Regulation of the formation of osteoclastic actin rings by proline-rich tyrosine kinase 2 interacting with gelsolin

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Osteoclast activation is important for bone remodeling and is altered in multiple bone disorders. This process requires cell adhesion and extensive actin cytoskeletal reorganization. Proline-rich tyrosine kinase 2 (PYK2), a major cell adhesion–activated tyrosine kinase in osteoclasts, plays an important role in regulating this event. The mechanisms by which PYK2 regulates actin cytoskeletal organization and osteoclastic activation remain largely unknown. In this paper, we provide evidence that PYK2 directly interacts with gelsolin, an actin binding, severing, and capping protein essential for osteoclastic actin cytoskeletal organization. The interaction is mediated via the focal adhesion–targeting domain of PYK2 and an LD motif in gelsolin’s COOH terminus. PYK2 phosphorylates gelsolin at tyrosine residues and regulates gelsolin bioactivity, including decreasing gelsolin binding to actin monomer and increasing gelsolin binding to phosphatidylinositol lipids. In addition, PYK2 increases actin polymerization at the fibroblastic cell periphery. Finally, PYK2 interacts with gelsolin in osteoclasts, where PYK2 activation is required for the formation of actin rings. Together, our results suggest that PYK2 is a regulator of gelsolin, revealing a novel PYK2–gelsolin pathway in regulating actin cytoskeletal organization in multiple cells, including osteoclasts.

Introduction

Osteoclastic bone resorption is a fundamental process associated with bone remodeling. It is altered in multiple bone disorders, including osteopetrosis and osteoporosis. Adhesion to bone matrix initiates osteoclast activation that is characterized by cytoskeletal reorganization, formation of sealing zones and polarized ruffled membranes, and directional secretion of acids and lysosomal enzymes onto the resorbing surface. Osteoclasts cultured on glass are characterized by unique cell adhesion structures called podosomes; dotlike aggregations of actin that are surrounded by many focal adhesion proteins (Marchisio et al., 1984). Podosomes are either clustered in a ring around the cell periphery corresponding to the sealing zone, or localized in central regions of cells (Marchisio et al., 1984; Akisaka et al., 2001). Podosomes are similar to focal adhesions in fibroblasts in terms of the molecular composition. Numerous signaling molecules at focal adhesions are present in podosomes, including integrins, cytoskeletal proteins (paxillin, vinculin, and talin), tyrosine kinases (c-Src; Boyce et al., 1992; Schwartzberg et al., 1997) and proline-rich tyrosine kinase 2 (PYK2; Duong et al., 1998; Pfaff and Jurdic, 2001), adaptor-like proteins p130Cas (Cas, Crk-associated substrate; Lakkakorpi et al., 1999) and Cbl (a negative regulator of multiple tyrosine kinases; Tanaka et al., 1996), and phosphatidylinositol 3-kinase (PI 3-kinase; Chellaiah and Hruska, 1996). Unlike focal adhesions that are arrow shaped and located at the ends of actin stress fibers, the core of podosomes is composed of actin aggregates surrounded by focal adhesion proteins. Thus, actin-binding proteins including gelsolin and cortactin are localized at podosomes, but not focal adhesions (Chellaiah et al., 2000; Akisaka et al., 2001). In addition, podosomes have a shorter life span and faster turnover than focal adhesions, undergoing assembly and disassembly within minutes (Tarone et al., 1985; Akisaka et al., 2001). The assembly and disassembly of podosomes allow osteoclast migration, adhesion, and bone resorption (Kanehisa et al., 1990).

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Key words: FAK; PYK2; actin cytoskeleton; focal adhesions; podosomes

© The Rockefeller University Press, 0021-9525/2003/02/565/11 $8.00
The Journal of Cell Biology, Volume 160, Number 4, February 17, 2003 565–575
http://www.jcb.org/cgi/doi/10.1083/jcb.200207036
Several lines of evidence indicate that PYK2 plays a key role in regulating osteoclastic actin cytoskeletal organization and bone resorption. PYK2 is highly homologous to FAK, a major cell adhesion–activated tyrosine kinase in fibroblastic cells (Schaller et al., 1992; Lev et al., 1995; Sasaki et al., 1995). PYK2, but not FAK, is highly expressed in osteoclasts (Duong et al., 1998; Pfaff and Jurdic, 2001). On adhesion, PYK2 translocates into the Triton X-100–insoluble cytoskeletal fraction and localizes to podosomes and tight-sealing zones in resorbing osteoclasts plated on bone (Duong et al., 1998). Moreover, PYK2 becomes tightly associated with c-Src, an important cytoplasmic tyrosine kinase implicated in bone resorption in adherent osteoclasts (Soriano et al., 1991). When PYK2 expression is inhibited by an adenoviral antisense approach, osteoclasts display defects in bone resorption, which are similar to that in Src−/− osteoclasts (Duong et al., 2001). How PYK2 functions in osteoclasts remains largely unknown.

