PYK2 Autophosphorylation, but Not Kinase Activity, Is Necessary for Adhesion-induced Association with c-Src, Osteoclast Spreading, and Bone Resorption*

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Proline-rich tyrosine kinase 2 (PYK2) is the main adhesion-induced kinase in bone-resorbing osteoclasts. Previous studies have shown that ligation of αvβ3 integrin in osteoclasts induces c-Src-dependent tyrosine phosphorylation and PYK2 activation, leading to cytoskeletal rearrangement, migration, and polarization of these cells. In this study, we examined the role of PYK2 kinase activity and its major autophosphorylation site in adhesion-dependent signaling and cytoskeletal organization during osteoclast spreading and migration. By infecting pre-fusion osteoclasts using recombinant adenovirus expressing PYK2 and its mutants, we demonstrated that mutation at the autophosphorylation site (Y402F) abolishes PYK2 association with c-Src and reduces significantly phosphorylation at tyrosines 579/580 and 881 resulting in inhibition of osteoclast spreading and bone resorption. Overexpression of the kinase-dead PYK2(K475A) mutant had no effect on cell spreading, interaction with c-Src, or the phosphorylation level of Tyr-402, Tyr-579/580, and Tyr-881 relative to wild-type PYK2-expressing cells. Taken together these findings suggest that Tyr-402 is the major docking site for c-Src and can be phosphorylated by another tyrosine kinase in osteoclasts but not in HEK293 cells. Interestingly, both PYK2(Y402F) and PYK2(K457A) translocate normally to podosomes and have no effect on macrophage colony-stimulating factor-induced osteoclast migration. Whereas PYK2(Y402F) dominant negatively blocks osteoclast spreading and bone resorption, PYK2(K457A) may function in part as an adaptor by initially recruiting c-Src to the adhesion complex, which appears to activate PYK2 by phosphorylating additional tyrosines in its regulatory and C-terminal domains. We thus concluded that phosphorylation at Tyr-402 in PYK2 is essential in the regulation of adhesion-dependent cytoskeletal organization in osteoclasts.

PYK2 is highly expressed in osteoclasts, terminally differentiated bone-resorbing cells of hematopoietic origin, and participates in the signaling initiated by osteoclast interaction with bone. Adhesion to bone matrix induces osteoclast differentiation, cytoskeletal reorganization, and cellular polarization, leading to formation of unique membrane areas for active bone resorption. These include the following: (i) the sealing zone, for tight adhesion to bone matrix; (ii) the ruffled border, for directional secretion of protons and proteases into the resorption lacuna; and (iii) functional secretory domain toward bone marrow space, for transcytosis and release of degraded bone matrix (1–3). αvβ3 integrin is highly expressed in osteoclasts (4) and was suggested to mediate osteoclast adhesion to various bone matrix proteins and to regulate cytoskeletal organization required for migration and formation of the sealing zone (5, 6). Interference with αvβ3 integrin function by blocking antibodies or by RGD-containing peptides or proteins leads to inhibition of osteoclast migration and of bone resorption in vitro and in vivo (6–12). In addition, targeted disruption of β3 integrin in mice induces progressive osteosclerosis without apparent reduction in osteoclast number (13). These data suggest that αvβ3 integrin-mediated adhesion and the signaling initiated by it may play a key role in the regulating of osteoclastic bone resorption.

The primary adhesion structures in osteoclasts and monocytes are podosomes, which consist of F-actin core surrounded by a small circle or rosette of cytoskeletal and signaling proteins as well as αvβ3 integrin (14–17). Dynamic regulation of podosomes and osteoclast spreading has been shown to require the non-receptor tyrosine kinases c-Src and the proline-rich tyrosine kinase 2 (PYK2; also known as CAKβ, RAFTK, or CADTK) (18–20). Src-deficient mice are osteopetrotic due to osteoclast dysfunction, which can be partially rescued by expression of kinase-defective c-Src (21, 22). We reported previously (16, 19) that PYK2 localizes to podosomes as well as to sealing zone in bone-resorbing osteoclasts, and cells treated with antisense PYK2 are not able to resorb bone in vitro. Furthermore, engagement of αvβ3 integrin by ligands or antibodies in osteoclasts leads to PYK2 tyrosine phosphorylation in a c-Src-dependent manner (16). The role of PYK2 phosphorylation and the activity to carry these functions have not been fully defined. Similar to the focal adhesion kinase (FAK), PYK2 lacks SH2 and SH3 domains but contains other functional domains, including two proline-rich regions in its C terminus and several phosphorylated tyrosine residues, which can mediate specific protein-protein interactions (23). Tyrosine 402 in PYK2, anal-

