Stable Association of PYK2 and p130Cas in Osteoclasts and Their Co-localization in the Sealing Zone*

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Bone resorption is initiated by osteoclast attachment to the mineralized matrix, cytoskeletal reorganization, cellular polarization, and the formation of the sealing zone. The present study examines the interaction between PYK2 and p130Cas (Crk-associated substrate), suggested to be a part of the signaling pathway initiated by osteoclast adhesion. Using murine osteoclast-like cells (OCLs) and their mononuclear precursors (pOCs), generated in a co-culture of bone marrow and osteoblastic MB1.8 cells, we show that: 1) p130Cas is tyrosine-phosphorylated upon adhesion of pOCs to vitronectin or ligation of β3 integrins; 2) p130Cas colocalizes with PYK2 and the cytoskeletal proteins F-actin, vinculin, and paxillin in the podosomal-rich ring-like structures of OCLs plated on glass and in the sealing zone in actively resorbing OCLs on bone; 3) p130Cas and PYK2 form a stable complex in pOCs, independent of tyrosine phosphorylation of either molecule, and this complex is present in Src (−/−) OCLs, in which neither protein is phosphorylated or associated with the osteoclast adhesion structure; 4) the association of p130Cas and PYK2 is mediated by the SH3 domain of p130Cas and the C-terminal domain of PYK2. These findings suggest that p130Cas and its association with PYK2 may play an important role in the adhesion-dependent signaling that leads to cytoskeletal reorganization and formation of the sealing zone during osteoclast activation.

Adhesion of cells to the extracellular matrix (ECM) initiates signaling pathways that lead to cellular spreading and migration as well as to modulation of growth and differentiation. Integrins belong to a major class of adhesion receptors that mediate these cellular functions. Integrin engagement induces a cascade of tyrosine phosphorylation and the recruitment of structural and signaling molecules to multimeric complexes associated with the actin cytoskeleton, called focal adhesion contacts (1, 2). Proline-rich tyrosine kinase 2 (PYK2, also known as RAFTK and CASKβ) (3–5) is related to FAK, known to play an important role in cell adhesion (6). Similar to FAK, PYK2 lacks a transmembrane region and SH2 and SH3 domains but has two proline-rich regions in its C terminus. PYK2 is highly expressed in brain and various hematopoietic cells (3). 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 JNK signaling pathway (4, 7, 8). In addition, stimulation of G-protein-coupled receptors induces tyrosine phosphorylation of PYK2 and complex formation between PYK2 and Src via the SH2 domain of Src, leading to activation of the MAP kinase signaling pathway (9). Similar to FAK, PYK2 is tyrosine-phosphorylated and is activated by adhesion-mediated signaling in platelets and B cells (10, 11). In addition, PYK2 interacts with and phosphorylates the focal adhesion-associated protein paxillin in vitro (12). PYK2 is thus suggested to participate in the transfer of signals from the cell surface to the cytoskeleton.

p130Cas was first identified as a major tyrosine-phosphorylated protein in v-src and v-crk transformed cells (13, 14). p130Cas contains an N-terminal SH3 domain, a substrate domain, a proline-rich region, and a C-terminal domain with multiple tyrosine residues (14). This unique structure suggested that p130Cas may serve as a docking protein for multiple SH2 and SH3 domain-containing molecules. In addition, p130Cas is tyrosine-phosphorylated during integrin-mediated adhesion and localizes to focal adhesions in fibroblasts (15–17). p130Cas was shown to bind to FAK both in vitro and in vivo (15, 18). Integrin-dependent p130Cas phosphorylation was absent in c-Src deficient fibroblasts (19). Recently, PYK2 was shown to be tyrosine phosphorylated and associated with p130Cas upon B cell adhesion to fibronectin and stimulation of the antigen receptor (10).

