

# 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 p130<sup>Cas</sup> (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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\*Abbreviations used in this paper: C-gelsolin, COOH-terminal domain of gelsolin; FAT, focal adhesion targeting; N-gelsolin, NH<sub>2</sub>-terminal domain of gelsolin; PI 3, phosphatidylinositol 3; PY402, phosphotyrosine 402; PYK2, proline-rich tyrosine kinase 2; TRAP, tartrate-resistant acid phosphatase.

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<sup>-/-</sup> 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 Ca<sup>2+</sup> 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 Ca<sup>2+</sup> (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<sup>-/-</sup> 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.

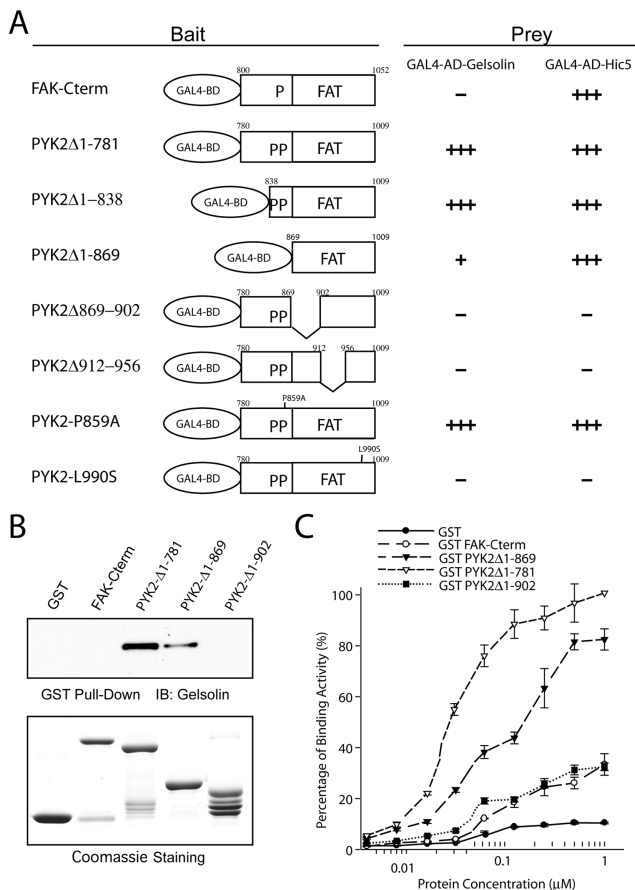
## Results

### 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  $\beta$ -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 (PYK2 $\Delta$ 869–902 and PYK2 $\Delta$ 912–956) 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 PYK2 $\Delta$ 1–902 or FAK-Cterm (Fig. 1 B). In contrast, GST-fusion proteins containing the FAT domain (i.e., PYK2 $\Delta$ 1–781 and PYK2 $\Delta$ 1–869) 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.

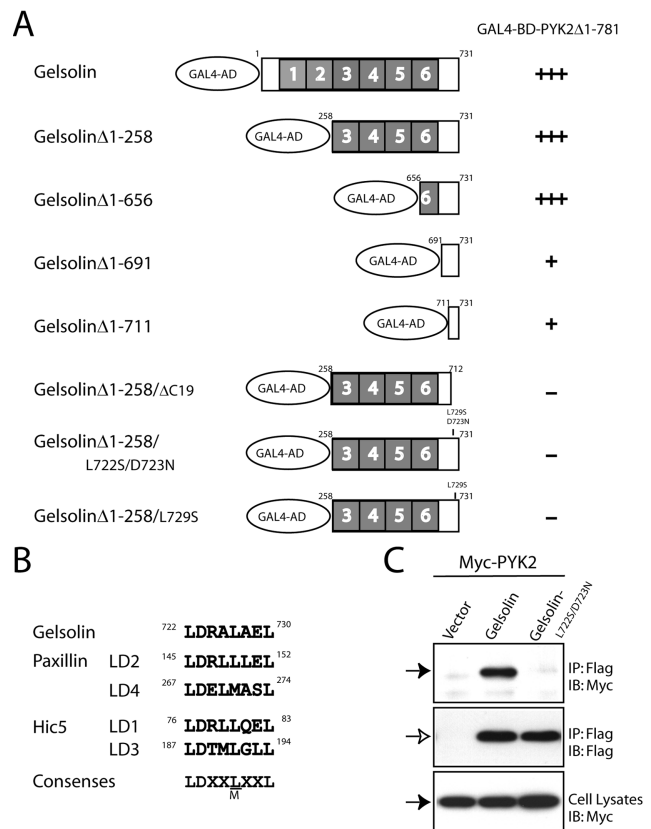
To further characterize the interaction, we assessed the relative binding affinity by ELISA-based binding assay. Gelsolin was coated in a 96-well plate and incubated with increasing concentrations of GST-PYK2 fusion proteins. Bound PYK2 or mutants were detected by monoclonal anti-GST antibody, followed by anti-mouse secondary antibody conjugated to alkaline phosphatase. As shown in Fig. 1 C, PYK2 $\Delta$ 1–781 bound to gelsolin in dose-dependent and saturable manners with an EC<sub>50</sub> value of  $30 \pm 4$  nM. Confirming the results from studies above, partial deletion of the FAT domain (as in PYK2 $\Delta$ 1–902) inhibited the binding (Fig. 1 C).



**Figure 1. Dependence of the PYK2-gelsolin interaction on the FAT domain in PYK2.** (A) Analyses of PYK2 domains essential for binding to gelsolin by yeast two-hybrid assays. Yeast cells were cotransformed with a vector encoding the Gal4 DNA binding domain (Gal4-BD) fused to PYK2 or FAK constructs and Gal4 activation domain (Gal4-AD) fused to gelsolin or Hic 5. Transformed yeast cells were scored for growth on Leu<sup>-</sup>, Trp<sup>-</sup>, and His<sup>-</sup> plates and for β-gal activity; (-) no blue after 24 h, (+) blue after 4 h, (+++) blue within 30 min. (B) Direct interaction between PYK2 and gelsolin in vitro. The purified gelsolin was incubated with indicated GST fusion proteins (10 μg) immobilized on agarose beads. Bound gelsolin was resolved on SDS-PAGE and immunoblotted with anti-gelsolin antibodies (top). Similar amount of GST fusion proteins was revealed by Coomassie staining (bottom). (C) ELISA analysis of the interaction between PYK2 and gelsolin. Gelsolin was coated on 96-well plates (5 pmol/well) and incubated with increasing concentrations of GST-fusion proteins of indicated PYK2 or FAK mutants. Bound PYK2 and mutants were detected by anti-GST antibodies, followed by AP-conjugated secondary antibody. The binding activity was determined by measuring the absorbance at 405 nm. The values were converted into relative binding with the value of maximal 405 nm absorbance as 100%.