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¶The abbreviations used are: PYK2, proline-rich tyrosine kinase 2; pO/C, pre-fusion osteoclast; wt, wild type; M-CSF, macrophage colony-stimulating factor; m.o.i., multiplicity of infection; α-MEM, α-minimum Eagle’s medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; 1α,25(OH)2D3, 1α,25-dihydroxyvitamin D3; TRITC, tetramethylrhodamine isothiocyanate; mAb, monoclonal antibody; HSV, herpes simplex virus; FAK, focal adhesion kinase; SH, Src homology; OCL, osteoclast-like cells.
ogous to tyrosine 397 in FAK, has been suggested as the primary autophosphorylation site that provides a docking site for the SH2 domain of c-Src (23-25). Association of PYK2 with c-Src leads to further phosphorylation of PYK2 at tyrosines 579 and 580 in the kinase domain activation loop, assumed to be required for its maximal catalytic activity, and at tyrosine 881 at the C terminus, which mediates the association of PYK2 with Grb2 (25). Association of PYK2 with Grb2 (25) is facilitated by the C-terminal proline-rich region, which mediates the association of PYK2 with p130Cas and Graf (17, 23). The proline-rich C-terminal region is also required for its maximal catalytic activity, and at tyrosine 881 at the C terminus, which mediates the association of PYK2 with Grb2 (25). In addition, proline-rich regions in the PYK2 C terminus mediate interactions with yet other adaptor molecules, p130Cas and Graf (17, 23).

The objective of this study was to examine if PYK2 kinase activity and its ability to associate with c-Src are essential for mediating osteoclastic cytoskeletal organization. We examined the effects of overexpression of PYK2 kinase-defective (K457A) mutant and of an autophosphorylation site (Y402F) mutant on the adhesion-mediated signaling, cell spreading, and migration. We found that Tyr-402 phosphorylation, but not its kinase activity, is important for osteoclast spreading and adhesion-induced association of PYK2 with c-Src. Furthermore, expression of PYK2-Y402F in osteoclasts prevented normal bone resorption. Whereas both PYK2(K457A) and PYK2(Y402F) translocate to podosomes, PYK2(Y402F) acts in a dominant negative fashion to inhibit adhesion-dependent signaling in osteoclasts by blocking binding to c-Src and phosphorylation of additional tyrosines in the regulatory and C-terminal domains of PYK2. On the other hand, we observe normal phosphorylation of the kinase-defective PYK2(K457A) at Tyr-402, followed by Src recruitment. We thus suggest here that PYK2 functions as an adaptor for c-Src which allows full activation of PYK2 and other signaling molecules in the adhesion complexes during osteoclastic cytoskeletal organization.

MATERIALS AND METHODS

Reagents—Tissue culture media and cell dissociation buffer were purchased from Invitrogen; fetal bovine serum (FBS) was from JRH Bioscience (Lenexa, KS); collagenase was from Wako Chemicals (Richmond, VA); dispase was from Roche Molecular Biochemicals; and macrophage colony-stimulating factor (M-CSF) was from R & D Systems Inc. (Minneapolis, MN). 1α,25-Dihydroxyvitamin D3, (1α,25- (OH)2D3) was a gift of Dr. Milan R. Uskokovic (Hoffmann-La Roche). Phosphorylation site-specific polyclonal antibodies for PYK2 were from BIOSOURCE International (Camarillo, CA). Anti-PYK2 polyclonal antibodies were as described previously (16). Anti-pp60c-Src (mAb GD11) and anti-phosphotyrosine (mAb 4G10) were from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibodies to paxillin were from BD Transduction Laboratories (San Diego, CA), and anti-HSV-Tag was from Novagen (Madison, WI). Polyclonal antibodies raised against human β3 integrins were a gift from Dr. B. Bednar (Merck). Fluorescein isothiocyanate- and TRITC-conjugated IgGs were from The Jackson Laboratory (West Grove, PA); horseradish peroxidase-conjugated IgG was from Amersham Biosciences, and Oregon Green 488 phalloidin was from Molecular Probes (Eugene, OR).

Cell Cultures—Pre-fusion osteoclasts (pOCs) were prepared as described previously (26). Briefly, pre-fusion osteoclasts were obtained from co-cultures of osteoblastic MB1.8 cells and murine bone marrow cells in α-MEM containing 10% FBS and 10 nM 1α,25(OH)2D3. After 5 days in co-culture, pOCs were released from dishes with enzyme-free cell dissociation buffer after removing MB1.8 cells with 0.1% (w/v) collagenase and dispase in PBS.

