Inhibition of Osteoclast Function by Adenovirus Expressing Antisense Protein-tyrosine Kinase 2*

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Osteoclast activation is initiated by adhesion to bone, cytoskeletal rearrangement, formation of the sealing zone, and formation of the polarized ruffled membrane. Previous findings suggest that protein-tyrosine kinase 2 (PYK2), a cytoplasmic kinase related to focal adhesion kinase, participates in these events. This study examines the role of PYK2 in adhesion-mediated signaling and osteoclast function, using PYK2 antisense. We produced a recombinant adenovirus containing a 300-base pair reversed 5′-coding region of PYK2 and used full-length PYK2 as a control. Murine osteoclast-like cells or their mononuclear precursors were generated in a co-culture of bone marrow and osteoblasts. Infection with antisense adenovirus significantly reduced the expression of endogenous PYK2 protein relative to uninfected cells or to cells infected with sense PYK2 and caused: 1) a reduction in osteoclast formation in vitro; 2) inhibition of cell spreading and of actin ring formation in osteoclasts plated on glass or bone and of attachment and spreading of osteoclast precursors plated on vitronectin; 3) inhibition of bone resorption in vitro; 4) marked reduction in p130Cas tyrosine phosphorylation; and 5) no change in αβ3 integrin expression or c-Src tyrosine phosphorylation. Taken together, these findings support the hypothesis that PYK2 plays a central role in the adhesion-dependent cytoskeletal organization and sealing zone formation required for osteoclastic bone resorption.

Osteoclasts are multinucleated, terminally differentiated cells that degrade mineralized matrix. Osteoclast adhesion to bone matrix is an essential prerequisite for osteoclast differentiation, migration, and polarization, including formation of a tight sealing zone and directional secretion of protons and lysosomal proteases into the resorption lacuna (1, 2). Integrins have been suggested to mediate osteoclast adhesion to the matrix and regulate the cytoskeletal organization required for migration and formation of the sealing zone (3, 4). αβ3 integrin is highly expressed in osteoclasts in vitro and in vivo (5). Interference with αβ3 integrin function by blocking antibodies, disintegrins, or small molecular weight RGD mimetics lead to inhibition of bone resorption in vitro and in vivo, supporting the key role of this integrin in adhesion-dependent osteoclastic activation (6–9).

The proline-rich tyrosine kinase (PYK2; also known as cell adhesion kinase β, related adhesion focal tyrosine kinase, or calcium-dependent tyrosine kinase)1 and focal adhesion kinase (FAK) are members of a distinct family of non receptor protein-tyrosine kinases that are regulated by a variety of extracellular stimuli (10, 11). Although FAK is widely distributed, PYK2 is predominantly expressed in the central nervous system and in hematopoietic lineage cells. The alternative spliced isoform PYK2-H is specifically expressed in T and B lymphocytes, monocytes, and natural killer cells (12–15). Similar to FAK, PYK2 lacks SH2 and SH3 domains but possesses several functional domains, including two proline-rich regions in its C terminus and several phosphorylated tyrosine residues, through which specific protein-protein interactions can occur. PYK2 was shown to play an important role in the integration of signals initiated by a diverse group of extracellular stimuli, including integrin binding, growth factors, cytokines, chemokines, and certain stress stimuli (11). In PC12 cells, PYK2 tyrosine phosphorylation and activation are stimulated by neuronal stimuli and stress signals, leading to modulation of a potassium channel and activation of the c-Jun N-terminal kinase signaling pathway (16). In addition, stimulation of G protein-coupled receptors induces tyrosine phosphorylation of PYK2 and complex formation between PYK2 and Src via the SH2 domain, leading to activation of the MAP kinase signaling pathway (10, 11). PYK2 was also suggested to participate in the transfer of signals from the cell surface to the cytoskeleton, because it is tyrosine phosphorylated and activated by adhesion-initiated signaling in osteoclasts, monocytes, platelets, and B lymphocytes (14, 17, 18). PYK2 was also shown to interact with and phosphorylate the focal adhesion-associated protein Paxillin (19, 20).

In osteoclasts, PYK2 tyrosine kinase has been suggested to mediate integrin-initiated Src-dependent signaling, following adhesion (5). Furthermore, PYK2 localizes to podosomes and the sealing zone, the primary adhesion structures in osteoclasts during resorption (17). It has been shown that Src deficiency is associated with osteopetrosis in mice, because of loss of osteoclast function (21). Recently, targeted disruption of β3 integrin in mice was shown to cause progressive osteosclerosis without an apparent reduction in the number of osteoclasts on bone (22). Consistent with this observation, interfering with αβ3 integrin function in vitro, using the disintegrin echistatin,
results in loss of osteoclast migration and formation of the sealing zone (3). On the other hand, inhibition of bone resorption in vivo by echistatin was not accompanied by a reduction in the number of osteoclasts on the bone surface (23). Because PYK2 is a down stream mediator of the αββ3 and Src-dependent signaling pathway in osteoclasts, we therefore tested whether PYK2 plays a rate-limiting role in osteoclast activity. In this study, we inhibited PYK2 expression using adenoviral vectors expressing PYK2 antisense. We demonstrate that reduction in PYK2 protein level results in inhibition of osteoclast formation in vitro, primarily because of reduced osteoclast adhesion and spreading. Moreover, expression of PYK2 antisense inhibits formation of the sealing zone in osteoclasts and blocks their bone resorption activity. These findings suggest that PYK2 plays an essential role in the adhesion-dependent cytoskeletal organization that leads to osteoclast polarization and activity.