### 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 NH<sub>2</sub> 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

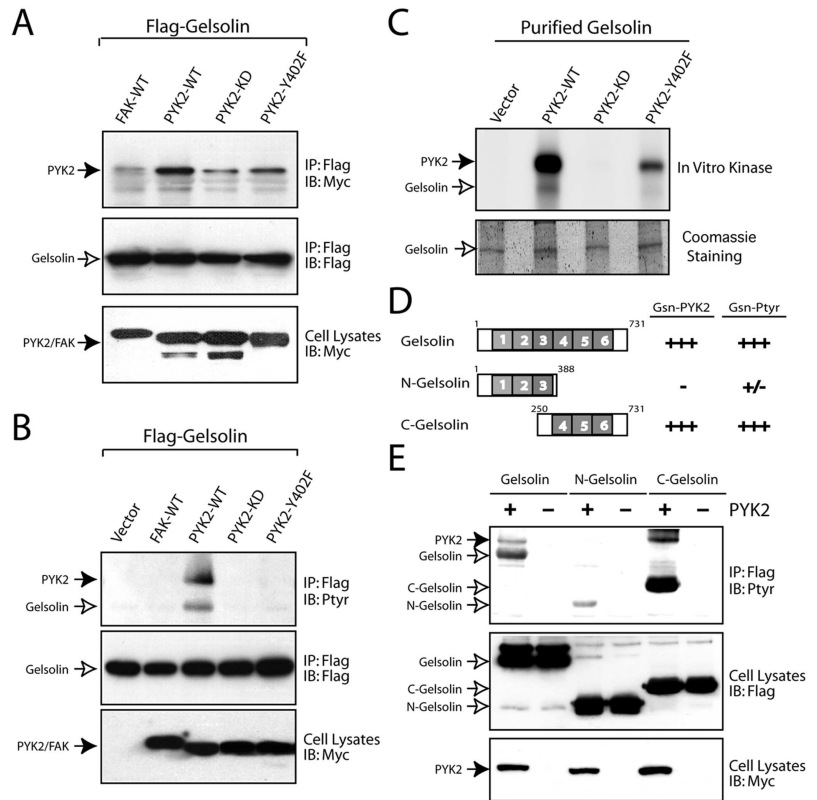


**Figure 2. Dependence of the PYK2-gelsolin interaction on the LD motif in gelsolin COOH-tail.** (A) Analyses of gelsolin domains essential for binding to PYK2 by yeast two-hybrid assays. Yeast cells were cotransformed with Gal4-BD-PYK2Δ1-781 and Gal4-AD fused to different fragments or mutants of gelsolin. Transformed yeast cells were scored for growth in Leu<sup>-</sup>, Trp<sup>-</sup>, and His<sup>-</sup> plates with 30 mM 3-aminotriazole and for β-gal activity; (-) no blue after 24 h, (+) blue after 4 h, (+++) blue within 30 min. The numbers represent the amino acid residues in mouse gelsolin. (B) Consensus sequences of LD motifs. (C) Requirement of the LD motif for PYK2-gelsolin interaction. HEK 293 cells were transiently transfected with Flag-tagged gelsolin or gelsolin-L722S/D723N with Myc-tagged PYK2. Cell lysates were incubated with anti-Flag antibodies to immunoprecipitate the gelsolin complex, which was resolved on SDS-PAGE and immunoblotted with antibodies against Myc (top) and Flag (middle). Expression of PYK2 was revealed by immunoblotting with anti-Myc antibodies (bottom).

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-

### Figure 3. Regulation of PYK2–gelsolin interaction and gelsolin tyrosine phosphorylation by PYK2.

(A) Regulation of PYK2–gelsolin interaction by PYK2. HEK 293 cells cotransfected with 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).



for interaction with PYK2, we generated point mutations in this motif and assayed their binding activities (Fig. 2 A). Mutations of conserved residues including leucine 722, aspartic acid 723, or leucine 729 abolished the interaction (Fig. 2 A). These results indicate that the LD-motif in the COOH terminus of gelsolin is critical for the interaction with PYK2.

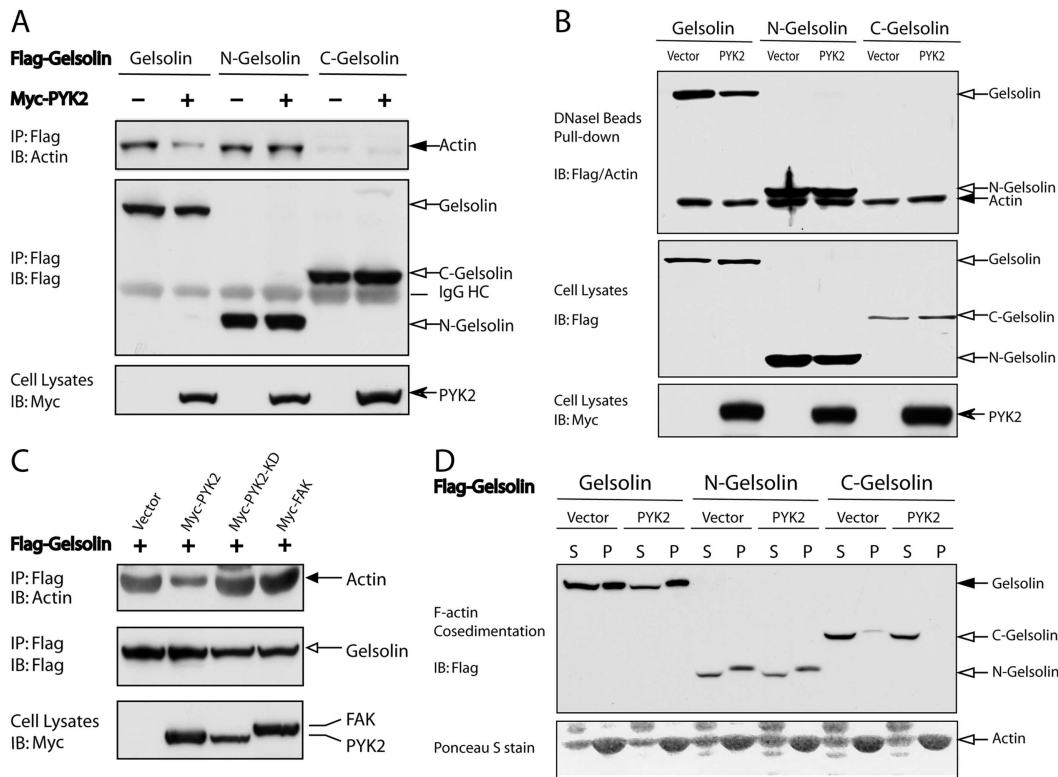
To determine whether PYK2 interacts with gelsolin in mammalian cells, we examined whether they associated in HEK 293 cells transfected with Flag-gelsolin and Myc-PYK2. As shown in Fig. 2 C, PYK2 was detected in immunoprecipitates of gelsolin, suggesting that PYK2 binds to gelsolin in mammalian cells. As observed in yeast, the interaction required the LD motif of gelsolin because gelsolin-L722S/D723N, with a mutation in the LD motif, did not coimmunoprecipitate with PYK2 (Fig. 2 C).