Construction of Recombinant Adenoviruses—The recombinant, replication-deficient adenovirus vectors were constructed using pAElsp1 plasmid consisting of human cytomegalovirus promoter and the bovine

Fig. 1. Kinase activity and tyrosine phosphorylation of PYK2 mutants expressed in HEK293 cells. A, uninfected HEK293 cells (Control) and HEK293 cells infected with the indicated m.o.i./cell of adenovirus expressing wild type PYK2 (wt PYK2), PYK2(K457A), or PYK2-(Y402F) for 24 h were lysed and subjected for PYK2 immunoprecipitation (IP) and in vitro kinase assay as described under “Materials and Methods.” Half the immunoprecipitates were used for blotting for HSV-Tag, stripped and re-blotted for PYK2, to normalize for the amounts of expressed protein.
growth hormone polyadenylation site as described previously (27). Before transfer into adenovirus vector, full-length murine PYK2 cDNA was cloned into pCDNA3 (Invitrogen) as described previously (16), and Lys-457 → Ala and Tyr-402 → Phe mutations were done using U. S. E. mutagenesis kit from Amersham Biosciences. All the constructs had HSV-Tag at their C terminus. Recombinant viruses were produced in HEK293 cells, purified, and titrated according to standard methods (28).

**Infection of Cells—Expression, kinase activity, and tyrosine phosphorylation of PYK2** and its mutants were first tested by infecting HEK293 cells. The cells were infected with recombinant viruses at multiplicity of infection (m.o.i.) of 1–100. After 24 h, cells were washed once with PBS, lysed into modified RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.2% sodium deoxycholate, 1 mM EDTA, 5 mM sodium fluoride, 1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonfluoride, and a mixture of protease inhibitors) on ice. Lysates at equal protein concentrations were incubated for 2 h with anti-c-Src antibody, followed by G-protein-Sepharose for 1 h at 4 °C, and finally washed with ½ times Nonidet P-40 lysis buffer (4 times). Immunoprecipitated proteins were separated on 8% SDS-PAGE, blotted, and stained as described above. The levels of tyrosine phosphorylation and PYK2 were quantitated using an imaging densitometer (model GS-700; Bio-Rad) and expressed as fold of phosphorylated PYK2 in uninfected cells. Each was normalized to total levels of PYK2 in respective sample.

**Kinase Assay—HEK293 cells or OCLs expressing PYK2 mutants or uninfected control cells were lysed into modified RIPA buffer and precipitated with anti-PYK2 or anti-HSV antibodies for 1 h at 4 °C, followed by G-protein-Sepharose for 1 h at 4 °C, and washed with RIPA buffer (3 times). Half of the immunoprecipitates were subjected to blotting with anti-PYK2 or anti-HSV antibodies, and the other half was washed once with low salt buffer (100 mM NaCl, 10 mM Tris, pH 7.4, 5 mM MnCl₂), and incubated with kinase reaction mixture (10 mM Tris, pH 7.4, 5 mM MnCl₂, 1 μM cold ATP, 5 μCi of [γ-32P]ATP) for 15 min at 30 °C. The samples were subjected to SDS-PAGE and autoradiography.

**Cell Attachment and Spreading—pOCs expressing PYK2 and its mutants were allowed to attach to vitronectin-coated dishes (20 μg/ml, Invitrogen) at indicated times in the absence of serum. After gently washing with PBS, attached cells were fixed and stained for TRAP, and the cell area was counted using an image-analysis system (Empire Image System, Milford, NJ).**

**Bone Resorption—Isolated pOCs were cultured together with 10 nM 1a,25(OH)₂D₃-pretreated (48 h) MB1.8 cells on dentine slices in the presence of 10 nM 1a,25(OH)₂D₃ for 24 h. Cells were then removed by ultrasonication in NH₄OH, and resorption pits on dentine slices were examined by scanning electron microscopy (ElectroScan model 2010).**

**Cell Migration—Cell migration was assayed using a Boyden chamber type apparatus (Neuroprobes, Cabin John, MD) as described before (29). Briefly, 5 nM M-CSF was placed in the bottom chamber and isolated pOCs at a density of 20,000 cells/well in the upper chamber. Cells were allowed to migrate through a polycarbonate filter for 9 h in a humidified incubator at 37 °C. Then the cells that migrated to the bottom of the filter were stained for TRAP and counted from 10 sequentially selected fields using an Olympus IX70 microscope with a ×10 objective.**

**Immunofluorescence—pOCs expressing PYK2 and its mutants were allowed to attach onto vitronectin-coated glass coverslips for 1–2 h,**

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**Fig. 2. Expression levels of PYK2 mutants in osteoclast-like cells.** Isolated pOCs were left untreated (C) or infected with various m.o.i./pOC of adenovirus expressing wt PYK2, PYK2(K457A), or PYK2(Y402F) for 1 h and cultured with the osteoclast-inducing MB1.8 cells for 3 days. Then MB1.8 cells were removed with collagenase-dispase as described under “Materials and Methods,” and osteoclast-like cells were lysed and subjected to PYK2 immunoprecipitation (IP) followed by blotting for HSV-Tag to illustrate the expression of exogenous PYK2 and then for PYK2 to illustrate both exogenous and endogenous PYK2. PYK2 fold expression was calculated as the ratio of the PYK2 amount in controls (C). 1–6 in the graphs correspond to lanes 1–6 in the blots.
fixed with 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100 in PBS. Cells were stained using anti-β, or anti-PYK2 polyclonal antibodies, and anti-HSV, anti-phosphotyrosine, and anti-paxillin mAbs, followed with the appropriate secondary antibodies, or with Oregon-Green 488 phalloidin. Stainings were viewed with a Leica TCS SP Spectral confocal laser scanning microscope equipped with Argon-Krypton laser (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany).