MATERIALS AND METHODS

Antibodies—Anti-PYK2 polyclonal antibodies were previously described (17). Antibodies to phosphorysorine (monoclonal antibody 4G10) and anti-Src were from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-paxillin was from Transduction Labs (Lexington, KY). Monoclonal antibody to adenoviral penton protein (clone 1431) was from Accurate Chemicals. Polyclonal antibodies raised against human β3 integrins were a gift from Dr. B. Bednar (Merck). Fluorescein isothiocyanate-conjugated goat anti-mouse IgG and tetramethylrhodamine isothiocyanate-conjugated donkey anti-rabbit IgG were purchased from Jackson Labs. (West Grove, PA). All horseradish peroxidase-conjugated secondary antibodies and protein G-Sepharose were from Amersham Pharmacia Biotech.

Cell Culture—Murine osteoclast-like multinucleated cells (OCLs) were prepared on collagen gels as reported (24) with some modifications. Briefly, the osteoblastic MB1.8 cells were plated at 1 × 10^5 cells/cm² on culture dishes precoated with 5 ml of 0.2% collagen gel (Nitta Gelatin Co., Osaka, Japan). After 24 h, bone marrow cells isolated from tibia of 6–8-week old Balb/c mice were added (2.5 × 10^4 cells/cm²) to the monolayer of MB1.8 cells. The co-culture was maintained in α-minimal essential medium containing 10% fetal bovine serum and 10 nM 1,25(OH)D3. OCLs were formed within 7 days and released from the dishes by treatment with 5 ml of 0.2% collagenase (Wako Pure Chemical Co.,) and collected by centrifugation at 250 × g for 5 min. Alternatively, OCLs and mononuclear preadipogenic osteo-clasts (pOCs) were prepared as described previously (25), except that at 6 days in culture, pOCs were detached using 10 mM EDTA solution, after removing MB1.8 cells with collagenase-dispase, followed by washing three times with α-minimal essential medium. pOCs, 95% pure as assessed by flow cytometry using polyclonal antibodies as described previously (3), were infected with recombinant adenovirus vectors expressing PYK2/AS for 4 days. Purified pOCs were then plated on vitronectin-coated glass coverslips for 1 h in the absence of serum. Cells were then fixed with 4% paraformaldehyde in phosphate-buffered saline and stained with Oregon green-labeled phalloidin and Hoechst 33342 stain (Molecular Probes Inc., Eugene, OR). Apoptotic pOCs were assessed by staining with Oregon green-labeled phalloidin and Hoechst 33342 stain (Molecular Probes Inc., Eugene, OR). Actin was stained with Oregon green-labeled phalloidin and DAPI. Actin was stained with Oregon green-labeled phalloidin and DAPI.

RESULTS

Efficiency of Adenovirus-mediated Gene Transfer in Osteoclasts—The recombinant replication-deficient adenovirus vectors were constructed using pdAE1sp1 plasmid consisting of the human cytomegalovirus promoter and the bovine growth hormone polyadenylation site as previously described (26). Adenovirus (Ad) expressing the Escherichia coli β-galactosidase gene (Ad-β-galactosidase) was used to optimize infection conditions. The pAdE1-PYK2/wt recombinant plasmid was constructed using the full-length murine PYK2 cDNA, which was cloned from a mouse spleen λ-ZAPII cDNA library (Stratagene, La Jolla, CA) using the specific PYK2 probe (17). We also produce a recombinant adenovirus (Ad-PYK2/AS) carrying an antisense sequence of PYK2 consisting of 300 base pairs of the reverse 5'-coding region of the murine PYK2 cDNA. Recombinant viruses were produced in the human embryonic kidney cell line, purified, and titrated according to standard methods (27).

Infection of Osteoclast-like Cells—The co-cultures of MB1.8 cells and murine bone marrow cells were infected with recombinant virus at different multiplicities of infection (MOI: 1000, 100, 10, 0.1, and 0.01). Viral stock was diluted with α-minimal essential medium and added directly to the co-cultures. Adenovirus was normally added to cultures at day 3, for 24 h, followed by a change of medium. To determine the efficiency of infection, cells were infected with Ad-β-galactosidase for 24 h. At day 7, cells were washed once with phosphate-buffered saline and fixed with 3.7% formaldehyde solution in phosphate-buffered saline. The co-cultures were either stained with X-gal histochemical staining or with tartrate-resistant acid phosphatase (TRAP), a marker enzyme for osteoclasts (28). The number of X-gal-positive cells and TRAP-positive cells was counted in triplicate cultures. Efficiency of adenoviral infected osteoclasts was expressed as the ratio of X-gal-positive to total TRAP-positive cells. Infected pOCs or OCLs were harvested from the infected co-cultures at day 5 or 7, respectively. pOCs were used for biochemical analyses, cell adhesion, and spreading on vitronectin. OCLs were used in immunostaining and pit formation.

