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βPix-enhanced p38 Activation by Cdc42/Rac/PAK/MKK3/6-mediated Pathway

IMPLICATION IN THE REGULATION OF MEMBRANE RUFFLING*

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βPix (PAK-interacting exchange factor) is a recently identified guanine nucleotide exchange factor for Rho family small G protein Cdc42/Rac. The protein interacts with p21-activated protein kinase (PAK) through its SH3 domain. We examined the effect of βPix on MAP kinase signaling and cytoskeletal rearrangement in NIH3T3 fibroblast cells. Overexpression of βPix enhanced the activation of p38 in the absence of other stimuli and also induced translocation of p38 to the nucleus. This βPix-induced p38 activation was blocked by coexpression of dominant-negative Cdc42/Rac or kinase-inactive PAK, indicating that the effect of βPix on p38 is exerted through the Cdc42/Rac-PAK pathway and requires PAK kinase activity. The essential role of βPix in growth factor-stimulated p38 activation was evidenced by the blocking of platelet-derived growth factor-induced p38 activation in the cells expressing βPix SH3m (W43K) and βPix DHm (L238R,L239R). In addition, SB203580, a p38 inhibitor, and kinase-inactive p38 (T180A,Y182F) blocked membrane ruffling induced by βPix, suggesting that p38 might be involved in mediating βPix-induced membrane ruffling. The results in this study suggest that βPix might have a role in nuclear signaling, as well as in actin cytoskeleton regulation, and that some part of these cellular functions is possibly mediated by p38 MAP kinase.

The Rho family GTPases have emerged as key regulators that mediate extracellular signaling pathways leading to the formation of polarized actin-containing structures such as stress fibers, membrane ruffles, lamellipodia, and filopodia. Besides changes in cytoskeletal architecture, these GTPases mediate diverse biological events, including stimulation of DNA synthesis, cellular transformation, and signaling to the nucleus (for recent reviews, see Refs. 1 and 2). Although direct association of serine/threonine kinases or signaling proteins with the individual GTPases is critical for downstream effects, the precise mechanism by which these GTPases regulate actin cytoskeleton and exhibit these biological activities remains to be determined (3, 4). Among these effectors, the binding partner of Cdc42 and Rac, p21-activated kinase (PAK)[1] activity has been linked to Cdc42 and Rac-mediated cytoskeletal changes. The important roles that PAK plays as an effector of Cdc42/Rac signaling have been established from genetic and biochemical studies in yeasts and mammalian cells (reviewed in Refs. 5 and 6). The repertoire of signaling pathways that are responsive to activation by Rho family members is rapidly expanding, and many of these have shown apparent multiplicity of PAK-mediated signaling pathways (7).

Recently, the PAK-interacting exchange factor (Pix/COOL/p85SPR) family was cloned by several groups, including our laboratory (8–11). The Pix family has Dbl homology and a flanking PH domain, which are conserved in all of the guanine exchange factors for Rho GTPases. Pix can induce membrane ruffling, with an associated activation of Rac1 (9); however, the extent and selectivity between Pix isoforms to activate Cdc42 and Rac are not clear (10, 12). Pix interacts tightly with the regulatory N terminus of PAK via its SH3 domain. As shown by Daniels *et al.* (12), αPix stimulates PAK kinase activity through exchange factor-dependent and -independent mechanisms. Additionally, the guanine nucleotide exchange activity was positively modulated by PAK binding. However, the exact role of Pix in regulating PAK localization and function in the actin rearrangement remains to be clarified. Pix was included in a protein complex containing paxillin, PAK, Nck, and p95PKL (paxillin-kinase linker), a member of ARF-GAP family, and this suggests a possibility for the integration of the ARF and Rho family signal transduction at the cytoskeleton (13, 14). It was also reported that the guanine nucleotide exchange activity of Pix could be activated by direct association of the p85 regulatory subunit of phosphatidylinositol 3-kinase (15). Interaction of Pix with a variety of signaling proteins suggests that Pix might have an important role in mediating the effects of extracellular signals, *e.g.* growth factors, extracellular matrix, stress, and cytokine, to cytoskeletal rearrangement.

It was reported that Cdc42 and Rac also regulate the JNK/SAPK and p38 signaling pathway and the ability of PAK to regulate these same MAP kinases pathways (16–18). There are several reports showing that Dbl-related proteins, the activators of Rho GTPases, have roles in the regulation of several biological activities, including transformation, metastasis, cy-

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[1] The abbreviations used are: PAK, p21-activated kinase; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; MAP, mitogen-activated protein; DMEM, Dulbecco's modified Eagle's medium; PID, PAK inhibitory domain; HA, hemagglutinin; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; GEF, guanine nucleotide exchange factor; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.

toskeletal reorganization, and transcriptional activation (7). However, relatively little was known of the exact roles of these GEFs in the MAP kinase pathway, whereas Cdc42 and Rac were known as potent SAPK and p38 activators (19–21). Despite the coincidence of two signalings leading to actin reorganization and MAP kinase activity by Cdc42/Rac/PAK in many cases, there were few reports about cross-talk between these two signalings (22).