RESULTS

Kinase Activity and Tyrosine Phosphorylation of PYK2 and Its Mutants in HEK293 Cells—Kinase activity and tyrosine phosphorylation levels of exogenous murine PYK2( wt), PYK2( Y402F), and PYK2( K457A) were first characterized in HEK293 cells, which do not express endogenous PYK2. Cells were infected with recombinant adenovirus constructs expressing HSV-tagged PYK2( wt) or the mutants at m.o.i. of 1–10 for 24 h and lysed for biochemical analyses. In vitro kinase assay of immunoprecipitated PYK2 shows autophosphorylation activity of wild type PYK2 and of PYK2( Y402F). This suggests that additional tyrosines in PYK2, beside the known major autophosphorylation site Tyr-402, can be phosphorylated in vitro. In contrast, the ATP-binding site defective PYK2( K457) mutant shows no kinase activity under the same condition (Fig. 1A). Western blots for HSV-Tag and PYK2 reveal expression levels of exogenous PYK2 and the mutants in HEK293 cells (Fig. 1A, lower panels).

Phosphorylation of various tyrosines in PYK2( wt) and its mutants was investigated using phosphorylation site-specific antibodies for PYK2. Fig. 1B shows that in HEK293 cells, when expressed at the similar concentrations, PYK2( wt) was highly phosphorylated at tyrosines 402, 579/589, and 881, whereas only weak phosphorylation in PYK2(K457A) was detected at these tyrosines. Undetectable phosphorylation at Tyr-402, Tyr-579/Tyr-580, and Tyr-881 was observed in PYK2(Y402F). This suggests that phosphorylation of PYK2 at these tyrosines was initiated by the autophosphorylation at Tyr-402, which was previously suggested to recruit an Src kinase for further phosphorylation at the C-terminal tyrosines. Interestingly, the kinase-defective PYK2 appears to interact with another tyrosine kinase in HEK293 cells, which weakly phosphorylates Tyr-402. Our results also suggest that tyrosines other than 402, 579/589, or 881 is a substrate for in vitro kinase activity of PYK2(Y402F) as observed in Fig. 1A.

Expression and Kinase Activity of Exogenous PYK2 and Its Mutants in Osteoclast-like Cells—Expression of PYK2 and its mutants in OCLs was characterized by infecting isolated pOCs at increased m.o.i. of adenovirus constructs and then culturing pOCs with osteoblastic MB1.8 cells to support osteoclast survival and fusion. After 3 days, MB1.8 cells were removed with collagenase-dispase, and OCLs were lysed for biochemical analysis. Immunoprecipitation analysis shows dose-dependent increase in the expression of all exogenous HSV-tagged PYK2 forms (Fig. 2). Concentrations of adenovirus resulting in ~3–4-fold expression of exogenous over endogenous PYK2 were used in all following experiments. No defects in OCL growth or survival were observed at the highest expression levels of exogenous PYK2.

Next kinase activities of PYK2( wt) and its mutants overexpressed in osteoclast-like cells were characterized in in vitro kinase assay. Fig. 3 demonstrates high autophosphorylation activity of exogenously expressed PYK2( wt) in immunoprecipitates using anti-HSV antibodies. PYK2(Y402F) clearly displayed lower autophosphorylation activity as compared with the wild type, and no activity was found to associate with PYK2(K457A). Western blots for HSV and PYK2 demonstrate similar expression levels of exogenous PYK2 forms recovered in HSV immunoprecipitates (Fig. 3, left lanes). These results confirm the functional expression of PYK2 and its mutants in osteoclast-like cells, similar to that observed in HEK293 cells. In PYK2 immunoprecipitates (Fig. 3, right lanes), expression levels of exogenous PYK2 forms were similar (Fig. 3, blot: HSV) and 3–4 times that of endogenous PYK2 (Fig. 3, blot: PYK2, as comparing control to the last three right lanes in IP: PYK2).
Tyrosine Phosphorylation of PYK2 in Osteoclast Function