Cell Attachment and Spreading—Polystyrene dishes (35 mm, Becton Dickinson) were coated overnight at 4 °C with 10 μg/ml human vitronectin (Life Technologies, Inc.). Uninfected pOCs or cells infected with Ad-PYK2/wt or Ad-PYK2/AS were isolated as described above. For biochemical analyses, cells were either kept in suspension (0.5 × 10^6 cells/ml) or allowed to attach to vitronectin-coated plates for 1 h at 37 °C. Cells were solubilized in modified RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.2% sodium deoxycholate, 1 mM EDTA, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonfluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and prepared for immunoprecipitation. To quantify the planar area of cell spreading, cells were allowed to adhere and spread on vitronectin for 1 h. After fixing with 4% paraformaldehyde in phosphate-buffered saline, the peripheral of each cell was outlined, and the total planar area was calculated using an image analyzing system (Empire Imaging System, Milford, NJ).

Assessment of Osteoclast Apoptosis—Because it is difficult to determine assessment of osteoclast apoptosis in the co-culture system in the presence of serum, growth factors and extracellular matrix proteins derived from MB1.8 cells, pOCs (25,000 cells/well) were therefore isolated from uninfected co-cultures or cells infected with Ad-PYK2/AS for 4 days. Purified pOCs were then plated on vitronectin-coated glass coverslips for 1 h in the absence of serum. Cells were then fixed with 4% paraformaldehyde in phosphate-buffered saline and stained with Oregon green-labeled phalloidin and Hoechst 33342 stain (Molecular Probes Inc., Eugene, OR). Apoptotic pOCs were assessed by counting the number of nuclei with changes in nuclear condensation and fragmentation. Results are expressed as the percentages of apoptotic cells over total counted pOCs (n = 100), viewed with a 20× objective and a Zeiss Axiopt fluorescence microscope.

Immunoprecipitation—Cell extracts were prepared with modified RIPA buffer and immunoprecipitated with antibodies to PYK2, pp60-syn, p130cas, or paxillin. Immunoprecipitation was carried out for 4 h at 4 °C, followed by addition of protein G-Sepharose. Immunoprecipitated proteins were separated on SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P membranes. Blots were first immunoblotted with horseradish peroxidase-conjugated anti-phosphorysorine monoclonal antibody 4G10 and then with the respective immunoprecipitating antibodies, followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG. Blots were developed by ECL (Amersham Pharmacia Biotech). Image densitometry (model GS-700; Bio-Rad) was used to estimate the phosphorysorine contents and the protein levels of each protein, from which the specific activity of tyrosine phosphorylated protein was calculated. Relative specific activity of phosphorylated protein was normally determined from triplicate experiments.

FIT Assay—PI-forming activity of OCLs was determined as described (3). Aliquots (1,000 cells/slice) of the OCLs were placed on dentine slices in 96-well culture plates. After 20 h, dentine slices were either stained with Mayer's hematoxylin for resorption pits. Pit area was measured by image analysis. The results were expressed as the means ± S.D. (n = 5) of resorbed area per whole dentine surface area.

Immunofluorescence Microscopy—Immunofluorescent labeling of actin filaments in OCLs was performed essentially as previously described (17). OCLs were placed on serum-coated glass coverslips or on bone slices for 20 h and fixed in 4% paraformaldehyde. Cells were then double-stained for TRAP and F-actin (3). PYK2 was visualized using the affinity purified anti-PYK2 antibodies, followed by tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit IgG. Actin was stained with Oregon green 514 phalloidin (Molecular Probes). Paxillin was stained with monoclonal antibody 349 and visualized using fluorescein isothiocyanate goat anti-mouse IgG. The αβ3 integrins were immunostained using polyclonal antibodies as described previously (3), followed by fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG. Immunofluorescence-labeled cells were photographed through an 100× objective using a Zeiss Axioskop epifluorescence microscope or with a Leica TCS SP Spectral confocal laser scanning microscope equipped with an Argon-crypton laser (Leica Microsystems Heidelberg GmbH).

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Efficiency of Adenovirus-mediated Gene Transfer in Osteoclast-like Cells—The co-cultures of murine osteoblastic MB1.8...
cells and bone marrow cells were first infected with Ad-β-galactosidase virus at MOI of 10 for every 24 h from day 1 to day 5 in culture. At day 7, parallel co-cultures were stained with either X-gal or TRAP stains. Frequency of adenovirus-mediated gene transfer in OCLs was quantitated as the ratio of β-gal(1) cells to TRAP(1) cells. As shown in Fig. 1A, 75–80% of the total TRAP(1) cells were infected with Ad-β-galactosidase when virus was added to co-cultures at either day 3 or day 4. We also noted that the fraction of β-gal expressing multinucleated OCLs with three or more nuclei, was the same as in the TRAP(1) cells, suggesting that adenoviral gene transfer does not affect osteoclast fusion. A dose-response relationship between viral dose (MOI) and the fraction of infected OCLs was also observed (Fig. 1B) when Ad-β-gal was added at day 3 and quantitated for β-gal(1) and TRAP(1) cells at day 7. Although the co-cultures contain a confluent layer of osteoblastic MB1.8 cells, β-gal was expressed preferentially in OCLs and pOCs, as shown in Fig. 1C.