### 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 examined 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  $\gamma$ [ $^{32}$ P]ATP. This reaction generated two major  $^{32}$ P-labeled proteins at 120 and 95 kD, respectively (Fig. 3 C). The 120-kD protein was presumably PYK2 because it comigrated 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). The NH<sub>2</sub>-



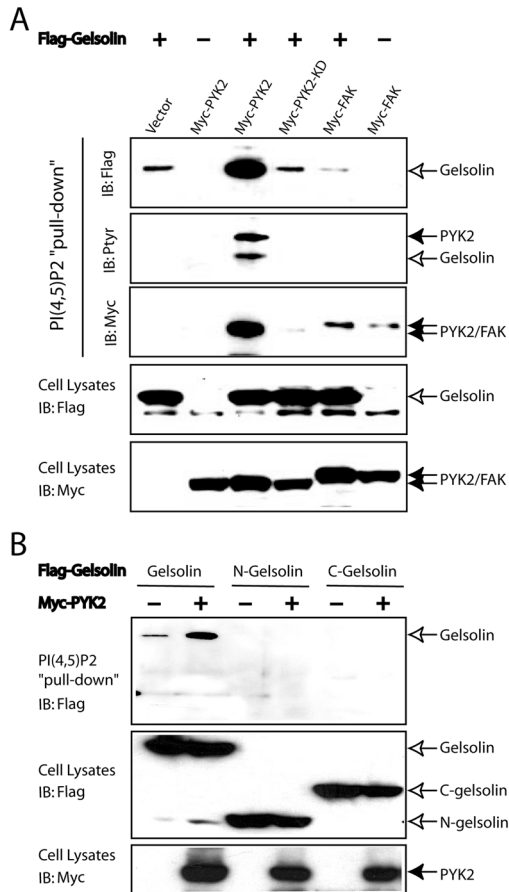
**Figure 4. Inhibition of gelsolin-actin binding by PYK2.** HEK 293 cells expressing gelsolin, N-gelsolin, or C-gelsolin with or without PYK2 were lysed, and cell lysates were subjected to analyses of gelsolin-actin monomer binding by coimmunoprecipitation of gelsolin with actin (A) and DNaseI-Sepharose pull-down (B). (C) Coimmunoprecipitation of gelsolin with actin in HEK 293 cell lysates expressing gelsolin with PYK2, PYK2-KD, or FAK. (D) Gelsolin-F-actin cosedimentation. HEK 293 cells expressing the indicated proteins were lysed in a buffer containing 1 mM EGTA to chelating calcium. In coimmunoprecipitation assays, 30 mM MgCl<sub>2</sub> was added in the washing buffer to depolymerize actin filaments.

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 NH<sub>2</sub>-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 MgCl<sub>2</sub> to depolymerize F-actin, and subsequent immunoblotting for actin. Because of the presence of EGTA in the

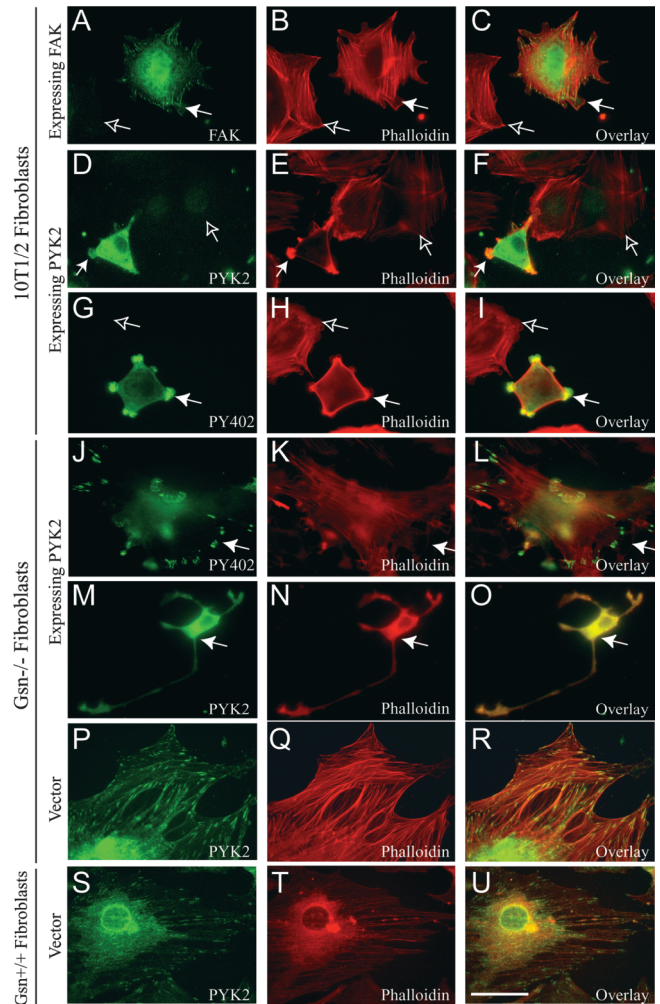
assay, the gelsolin-actin binding detected is believed to be calcium-independent, and thus mediated by the NH<sub>2</sub>-terminal region. Indeed, under these conditions, C-gelsolin did not bind to actin, whereas N-gelsolin did (Fig. 4 A). Interestingly, actin interaction with the full-length gelsolin 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 NH<sub>2</sub>-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).