Gelsolin is an actin-binding protein important for actin cytoskeletal organization in multiple cells including osteoclasts (Kwiatkowski, 1999; Chellaiah et al., 2000; Robinson et al., 2000; Sun et al., 2000). It contains six tandem homologous repeats (S1–6) which can be separated into the N-half (S1–3) and the C-half (S4–6; Robinson et al., 2000; Sun et al., 2000). The S4 domain in the C-half binds to a single actin molecule when Ca2+ is present, whereas the N-half binds to an actin monomer (G-actin) via the S1 domain and actin filaments (F-actin) via the S2 domain, even in the absence of Ca2+ (Robinson et al., 2000; Sun et al., 2000). Per the tail latch hypothesis, the C-tail in the C-half acts as a latch to inhibit actin binding by the N-half (Kwiatkowski et al., 1989; Robinson et al., 2000; Sun et al., 2000). In the presence of calcium, the C-tail releases inhibition of the N-half, which caps and severs actin filaments. Although calcium regulates gelsolin-actin binding positively, polyphosphoinositides negatively regulate gelsolin–actin binding (Janmey and Stossel, 1987; Robinson et al., 2000; Sun et al., 2000). Functionally, gelsolin is involved in osteoclastic podosome formation and bone resorption (Chellaiah et al., 2000). Osteopontin, a major bone matrix protein, stimulates gelsolin-associated Src and PI 3-kinase activities, leading to increased actin filament formation in avian osteoclasts (Chellaiah and Hruska, 1996). In osteoclasts derived from gelsolin−/− mice, podosome assembly and osteopontin-stimulated signaling related to motility and bone resorption are blocked (Chellaiah et al., 2000).

In this paper, we report the interaction of PYK2 with gelsolin in yeast two-hybrid system, in vitro, and in osteoclasts. We demonstrate that the focal adhesion–targeting (FAT) domain of PYK2 interacts with the LD motif in the C-tail of gelsolin. PYK2 phosphorylates gelsolin, decreases gelsolin binding to actin monomer, yet increases gelsolin binding to phosphatidylinositol. In addition, overexpression of PYK2 in fibroblasts results in increased actin polymerization at the cell periphery; this event depends on the presence of gelsolin protein. Finally, we demonstrate that PYK2 activation is required for the formation of actin rings in osteoclasts. These results suggest that PYK2 regulates actin cytoskeletal organization in osteoclasts by interacting with and regulating gelsolin.

**Results**

**Dependence of the PYK2–gelsolin interaction on the FAT domain of PYK2**

To understand the potential mechanisms by which PYK2 regulates actin cytoskeletal organization, we used the yeast two-hybrid cloning system to identify proteins that interact with PYK2 with the idea that they may participate in PYK2 signaling. Using PYK2 COOH terminus (amino acid 781 to 1009) as bait, we identified nearly 75 β-galactosidase–positive clones, of which 10 clones encoded different regions of gelsolin. To identify the region in PYK2 that interacts with gelsolin, we characterized several PYK2 mutants. Because the bait contains a focal adhesion–targeting (FAT) domain and a proline-rich sequence, we first tested whether these two domains are involved in interacting with gelsolin. As shown in Fig. 1 A, PYK2 constructs with the intact FAT domain interacted with gelsolin, indicating the importance of this domain in binding to gelsolin. Further studies demonstrated that partial deletions of the FAT domain (PYK2Δ869–902 and PYK2Δ912–956) abolished the interaction, suggesting the dependence of this domain for gelsolin interaction (Fig. 1 A). Previous studies have demonstrated that paxillin interacts with the FAT domain via a secondary structure motif that requires the conserved leucine residue (L990 in PYK2 and L1024 in FAK; Tachibana et al., 1995; unpublished data). We investigated whether the PYK2–gelsolin interaction requires L990. As shown in Fig. 1 A, a point mutation in L990 (PYK2-L990S) prevented PYK2 from binding to gelsolin, suggesting a similar interaction mechanism. Moreover, the interaction is PYK2 specific because gelsolin did not interact with FAK, although its FAT domain is highly homologous to that of PYK2 (Fig. 1 A). In the control, FAK did interact with Hic5 (Fig. 1 A), a paxillin-related protein interacting with the FAT domain of both PYK2 and FAK (Matsuya et al., 1998), ruling out the possibility of improper expression of FAK construct. These results demonstrate that PYK2 specifically interacts with gelsolin via the FAT domain.

The PYK2–gelsolin interaction occurs not only in yeast cells, but also in in vitro pull-down assays using purified proteins. When gelsolin was incubated with various GST–PYK2 fusion proteins immobilized on agarose beads, GST alone did not precipitate with gelsolin, nor did the PYK2Δ1–902 or FAK-CterM (Fig. 1 B). In contrast, GST–fusion proteins containing the FAT domain (i.e., PYK2Δ1–781 and PYK2Δ1–869) pulled down gelsolin. These results not only demonstrate a direct interaction between gelsolin and PYK2, but also further indicate the requirement for and the sufficiency of the PYK2 FAT domain to mediate the interaction with gelsolin.