**Infection of Osteoclast-like Cells with Adenovirus Expressing PYK2 Antisense Reduces PYK2 Protein Expression**—To analyze the effect of Ad-PYK2/AS on PYK2 expression in OCLs, we infected the co-cultures with adenoviral vectors (MOI = 10) expressing either full-length PYK2 (Ad-PYK2/wt) or PYK2 antisense (Ad-PYK2/AS) at day 3 or 4 of the co-cultures. OCLs were then purified at day 7, as described under “Materials and Methods.” Approximately equal amounts of protein were isolated from untreated and infected OCLs, as illustrated by Western blotting with anti-β3 integrin antibodies (Fig. 2, lower panel). This suggested that adenoviral infection did not significantly affect osteoclast differentiation using markers such as β3 integrin expression (Fig. 2) or TRAP activity (Figs. 1C and 3A). PYK2 protein expression in OCLs treated with Ad-PYK2/wt at day 3 of the co-culture was increased about 2-fold, as compared with untreated cells (Fig. 2). In contrast, there was a significant reduction (50–70%) in PYK2 protein levels, in OCLs treated with Ad-PYK2/AS at day 3 or 4 as described under “Materials and Methods.” PYK2 or β3 integrin were immunoprecipitated from equal protein concentrations of these lysates, followed by immunoblotting with anti-PYK2 (upper panel) or anti-β3 integrin (lower panel) antibodies.

**Adenovirus Expressing Antisense PYK2 Inhibits the Formation of TRAP(+) Cells**—To determine if adenovirus expressing antisense PYK2 inhibits osteoclastogenesis, we infected the co-cultures with adenovirus expressing either wild type PYK2 (Ad-PYK2/wt) or Ad-PYK2/AS at MOI = 100 (Fig. 3B).
Fig. 3. Infection of the co-culture system with adenovirus expressing PYK2 antisense inhibits osteoclastogenesis. Numbers and Area of TRAP(+) cells were quantitated from co-cultures infected with increased concentrations of either Ad-PYK2/wt (A) or with Ad-PYK2/AS (B). Each condition was run in quadruplicate samples, and the results are expressed as the means ± S.D. C, morphological analyses of co-cultures infected with Ad-PYK2/wt and Ad-PYK2/AS. Note that area of TRAP(+) cells was more sensitive to the increase in expression of PYK2 antisense than the number of these cells. Bars, 50 μm.

Adenovirus Expressing Antisense PYK2 Inhibits pOC Cell Spreading without Affecting pOC Apoptosis—To further confirm the role of PYK2 in the regulation of osteoclast spreading, we studied pOCs isolated from cocultures, which were either untreated or pOCs infected with Ad-PYK2/wt and Ad-PYK2/AS. As shown in Fig. 2, adenoviral gene transfer did not alter the expression of the β3 integrin. We plated the pOCs on vitronectin-coated dishes under serum-free conditions and determined the increase in cellular planar area with time. Planar area of untreated or pOCs infected with Ad-PYK2/wt increased 4-fold within 60 min (Fig. 4A). However, pOCs infected with Ad-PYK2/AS failed to spread on vitronectin (Fig. 4, A and B). On the other hand, several TRAP(−) cells occasionally found in this pOC preparation normally spread on VN (Fig. 4B, panel c, arrowhead). We observed a small increase in the rate of cell spreading in pOCs infected with Ad-PYK2/wt (Fig. 4A), consistent with the 2-fold increase in PYK2 protein expression in OCLs infected with Ad-PYK2/wt (Fig. 2). These cells seem very well spread by comparison with the normal controls (Fig. 4B, panels a and b).

Because Ad-PYK2/AS inhibited osteoclast spreading and subsequently reduced the number of TRAP(+) cells in the co-culture system (Fig. 3), we examined the effect of Ad-PYK2/AS on purified pOCs plated on vitronectin-coated dishes for 60 min under serum-free conditions. Because it is difficult to assess adhesion-dependent osteoclast apoptosis in coculture systems where osteoclastogenesis were influenced by serum and growth factors, cytokines, and extracellular matrix proteins derived from the osteoblastic/stromal MB1.8 cells, we therefore examine apoptotic events in pOCs isolated from cocultures uninfected and infected for 4 days prior to optimal fusion (25). Typical preparations of pOCs contain mainly mononuclear cells (~80%) and cells with two to four nuclei (Fig. 4C). Uninfected pOCs and pOCs infected with Ad-PYK2/wt appeared as spread cells with organized podosomal adhesion contacts and with flattened nuclei (Fig. 4C, panels a–d). On the other hand, pOCs infected with Ad-PYK2/AS were associated with reduced adhesion and cell rounding and compacted nuclei (Fig. 4C, panels e and f). Because of thick rounding cell shape, pOCs expressing PYK2 antisense often appeared to have overlapping nuclei at one plane of focus. However, we did not detect significant increase in number of apoptotic cells associated with chromatin condensation and nuclear fragmentation in pOCs infected with Ad-PYK2/AS. We indeed found that during the initial phase of cell adhesion, the percentage of apoptotic pOCs are 4.8% in uninfected pOCs, 5.7% in cells infected with Ad-PYK2/wt, and 6.2% in pOCs infected with Ad-PYK2/AS.