In the present study, we demonstrate that the overexpression of βPix in NIH3T3 fibroblast cells induces activation of p38 through a Cdc42/Rac-PAK-MKK3/6-mediated pathway, and this activation of p38 is necessary for the formation of membrane ruffles induced by βPix overexpression.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—All materials for culture were purchased from Life Technologies, Inc. NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% bovine calf serum and maintained in 10% CO_2 at 37 °C. Transient transfection of cells with mammalian expression vectors was performed using LipofectAMINE^{PLUS} according to manufacturer's instructions.

Expression Vectors—The coding region of βPix was subcloned into pFLAGCMV2 (Eastman Kodak Co.) and pcDNA3.1MycHis (Invitrogen), respectively. pEBB/HA-PAK1 was from Bruce J. Mayer. PAK inhibitory domain (PID: corresponding to 83–149 of hPAK1) was amplified by polymerase chain reaction using PID^{forward} (GGAATTCCACACAATTCATGTCGG) and PID^{reverse} (CGTCTAGATGACTTATCTGTAAAGC) primers, cut with *Eco*RI and *Xba*I, and inserted into the *Eco*RI-*Xba*I site of HA-linked pcDNA3. Site-directed mutagenesis was performed using a QuikChange^{TM} site-directed mutagenesis kit (Stratagene). The following mutagenic primers were used: pFLAGCMV2-βPix (W43K), 5′-GTGGAGGAAGGAGGCaaGTGGGAGGGCACACA C-3′ and 5′-GTGTGTGCCCTCCCACttGCCTCCTTCCTCCAC-3′; pFLAGCMV2-βPix (L238R,L239S), 5′-GTCTCTCCAGCTCCTTTgaCaGTGTGGGGTACTT GT-3′ and 5′-ACAAGTACCCCACA CtGtcAAAGGAGCTGGAGAGAC-3′; pEBB/HA-PAK1 (K299R), 5′-CAGGAGGTGGCCATTAgGCAGATGAATCTTCAG-3′ and 5′-CTGAAGATTCATCTGCcTAATGGTCACCTCTG-3′; pcDNA3/FLAG-p38 (T180A,Y182F), 5′-CAGATGATGAAATGgCAGGCTtCGTGGCCACTAGGTG-3′ and 5′-CACCTAGTGGCCACGaAGCCTGcCATTTCATCATCTG-3′; pcDNA3/HA-PID (L107F), 5′-AGCAGTGGGCCCGCTTGtTTCAGACATCAAATATC-3′ and 5′-GATATTTGATGTCTGAAaCAAGCGGGCCCACTGCT-3′.

Mismatches are indicated by lowercase letters. The mutation was verified by automatic DNA sequencing (Applied Biosystems).

Immunocytochemistry—35-mm dish-cultured cells were transfected on 0.1% gelatin-coated coverslip. After 24 h, the medium was removed, and the cells were starved in serum-free medium for 16 h before fixation. SB203580 (Calbiochem, Inc.) was treated for 2 h before fixation at 10 μM concentration. Cells were fixed in 3.7% paraformaldehyde solution in phosphate-buffered saline (PBS) for 10 min and permeabilized with 0.5% Triton X-100 in PBS for 5 min. Cells were incubated in 10% goat serum (Vector Laboratories, Inc.) and 3% bovine serum albumin-containing 0.1% Triton X-100 in PBS for 1 h. Primary antibodies were diluted as follows: Anti-FLAG M2 antibody (Sigma), 5000:1; anti-Myc 9E10 hybridoma culture medium, 200:1; anti-phospho-specific p38 rabbit antibody (New England Biolabs, Inc.), 500:1. Rhodamine-phalloidin (Molecular Probes) was diluted at 2000:1 and treated for 15 min after 1 h primary antibody incubation. Cells were then stained with fluorescein isothiocyanate or rhodamine-conjugated secondary antibodies (100:1, Jackson ImmunoResearch Laboratories, Inc.). 4,6-Diamidino-2-phenylindole (Molecular Probes) was added to VECTASHIELD mounting solution (Vector Laboratories, Inc.) at 2 μg/ml concentration. Cells were observed under the fluorescence microscope (Axioplan2, Zeiss) equipped with a 63× (1.4 NA) Planapochromat objective lens. Fluorescence micrographs were taken on T-max P3200 film (Kodak).