Osteoclasts are highly differentiated bone resorbing cells. Osteoclast activation is initiated by adhesion to bone matrix and formation of the sealing zone, a specialized adhesion structure responsible for the tight attachment of osteoclasts to mineralized bone matrix (20, 21). We found that PYK2 is highly expressed in osteoclasts and is tyrosine-phosphorylated upon integrin-mediated adhesion and is activated by osteoblastic MB1.8 cells, which are essential for osteoclast function in vitro (22, 23). Furthermore, PYK2 localizes to podosomes, the primary adhesion structures in osteoclasts which become the sealing zone in actively resorbing osteoclasts (22). We also showed that tyrosine phosphorylation of p130Cas is involved in the organization of the podosome-rich ring structures in osteoclasts (24). Tyrosine phosphorylation of p130Cas was markedly reduced in osteoclasts derived from Src(−/−) mice, in which
osteoclast activity is severely compromised (24).

In this study, we demonstrate that p130Cas participates in adhesion-mediated signaling in osteoclasts. p130Cas is tyrosine-phosphorylated upon β3-integrin engagement by ligand binding or antibody-induced clustering. In osteoclasts, p130Cas is stably associated with PYK2, via the SH3 domain of p130Cas and the C-terminal domain of PYK2, independent of tyrosine phosphorylation. Furthermore, p130Cas co-localizes with PYK2 in the sealing zone of resorbing osteoclasts on bone. These findings suggest that the engagement of β3-integrin initiates the activation of the p130Cas-PYK2 complex which plays a role in osteoclast activation.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Fibronectin was from NY Blood Center (New York, NY); vitronectin and laminin were from Life Technologies, Inc.; and collagen was from Collaborative Biomedical Products (Bedford, MA). Anti-PYK2 and anti-p130Cas polyclonal antibodies were developed as described (15, 22). Anti-N-domain of PYK2 antibodies was from Sigma. Anti-β3 (mAb 18/2) was from ATCC (Manassas, VA), and anti-β1 (mAb 9E7G7) and anti-β2 (mAb 2C9.G2) integrins were from PharMingen (San Diego, CA). Horseradish peroxidase-conjugated anti-phosphotyrosine antibodies were from Transduction Labs and Upstate Biotechnology. Other conjugated secondary antibodies were from Jackson Laboratories (Bar Harbor, ME), and Sigma. Collagenase was from Wako Chemicals U.S.A., Inc. (Dallas, TX), and dispase was from Boehringer-Mannheim. 1α,25-Dihydroxyvitamin D3 (1α,25(OH)2D3) was a gift from Dr. M. Uskokovic, Hoffmann-LaRoche (Nutley, NJ). GST fusion proteins of Fyn, Lyn, and P13-kinase were from PharMingen and Santa Cruz Biotechnology. GST-fusion proteins of c-Src, PYK2, and p130Cas were generated as described (15, 22).

**Animals**—Heterozygote Src(+/-) mice were obtained from Jackson Laboratory (Bar Harbor, ME), and Src(-/-) mice were phenotypically distinguished from their Src(+/-) siblings by lack of tooth eruption. All animals were cared for according to Institutional Animal Care and Use Committee (IACUC) Guide.

**Cell Cultures**—Prefusion osteoclasts (pOCs) were prepared as described previously (22, 23). Briefly, mouse bone marrow cells were co-cultured with osteoblastic MB1.8 cells for 5–6 days in the presence of 10 nm 1α,25(OH)2D3. pOCs were released from dishes with 10 mM EDTA after removing MB1.8 cells with collagenase-dispase. Alternatively, the co-cultures were kept for 7–8 days to achieve multinucleated osteoclast-like cells (OCLs) and purified as described (22). Src(-/-) and Src(+/-) OCLs were obtained from co-cultures of MB1.8 cells and spleen cells isolated from 2–3 wk old Src(-/-) or their normal littermates as described above.