Adenovirus Expression of Antisense PYK2 Inhibits Tyrosine
Phosphorylation of p130Cas and Modulates the Tyrosine Phosphorylation of Paxillin—We have previously shown that PYK2 and p130Cas form a stable complex independent of c-Src (29). However, in Src(−/−) OCLs, tyrosine phosphorylation levels of PYK2 and p130Cas were greatly reduced (29). In this study, we examined the kinetics of tyrosine phosphorylation of c-Src, p130Cas, and paxillin in pOCs which were either untreated or infected with Ad-PYK2/wt or Ad-PYK2/AS and were allowed to attach to VN-coated dishes. The level of tyrosine phosphorylated proteins was determined in cells adhering for 15, 30, or 60 min and was compared with cells maintained in suspension. First, tyrosine phosphorylated proteins were compared in total cell lysates isolated from uninfected pOCs and cells infected with either Ad-PYK2/wt and Ad-PYK2/AS, adhering on VN for 60 min (Fig. 5A). At equal amounts of protein, the level of tyrosine phosphorylated proteins was generally lower in cells expressing PYK2/AS as compared with the those in controls. As previously reported (17), PYK2 became significantly tyrosine phosphorylated upon pOC adhesion to VN-coated surfaces for 15 min in control cells (Fig. 5B). Overexpression of PYK2 in pOCs infected with Ad-PYK2/wt was consistently found to yield a slightly faster rate of tyrosine phosphorylation relative to normal controls, full phosphorylation being reached at 15 min. In contrast, the levels of PYK2 expression in pOCs treated with Ad-PYK2/AS were reduced by 60% as quantitated by densitometry (data not shown). Consequently, tyrosine phosphorylated PYK2 was also reduced (Fig. 5B, upper panel). Tyrosine phosphorylation levels of c-Src in these cells were not different in pOCs transfected with wild type PYK2 or PYK2 antisense (Fig. 5B, lower panel). Nor did we find a significant change in c-Src activity in these cells, as determined by in vitro kinase assays (data not shown).

On the other hand, the tyrosine phosphorylation levels of p130Cas were greatly reduced in pOCs treated with Ad-PYK2/AS, as compared with controls or to pOCs infected with Ad-PYK2/wt (Fig. 5C, upper panel). The kinetics of p130Cas tyrosine phosphorylation were also faster in pOCs overexpressing wild type PYK2 (maximal phosphorylation at 15 min). This is consistent with increased spreading of these cells on VN. Taken together, these observations suggest that PYK2 might serve as a rate-limiting signaling molecule on the path to the cytoskeletal organization required for osteoclast adhesion and spreading.

We also examined the tyrosine phosphorylation of paxillin under the same conditions. Although paxillin appeared to be phosphorylated normally at 15 and 30 min in pOCs infected with Ad-PYK2/AS, we consistently observed diminished levels of paxillin phosphorylation at 60 min relative to control cells or cells infected with Ad-PYK2/wt (Fig. 5C, lower panel).

Adenovirus Expressing Antisense PYK2 Inhibits Ring Formation in Osteoclast-like Cells—Because PYK2 appears to play a role in pOC spreading, we analyzed the effect of antisense infected with either Ad-PYK2/wt or Ad-PYK2/AS. To examine the planar area of cell spreading, pOCs were allowed to adhere and spread on VN-coated surfaces for 1 h, fixed, and quantitated for cell area (A) and stained for TRAP activity (B), as described under “Materials and Methods.” Each condition was quantitated from quadruplicate samples. The results were expressed as the means ± S.D. The arrowhead points to a TRAP(−) cell spreading on VN. C, pOCs were isolated from uninfected cultures (panels a and b) or cultures infected with either Ad-PYK2/wt (panels c and d) or Ad-PYK2/AS (panels e and f) for 4 days. Cells were then allowed to attach to VN-coated glass coverslips for 60 min as described above, followed by staining for F-actin using Oregon-green phalloidin (panels a, c, and e) and stained for DNA using Hoechst 33342 (panels b, d, and f). Noted that nuclei in pOCs expressing PYK2 AS are in different plane of focus (arrowhead), because these cells have thick rounding cell bodies. Apoptotic cells were carefully examined for nuclear condensation and fragmentation (arrow). Bars, 10 μm.