**Expression of PYK2(Y402F) Inhibits Osteoclast-like Cell Spreading and Bone Resorption**—Because we demonstrated previously (19) the role of PYK2 antisense in inhibiting cytoskeletal reorganization in osteoclasts during cell adhesion and spreading, we thus examined the effects of PYK2 mutants on spreading of pOCs on vitronectin under serum-free conditions. From the time course of pOC spreading as shown in Fig. 5A, there were no statistically significant differences between the rate of cell spreading in control pOCs expressing only endogenous PYK2 and pOCs overexpressing wild type or kinase-defective PYK2. In contrast, spreading of pOCs overexpressing PYK2(Y402F) was severely impaired (Fig. 5A and B, p < 0.001 at 30- and 60-min time points). These results suggest that the poorly tyrosine-phosphorylated PYK2(Y402F) functions as dominant negative PYK2 preventing cytoskeletal reorganization during osteoclast spreading. On the other hand, the kinase activity of PYK2 appears to be unnecessary for supporting osteoclast spreading.

Next we investigated if reduced cell spreading, caused by overexpressing PYK2(Y402F), affects bone resorption. Control and PYK2(Y402F)-expressing pOCs were cultured on dentine slices together with MB1.8 cells for 24 h. The cells were removed, and resorption pits were examined by S.E.M. Only very shallow resorption pits were made by PYK2(Y402F)-expressing osteoclasts (Fig. 5C, panels C and D), when compared with uninfected cells (Fig. 5C, panels A and B). This indicates that expression of PYK2(Y402F) prevents both cell spreading and bone resorption, similar to cells expressing PYK2 antisense or to c-Src-deficient osteoclasts.

c-Src Tyrosine Phosphorylation and Its Association with PYK2 Mutants in Osteoclast-like Cells—Since the rate of cell

**Fig. 4. Defective tyrosine phosphorylation of autophosphorylation site-mutated PYK2(Y402F) but not of kinase-dead PYK2(K457A) in osteoclast-like cells.** Isolated pOCs were left untreated (Control) or infected with adenovirus expressing wt PYK2, PYK2(K457A), or PYK2(Y402F) for 1 h and cultured with MB1.8 cells for 3 days. Osteoclast-like cells were lysed, and half of the lysates were subjected to HSV-Tag immunoprecipitation (IP) and half to PYK2 immunoprecipitation. Immunoprecipitates were then subjected for blotting for site-specific anti-phosphotyrosine PYK2 antibodies, pY402 (A), pY579/580 (B), or pY881 (C). Membranes were then stripped and re-blotted for HSV-Tag and then for PYK2. HSV immunoprecipitates visualize tyrosine phosphorylation of exogenously expressed PYK2 forms (left lanes), whereas PYK2-immunoprecipitates visualize tyrosine phosphorylation of both exogenously and endogenously expressed PYK2 (right lanes). Tyr(P)/PYK2 ratio demonstrates relative amounts of phosphorylation at each tyrosine in the samples.
spreading of osteoclasts overexpressing PYK2(Y402F) was inhibited in a similar manner as found in the Src-deficient osteoclasts and phosphorylated Tyr-402 has been suggested to mediate the recruitment of c-Src to PYK2 via its association to the Src-SH2 domain, we investigated the ability of PYK2 mutants binding to endogenous c-Src in osteoclasts by co-immunoprecipitation methods. Fig. 6 demonstrates the association of PYK2(wt) and PYK2(K457A), but not PYK2(Y402F) with c-Src (Fig. 6A), suggesting that phosphorylated tyrosine 402 of PYK2 is the major docking site for c-Src in this cell type. In addition, we could not detect any differences in the levels of tyrosine phosphorylation (Fig. 6B) or kinase activity (data not shown) of c-Src in osteoclast-like cells overexpressing various PYK2 mutants, confirming our previous results that c-Src is not the substrate of PYK2 in osteoclasts.

Localization of Exogenous PYK2 and Its Mutants to Podosomes in Osteoclast-like Cells—Since cell spreading was impaired in pOCs expressing PYK2(Y402F), we next characterized the localization of PYK2(wt) and its mutants in pOCs. Double stainings of HSV, showing the localization of exogenous PYK2, and F-actin showed that both PYK2(wt) and PYK2(K457A) can be readily localized to podosomes in pOCs adhered to vitronectin (Fig. 7, B, C, F, and G). Although the spreading of PYK2(Y402F) expressing pOCs were greatly reduced, the cells adhere to vitronectin, and a few of them started to form podosomes containing lamellipodia, and PYK2(Y402F) could be found to translocate into the podosomes in these cells as seen with anti-HSV antibodies (Fig. 7, D and H; Fig. 8, C and D). Double stainings of HSV and PYK2 demonstrated that in addition to peripheral podosomes substantial amounts of all exogenously expressed PYK2 forms were localized in the cytoplasm (Fig. 8). In addition, β3 integrin and paxillin co-localized with HSV and surrounded F-actin stained podosomes, respectively, in pOCs expressing PYK2 forms (Fig. 9). This demonstrates that pOCs expressing kinase-dead PYK2(K457A) or PYK2(Y402F) are able to form normal podosomes. In addition, the results suggest that PYK2 might be constitutively recruited to the podosomes upon cell adhesion, and phosphorylation of Tyr-402, Tyr-579/580, or Tyr-881 or association with c-Src might not be necessary for podosomal targeting of PYK2.