To further characterize the interaction, we assessed the relative binding affinity by ELISA-based binding assay. Gelsolin was coated in a 96-well plate and incubated with increasing concentrations of GST–PYK2 fusion proteins. Bound PYK2 or mutants were detected by monoclonal anti-GST antibody, followed by anti–mouse secondary antibody conjugated to alkaline phosphatase. As shown in Fig. 1 C, PYK2Δ1–781 bound to gelsolin in dose-dependent and saturable manners with an EC50 value of 30 ± 4 nM. Confirming the results from studies above, partial deletion of the FAT domain (as in PYK2Δ1–902) inhibited the binding (Fig. 1 C).
Dependence of the PYK2–gelsolin interaction on the COOH-terminal region of gelsolin

The original clones isolated from the yeast two-hybrid screen contain the COOH-terminal region of gelsolin (Fig. 2 A; GelsolinΔ1–258 and GelsolinΔ1–656), suggesting that the NH2 terminus and the first five gelsolin repeats were not essential for the interaction and instead, the COOH terminus may be critical. To identify the sequence in gelsolin responsible for PYK2 interaction, we generated a gelsolin mutant with a deletion of COOH-terminal 19 amino acids (GelsolinΔ1–258/ΔC19) and examined its binding to PYK2. This mutant did not interact with PYK2 (Fig. 2 A), suggesting that the COOH terminus was required for the interaction. Previous studies have shown that the FAT domain interacts with paxillin and its family members via the LD motif. This motif is characterized by leucine-rich sequences that begin with a leucine (L) and an aspartate (D; Brown et al., 1996; Turner, 2000). The dependence of the PYK2–gelsolin interaction on the FAT domain led us to investigate whether there is a similar motif in gelsolin’s COOH terminus. Sequence analyses revealed an LD-like motif (LDxxLxxL) in this region (residues 721 to 732) that is homologous to those in paxillin and Hic5 (Fig. 2 B). To determine whether the LD-like motif is criti-
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Correlation of gelsolin tyrosine phosphorylation with PYK2–gelsolin interaction

We investigated whether PYK2 kinase activity regulates its binding to gelsolin. Flag-tagged gelsolin was cotransfected with Myc-tagged wild-type (PYK2-WT), catalytically inactive (PYK2-KD), autophosphorylation mutant (PYK2-Y402F) of PYK2, or FAK into HEK 293 cells. The interaction of gelsolin with PYK2 was assayed by coimmunoprecipitation. PYK2-WT associated with gelsolin as expected (Fig. 3 A). However, PYK2-KD, PYK2-Y402F, or FAK had significantly reduced binding with gelsolin (Fig. 3 A), indicating that the catalytic activity of PYK2 may regulate the interaction between PYK2 and gelsolin, and that gelsolin may be a substrate of PYK2 kinase. To test this hypothesis, we examined tyrosine phosphorylation of gelsolin by PYK2. As shown in Fig. 3 B, gelsolin was tyrosine phosphorylated in cells coexpressing PYK2. However, tyrosine phosphorylation of gelsolin was diminished in cells coexpressing PYK2-KD or PYK2-Y402F, suggesting the involvement of PYK2 kinase activity in this event. The specificity of phosphorylation was demonstrated in that gelsolin was not phosphorylated by FAK (Fig. 3 B), probably due to its inability to interact with gelsolin.

To determine whether gelsolin phosphorylation was mediated by PYK2, we immunopurified PYK2 and incubated it with gelsolin in an in vitro kinase reaction containing γ[32P]ATP. This reaction generated two major 32P-labeled proteins at 120 and 95 kD, respectively (Fig. 3 C). The 120-kD protein was presumably PYK2 because it co-migrated with the band that was recognized by anti-PYK2 antibody. The lower molecular weight band co-migrated with gelsolin, suggesting that it could be phosphorylated by PYK2. This notion was further attested by the finding that the kinase-dead mutant was unable to phosphorylate gelsolin (Fig. 3 C). In a typical reaction, there was 20 pmol of gelsolin. 1.6 pmol of hot ATP was covalently associated with gelsolin. Assuming one tyrosine phosphorylation site, ~8% of gelsolin is phosphorylated by PYK2 in the in vitro kinase assay. Phosphorylation by PYK2 most occurred in the COOH-terminal domain of gelsolin (C-gelsolin), a domain that interacts with PYK2 (Fig. 3, D and E).

Figure 3 Regulated PYK2–gelsolin interaction and gelsolin tyrosine phosphorylation by PYK2.

(A) Regulation of PYK2–gelsolin interaction by PYK2.