**Cell Adhesion**—After isolation, pOCs (10⁵ cells/plate) were washed twice with serum-free α-minimal essential medium containing 0.1% bovine serum albumin (Sigma) and kept in suspension or allowed to

**Fig. 1. Expression of p130Cas and PYK2 in osteoclasts and tyrosine phosphorylation of p130Cas in pOCs on various matrices.** A, Western blot analysis for p130Cas (top panel) and PYK2 (bottom panel) in cell lysates (6 μg) from IC21 macrophages, isolated prefusion osteoclasts (pOC), and multinucleated osteoclast-like cells (OCL) was carried out as described under “Experimental Procedures.” B, pOCs were allowed to attach to plates coated with serum, vitronectin (VN), osteopontin (OPN), fibronectin (FN), type I collagen (Col I), type IV collagen (Col IV), or laminin (LN) for 1 h. Cell lysates were subjected to p130Cas immunoprecipitation, followed by blotting for phosphotyrosine (PY) and p130Cas, as described under “Experimental Procedures.”

**Fig. 2. Tyrosine phosphorylation of p130Cas correlates with pOC adhesion and spreading.** A, pOCs were kept in suspension or allowed to attach to vitronectin-coated plates in the absence of serum at 37 °C for indicated times, 5–60 min. Cell lysates were subjected to p130Cas immunoprecipitation, followed by blotting for phosphotyrosine (PY) and p130Cas, as described under “Experimental Procedures.” B and C, in the parallel cultures, pOCs were fixed and stained for TRAP at the indicated times, and the number and area of attached TRAP(+) cells were quantitated as described under “Experimental Procedures.” Data are expressed as the means ± S.E. of four fields in panel B and as the means ± S.E. of more than 300 pOCs in panel C.
attach to polystyrene dishes, coated with ECM proteins (fetal bovine serum, fibronectin (25 μg/ml), vitronectin (10 μg/ml), osteopontin (50 μg/ml), laminin (25 μg/ml), type I or type IV collagen (25 μg/ml)). After 5–60 min at 37 °C, an equal volume of 2× TNE lysis buffer (20 mM Tris, pH 7.8, 300 mM NaCl, 2 mM EDTA, 2% Nonidet P-40, 2 mM NaVO₃, 20 mM NaF, 20 μg/ml leupeptin, 1 trypsin inhibitory units/ml aprotinin and 2 mM phenylmethanesulfonyl fluoride) was added to the plates. Clarified lysates were subjected to immunoprecipitation and -blotting. Alternatively, pOCs were allowed to attach to vitronectin-coated plates in serum-free medium for the indicated times and then fixed and stained for TRAP as described (24). Numbers and area of attached pOCs were measured using the Empire Imaging Analyzer System (Milford, NJ). Results are expressed as the means (± S.E.) of four fields for the number of attached cells and of more than 300 cells for the area of pOCs.

Immunoblotting and Immunoprecipitation—Lysates were separated on a 4–20% gradient or 8% SDS-PAGE (Novex, San Diego, CA) and electrotransferred to Immobilon-P membrane (Millipore, Bedford, MA). Lysates were precleared with Sepharose-4B and precipitated with anti-p130Cas, anti-vinculin and anti-paxillin antibodies, followed with the appropriate secondary antibodies, or with FITC- or rhodamine-conjugated phallolidin. Immunofluorescent stainings were viewed with a fluorescence microscope or with a confocal laser scanning microscope (Leica, Heidelberg, Germany), equipped with a multilinie Omnichron argon-crypton laser (Chino, California) (26).

RESULTS

Adhesion-dependent Tyrosine Phosphorylation of p130Cas—Expression of p130Cas and PYK2 in osteoclasts was examined using Western blot analysis. Both pOCs and multinucleated OCLs express significant levels of p130Cas and PYK2 similar to that of IC-21 macrophages (Fig. 1A). We have previously found that the osteoblastic MB1.8 cells do not express detectable...
levels of PYK2 (22).