Cell Lysis, Immunoprecipitation, Immunoblotting, and Immune Complex Kinase Assay—To measure the activities of p38 and Erk, cells on a 100-mm dish were transfected with pcDNA3FLAG-p38 or -Erk together with other mammalian expression vectors of interest. At 40 h after transfection, cells were washed three times with ice-cold PBS and lysed in immunoprecipitation buffer (1% Triton X-100, 50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 10 μM leupeptin, and 1.5 μM pepstatin). Lysates were clarified by centrifugation at 15,000 × *g* for 10 min. Protein concentration in the supernatant was determined using Bio-Rad protein assay reagent (Bio-Rad). Clarified lysates of 500 μg of total protein were incubated with 4 μg of anti-FLAG M2 antibody for 4 h, followed by additional incubation with protein A-Sepharose (Amersham Pharmacia Biotech) for 1 h. The immunoprecipitates were washed three times with immunoprecipitation buffer, once with kinase buffer (50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 0.2 mM dithiothreitol), and resuspended in 20 μl of kinase buffer containing 5 μCi of [γ-^{32}P]ATP (PerkinElmer Life Sciences), 100 μM ATP, and 4 μg of glutathione *S*-transferase-ATF2 (for p38 assay) or

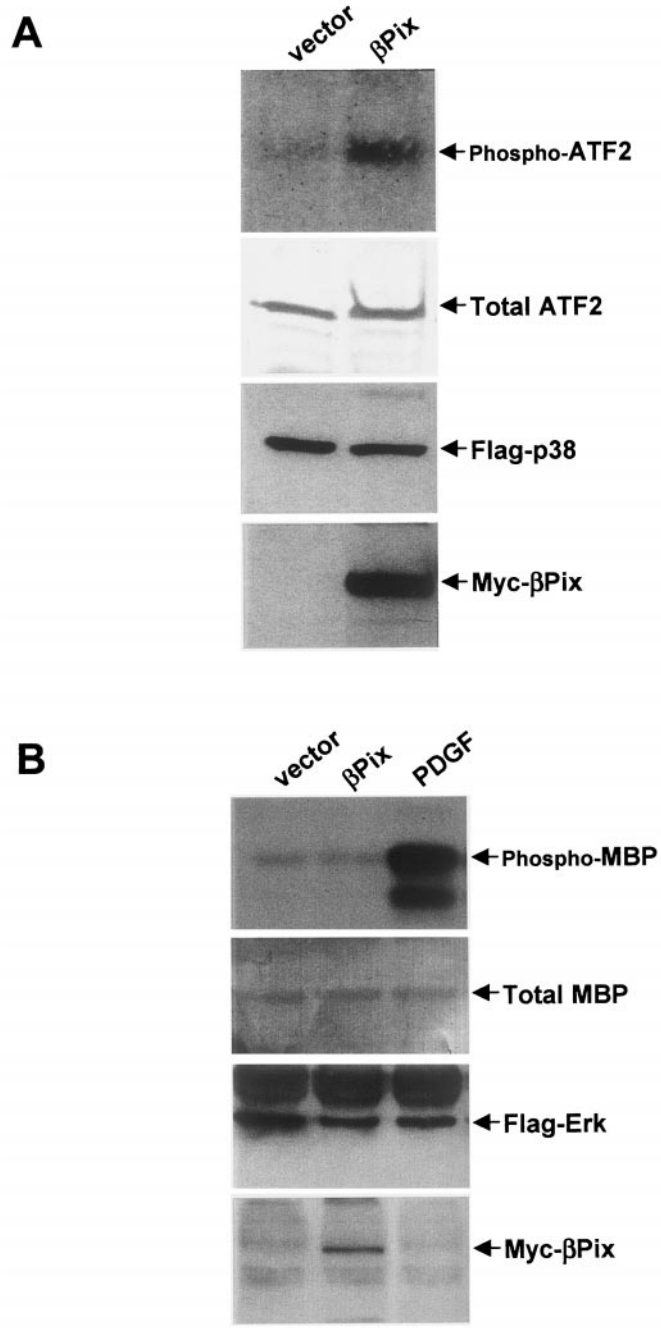


FIG. 1. **βPix augmented p38 activity, but not Erk kinase activity.** NIH3T3 fibroblast cells were transfected with empty vector or Myc-tagged βPix together with FLAG-tagged p38 or Erk. After serum starvation for 16 h in DMEM, cells were lysed, and immune complex kinase assays of p38 (*A*) and Erk (*B*) were performed as described under "Experimental Procedures." For a positive control in *B*, cells were treated with 20 ng/ml PDGF for 15 min before lysis (*third lane* in *B*). After visualization of phosphorylated ATF2 or MBP by autoradiography (*first panels*), the same membranes were probed with anti-FLAG M2 antibody (*third panels*) and then stained with Coomassie Brilliant Blue to normalize the amount of total ATF2 or MBP (*second panels*). The *fourth panels* show the amount of transfected Myc-βPix in 20 μg of total cell lysate using anti-Myc 9E10 antibodies. Data shown are representative of three independent experiments.