Overexpressing of PYK2 and Its Mutants in Osteoclast-like Cells Did Not Affect Cell Migration toward M-CSF—We demonstrated previously (30) that although cell spreading of Src-deficient osteoclasts is severely impaired, their migration toward M-CSF is normal. Similar to Src-deficient osteoclasts, we showed here that the migration of osteoclasts overexpressing PYK2(Y402F) toward M-CSF is comparable with that of uninfected pOCs or of cells infected with adenovirus expressing PYK2(wt) or PYK2(K457A) (Fig. 10). However, we observed a consistent increase in the number of migrated pOCs that overexpress exogenous PYK2(wt) or kinase-defective PYK2 as compared with control uninfected cells, suggesting that an increase in PYK2 expression over the endogenous levels might accelerate the rate of osteoclast migration toward M-CSF. It should also be noted that pOCs expressing PYK2(Y402F) when cultured together with MB1.8 cells in the presence of serum for 3
days were normally spread (data not shown). This suggested that M-CSF secreted by MB1.8 cells can also induce normal spreading of PYK2(Y402F) expressing osteoclasts.

**DISCUSSION**

PYK2 has been shown to be one of the major adhesion-induced tyrosine kinase in osteoclasts, necessary for osteoclastic bone resorption (16, 19). The present data characterize the importance of various PYK2 domains for its function in osteoclasts. First, we verified adenovirus expression of wild type PYK2 and its mutants in HEK293 cells, which do not express endogenous PYK2. In HEK293 cells, both kinase-defective PYK2(K457A) and autophosphorylation site-mutated PYK2(Y402F) were very poorly phosphorylated at tyrosines 402, 579/580, and 881. This suggests that autophosphorylation of tyrosine 402 is necessary for further tyrosine phosphorylation of PYK2 in HEK293 cells. Similar observations using transfection of HEK293 cells (20, 31) and endothelial cells (32) have been described recently. Interestingly, Tyr-402 is not the only autophosphorylation site of PYK2, because we consistently observe weak phosphorylation of tyrosines other than Tyr-402, Tyr-579/580, and Tyr-881 in PYK2(Y402F) in the in vitro kinase assay and by blotting with antibodies that recognize any phosphotyrosine residues (data not shown). The identity and potential role of the additional autophosphorylation sites of PYK2 in the adhesion-dependent signaling pathway in osteoclasts or other cells are subjects of future investigations.

We and others (33, 34) have demonstrated previously the use of replication-defective recombinant adenovirus as an effective method to highly introduce exogenous genes into osteoclasts and that adenovirus expressing PYK2 antisense inhibits osteoclast spreading and function (19). By using the same method, this study describes dose-dependent expression of wild type PYK2 and two mutants PYK2(K457A) and PYK2(Y402F) in osteoclast-like cells. Although endogenous PYK2 is an abundantly expressed kinase in osteoclasts, 3–4-fold higher expression of exogenous PYK2 over the endogenous level could readily be achieved without affecting osteoclast survival.

In contrast to HEK293 cells, overexpression of the kinase-defective PYK2(K457A) mutant in osteoclasts showed no reduction in phosphorylation at the tyrosines 402, 579/580, and 881 and did not prevent cell spreading on vitronectin, suggesting that its kinase activity is not necessary for the integrin αvβ3-dependent signaling in osteoclasts mediated by PYK2. Interestingly, phosphorylation of Tyr-402, which leads to its association with c-Src, is normal in PYK2(K457A). Given PYK2 homodimerization, it is highly unlikely that phosphorylation of the overexpressed mutant was due to the endogenous wild type PYK2, suggesting that Tyr-402 was phosphorylated by another tyrosine kinase(s) expressed in osteoclasts. Src family of kinases as well as the tyrosine receptor kinases, platelet-derived growth factor or vascular endothelial growth factor receptors, have been implicated in the activation of FAK independently of