The effect of adhesion on p130 Cas tyrosine phosphorylation was examined in isolated pOCs, which can be reseeded on various ECM proteins in the absence of serum. Attachment to vitronectin, osteopontin, fibronectin, and serum stimulated p130Cas tyrosine phosphorylation, whereas lower levels of tyrosine phosphorylation were detected in pOCs adhering to type I or type IV collagen, or to laminin (Fig. 1B). Relative to pOCs maintained in suspension, p130 Cas was tyrosine-phosphorylated in a time-dependent manner upon attachment to vitronectin. An increase in p130 Cas tyrosine phosphorylation was detected within 5 min of seeding and reached the maximum around 30 min (Fig. 2A). Interestingly, pOC attachment to vitronectin-coated plates appears to precede the peak phosphorylation of p130Cas (Fig. 1B), suggesting that tyrosine phosphorylation of p130Cas may play a role in the cytoskeletal organization of osteoclast precursors upon adhesion. Furthermore, tyrosine phosphorylation parallels to the time course of osteoclast spreading (Fig. 2C).

β3-Integrin Clustering Induces Tyrosine Phosphorylation of p130Cas—Matrix preferences for inducing p130Cas tyrosine phosphorylation suggested a selective integrin-mediated phenomenon. Osteoclasts express αvβ3, αvβ1, and αvβ2-integrins (26–29). Immature osteoclast precursors were shown to express β3- and β5-integrins (30, 31). Integrin involvement and specificity for p130Cas tyrosine phosphorylation was examined by antibody-mediated clustering of integrins in pOCs in suspension. Clustering with anti–β3-antibody strongly induced p130Cas tyrosine phosphorylation, whereas clustering with anti-β1- or anti-β2-antibody induced low levels of p130Cas phosphorylation in pOCs (Fig. 3), indicating that the vitronectin receptor αvβ3 is the major integrin responsible for this effect. Clustering of β5-integrin was not examined in this study because antibodies that recognize the extracellular domain of the murine β5-integrin are not available.

Localization of p130Cas in Podosomes and in the Sealing Zone of Osteoclasts and Co-localization with PYK2—Because β3-mediated adhesion is thought to be involved in osteoclast activation, the subcellular localization of p130Cas was examined in OCLs plated on glass coverslips and on bone. On glass coverslips, p130Cas was localized in podosomes (Fig. 4) and, along with F-actin, organized in a typical podosomal-rich adhesion structure at the periphery of OCLs (24). Double staining of p130Cas and PYK2 in OCLs on glass showed that p130Cas also co-localized with PYK2 (Fig. 4). On mineralized bone matrix, osteoclasts polarize and form the sealing zone that circumscribes the resorptive ruffled border membrane, where protons and proteases are secreted. Sealing zone formation can be followed using vinculin immunostaining to visualize the transition from small rings in podosomes to a double circle structure in the mature sealing zone (21). Similarly, on bone, p130Cas localized in OCLs to podosomes and to the nascent...
sealing zone, as well as to the double circle structure in the mature sealing zone (Fig. 5A). p130Cas also co-localized with the cytoskeletal proteins F-actin, vinculin, and paxillin (Fig. 6). The distribution of paxillin (Fig. 6h), shown here for the first time in osteoclasts on bone, follows the staining pattern of vinculin. However, in resorbing OCLs, p130Cas also co-localized with PYK2 in the nascent sealing zone, as well as in the mature sealing zone (Fig. 5B). This morphological evidence for the co-localization of p130Cas and PYK2 in osteoclasts in vivo supports a role for p130Cas in the morphological changes associated with osteoclast activity.