FIG. 4. Adenovirus expressing PYK2 antisense inhibits fusion osteoclast-like cell spreading without affecting apoptosis. pOCs were purified from control co-cultures (cont.) or from cultures
PYK2 on the organization of actin rings in osteoclasts. OCLs were previously shown to adhere to glass or plastic surfaces via an actin-rich ring structure (24). OCLs treated with Ad-PYK2/wt or Ad-PYK2/AS were plated on glass coverslips and costained for F-actin and TRAP activity. As shown in Fig. 6, OCLs expressing wild-type PYK2 formed actin rings similar to control OCLs. On the other hand, the multinucleated TRAP(+) cells isolated from the cocultures infected with Ad-PYK2/AS adhered but did not spread to form actin rings. We then plated the same number of OCLs on bone slices in the presence of the osteoblastic MB1.8 cells to investigate distribution of microfilaments on bone. After 24 h, the cells were fixed, and we analyzed the organization of the sealing zone in these cells (Fig. 7). OCLs infected with Ad-PYK2/AS attached to bone surfaces via randomly distributed adhesion contacts and appeared to have a retracted morphology (Fig. 7, panels a, b, and g–l) by comparison with the normal actin-rich sealing zone, formed by normal OCLs (Fig. 7, panel c) or cells infected with Ad-PYK2/wt during resorption (Fig. 7, panel d). These cells formed small, punctated, and disorganized ring structures visualized by staining for F-actin (Fig. 7, panels g and j) and paxillin (Fig. 7, panel k). We previously reported that αvβ3 integrins preferentially distribute to the basal plasma membrane of osteoclasts during resorption (3). Similarly, localization of αvβ3 integrins in OCLs infected with Ad-PYK2/wt was normally distributed to the basal membrane (Fig. 7, panel e). Here, we found that αvβ3 integrins appear to cluster into small punctated structures on the basal plasma membrane in OCLs expressing antisense PYK2 (Fig. 7, panel h) and partially colocalize with F-actin (Fig. 7, panel i).

**Adenovirus Expression of Antisense PYK2 Inhibits Osteoclastic Bone Resorption**—Because OCLs expressing antisense PYK2 do not form normal sealing zones on bone (Fig. 7), we assumed that OCLs with diminished PYK2 expression will not efficiently resorb bone. The same number of OCLs, either untreated or treated with Ad-PYK2/wt or Ad-PYK2/AS, were plated on dentine slices, and the area of resorption pits made by these cells was determined after 24 h. As shown in Fig. 8, pit forming activity was inhibited by 90% in OCLs expressing antisense PYK2. This finding suggests that PYK2 mediates the cytoskeletal organization required for osteoclastic polarization and active bone resorption.

**DISCUSSION**

This study reports the effective use of a replication-defective recombinant adenovirus as a vector for expressing PYK2-wt and AS in osteoclasts and the inhibition of osteoclast adhesion, spreading, and function by PYK2 AS. Osteoclasts are terminally differentiated cells that have been notoriously difficult to transfect using conventional methods. The advantage of recombinant adenovirus is that it normally has a high efficiency of gene transfer and can infect nonreplicating cells (27). In addition, adenoviruses enter cells by means of αvβ3 or αvβ5 integrins, which serve as receptors for the adenoviral penton (30). We have previously shown that expression of αvβ3 integrins in pOCs is induced around day 3 or 4 of the osteoclast-generating coculture system (31). Consistent with this observation, we found, using β-galactosidase expressing virus, that adenovirus efficiently mediated gene transfer into osteoclasts around day 3 of the coculture. Although osteoblastic MB1.8 cells express αvβ5 integrins (31), we did not observe significant osteoblast...
infecitivity with this adenoviral vector at low virus concentration (MOI = 10); however, at higher virus concentrations (MOI = 100), a few MB1.8 cells were infected (data not shown). Thus, an MOI = 10 the adenovirus vector appears to mediate in our co-culture system gene transfer preferentially into OCLs, in agreement with previous reports on the use of recombinant adenovirus to transfer epidermal growth factor receptor and Csk into osteoclast-like cells (28, 32).

Despite its structural similarity to FAK, PYK2 appears to have different or additional cellular roles. In several cell types including fibroblasts, epithelial, and neuronal cells, PYK2 seems to integrate adhesion-dependent signaling events with MAP kinase activation (16, 33, 34), with stress-induced c-Jun N-terminal kinase activation (35, 36), or with Src-mediated MAP kinase activation (37). PYK2 is also activated by various stimuli that elevate the intracellular calcium concentration via the nicotine acetylcholine receptor, the voltage-gated calcium channels, the G protein-coupled receptors, e.g. bradykinin and angiotensin II, or via stimuli that promote calcium release from intracellular stores (37, 38). Although FAK was shown to promote cell survival, PYK2 overexpression induced apoptosis in fibroblasts (15). However, in thymocytes, where PYK2 is physically associated with Janus tyrosine kinase 1 and participates in interleukin-7-dependent signaling, PYK2 antisense-induced apoptosis (39).

Most information on PYK2 function has been obtained by overexpressing PYK2 and its mutants in cells, which also express FAK. It is possible that overexpression of PYK2 and its mutants could have disrupted, in a dominant negative fashion, not only PYK2-mediated but FAK-mediated signaling pathways as well. Targeted disruption of FAK in mice results in embryonic lethality (40), a reflection of its broad cellular functions (11), whereas PYK2 knockout mice seem to develop normally. Thus far, the only phenotype identified in PYK2 null mice was mild osteopetrosis without apparent reduction of osteoclastogenesis. PYK2 expression was elevated in FAK-deficient fibroblasts but did not rescue the cell migration defects caused by FAK deletion, nor did PYK2 localize to focal adhesion contacts in these cells (33, 40). Furthermore, tyrosine phosphorylation of wild type PYK2 expressed in Chinese hamster ovary cells was not altered by plating the cells on fibronectin. On the other hand, chimeric PYK2, which contained FAK C-terminal domain, exhibited enhanced tyrosine phosphorylation and localization to focal adhesion contacts upon attachment to fibronectin (41). These observations indicate that localization of PYK2 to sites of integrin receptor clustering seems to play an important role in the adhesion-dependent activation of this protein-tyrosine kinase.