HEK 293 cells cotransfected with Flag-gelsolin and Myc-tagged PYK2 or mutants with Flag-gelsolin were lysed. Immunoprecipitations (IP) with anti-Flag antibodies were immunoblotted (IB) with anti-Myc (top) or anti-Flag (middle) antibodies. Expression of PYK2 was demonstrated in the bottom panel. (B) Tyrosine phosphorylation of gelsolin by PYK2. HEK 293 cells were cotransfected Flag-gelsolin with Myc-tagged PYK2-WT, PYK2-KD, or PYK2-Y402F and FAK. Cell lysates were incubated with anti-Flag antibodies and resulting immunocomplexes immunoblotted with anti-phosphotyrosine antibodies (Ptyr, RC 20). (C) Tyrosine phosphorylation of gelsolin by PYK2 in in vitro kinase assays. PYK2-WT, PYK2-KD, and PYK2-Y402F protein were immunoprecipitated from transfected HEK 293 cells and subjected to an in vitro kinase assay using gelsolin as a substrate. (D) Schematic diagrams of gelsolin mutants. The six repeats in gelsolin are indicated. The strength of the PYK2–gelsolin interaction (Gsn-PYK2) and gelsolin tyrosine phosphorylation (Gsn-Ptyr) in response to PYK2 are indicated in the right columns. ++++, strong interaction or tyrosine phosphorylation; –, no detectable interaction or tyrosine phosphorylation. (E) Tyrosine phosphorylation of gelsolin deletion mutants by PYK2. HEK 293 cells were transfected Flag-gelsolin and gelsolin mutants (N-gelsolin and C-gelsolin) with or without Myc-PYK2. Gelsolin or gelsolin mutants were immunoprecipitated with anti-Flag antibodies and immunoblotted with anti-Ptyr (RC20).
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terminal domain of gelsolin (N-gelsolin) did not interact with PYK2 (unpublished data), and was thus less or not phosphorylated (Fig. 3, D and E). These results suggest the requirement of PYK2–gelsolin interaction for gelsolin’s tyrosine phosphorylation and that tyrosine residues to be phosphorylated by PYK2 are located in the COOH-terminal region of gelsolin.

Regulation of gelsolin association with G-actin

Gelsolin is implicated in actin filament severing and capping in a manner dependent on gelsolin binding to actin. Three actin binding sites are identified in gelsolin; two in the NH₂-terminal region (one for F-actin and one for G-actin) and one in the COOH-terminal region that binds G-actin in a calcium-dependent manner (Robinson et al., 2000; Sun et al., 2000). To understand the possible role of PYK2 in regulating gelsolin function, we examined the effect of PYK2 on gelsolin-actin monomer binding using two methods; coimmunoprecipitation and DNaseI-Sepharose pull-down. For coimmunoprecipitation assay, HEK 293 cells expressing gelsolin with or without PYK2 were lysed in an extraction buffer containing 1 mM EGTA to chelate calcium. Cell lysates were subjected to immunoprecipitation for gelsolin with a washing buffer containing 30 mM MgCl₂ to depolymerize F-actin, and subsequent immunoblotting for actin. Because of the presence of EGTA in the assay, the gelsolin-actin binding detected is believed to be calcium-independent, and thus mediated by the NH₂-terminal region. Indeed, under these conditions, C-gelsolin did not bind to actin, whereas N-gelsolin did (Fig. 4 A). Interestingly, actin interaction with the full-length gelsolin was significantly reduced in HEK 293 cells expressing PYK2 (Fig. 4 A), suggesting that PYK2 inhibits calcium-independent gelsolin-actin monomer binding. Similar results were obtained in experiments using the DNaseI-Sepharose beads, which pull down actin monomers and associated proteins (Yamamoto et al., 2001). As shown in Fig. 4 B, actin as well as full-length and N-gelsolin, but not C-gelsolin, were detected in the pull-down complexes. Again, PYK2 reduced the amount of actin associated with full-length gelsolin (Fig. 4 B). Note that the actin association with N-gelsolin was not inhibited by PYK2 (Fig. 4, A and B), presumably because it did not interact directly with PYK2. Together with the finding that COOH-terminal gelsolin interacts with PYK2, these results suggest that PYK2 interacts with the COOH-terminal region of gelsolin to regulate the calcium-independent actin binding by the NH₂-terminal gelsolin. Attesting the specificity of the regulatory effect, the gelsolin–actin interaction was not regulated by the kinase-dead PYK2 or wild-type FAK (Fig. 4 C). Moreover, PYK2 appeared to have little effect on gelsolin binding to F-actin (Fig. 4 D).
Regulation of gelsolin association with PI(4,5)P2 association

Previous studies have shown that gelsolin interacts with PI(4,5)P2, and that this interaction negatively regulates gelsolin-actin binding (Janmey and Stossel, 1987; Robinson et al., 2000; Sun et al., 2000). PYK2 may modulate PI(4,5)P2-gelsolin binding to inhibit gelsolin-actin binding. To test this hypothesis, we examined the effects of PYK2 on gelsolin–PI(4,5)P2 interaction. PI(4,5)P2 beads were incubated with HEK 293 cell lysates expressing Flag-gelsolin or gelsolin deletion mutants. HEK 293 cell lysates expressing indicated proteins were incubated with PI(4,5)P2 beads. PI(4,5)P2-bound proteins, such as gelsolin, PYK2, or FAK were detected by immunoblotting with indicated antibodies.