**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.

### 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 association (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 with or without PYK2. Gelsolin bound to the beads was detected by immunoblotting with anti-Flag antibodies. PI(4,5)P2-associated gelsolin was significantly increased when PYK2 was coexpressed with gelsolin (Fig. 5 A). This effect appeared to be PYK2 specific and require the kinase activity of PYK2 because FAK and the catalytically inactive PYK2-KD failed to increase in gelsolin-PI(4,5)P2 binding (Fig. 5 A). Furthermore, PI(4,5)P2-associated gelsolin appeared to be tyrosine phosphorylated (Fig. 5 A). Although PI(4,5)P2 beads failed to precipitate PYK2, the beads did precipitate PYK2 when gelsolin was coexpressed (Fig. 5 A), indicating the formation of a ternary complex



**Figure 6. Increase in actin polymerization at fibroblastic cell periphery by PYK2.** 10T1/2 or embryonic fibroblasts derived from gelsolin knock-out (*gsn*<sup>-/-</sup>) or wild type (*gsn*<sup>+/+</sup>) 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  $\mu$ m.

of PI(4,5)P2-gelsolin-PYK2. Next, we examined whether PYK2 regulation of PI(4,5)P2-gelsolin binding depends on PYK2-gelsolin interaction. N-gelsolin contains PI(4,5)P2, but not PYK2 binding site, whereas C-gelsolin has PYK2, but not PI(4,5)P2 binding site (Janmey and Stossel, 1987; Robinson et al., 2000; Sun et al., 2000). PYK2 increased PI(4,5)P2 binding to full-length gelsolin, but not N- or C-gelsolin (Fig. 5 B), suggesting the requirement of the COOH-terminal region of gelsolin. These results suggest that PYK2 regulation of gelsolin binding to PI(4,5)P2 may play a role in inhibiting gelsolin-G-actin interaction.

### PYK2 stimulation of actin polymerization at fibroblastic cell periphery

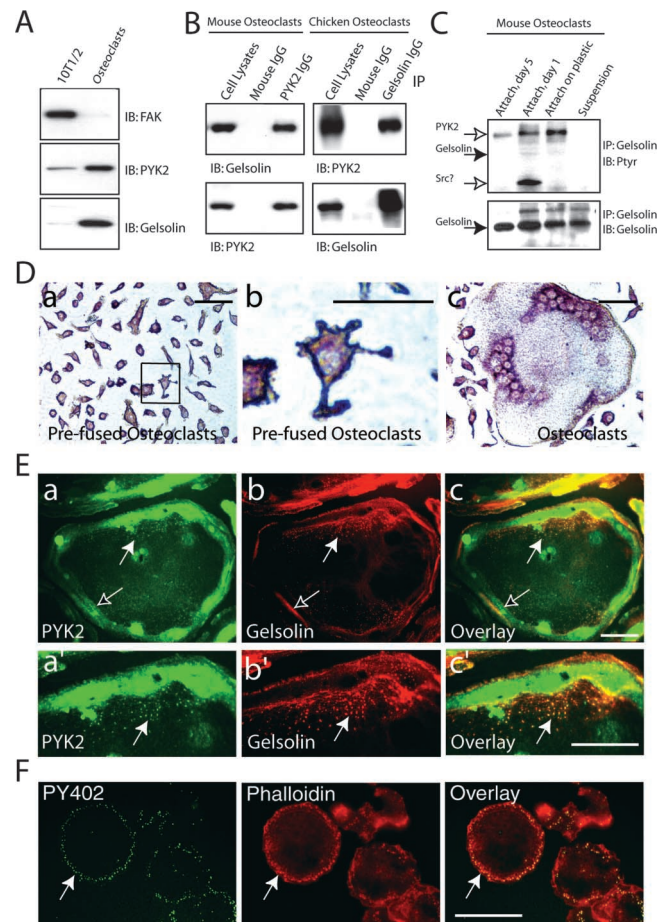
Gelsolin caps actin filaments at the barbed ends. This event requires gelsolin-G-actin binding and is negatively regulated by gelsolin-PI(4,5)P2 association (Janmey and Stossel, 1987;

Robinson et al., 2000; Sun et al., 2000). The reciprocal relationship between an increase in gelsolin-PI(4,5)P<sub>2</sub> binding and an inhibition of gelsolin-G-actin association by PYK2 suggests that PYK2 may lead to gelsolin uncapping and actin filament polymerization. To test this hypothesis, we examined actin polymerization in 10T1/2 fibroblasts expressing PYK2-WT by phalloidin staining. As shown in Fig. 6, fibroblasts expressing PYK2-WT, but not FAK-WT, exhibited increased phalloidin staining at the cell periphery. Interestingly, although expressed PYK2 was distributed in both cell periphery and cytoplasm (Fig. 6 D), activated PYK2 stained by phosphotyrosine 402 (PY402) antibody was mainly located at the cell periphery (Fig. 6 G). This staining pattern was in close proximity with that of phalloidin in PYK2 expressing cells, suggesting that activated PYK2 may contribute to the increased actin polymerization at the cell periphery. Moreover, in fibroblasts derived from *gsn*<sup>-/-</sup> or *gsn*<sup>+/+</sup> mice, endogenous PYK2 was localized at focal adhesions (Fig. 6, P–U), a finding consistent with previous report in smooth muscle cells or 10T1/2 fibroblasts (Brinson et al., 1998; Du et al., 2001). On PYK2 expression, *Gsn*<sup>-/-</sup> fibroblasts either remained unchanged in shape or had neurite-like processes (Fig. 6, M–R). However, PYK2 expression in *gsn*<sup>+/+</sup> cells led to apoptosis (unpublished data), a finding consistent with previous report (Xiong and Parsons, 1997). These results suggest that PYK2 induction of actin filaments at the cell periphery and apoptosis requires gelsolin.