Association of p130Cas with PYK2 in Osteoclasts—Because p130Cas colocalizes with PYK2 in the sealing zone of active osteoclasts, we investigated the interaction of the two proteins in these cells. Immunoprecipitation and immunoblotting experiments show association of p130Cas with PYK2 in pOCs in situ (Fig. 7). Furthermore, this association was present both in suspended and attached cells (Fig. 7), suggesting that it is independent of tyrosine phosphorylation.

c-Src Is Not Required for the Association of p130Cas and PYK2—This was confirmed in Src(−/−) osteoclasts. Src has been implicated in p130Cas and PYK2 tyrosine phosphorylation, and osteoclast function is severely compromised in Src(−/−) mice (9, 32). As observed previously, the level of tyrosine phosphorylation of PYK2 immunoprecipitated from Src(−/−) OCLs is markedly reduced, although the same level of PYK2 is expressed in Src(−/−) and Src(+/?) OCLs (Fig. 8) (22). Interestingly, a comparable level of p130Cas was immunoprecipitated with PYK2 from both Src(−/−) and Src(+/?) OCLs (Fig. 8, middle panel). We previously reported the expression of both p130Cas isoforms (Cas A and B) in Src(−/−) OCLs, as compared with the predominant expression of Cas B in Src(+/?) OCLs. Both isoforms of p130Cas appeared to associate with PYK2 in Src(−/−) OCL lysates (Fig. 7, middle panel). Furthermore, treatment of Src(+/?) OCLs with cytchalasin D for 20 min caused dephosphorylation of PYK2 and dissociation of the Src and PYK2 complex (Fig. 8, bottom panel) but had no significant effect on the association of p130Cas with PYK2 (Fig. 8, middle panel). These results indicate that the association of p130Cas and PYK2 is not dependent on an intact cytoskeleton or on c-Src function in osteoclasts.

Association of p130Cas and PYK2 Is Mediated by SH3 Domains of p130Cas and the C-terminal Domain of PYK2—To characterize the domains that mediate the association of p130Cas with PYK2, GST-fusion proteins encoding for SH3 domains from various signaling molecules, as well as the kinase and N- and C-terminal domains of PYK2 were incubated with lysates prepared from OCLs. GST-fusion protein encoding the SH3 domain of p130Cas binds to PYK2 from OCL lysates, whereas the GST-fusion proteins encoding the SH3 domains of Src, Lyn, Fyn, or PI3-kinase or GST alone do not bind to PYK2 in OCL lysates (Fig. 9A). On the other hand, the GST-fusion protein encoding the C-terminal domain of PYK2, but not the kinase or N-terminal domains or GST alone, interacts with p130Cas (Fig. 9B). Lysates from attached OCLs were used for this experiment, and the p130Cas that associated with the

Fig. 6. Co-localization of p130Cas with cytoskeletal proteins in OCLs on bone. Pseudocolored confocal images of double stainings of p130Cas (red, a, d, and g) with F-actin (b), vinculin (e), and paxillin (h) (green) in resorbing OCLs on bone. Colocalization is seen as yellow in overlaid images (c, f, and i). Note two osteoclasts in different phases of resorption cycle in p130Cas and vinculin double staining (d and e), both showing co-localization (f). Images merged from optical sections from 2.6 (a–c), 4.0 (d–f), and 1.8 (g–i) μm thickness close to the bone surface. Bars, 10 μm.
These findings suggest that both PYK2 and p130Cas may be osteoclasts plated on glass or on plastic culture dishes (24).

The adhesion-dependent tyrosine phosphorylation of p130Cas in osteoclasts is consistent with findings in other cell types although the kinetics are slightly different. In fibroblasts (17) and primary chicken embryo cells (15), maximal phosphorylation was seen at 15–20 min, whereas in osteoclasts, optimal phosphorylation is not reached until after 30 min, paralleling the time course of osteoclast spreading. The preferences for ECM proteins is also different, osteopontin, vitronectin, and fibronectin in osteoclasts rather than primarily fibronectin in fibroblasts. β3 seems to be the major integrin that mediates these effects in osteoclasts.