Figure 5. PYK2 stimulation of gelsolin-PI(4,5)P2 association. (A) PI(4,5)P2 binding to gelsolin or PYK2–gelsolin complexes in HEK 293 cells coexpressing Flag-gelsolin with or without PYK2-WT, PYK2-KD, or FAK. (B) Co-expressing PYK2 with Flag-gelsolin or gelsolin deletion mutants. HEK 293 cell lysates expressing indicated proteins were incubated with PI(4,5)P2 beads. PI(4,5)P2-bound proteins, such as gelsolin, PYK2, or FAK were detected by immunoblotting with indicated antibodies.

Figure 6. Increase in actin polymerization at fibroblastic cell periphery by PYK2. 10T1/2 or embryonic fibroblasts derived from gelsolin knock-out (gels−/−) or wild type (gels+/+) mice were transiently transfected with indicated constructs. Transfected cells were trypsinized, replated onto fibronectin-coated coverslips for 2–4 h, fixed, and stained with indicated antibodies. Phalloidin was used to stain polymerized actin filaments. Open arrows indicate cells without PYK2 expression; Closed arrows indicate PYK2 expressing cells. Bar, 50 μm.
Interaction of PYK2 with gelsolin and their colocalization in osteoclasts

As an initial step to understand possible roles of PYK2–gelsolin interaction in vivo, we examined expression and interaction of PYK2 and gelsolin in different cells including 10T1/2 fibroblasts and mouse osteoclasts. Western blot analysis demonstrated that both PYK2 and gelsolin were highly expressed in mouse osteoclasts, but less in 10T1/2 fibroblasts (Fig. 7 A). Thus, we determined whether endogenous PYK2 and gelsolin interact in mouse osteoclasts. As shown in Fig. 7 B, PYK2 was detected in gelsolin, but not in nonspecific IgG, immunocomplexes, indicating that gelsolin associated with PYK2 specifically in mouse osteoclasts. This interaction appeared to be strong with 5–10% of gelsolin associated with PYK2 immunocomplexes (Fig. 7 B). Moreover, this interaction was not only observed in mouse osteoclasts, but also in chicken osteoclasts (Fig. 7 B). Next, we assessed whether gelsolin is tyrosine phosphorylated in mouse osteoclasts when PYK2 is activated. Mouse osteoclast precursors cultured in suspension dishes were held in suspension for 2 h, remained in suspension dishes, or cultured in tissue culture dishes with M-CSF and Rank ligand for different times. PYK2 was activated in these cultured conditions except when cells were held in suspension (unpublished data). Gelsolin was immunoprecipitated from these cell lysates and its tyrosine phosphorylation was detected by immunoblotting with antibodies against phosphotyrosine (RC20). As shown in Fig. 7 C, three tyrosine phosphorylated proteins were detected in gelsolin immunoprecipitates. Two proteins appeared to co-migrate with PYK2 and Src, a kinase that phosphorylates gelsolin in vitro (De Corte et al., 1997, 1999), and one protein band co-migrated with gelsolin (Fig. 7 C). This result suggests that gelsolin in osteoclasts may be tyrosine phosphorylated when PYK2 and Src are activated.

Then, we examined subcellular localizations of PYK2 and gelsolin in mouse osteoclasts. Mouse osteoclasts, indicated by the tartrate-resistant acid phosphatase (TRAP) staining (Fig. 7 D), were plated on glass coverslips, fixed, and im-
munostained using antibodies against PYK2 (polyclonal; United Biomedical, Inc.) and gelsolin (monoclonal). The PYK2 antibody was specific and did not cross-react with overexpressed FAK (Du et al., 2001). PYK2 was distributed in dot- or ringlike podosomes at the cell periphery. Gelsolin was colocalized with PYK2 in dotlike podosomes (Fig. 7 E, solid arrow). However, the two proteins only partially overlap in ring-like podosomes (Fig. 7 E, open arrow). The staining did not appear to be nonspecific because immunostaining osteoclasts with secondary antibodies alone generated the background staining that was different from staining by PYK2 or PY402 antibodies (unpublished data). These results imply that PYK2 and gelsolin may function as a complex to regulate podosome and actin ring formation in mouse osteoclasts. In addition to mouse osteoclasts, chicken osteoclasts were used to examine PYK2 subcellular localization. The active PYK2 stained with the PY402 antibody was codistributed with F-actin labeled by phalloidin at podosomes in the cell periphery (Fig. 7 F), conforming the results from mouse osteoclasts.

**Role of PYK2 activity in the formation of actin rings and cell periphery podosomes in osteoclasts**

The interaction of PYK2 with gelsolin in osteoclasts suggests a role of PYK2 in regulating osteoclastic actin cytoskeletal organization. To address this hypothesis, adenoviruses encoding PYK2-KD, a dominant-negative mutant of PYK2, and GFP were generated and used to infect cultured mouse osteoclasts. Infected cells were fixed and immunostained with phalloidin to examine the actin ring and podosome structures, two important actin-associated structures in mouse osteoclasts. The active PYK2 stained with the PY402 antibody was codistributed with F-actin labeled by phalloidin at podosomes in the cell periphery (Fig. 7 F), conforming the results from mouse osteoclasts.