In this study, we directly examined the role of PYK2 in αβ3 integrin-dependent regulation of osteoclastic cytoskeletal organization and bone resorption. We previously found that in contrast to fibroblasts, osteoclasts and osteoclast-like cells expressing PYK2 antisense inhibit sealing zone formation in osteoclasts during polarization on bone. Localization of microfilaments and αβ3 integrins in uninfected and OCLs infected with Ad-PYK2/AS or Ad-PYK2/wt were examined after plating on bovine cortical bone slices for 20 h at 37 °C. Pseudocolored confocal microscopic images of osteoclasts infected with Ad-PYK2/AS double stained with anti-adenoviral penton proteins (panel a, red) and F-actin (panels a and b, green) as compared with F-actin localized in a typically sealing zone at the bone surface in normal OCLs (panel c, green) or in cells infected with Ad-PYK2/wt (panel d, green). OCLs infected with Ad-PYK2/wt (panels d–f) showed a sealing zone (panel d), localization of αβ3 integrins at the basal membrane (panel e, red) and an overlay image of F-actin and αβ3 integrations (panel f). The cells expressing PYK2/AS were also double stained for F-actin (panels g and j, green) and αβ3 integrins (panel h, red) or paxillin (panel k, red). Colocalization is seen as yellow in overlay images (panels i and l), respectively. Note small actin rings or aggregation of F-actin at bone surface in osteoclasts expressing PYK2/AS (arrows in panels b, g, and j) by comparison with well defined F-actin rings in an osteoclast expressing Ad-PYK2/wt (panel d) or in wild type osteoclasts (panel c). αβ3 integrins do not colocalize with actin at the bone surface, whereas paxillin and F-actin colocalize in podosome-containing rings at bone surface (arrows in panels j–l). On the other hand, αβ3 integrins colocalize with actin in punctated structures at the basal membrane (arrowheads in panels g–i). Images merged from optical sections from 4.5 μm (panel a and b), 10.5 μm (panel c), 5.4 μm (panels d–f), 6.8 μm (panels g–i), and 5.8 μm (panels j–l) thickness are shown. Bars, 10 μm.

Fig. 7. Adenovirus expressing PYK2 antisense inhibits sealing zone formation in osteoclasts during polarization on bone. Localization of microfilaments and αβ3 integrins in uninfected and OCLs infected with Ad-PYK2/AS or Ad-PYK2/wt were examined after plating on bovine cortical bone slices for 20 h at 37 °C. Pseudocolored confocal microscopic images of osteoclasts infected with Ad-PYK2/AS double stained with anti-adenoviral penton proteins (panel a, red) and F-actin (panels a and b, green) as compared with F-actin localized in a typically sealing zone at the bone surface in normal OCLs (panel c, green) or in cells infected with Ad-PYK2/wt (panel d, green). OCLs infected with Ad-PYK2/wt (panels d–f) showed a sealing zone (panel d), localization of αβ3 integrins at the basal membrane (panel e, red) and an overlay image of F-actin and αβ3 integrations (panel f). The cells expressing PYK2/AS were also double stained for F-actin (panels g and j, green) and αβ3 integrins (panel h, red) or paxillin (panel k, red). Colocalization is seen as yellow in overlay images (panels i and l), respectively. Note small actin rings or aggregation of F-actin at bone surface in osteoclasts expressing PYK2/AS (arrows in panels b, g, and j) by comparison with well defined F-actin rings in an osteoclast expressing Ad-PYK2/wt (panel d) or in wild type osteoclasts (panel c). αβ3 integrins do not colocalize with actin at the bone surface, whereas paxillin and F-actin colocalize in podosome-containing rings at bone surface (arrows in panels j–l). On the other hand, αβ3 integrins colocalize with actin in punctated structures at the basal membrane (arrowheads in panels g–i). Images merged from optical sections from 4.5 μm (panel a and b), 10.5 μm (panel c), 5.4 μm (panels d–f), 6.8 μm (panels g–i), and 5.8 μm (panels j–l) thickness are shown. Bars, 10 μm.