### 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 osteoclastic 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



**Figure 7. Interaction and colocalization of gelsolin with PYK2 in osteoclasts.** (A) Western blot analysis of the expression of PYK2, FAK, and gelsolin in 10T1/2 fibroblasts and mouse osteoclasts. Cell lysates (10  $\mu$ g of protein) were subjected to SDS-PAGE and immunoblotting using indicated antibodies. (B) Coimmunoprecipitation of gelsolin with PYK2 in both mouse and chicken osteoclasts. Cell lysates (500  $\mu$ g) were incubated with anti-PYK2 (PYK2 IgG), anti-gelsolin (Gelsolin IgG), or nonspecific mouse IgGs (Mouse IgG), and resulting immunocomplexes immunoblotted with antibodies against gelsolin or PYK2. Lysate inputs (50  $\mu$ g, cell lysates) were loaded. (C) Tyrosine phosphorylation of gelsolin in mouse osteoclasts. Mouse osteoclastic precursors cultured in suspension dishes were held in suspension for 2 h (Suspension), remained in suspension dishes (Attach on plastic), or cultured in tissue culture dishes with M-CSF and Rank ligand for 1 d (Attach, day 1) and 5 d (Attach, day 5). Immunoprecipitations with anti-gelsolin antibodies were immunoblotted with antibodies against phosphotyrosine (RC20) or gelsolin. Open arrows indicate the proteins that co-migrate with PYK2 or Src, and the closed arrows indicate the protein that co-migrates with gelsolin. (D) TRAP staining of mouse osteoclasts. Mouse osteoclasts were stained with TRAP, marker of osteoclasts. Both fused (c) and prefused osteoclasts (a and b) were positive for TRAP staining. (E) Coimmunostaining of the active PYK2 (PY402) with F-actin in chicken osteoclasts. (F) Coimmunostaining of the active PYK2 (PY402) with F-actin in chicken osteoclasts. Mouse or chicken osteoclasts were fixed with 4% PFA, permeabilized with 1% Triton X-100, and stained with indicated antibodies. Bars (D–F), 50  $\mu$ m.

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-

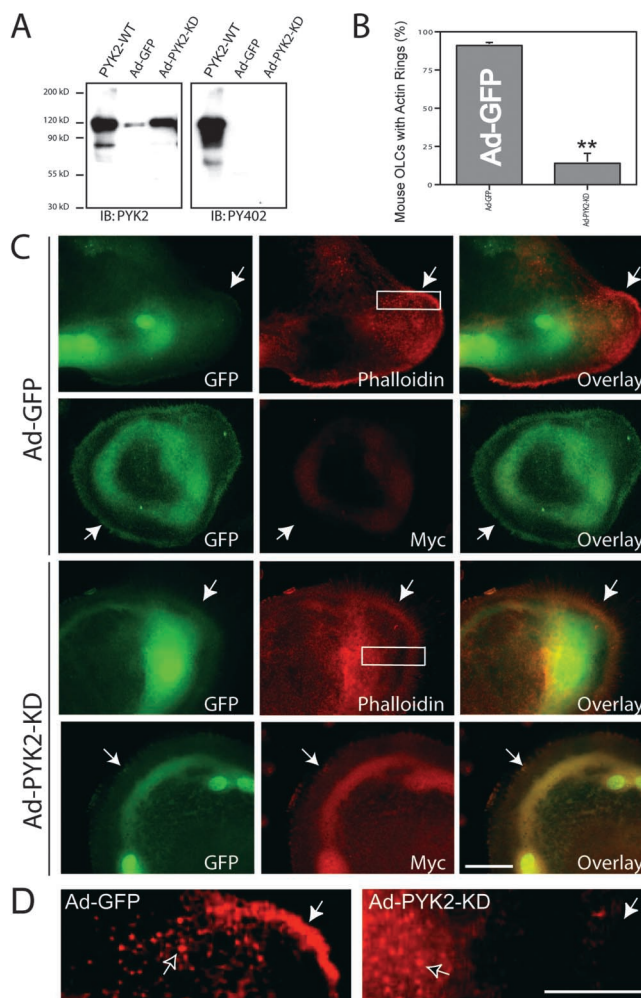
munostained using antibodies against PYK2 (polyclonal; United Biomedical, Inc.) and gelsolin (monoclonal). The PYK2 antibody was specific and did not cross-react with overexpressed FAK (Du et al., 2001). PYK2 was distributed in dot- or ringlike podosomes at the cell periphery. Gelsolin was colocalized 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. Fig. 8 A shows the expression and tyrosine phosphorylation of PYK2 and its mutant in virus-infected mouse osteoclasts. Although nearly all osteoclasts (>90%) infected with the adenovirus encoding GFP exhibited normal actin ring and podosome structures, the majority (>80%) of osteoclasts expressing PYK2-KD had no visible actin ring structures (Fig. 8, B and C), indicating that PYK2 activity is required for the actin ring formation in osteoclasts. Most dotlike podosomes were distributed at the cell periphery in osteoclasts expressing GFP adenovirus (Fig. 8 D). In contrast, podosomes were concentrated at central regions of the cytoplasm in PYK2-KD expressing osteoclasts (Fig. 8 D). These results are consistent with the previous report that osteoclasts expressing PYK2 anti-sense RNA exhibit major defects of actin ring formation (Duong et al., 2001). These results suggest that PYK2 activation may be required for the formation of cell periphery podosomes, or alternatively, translocation of podosomes from central regions to the cell periphery, which is important for the assembly of actin ring structures.

### 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)P<sub>2</sub> is increased, but gelsolin binding to actin monomer is decreased. We speculate that this event may lead to the uncapping of



**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  $\mu$ m.

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-depend-



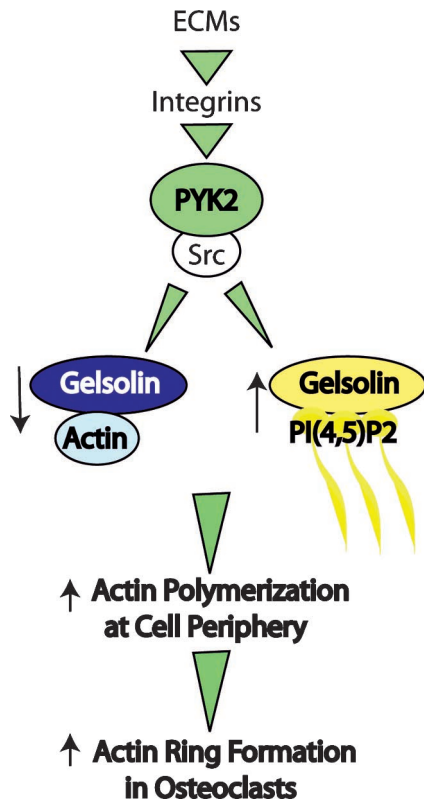


Figure 9. **A working hypothesis by which PYK2 regulates actin cytoskeletal organization in osteoclasts.** Osteoclastic adhesion to bone matrix activates PYK2 and increases PYK2–gelsolin interaction. Gelsolin binding to phosphatidylinositol is increased. At the same time, the gelsolin–actin monomer interaction is decreased, which prevents gelsolin from capping actin filaments at barbed ends. Subsequently, the growth of actin filaments at cell periphery is increased, which is important for actin ring formation in osteoclasts.

dent actin polymerization at the cell periphery. In addition, PYK2 may increase gelsolin-severing activity by decreasing the EGTA stable gelsolin–actin complex and increasing free gelsolin that can sever actin filaments in the presence of calcium (Janmey et al., 1985). Although these results suggest that gelsolin may be a target of PYK2 in regulating actin polymerization, they do not exclude gelsolin-independent mechanisms. In addition to gelsolin, we believe PYK2 may regulate functions of other proteins important for actin polymerization. These proteins include Rho family GTPases (Ren et al., 2001) and WASP family proteins.