**Figure 8. Requirement of PYK2 activity for the formation of actin rings and cell peripheral podosomes in mouse osteoclasts.**

(A) Western blot analysis of PYK2 expression in osteoclasts infected with adenoviruses expressing GFP, PYK2-WT, or PYK2-KD by anti-PYK2 and PY402 antibodies. (B) Quantitative analysis of mouse osteoclasts with intact actin rings after infection. (C) Immunostaining of mouse osteoclasts infected with indicated adenoviruses. (D) Amplified images from C. Bars (C and D), 50 μm.

**Discussion**

This paper presents evidence for a role of the PYK2–gelsolin interaction in regulating actin cytoskeletal organization, which lead us to propose a model detailed in Fig. 9. In this model, PYK2, in response to extracellular stimuli, interacts with gelsolin. The affinity of gelsolin for PI(4,5)P2 is increased, but gelsolin binding to actin monomer is decreased. We speculate that this event may lead to the uncappping of actin filaments at the barbed ends, increase in actin polymerization at the cell periphery, and regulation of osteoclastic actin ring formation (Fig. 9).

The identification of the PYK2–gelsolin interaction suggests that PYK2 is an important regulator of gelsolin in actin polymerization. Gelsolin binds to two actin monomers in the presence of calcium. One actin monomer remains bound after chelation of calcium with EGTA (Wang and Bryan, 1981; Lind et al., 1982; Kurth et al., 1983). The EGTA stable gelsolin–actin complex appears to have a higher affinity for barbed filament end than free gelsolin (Janmey et al., 1985). Our results demonstrate that PYK2 inhibits this EGTA stable gelsolin–actin monomer association. This inhibition may lead to the generation of free actin barbed ends and increase of actin polymerization at cell periphery. This notion is supported by the findings that active PYK2 is localized at the cell periphery in fibroblasts and osteoclasts and that cells expressing PYK2 exhibit increased gelsolin-depen-
There is a reciprocal relationship regulated by PI(4,5)P2 (Janmey and Stossel, 1987; Robinson et al., 2000; Sun et al., 2000). Interestingly, our studies show that PYK2 increases PI(4,5)P2 binding to gelsolin. It seems reasonable that PYK2 inhibits the gelsolin-actin binding by increasing the association with PI(4,5)P2. It is worth pointing out that the kinase-inactive mutant PYK2 also increased, albeit weakly, the gelsolin–PI(4,5)P2 interaction, which may suggest that the binding to PYK2 may regulate gelsolin’s conformation. Moreover, PYK2 stimulates PI4-kinase (unpublished data), and Nirs, Drosha sp rdgB homologues in human and mouse with phosphoinositol transfer activity (Lev et al., 1999). Both PI4-kinase and Nirs are important for generation of PI(4,5)P2 in the cell. Thus, an increase in PI(4,5)P2 supply in the PYK2 signaling complex may be a mechanism to increase the gelsolin-PI(4,5)P2 binding in vivo.

Actin rings at cell periphery of osteoclasts contain highly dynamic clustered F-actin filaments. Stimulation of integrin leads to activation of gelsolin-associated PI 3 kinase and decrease in gelsolin-actin association, both of which are important for actin cytoskeleton reorganization (Chellaiah and Hruska, 1996). Our studies may provide a link between integrin stimulation and downstream actin polymerization. Interestingly, PYK2 and gelsolin colocalize at the podosomes and actin rings at the cell periphery of osteoclasts. Osteoclasts expressing PYK2-KD exhibit defects in the formation of actin rings at the cell periphery, but not dotlike podosomes in the cytoplasm. These observations support a role of PYK2 as an important regulator of gelsolin in actin cytoskeletal organization induced by various stimuli that activate PYK2 in addition to integrin engagement (Fig. 9). It is worthy pointing out that some data in this paper were from in vitro and overexpression experiments, and thus need to be confirmed in vivo models.

### Materials and methods

**Reagents**

mAbs were purchased from Santa Cruz Biotechnology, Inc. (anti-Myc), Sigma-Aldrich (anti-Flag), and Transduction Laboratories (anti-PYK2 and anti-gelsolin). pAbs against PYK2 and PY402 in PYK2 were obtained from Upstate Biotechnology and Biosource International, respectively. The cDNA encoding mouse gelsolin was purchased from the American Type Culture Collection. Gelsolin knockout (Gsn−/−) mice were provided by Dr. David Kwiatkowski (Brigham and Women’s Hospital, Boston, MA; Witke et al., 1995). The Gsn+/− mouse was maintained in mixed Sv129-BALB/c and Sv129-C57/B1 backgrounds. Littermates that were Gsn−/− derived from Gsn+/− matings were used for generation of dermal fibroblasts.