Fig. 8. Adenovirus expressing PYK2 antisense inhibits bone resorption. Aliquots (20,000 cells/slice) of uninfected (cont) or infected pOClS with either Ad-PYK2/wt (wt) or Ad-PYK2/AS (AS) were plated on dentine slices for 24 h at 37 °C. Resorption pit area were measured using an image analyzing system. The results were expressed as the means ± S.D. (n = 5) of resorbed area per whole dentine surface area.
press high levels of PYK2 both in vivo and in culture and very little FAK protein (17). FAK-independent integrin-stimulated signaling events have also been demonstrated in other hemato-poietic cells, where FAK protein expression is low (42–44). PYK2 acted in osteoclasts as a cell adhesion-dependent kinase, being rapidly tyrosine phosphorylated upon ligand engagement of αβ3 integrins (17). In addition, PYK2 localizes to podosomal adhesion contacts of migrating osteoclasts (17). Furthermore, the involvement of PYK2 in the cytoskeletal organization associated with osteoclastic bone resorption is consistent with PYK2 localization in the sealing zone structures, a prerequisite for cell polarization (17).

Tyrosine phosphorylation of PYK2 is severely reduced in Src-deficient osteoclasts (17, 29), which are functionally compromised. Osteoclasts express high levels of c-Src and low levels of other Src family kinases (45), and Src deficiency is associated with osteopetrosis in mice. Loss of osteoclast function was shown to be due partly to defective polarization, leading to lack of ruffled border formation (21, 46). Furthermore, targeted disruption of β3 integrin in mice induces progressive osteoclasts, without an apparent reduction in osteoclast number (22). Here, we demonstrated that similar to Src deficiency, significant reduction in PYK2 expression in osteoclasts in vitro interfered with the cytoskeletal organization, required for formation of the osteoclast sealing zone on the bone surface. Expression levels of αβ3 integrins and of PYK2 were not altered in Src-deficient osteoclasts (17, 47). Similarly, we show here that the expression of integrin receptors, c-Src, and other PYK2-associated molecules such as p130Cas and paxillin were not changed in osteoclasts expressing PYK2 AS.

We further examined the effect of PYK2 deficiency on known αβ3 integrin-mediated signaling events in osteoclasts. Our previous findings suggested that the adhesion-dependent increase in PYK2 tyrosine phosphorylation in osteoclasts may occur downstream of αβ3 integrin-dependent Src activation (17). The findings of this study are consistent with this interpretation, because the adhesion-dependent tyrosine phosphorylation of c-Src was unaffected by PYK2 antisense expression in osteoclasts. We also found that the in vitro kinase activity of c-Src was not altered in these cells (data not shown). On the other hand, we have shown that tyrosine phosphorylated p130Cas is part of and probably involved in the organization of the podosome-rich ring structure in osteoclasts (24, 29). Similar to PYK2, p130Cas phosphorylation was also markedly reduced in osteoclasts derived from Src(-/-) mice (24). Moreover, PYK2 and p130Cas form a stable complex in osteoclasts. This complex is independent of tyrosine phosphorylation and is present in Src(-/-) osteoclasts, in which neither protein is phosphorylated or associated with the osteoclast adhesion structure (29). Here, we found that suppression of PYK2 expression reduced substantially p130Cas tyrosine phosphorylation in osteoclasts, suggesting that PYK2 might indeed directly phosphorylate p130Cas or serve as an adaptor molecule for recruiting Src kinases to p130Cas. This is consistent with p130Cas being a substrate for PYK2, as well as for Src kinase (48, 49). We and others have shown that integrin-mediated PYK2 activation leads to p130Cas tyrosine phosphorylation in a Src-dependent manner (29, 50), and PYK2 is constitutively associated with p130Cas in osteoclasts (29).

Our observations strongly suggested that PYK2 play a role in osteoclastic adhesion and spreading; we examined the effect of PYK2 antisense on osteoclast apoptosis. Because we could not assess osteoclast apoptosis in the co-culture systems where osteoclast formation and survival were influenced by various growth factors, cytokines, and extracellular matrix proteins derived from the osteoblastic/stromal cells, we examine apoptotic events in osteoclast precursors isolated from co-cultures uninfected and infected for 4 days (25). Under serum-free conditions, we could not detect a significant increase in the number of apoptotic pOCs expressing PYK2 antisense. However, we could not rule out the possibility that apoptotic cells could be lost during the long term co-cultures, because we observed a significant reduction in TRAP(+) cells in cultures infected 4 days earlier with high concentration of Ad-PYK2/AS. We routinely isolated pOCs infected with Ad-PYK2/AS that had 50–70% lower PYK2 protein levels. It is possible that osteoclasts with even lower levels of PYK2 expression could be detached and undergo anoikis in culture. A possible role for PYK2 in adhesion-dependent signaling that mediated osteoclast survival in vitro, will be the subject of future studies. On the other hand, targeted disruption of β3 integrin, c-Src, or PYK2 in mice were reported to develop osteopetrosis without a reduction in osteoclast number, suggesting that β3 integrin-mediated signaling might not play an important role in osteoclast survival in vivo (21, 22).

In summary, consistent with our previous findings suggesting that PYK2 serves as an adhesion-dependent kinase involved in the regulation of bone resorption, we demonstrate using adenovirus expressing PYK2 antisense that PYK2 is essential for integrin-mediated cell spreading and actin ring formation in osteoclasts. PYK2 also plays a central role in the cytoskeletal organization necessary for forming the osteoclast sealing zone during bone resorption. Taken together these observations suggest that, similar to αβ3 integrin and to its upstream regulator c-Src, PYK2 plays a rate-limiting role in osteoclastic bone resorption.

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