The exact mechanisms by which PYK2 decreases gelsolin–actin interaction remain to be identified. The dependence of the PYK2 regulation of gelsolin–actin interaction on the kinase activity suggests an important role of tyrosine phosphorylation. An intriguing hypothesis is that on phosphorylation by PYK2, gelsolin has a lower affinity for actin molecules due to a conformational change. Note that gelsolin tyrosine phosphorylation was very low when PYK2 was activated in osteoclasts. This may suggest that other mechanisms, in addition to tyrosine phosphorylation of gelsolin, are involved in PYK2 regulation of gelsolin activity. Earlier studies have shown that the gelsolin–actin interaction is regulated by PI(4,5)P2 (Janmey and Stossel, 1987; Robinson et al., 2000; Sun et al., 2000). There is a reciprocal relationship

between gelsolin binding to actin and that to PI(4,5)P2. It is believed that binding to PI(4,5)P2 causes a conformational change in gelsolin that causes the release of actin (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, *Drosophila* 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 (Chelliah 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 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*<sup>-/-</sup>) mice were provided by Dr. David Kwiatkowski (Brigham and Women's Hospital, Boston, MA; Witke et al., 1995). The *Gsn*<sup>-/-</sup> mouse was maintained in mixed Sv129-BALB/c and Sv129-C57/bl backgrounds. Littermates that were *Gsn*<sup>-/-</sup> derived from *Gsn*<sup>+/-</sup> 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 transcriptional 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<sup>+</sup> β-gal<sup>+</sup> colonies, and was retransformed 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 (MEQKLISEEDL) 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 (MDYKDDDDKGP) 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 (QuikChange<sup>®</sup>; 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  $\mu$ g/ml penicillin G, and 100  $\mu$ g/ml streptomycin (GIBCO BRL). The calcium phosphate precipitation method was used to transfect HEK 293 cells, and 20–30  $\mu$ l SuperFect<sup>®</sup> (Invitrogen) was incubated with the constructs to transfect 10T1/2 cells.

Dermal fibroblasts derived from Gsn<sup>-/-</sup> and Gsn<sup>+/+</sup> mice were generated as described previously (Witke 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<sup>®</sup> was used for transfection.

Mouse osteoclasts were generated by in vitro differentiation of mouse bone marrow macrophages (BMMs) by RANKL/M-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  $\alpha$ -MEM containing 10% FBS plus 10 ng/ml recombinant M-CSF (R&D Systems). Cells were incubated at 37°C with 5% CO<sub>2</sub> overnight. Nonadherent cells were harvested 12 h later and subjected to Ficoll-Hypaque gradient to purify BMMs. Isolated BMMs were then cultured in  $\alpha$ -MEM containing 10% FBS plus 10 ng/ml recombinant M-CSF. To generate osteoclasts, 10<sup>5</sup> BMMs were plated in one well of a 24-well plate in  $\alpha$ -MEM containing 10% FBS in the presence of 10 ng/ml recombinant M-CSF and 88 ng/ml recombinant GST-RANKL. Osteoclasts began to form 96 h later. The identity of osteoclasts was confirmed by TRAP staining using the Leukocyte Acid Phosphatase Kit (Sigma-Aldrich). Chicken osteoclasts were generated as described previously (Williams et al., 1996).

### Protein pull-down assays

GST-fusion protein pull-down assay was performed as described previously (Ren et al., 2001). DNaseI-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, 1 mM EGTA, 2 mM MgCl<sub>2</sub>, 1 mM sodium vanadate, 10  $\mu$ g/ml leupeptin, 40  $\mu$ g/ml aprotinin, 80  $\mu$ g/ml benzamide, and 1 mM PMSF. Cell lysates were incubated with DNaseI immobilized on Sepharose beads at 4°C for 1 h. Actin monomers bind DNaseI 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<sup>™</sup> immunoplates (Nunc) were coated with purified human gelsolin (5 pmol/well; Cytoskeleton, Inc.) in BBS (125 mM borate, 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 BBS, followed by incubating with mouse anti-GST mAb (1:20,000) for 2 h at RT. After washing four times in BBS and once with substrate buffer (0.1 mM MgCl<sub>2</sub>, 5% 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 coimmunoprecipitation 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 MgCl<sub>2</sub>, 1 mM sodium vanadate, 10  $\mu$ g/ml leupeptin, 40  $\mu$ g/ml aprotinin, 80  $\mu$ g/ml benzamide, and 1 mM PMSF. 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 MgCl<sub>2</sub> 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 MgCl<sub>2</sub>, and 0.5 mM DTT) supplemented with 1 mM EGTA, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml aprotinin, and 1  $\mu$ g/ml pepstatin 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 phalloidin (Molecular Probes, Inc.) to a final concentration of 25  $\mu$ g/ml. 5.5  $\mu$ M F-actin was added to clarified cell lysates (50  $\mu$ g total protein) to final volume of 200  $\mu$ l, 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  $\mu$ g purified gelsolin was incubated with immunoprecipitated PYK2 or mutants in 40  $\mu$ l phosphorylation buffer containing 10 mM Hepes, pH 7.6, 10 mM magnesium acetate, 5  $\mu$ Ci of  $\gamma$ [<sup>32</sup>P]ATP, and 50  $\mu$ M 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

The PI(4,5)P<sub>2</sub> beads were purchased from Echelon Biosciences Incorporated. Gelsolin-PI(4,5)P<sub>2</sub> binding 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 PI(4,5)P<sub>2</sub> beads for 4 h at 4°C. Gelsolin-bound beads were subjected to immunoblotting with indicated antibodies.