We further examined the localization of p130Cas in osteoclasts and found it to be present in podosomes, where it co-localizes with PYK2 and cytoskeletal proteins. More importantly, it colocalizes with PYK2 in the sealing zone. During bone resorption, osteoclast adhesion to bone matrix leads to reorganization of cytoskeletal structures and formation of the sealing zone, a tight attachment between the osteoclast plasma membrane and bone matrix, with specific organization of F-actin and associated proteins, such as vinculin and talin (21). Initial formation and accumulation of podosomes precedes these changes (21). In this study, we localized p130Cas by confocal microscopy in resorbing osteoclasts on bone and found it is present in the newly forming sealing zone and mainly at the edges (the double circle structure) of mature sealing zones. In these structures, p130Cas colocalizes with vinculin and paxillin and as mentioned with PYK2. Together with our recent results on PYK2 (22), these findings strongly suggest that p130Cas and p130Cas–PYK2 complex play a role in the cytoskeletal reorganization and formation of the sealing zone during osteoclast activation.

Furthermore, co-immunoprecipitation of p130Cas and PYK2 in osteoclasts shows stable association of the two proteins. This resembles the association of p130Cas with FAK (15, 18) and is consistent with recent findings in B cells (10). Using GST-fusion proteins, this association was found to be mediated by the SH3 domain of p130Cas and the C-terminal domain of PYK2, similar to the association of p130Cas with FAK (15). This is in agreement with recent findings by Ohba et al. (39), showing that the first proline-rich sequence in the C-terminal domain of PYK2 can bind SH3-domain of p130Cas. Interestingly, SH3 domains of Src, Fyn, Lyn, or PI3-kinase did not bind to or antibody cross-linking. However, whereas PYK2 appears to be selectively tyrosine-phosphorylated upon clustering of the β3-integrin (22), p130Cas tyrosine phosphorylation is also induced, albeit to a lower level by other osteoclast integrins including β1 and β2-integrins.

C-terminal domain of PYK2 was tyrosine-phosphorylated, suggesting that tyrosine-phosphorylated p130Cas can associate with PYK2.

**DISCUSSION**

Bone resoring osteoclasts are highly differentiated cells specialized in the digestion of mineralized matrix. Osteoclast activation is initiated by recognition of and adhesion to the bone surface, followed by cellular polarization and formation of the sealing zone, a specialized membrane structure mediating tight adhesion between the osteoclast cell membrane and the bone surface. It is well documented from pharmacological studies that αvβ3, the major integrin in osteoclasts, is important for osteoclast function (27, 33, 34). αvβ3 mediates osteoclast adhesion to several RGD matrix proteins including vitronectin, osteopontin, bone sialoprotein, and fibronectin (35). Although αvβ3 localizes to podosomes, the initial adhesion structure in osteoclasts (26, 36), the presence of this integrin in the mature sealing zone in resorbing osteoclasts is controversial (26, 34, 37, 38). The signaling mechanisms regulating the adhesion-dependent activation of osteoclasts are not well understood. We have recently found that PYK2 is the major adhesion kinase in osteoclasts, which undergoes tyrosine phosphorylation upon β3-integrin engagement and that localizes to the sealing zone (22).

Similarly, p130Cas tyrosine phosphorylation correlates with the formation of the podosomal-rich adhesion structures in osteoclasts plated on glass or on plastic culture dishes (24). These findings suggest that both PYK2 and p130Cas may be involved in the adhesion-dependent signaling that mediates cytoskeletal organization in osteoclasts. This is further supported by the present study which shows that both p130Cas and PYK2 are tyrosine-phosphorylated upon pOC adhesion to the same ECM proteins and with a similar time course. In addition, p130Cas, similar to PYK2, is highly tyrosine-phosphorylated in pOCs upon β3-integrin clustering by either ligand engagement or antibody cross-linking. However, whereas PYK2 appears to be selectively tyrosine-phosphorylated upon clustering of the β3-integrin (22), p130Cas tyrosine phosphorylation is also induced, albeit to a lower level by other osteoclast integrins including β1 and β2-integrins.
GST-fusion proteins (10 μg) containing the SH3 domain of p130Cas and the C-terminal domain of PYK2. The kinase (PYK2, followed by incubation with glutathione-Sepharose beads for photyrosine (phorylation of p130 Cas and PYK2 are not necessary for the downstream signaling events initiated by p130Cas.