**Yeast two-hybrid studies**

The PYK2 COOH terminus (amino acid residues 781–1009) subcloned into pGBT10 (pGBT10-PYK2Δ1–781) was used as bait to screen mouse brain cDNA libraries fused to the GAL4 transcripational activation domain (GAL4-AD; Ren et al., 2001). The Y190 yeast strain was first transformed with pGBT10-PYK2Δ1–781, and subsequently with the mouse brain cDNA library. Positive clones were screened out on plates lacking leucine, tryptophan, and histidine with 30 mM 3-aminotriazole and by filter assays for β-galactosidase activity. Plasmid DNA was purified from the His+ β-gal− colonies, and was transformed into yeast with different bait vectors to determine specificity. To characterize binding between PYK2 and gelsolin, plasmids encoding PYK2, gelsolin, or their mutants were cotransformed into yeast Y190; interactions were characterized by growth and by β-galactosidase activity (Ren et al., 2001).
Expression vectors

The cDNAs of FAK and PYK2 were subcloned into mammalian expression vectors downstream of a Myc epitope tag (MEOKLI/SEEDL) as described previously (Xiong and Parsons, 1997). The cDNAs encoding gelsolin or its mutants were amplified by PCR, and were subcloned into mammalian expression vectors downstream of a Flag epitope tag (MDYKDDDDDKGP) under the control of the CMV promoter (Ren et al., 2001). PYK2-KD (kinase inactive mutant [lysine 457 to alanine]) and PYK2-Y402F (autophosphorylation mutant [tyrosine 402 to phenylalanine]) were generated as described previously (Xiong and Parsons, 1997). Other point mutations in PYK2 or gelsolin were also generated by PCR amplification (QuickChange®; Stratagene). Mutations were verified by DNA sequencing.

Cell culture and transfection

HEK 293 cells and 10T1/2 fibroblasts were maintained in DME supplemented with 10% FCS, 100 μg/ml penicillin G, and 100 μg/ml streptomycin ( Gibco BRL). The calcium phosphate precipitation method was used to transfect HEK 293 cells, and 20–30 μl SuperFect® (Invitrogen) was incubated with the constructs to transfect 10T1/2 cells.

Dermal fibroblasts derived from Gsn+/− and Gsn−/− mice were generated as described previously (Wiske et al., 1995; Azuma et al., 1998). In brief, skin tissue extracts from the mice were prepared by homogenization. Dermal fibroblasts were maintained in DME supplemented with 10% FCS. SuperFect® was used for transfection.

Mouse osteoclasts were generated by in vitro differentiation of mouse bone marrow macrophages (BMMs) by RANKL-MS-CSF (Feng et al., 2001). In brief, whole bone marrow cells were flushed out of mouse long bones and plated on 150-mm tissue culture plates in 37 °C/H1002. Following the addition of recombinant M-CSF and 88 ng/ml recombinant GST-RANKL, osteoclasts began to form 96 h later. The identity of osteoclasts was confirmed by immunostaining or lysed for Western blotting analysis. Mouse osteoclasts were infected with recombinant adenoviruses expressing PYK2 and gelsolin protein bands were excised from gel, and radioactivity was measured in a liquid scintillation counter (Beckman Coulter).

In vitro phosphorylation of gelsolin

GST-fusion protein pull-down assay was performed as described previously (Ren et al., 2001). DNasel- Sepharose pull-down assay was performed as described previously (Yamamoto et al., 2001). In brief, transiently transfected HEK 293 cells were lysed in the extraction buffer containing 0.75% Triton X-100, 60 mM Pipes, 25 mM Hepes, pH 7.2, 2 mM MgCl2, 1 mM sodium vanadate, 10 μg/ml leupeptin, 40 μg/ml apo- actin, 50 μg/ml benzamidine, and 1 mM PMFS. Cell lysates were incubated with DNasel immobilized on Sepharose beads at 4°C for 1 h. Actin monomers bind DNasel and many proteins, such as gelsolin, that bind actin monomers are pulled down as well. The bound protein complexes were analyzed by SDS-PAGE followed by immunoblotting.

ELISA

ELISA based binding assay was performed as described previously (Luo et al., 2002). In brief, 96-well MaxiSorp™ immunoplates (Nunc) were coated with purified human gelsolin (5 pmol/well; Cytoskeleton, Inc.) in BBS (125 mM boric acid, 75 mM NaCl at pH 8.5) overnight at 4°C. Plates that were washed three times in PBS and preblocked were incubated with different fragments of GST-PYK2 or FAK fusion proteins overnight at 4°C, and were washed four times in PBS, followed by incubation with mouse anti-GST mAb (1:20,000) for 2 h at RT. After washing four times in PBS and once with substrate buffer (0.1 mM MgCl2, 3% vol/vol diethanolamine, pH 9.8), plates were subjected to color reaction by incubating with 1 mg/ml PNPP (para-nitrophenyl phosphate) in substrate buffer. Absorbance at 405 nm was read with a microplate reader. All values were converted to relative binding by defining maximal absorbance as 100%.