Fig. 6. c-Src tyrosine phosphorylation and its association with PYK2 mutants in osteoclast-like cells. Isolated pOCs were left untreated (Control) or infected with adenovirus expressing wt PYK2, PYK2(K457A), or PYK2(Y402F) for 1 h and cultured with MB1.8 cells for 3 days. Then MB1.8 cells were removed with collagenase-dispase as described under “Materials and Methods,” and osteoclast-like cells were lysed. A, lysates were subjected to c-Src immunoprecipitation (IP) and blotting for HSV-Tag. Membranes were then stripped and re-probed first for PYK2 and then for c-Src. Sample of total cell lysate of each treatment was used for Western blotting (TCL, right lanes). IgG is immunoglobulin heavy chain used for immunoprecipitation. B, similar lysates were subjected for c-Src immunoprecipitation and blotting for phosphotyrosine (pY) and c-Src.
FAK kinase activity (35, 36). Because unlike many cell types osteoclasts highly express c-Src, c-Src might be responsible for phosphorylation of Tyr-402 in PYK2, to promote additional Src docking to this site, leading to maximal activation of PYK2 via phosphorylation of Tyr-579/580 in the regulatory domain and Tyr-881 in its C-terminal domain. Phosphorylation of these tyrosines is thus suggested to regulate downstream signaling and osteoclast spreading. This notion is in agreement with recent findings (37) demonstrating that adhesion-induced FAK auto-phosphorylation at tyrosine 397 as well as phosphorylation at tyrosine 577 in the kinase domain require Src family kinase function. On the other hand, Sanjay and co-workers (20) suggested that integrin-induced PYK2 tyrosine phosphorylation is c-Src-independent but is mediated by an increase in cytoplasmic Ca\(^{2+}\) concentration. In addition, PYK2 kinase activity was found to be necessary for cell spreading and migra-
Pulmonary vascular endothelial cells express both PYK2 and FAK, and FAK expression as well as p130Cas were decreased following transfection with the kinase-defective PYK2. These findings suggested that the kinase activity-dependent role of PYK2 on focal adhesion formation and cytoskeletal reorganization in vascular pulmonary endothelial cells was mediated through FAK and p130Cas (32).

An important finding in the present paper is the dominant negative function of the major autophosphorylation site mutant PYK2(Y402F) in osteoclast spreading. PYK2(Y402F) displayed in vitro kinase activity, albeit lower than wild type or endogenous PYK2. This is in agreement with results observed in HEK293 cells (31) (Fig. 1) and in pulmonary vascular endothelial cells (32). The lower kinase activity of PYK2(Y402F) could be due to poor phosphorylation of kinase domain tyrosines 579/580, suggested to be required for full activity of PYK2 (23). PYK2(Y402F) was unable to associate with c-Src, which may be the reason of reduced phosphorylation of tyrosines 579/580 as well as tyrosine 881 at the C terminus in PYK2(Y402F). Reduced tyrosine phosphorylation may prevent association with and activation of downstream signaling molecules, such as association of Grb2 to phosphorylated Tyr-881, required for cytoskeletal organization and osteoclast spreading. More importantly, reduced cell spreading also reflected to the impaired bone resorption because only very shallow resorption pits were formed by osteoclasts expressing PYK2(Y402F).

Further studies are needed to identify important downstream factors, whose association to signaling complex and/or activation is prevented by PYK2(Y402F) in osteoclasts. In other systems, PYK2 phosphorylation at tyrosine 402 and association with c-Src leads to activation of mitogen-activated protein kinase cascades via distinct mediators (31). Phosphorylation of Shc and Grb2 and their association with PYK2 and Sos mediate activation of extracellular signal-regulated kinase, whereas tyrosine phosphorylation of p130Cas and its association to PYK2 and Crk and possible Crk effectors lead to activation of other mitogen-activated protein kinase cascades, e.g., the c-Jun N-terminal kinase cascade (31). In osteoclasts, α,β2-mediated adhesion induces c-Src-dependent phosphorylation of extracel-

**Fig. 9. Localization of β3 integrin (A') and paxillin (B') in podosomes in PYK2 mutants expressing pOCs.** A’, pOCs were isolated from control co-cultures (Control, A and E) or from co-cultures infected with adenovirus expressing wt PYK2 (B and F), PYK2(K457A) (C and G), or PYK2(Y402F) (D and H) as described under “Materials and Methods.” pOCs were then allowed to adhere and spread on vitronectin-coated surfaces and subjected for double staining for β3 integrin (A–D) and HSV-Tag (E–H). B’, pOCs were isolated from co-cultures infected with adenovirus expressing PYK2(K457A) (A and C) or PYK2(Y402F) (B and D) as described under “Materials and Methods,” allowed to adhere and spread on vitronectin-coated surfaces, and subjected for double staining for F-actin (A and B) and paxillin (C and D). Extended confocal images from the adhesion structures close to the glass surface show podosomal staining of β3 integrin and HSV-tag (A’, arrows) and paxillin surrounding punctate F-actin staining (B’, arrows) in adenovirus-transfected pOCs. Bars (A’) 20 (A, C–E, G, and H), 10 (B and F), (B’) and 10 μm.
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lular signal-regulated kinases (30), which points to Grb2-Sos complex as one possible mediator.