### Generation of adenovirus and infection of osteoclasts

The recombinant, replication-deficient adenoviruses expressing PYK2 and catalytically inactive PYK2 were generated using the AdEasy<sup>™</sup> 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<sup>®</sup> imaging software.

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## References

- Akisaka, T., H. Yoshida, S. Inoue, and K. Shimizu. 2001. Organization of cytoskeletal F-actin, G-actin, and gelsolin in the adhesion structures in cultured osteoclast. *J. Bone Miner. Res.* 16:1248–1255.
- Azuma, T., W. Witke, T.P. Stossel, J.H. Hartwig, and D.J. Kwiatkowski. 1998. Gelsolin is a downstream effector of rac for fibroblast motility. *EMBO J.* 17:1362–1370.
- Boyce, B.F., T. Yoneda, C. Lowe, P. Soriano, and G.R. Mundy. 1992. Requirement of pp60src expression for osteoclasts to form ruffled borders and resorb bone in mice. *J. Clin. Invest.* 90:1622–1627.
- Brinson, A.E., T. Harding, P.A. Diliberto, Y. He, X. Li, D. Hunter, B. Herman, H.S. Earp, and L.M. Graves. 1998. Regulation of a calcium-dependent tyrosine kinase in vascular smooth muscle cells by angiotensin II and platelet-derived growth factor. Dependence on calcium and the actin cytoskeleton. *J. Biol. Chem.* 273:1711–1718.
- Brown, M.C., J.A. Perrotta, and C.E. Turner. 1996. Identification of LIM3 as the principal determinant of paxillin focal adhesion localization and characterization of a novel motif on paxillin directing vinculin and focal adhesion kinase binding. *J. Cell Biol.* 135:1109–1123.
- Chaponnier, C., H.L. Yin, and T.P. Stossel. 1987. Reversibility of gelsolin/actin interaction in macrophages. Evidence of  $Ca^{2+}$ -dependent and  $Ca^{2+}$ -independent pathways. *J. Exp. Med.* 165:97–106.
- Chellaiah, M., and K.A. Hruska. 1996. Osteopontin stimulates gelsolin associated phosphoinositide levels and PtdIns 3-hydroxyl kinase. *Mol. Biol. Cell.* 7:743–753.
- Chellaiah, M., N. Kizer, M. Silva, U. Alvarez, D. Kwiatkowski, and K.A. Hruska. 2000. Gelsolin deficiency blocks podosome assembly and produces increased bone mass and strength. *J. Cell Biol.* 148:665–678.
- De Corte, V., J. Gettemans, and J. Vandekerckhove. 1997. Phosphatidylinositol 4,5-bisphosphate specifically stimulates PP60(c-src) catalyzed phosphorylation of gelsolin and related actin-binding proteins. *FEBS Lett.* 401:191–196.
- De Corte, V., H. Demol, M. Goethals, J. Van Damme, J. Gettemans, and J. Vandekerckhove. 1999. Identification of Tyr438 as the major in vitro c-Src phosphorylation site in human gelsolin: a mass spectrometric approach. *Protein Sci.* 8:234–241.
- Du, Q.-S., X.-R. Ren, Y. Xie, Q. Wang, L. Mei, and W.-C. Xiong. 2001. Inhibition of PYK2-induced cytoskeletal reorganization, PYK2 autophosphorylation, and focal adhesion targeting by FAK. *J. Cell Sci.* 114:2977–2987.
- Duong, L.T., P.T. Lakkakorpi, I. Nakamura, M. Machwate, R.M. Nagy, and G.A. Rodan. 1998. PYK2 in osteoclasts is an adhesion kinase, localized in the sealing zone, activated by ligation of  $\alpha v \beta 3$  integrin, and phosphorylated by Src kinase. *J. Clin. Invest.* 102:881–892.
- Duong, L.T., I. Nakamura, P.T. Lakkakorpi, L. Lipfert, A.J. Bett, and G.A. Rodan. 2001. Inhibition of osteoclast function by adenovirus expressing antisense PYK2. *J. Biol. Chem.* 276:7484–7492.
- Feng, X., D.V. Novack, R. Faccio, D.S. Ory, K. Aya, M.I. Boyer, K.P. McHugh, F.P. Ross, and S.L. Teitelbaum. 2001. A Glanzmann's mutation in beta 3 integrin specifically impairs osteoclast function. *J. Clin. Invest.* 107:1137–1144.
- He, T.-C., S. Zhou, L.T. Da Costa, J. Yu, K.W. Kinzler, and B. Vogelstein. 1998. A simplified system for generating recombinant adenoviruses. *Proc. Natl. Acad. Sci. USA.* 95:2509–2514.
- Janmey, P.A., and T.P. Stossel. 1987. Modulation of gelsolin function by phosphatidylinositol 4,5-bisphosphate. *Nature.* 325:362–364.
- Janmey, P.A., C. Chaponnier, S.E. Lind, K.S. Zaner, T.P. Stossel, and H.L. Yin. 1985. Interactions of gelsolin and gelsolin-actin complexes with actin. Effects of calcium on actin nucleation, filament severing, and end blocking. *Biochemistry.* 24:3714–3723.
- Kanehisa, J., T. Yamataka, S. Doi, K. Turksen, J.N.M. Heersche, J.E. Aubin, and H. Takeuchi. 1990. A band of F-actin containing podosomes is involved in bone resorption by osteoclasts. *Bone.* 11:287–293.
- Kurth, M.C., L.L. Wang, J. Dingus, and J. Bryan. 1983. Purification and characterization of a gelsolin-actin complex from human platelets. Evidence for  $Ca^{2+}$ -insensitive functions. *J. Biol. Chem.* 258:10895–10903.
- Kwiatkowski, D.J. 1999. Functions of gelsolin: motility, signaling, apoptosis, and cancer. *Curr. Opin. Cell Biol.* 11:103–108.
- Kwiatkowski, D.J., P.A. Janmey, and H.L. Yin. 1989. Identification of critical functional and regulatory domains in gelsolin. *J. Cell Biol.* 108:1717–1726.
- Lakkakorpi, P.T., I. Nakamura, R.M. Nagy, J.T. Parsons, G.A. Rodan, and L.T. Duong. 1999. Stable association of PYK2 and p130(Cas) in osteoclasts and their co-localization in the sealing zone. *J. Biol. Chem.* 274:4900–4907.
