tyrosine phosphorylation of PYK2 and induced the association of PYK2 and paxillin, a 68-kD cytoskeletal protein (15). In resorbing OCLs on bone, paxillin colocalizes with PYK2 in the sealing zone, suggesting possible interaction between these two proteins.

In conclusion, although many questions remain to be answered regarding the role of PYK2 in osteoclast activation, in vitro and in vivo evidence presented in this study strongly sug-

gests that PYK2 is a major cell adhesion–dependent kinase in osteoclasts. PYK2 localizes to the podosome-containing ring-like adhesion structures in OCLs on plastic and to the sealing zone on bone. In OCLs, PYK2 was found to associate tightly with Src via its SH2 domain. In addition, in OCLs from Src (−/−) mice, tyrosine phosphorylation and kinase activity of PYK2 was greatly reduced. Taken together these data suggest that ligand binding to $\alpha_v\beta_3$ on the osteoclast surface leads to Src-dependent tyrosine phosphorylation of PYK2. Furthermore, osteoblast-dependent effects enhance PYK2 association with the actin cytoskeleton and increase the kinase activity of PYK2 and its associated proteins, leading to the formation of the actin-rich sealing zone. There are certainly many molecules involved in this process, but PYK2 seems to play a major role as an adhesion kinase that participates in the initial events of osteoclast activation.

Acknowledgments

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