It is well known that in fibroblastic cells, which do not express endogenous PYK2 but use FAK as the main adhesion-related tyrosine kinase, overexpression of PYK2 induces apoptosis (38). Apparently, competition of PYK2 to endogenous FAK in fibroblasts results in different biological effect compared with what we observed here with osteoclasts, expressing high level of endogenous PYK2 but little FAK. Furthermore, although PYK2 expression is elevated in FAK null fibroblasts, it is not able to fully substitute FAK function or localize to focal adhesions (39). In FAK null fibroblasts, PYK2 is targeted to focal adhesions only when it is expressed as a chimera containing FAK C-terminal domain (40). Thus PYK2 localization to adhesion contacts is a fairly unique feature. In addition to osteoclasts, PYK2 has been reported to localize to adhesion contacts only in monocytes and during nerve growth factor-induced neuronal cell differentiation (41–43). The present results of the dominant negative effect of PYK2(Y402F) on cytoskeletal organization, osteoclast spreading, and bone resorption further support the specific role of PYK2 function in osteoclasts.

Interestingly, osteoclasts expressing any of the PYK2 forms were able to form podosomes, and all the PYK2 forms were able to localize to podosomes in osteoclasts on vitronectin. This suggests that the kinase activity or tyrosine phosphorylation of PYK2 is not necessary for its localization to podosomes. Similarly, autophosphorylation site- or kinase domain-mutated FAK localizes to focal adhesions in fibroblasts (44). The mechanism for podosome-targeting of PYK2 is not well understood. The presented data are in agreement with recent results demonstrating direct binding of PYK2 to the β3 cytoplasmic tail in vitro regardless of PYK2 tyrosine phosphorylation or paxillin association (45). Thus it is unlikely that PYK2 tyrosine phosphorylation provides a regulatory mechanism for its targeting to podosomes. However, PYK2 tyrosine phosphorylation may affect its association with and recruitment of other components to podosomes or activity of downstream targets that regulate podosome dynamics. Evidently this could have crucial effects on cell spreading. As a matter of fact, targeting of PYK2(Y402F) to podosomes may be necessary for its dominant negative action on osteoclast spreading or enhance that effect. The molecular mechanism by which PYK2(Y402F) prevents osteoclast spreading remains to be elucidated. In the case of c-Src, it has been suggested that the reduced spreading and migration of the c-Src-deficient osteoclasts are caused by defect in podosome disassembly rather that assembly (20). Further studies are required to characterize cytoskeletal or other morphological changes preventing bone resorption of PYK2(Y402F) overexpressing osteoclasts.

Finally, migration of osteoclasts toward M-CSF is not disturbed by expression of the PYK2 mutants used in this study. Similar observations have been made recently (37) showing that FAK tyrosine phosphorylation by G-protein-coupled receptor agonists is not c-Src-dependent. One possibility is that the M-CSF stimulates osteoclast cytoskeletal organization and migration by activating the pathway downstream of the c-Src-PYK2 complex, consistent with our previous finding (30) that migration of c-Src-deficient osteoclasts toward M-CSF is not impaired. Phospholipase C-γ was found to be a common downstream mediator for αβ3 and M-CSF signals in osteoclasts and M-CSF-induced cytoskeletal reorganization and modulated the αβ3 integrin ligand interaction and recruitment of signaling molecules, including PYK2, to adhesion structures in the absence of c-Src (30). The present study suggests that phosphorylation of Tyr-402 in PYK2 is not required for M-CSF-induced cytoskeletal organization in osteoclasts. Although the previous study (30) showed that M-CSF did not highly induce PYK2 tyrosine phosphorylation in c-Src-deficient osteoclasts, we cannot rule out an effect of M-CSF on the binding of the C-terminal domain of PYK2 to phospholipase C-γ, which depends on both SH2 and SH3 domains of phospholipase C-γ (30).

In summary, our results show that the mutant of major autophosphorylation site PYK2(Y402F) functions as a dominant negative PYK2 by preventing its association with c-Src, which has been shown to regulate both PYK2 kinase activity and its ability to recruit downstream adhesion-dependent signaling molecules (16, 17, 30). Interestingly, although overexpression of PYK2(Y402F) results in inhibition of osteoclast spreading, it can be readily localized to a few forming podosomes, suggesting that c-Src recruitment to the adhesion contacts plays a significant role in mediating cytoskeletal organization in osteoclasts. Furthermore, overexpression of PYK2(Y402F) leads to impaired osteoclastic bone resorption in vitro. On the other hand, tyrosine phosphorylation of the kinase-defective PYK2(K457A) is similar to wild type PYK2 in osteoclasts, has normal interaction with c-Src, localizes to podosome, and does not affect osteoclast spreading. Taken together, our data thus suggest that PYK2 may function as an adaptor for c-Src which allows downstream signaling, required for normal osteoclast spreading and bone resorption.

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