- Lev, S., H. Moreno, R. Martinez, P. Canoll, E. Peles, J.M. Musacchio, G.D. Plowman, B. Rudy, and J. Schlessinger. 1995. Protein tyrosine kinase PYK2 involved in  $Ca^{2+}$ -induced regulation of ion channel and MAP kinase functions. *Nature.* 376:737–745.
- Lev, S., J. Hernandez, R. Martinez, A. Chen, G. Plowman, and J. Schlessinger. 1999. Identification of a novel family of targets of PYK2 related to *Drosophila* retinal degeneration B (rdgB) protein. *Mol. Cell. Biol.* 19:2278–2288.
- Lind, S.E., H.L. Yin, and T.P. Stossel. 1982. Human platelets contain gelsolin. A regulator of actin filament length. *J. Clin. Invest.* 69:1384–1387.
- Lind, S.E., P.A. Janmey, C. Chaponnier, T.J. Herbert, and T.P. Stossel. 1987. Reversible binding of actin to gelsolin and profilin in human platelet extracts. *J. Cell Biol.* 105:833–842.
- Luo, Z.G., Q. Wang, J.Z. Zhou, J. Wang, Z. Luo, M.Y. Liu, X. He, A. Wynshaw-Boris, W.C. Xiong, B. Lu, and L. Mei. 2002. Regulation of AChR clustering by Dishevelled interacting with MuSK and PAK1. *Neuron.* 35:489–505.
- Marchisio, P.C., D. Cirillo, L. Naldini, M.V. Primavera, A. Teti, and A. Zamboni-Zallone. 1984. Cell-substratum interaction of cultured avian osteoclasts is mediated by specific adhesion structures. *J. Cell Biol.* 99:1696–1705.
- Matsuya, M., H. Sasaki, H. Aoto, T. Mitaka, K. Nagura, T. Ohba, M. Ishino, S. Takahashi, R. Suzuki, and T. Sasaki. 1998. Cell adhesion kinase beta forms a complex with a new member, Hic-5, of proteins localized at focal adhesions. *J. Biol. Chem.* 273:1003–1014.
- Pfaff, M., and P. Jurdic. 2001. Podosomes in osteoclast-like cells: structural analysis and cooperative roles of paxillin, proline-rich tyrosine kinase 2 (PYK2) and integrin  $\alpha v \beta 3$ . *J. Cell Sci.* 114:2775–2786.
- Ren, X.R., Q.S. Du, Y.Z. Huang, S.Z. Ao, L. Mei, and W.C. Xiong. 2001. Regulation of CDC42 GTPase by PYK2 interacting with PSGAP, a novel PH and SH3 domain containing rhoGAP protein. *J. Cell Biol.* 152:971–983.
- Robinson, R.C., M. Meijillano, V.P. Le, L.D. Burtnick, H.L. Yin, and S. Choe. 2000. Domain movement in gelsolin: a calcium-activated switch. *Science.* 286:1939–1942.
- Sasaki, H., K. Nagura, M. Ishino, H. Tobioka, K. Kotani, and T. Sasaki. 1995. Cloning and characterization of cell adhesion kinase  $\beta$ , a novel protein-tyrosine kinase of the focal adhesion kinase subfamily. *J. Biol. Chem.* 270:21206–21219.
- Schaller, M.D., C.A. Borgman, B.S. Cobb, R.R. Vines, A.B. Reynolds, and J.T. Parsons. 1992. pp125<sup>FAK</sup>, a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc. Natl. Acad. Sci. USA.* 89:5192–5196.
- Schwartzberg, P.L., L. Xing, O. Hoffmann, C.A. Lowell, L. Garrett, B.F. Boyce, and H.E. Varmus. 1997. Rescue of osteoclast function by transgenic expression of kinase-deficient Src in Src<sup>-/-</sup> mutant mice. *Genes Dev.* 11:2835–2844.
- Soriano, P., C. Montgomery, R. Geske, and A. Bradley. 1991. Targeted disruption of the c-Src proto-oncogene leads to osteopetrosis in mice. *Cell.* 64:693–702.
- Sun, H.Q., M. Yamamoto, M. Meijillano, and H.L. Yin. 2000. Gelsolin, a multifunctional actin regulatory protein. *J. Biol. Chem.* 274:33179–33182.
- Tachibana, K., T. Sato, N. D'Avirro, and C. Morimoto. 1995. Direct association of pp125<sup>FAK</sup> with paxillin, the focal adhesion-targeting mechanism of pp125<sup>FAK</sup>. *J. Exp. Med.* 182:1089–1100.
- Tanaka, S., M. Amling, L. Neff, A. Peyman, E. Uhlmann, J.B. Levy, and R. Baron. 1996. c-Cbl is downstream of c-Src in a signaling pathway necessary for bone resorption. *Nature.* 383:528–531.
- Tarone, G., D. Cirillo, F.G. Giancotti, P.M. Comoglio, and P.C. Marchisio. 1985. Rous sarcoma virus-transformed fibroblasts adhere primarily as discrete protrusions of the ventral membrane called podosomes. *Exp. Cell Res.* 159:141–157.
- Turner, C.E. 2000. Paxillin and focal adhesion signaling. *Nat. Cell Biol.* 2:E231–E236.
- Wang, L.L., and J. Bryan. 1981. Isolation of calcium-dependent platelet proteins that interact with actin. *Cell.* 25:637–649.
- Williams, J.P., H.C. Blair, M.A. McKenna, S.E. Jordan, and J.M. McDonald. 1996. Regulation of avian osteoclastic H<sup>+</sup>-ATPase and bone resorption by tamoxifen and calmodulin antagonists. Effects independent of steroid receptors. *J. Biol. Chem.* 271:12488–12495.
- Witke, W., A.H. Sharpe, J.H. Hartwig, T. Azuma, T.P. Stossel, and D.J. Kwiatkowski. 1995. Hemostatic, inflammatory, and fibroblast responses are blunted in mice lacking gelsolin. *Cell.* 81:41–51.
- Xiong, W.C., and J.T. Parsons. 1997. Induction of apoptosis after expression of PYK2, a tyrosine kinase structurally related to focal adhesion kinase. *J. Cell Biol.* 139:529–539.
- Yamamoto, M., D.H. Hilgemann, S. Feng, H. Bito, H. Ishihara, Y. Shibasaki, and H.L. Yin. 2001. Phosphatidylinositol 4,5-bisphosphate induces actin stress-fiber formation and inhibits membrane ruffling in CV1 cells. *J. Cell Biol.* 152:867–876.