Tyrosine phosphorylation of the 26 fragment of c-Src which includes its SH3 and SH2 domains was not readily detected in lysates from Src(−/−) fibroblasts. In addition, both PYK2 and c-Src association of these two molecules.

phosphorylation by c-Src was not readily detected in lysates from Src(−/−) fibroblasts. In addition, both PYK2 and c-Src (24) are markedly reduced, but p130 Cas still associates with PYK2 in Osteoclasts. These results suggest that tyrosine phosphorylation of p130Cas and PYK2 in osteoclasts for the integrin-mediated activation of the FAK interaction (40). Our results indicate that in osteoclasts p130Cas and PYK2 associate in the absence of c-Src but do not localize to the podosomes and ring-like structures (22, 24).

In osteoclasts, c-Src is highly expressed and is essential for osteoclast function (32). Src-deficient mice have an osteoporotic phenotype caused by nonfunctional osteoclasts (32). The majority of c-Src is localized in the osteoclast ruffled border and in vacuoles rather than in the sealing zone (41, 42), suggesting that the interaction of c-Src and the p130Cas-PYK2 complex precedes formation of the actin ring and sealing zone. However, the direct involvement of c-Src in this process is clearly indicated by our recent findings showing adhesion-induced association of PYK2 with c-Src in osteoclasts (22) and PYK2 phosphorylation by c-Src in vitro. In addition, both PYK2 and c-Src were shown to translocate to the cytoskeleton upon osteoclast adhesion (22, 43), pointing to Src interaction with PYK2 in vivo. Furthermore, integrin-mediated p130Cas tyrosine phosphorylation is substantially diminished in both Src-deficient fibroblasts and osteoclasts (19, 24). In addition, c-Src was shown to bind directly to p130Cas (44), suggesting that p130Cas could be a substrate of c-Src in vivo. In human B cells, PYK2 phosphorylates p130Cas following β2-mediated stimulation (45). Taken together, these observations suggest that cooperation between PYK2 and Src kinases may be required for the complete phosphorylation of p130Cas, which is probably involved in downstream signaling.

In summary, the findings presented here show adhesion- and β2-integrin-mediated, Src-dependent tyrosine phosphorylation of p130Cas in osteoclasts with similar kinetics as recently shown for PYK2. Furthermore, p130Cas and PYK2 are stably associated via the SH3 domain of p130Cas and the proline-rich C-terminal domain of PYK2, independent of tyrosine phosphorylation or c-Src. Finally, p130Cas localizes to podosomes and the sealing zone, the functionally important adhesion structures in osteoclasts, where it co-localizes with PYK2. Taken together these findings suggest a role for p130Cas and the p130Cas-PYK2 complex in the c-Src and adhesion-dependent signaling that leads to osteoclast activation, cytoskeletal reorganization, and formation of the sealing zone.

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FIG. 9. Interaction between p130Cas and PYK2 is mediated by the SH3 domain of p130Cas and the C-terminal domain of PYK2. A, OCL lysates (1 mg/ml) were incubated with GST alone or with GST-fusion proteins (10 μg) containing the SH3 domains of Src, Lyn, Fyn, P62-kinase, or p130Cas. B, lysates were incubated with GST or with GST-fusion proteins (10 μg) containing the N-terminal (GST-PYK2(N)), the kinase (GST-PYK2(K)), or the C-terminal domain (GST-PYK2(C)) of PYK2, followed by incubation with glutathione-Sepharose beads for 2 h at 4°C. After washings, precipitated proteins were separated on SDS-PAGE and subjected to immunoblotting for PYK2 (A) or for photophos-phototyrosine (PY) and p130Cas (B).
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