Immunoprecipitation

PYK2-gelsolin immunoprecipitation was performed as described previously (Ren et al., 2001). Assay of gelsolin-actin association was performed as described previously (Chaponnier et al., 1987; Lind et al., 1987) with modification. Gelsolin constructs were cotransfected with empty vector or PYK2 constructs in HEK 293 cells, 48 h after transfection, cells were lysed in extraction buffer containing 0.75% Triton X-100, 60 mM Pipes, 25 mM Hepes, pH 7.2, 1 mM EGTA, 2 mM MgCl2, 1 mM sodium vanadate, 10 μg/ml leupeptin, 40 μg/ml aprotinin, 80 μg/ml benzamidine, and 1 mM PMFS. Flag-tagged gelsolin was immunoprecipitated with anti-Flag M2 affinity agarose beads (Sigma-Aldrich), washed three times with the extraction buffer, and once with 0.3 M MgCl2 included in the extraction buffer. The immunoprecipitates were analyzed by SDS-PAGE followed by immunoblotting.

Actin co-sedimentation assay

Transfected HEK 293 cells were rinsed twice with ice cold PBS and then collected in 0.5 ml of the binding buffer (10 mM imidazole, pH 7.2, 75 mM KCl, 5.0 mM MgCl2, and 0.5 mM DTT) supplemented with 1 mM EGTA, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml peptatin A. Cells were homogenized, followed by centrifugation at 100,000 g for 1 h at 4°C in a TLA-120.1 rotor (Beckman Coulter). Muscle monomer actin (Cytoskeleton, Inc.) was diluted to 2.5 mg/ml in binding buffer and incubated for 1 h at RT. Polymerized actin was stabilized by adding phallolidin (Molecular Probes, Inc.) to a final concentration of 25 μg/ml. 5 μM F-actin was added to clarified cell lysates (50 μg total protein) to final volume of 200 μl, and the mixtures were incubated at RT for 30 min. Samples were centrifuged at 100,000 g for 30 min at 20°C. Pellet and supernatant were analyzed by SDS-PAGE and Western blotting.

In vitro phosphorylation of gelsolin

2 μg purified gelsolin was incubated with immunoprecipitated PYK2 or mutants in 40 μl phosphorylation buffer containing 10 mM Hepes, pH 7.6, 10 mM magnesium acetate, 5 μCi of [γ-32P]ATP, and 50 μM ATP for 30 min at 30°C. Phosphorylated gelsolin and PYK2 were subjected to SDS-PAGE analysis. The dried gel was exposed to x-ray film. The corresponding PYK2 or gelsolin protein bands were excised from gel, and radioactivity was measured in a liquid scintillation counter (Beckman Coulter).

Assay of gelsolin-PIP2 binding

Three 5 μl beads were purchased from Echelon Biosciences Incorporated. Gelsolin-P1,5P2 binding assay was performed per the manufacturer’s instructions. In brief, HEK 293 cell lysates expressing Flag-gelsolin with or without PYK2, PYK2-KD, or FAK were incubated with P1,5P2 beads for 4 h at 4°C. Gelsolin-bound beads were subjected to immuno blotting with indicated antibodies.

Generation of adenovirus and infection of osteoclasts

The recombinant, replication-deficient adenoviruses expressing PYK2 and catalytically inactive mutant of PYK2 were generated using the AdEasy™ system (He et al., 1998). Wild-type and catalytically inactive mutant of PYK2 were constructed into pTRACK-GFP vector. The recombinant adenoviruses were generated by homologous recombination between the parental virus genome and the expression shuttle vector. Recombinant viruses were produced in the HEK 293 cell line. Mouse osteoclasts were infected with recombinant adenoviruses at different multiplicities of infection. Adenoviruses were usually applied at day 5 of differentiation, then incubated for 24 h followed by a change of medium. At day 7, cells were washed with PBS, then fixed with 4% PFA for immunostaining or lysed for Western blotting analysis.

Immunocytochemistry

Cells were fixed with 4% PFA for 20 min, blocked with 10% BSA, and incubated with antibodies against PYK2 (goat polyclonal; Santa Cruz Biotechnology, Inc.) or gelsolin (monoclonal; Transduction Laboratories). Double labeled immunostaining was done with appropriate fluorochrome-conjugated secondary antibodies. Fluorescent images of cells were captured on a Sony CCD camera mounted on a microscope (model E600; Nikon) using Photoshop® imaging software.

We are grateful to Dr. David Kwiatkowski for providing reagents and Maggie McKenna for technical help with chicken osteoclast cultures.

This work was supported in part by the National Institutes of Health (AR48120 to W.-C. Xiong; AR43225 and AR46031 to J.M. McDonald; and AR47830 to X. Feng; and NS40480 to L. Mei) and the American Heart Association Southeastern Affiliate Grant In Aid (051566B to W.-C. Xiong; AR48120 to W.-C. Xiong; AR43225 and AR46031 to J.M. McDonald; and AR47830 to X. Feng; and NS40480 to L. Mei) and the American Heart Association Southeastern Affiliate Grant In Aid (051566B to W.-C. Xiong; AR48120 to W.-C. Xiong; AR43225 and AR46031 to J.M. McDonald; and AR47830 to X. Feng; and NS40480 to L. Mei) and the American Heart Association Southeastern Affiliate Grant In Aid (051566B to W.-C. Xiong; AR48120 to W.-C. Xiong; AR43225 and AR46031 to J.M. McDonald; and AR47830 to X. Feng; and NS40480 to L. Mei).
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