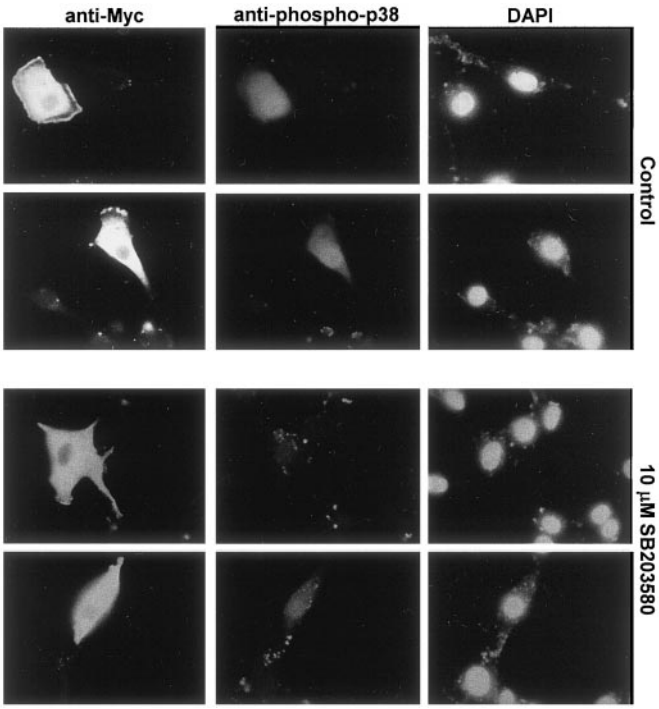


FIG. 2. **Effect of βPix overexpression on p38 activity at a single cell level.** NIH3T3 fibroblast cells were transiently transfected with Myc-tagged βPix. At 40 h after transfection, cells were treated without (*Control*) or with 10 μM SB203580 for 1 h and then triple-immunofluorescence staining was carried out using anti-Myc, anti-phospho-specific p38 antibodies, and 4,6-diamidino-2-phenylindole. Fluorescein isothiocyanate-labeled anti-mouse and tetramethylrhodamine B isothiocyanate-labeled anti-rabbit antibodies were used as second antibodies for anti-Myc and anti-p38 antibodies, respectively. Two representative examples of each result are presented. Three independent experiments were performed and two examples representative of all three experiments are presented.

MBP (for Erk kinase assay) and incubated at 30 °C for 30 min. Reactions were stopped by the addition of SDS-sample buffer (100 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.25% bromphenol blue). After SDS-polyacrylamide gel electrophoresis, proteins were transferred onto a polyvinylidine difluoride membrane (Millipore), and the membrane was subjected to autoradiography. The same membrane was then washed with 0.1% Triton X-100 containing PBS and immunoblotted using anti-FLAG M2 antibody. After visualization of kinase proteins by ECL reagent (Amersham Pharmacia Biotech), the membrane was stained with Coomassie Brilliant Blue to normalize the amount of substrates. To visualize active MKK3/6 protein, phosphorylation-specific MKK3/6-specific antibody (500:1) and MKK3 antibody (500:1) were used, respectively (New England Biolabs, Inc.). Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc.

RESULTS AND DISCUSSION

The effect of βPix on MAP kinases was examined using NIH3T3 fibroblast cells that were double transfected with FLAG-tagged MAP kinase and Myc-tagged βPix. Transfectants were subject to immune complex kinase assay and phosphorylation of each substrate was visualized by autoradiography. As shown in Fig. 1*A*, βPix augmented p38 activity about 3.5 times. We observed similar results in the immunoblotting analysis using phosphorylation-specific p38 antibody (data not shown). βPix had no effect on Erk kinase activity in several separated experiments (Fig. 1*B*), and JNK activity was slightly increased upon βPix overexpression (data not shown). Under these conditions, strong activation of Erk kinase by PDGF was observed. Next, we examined the effect of βPix overexpression on the p38 activation in a single cell level using phosphorylation-specific p38 antibody. In βPix-overexpressing cells, we could observe

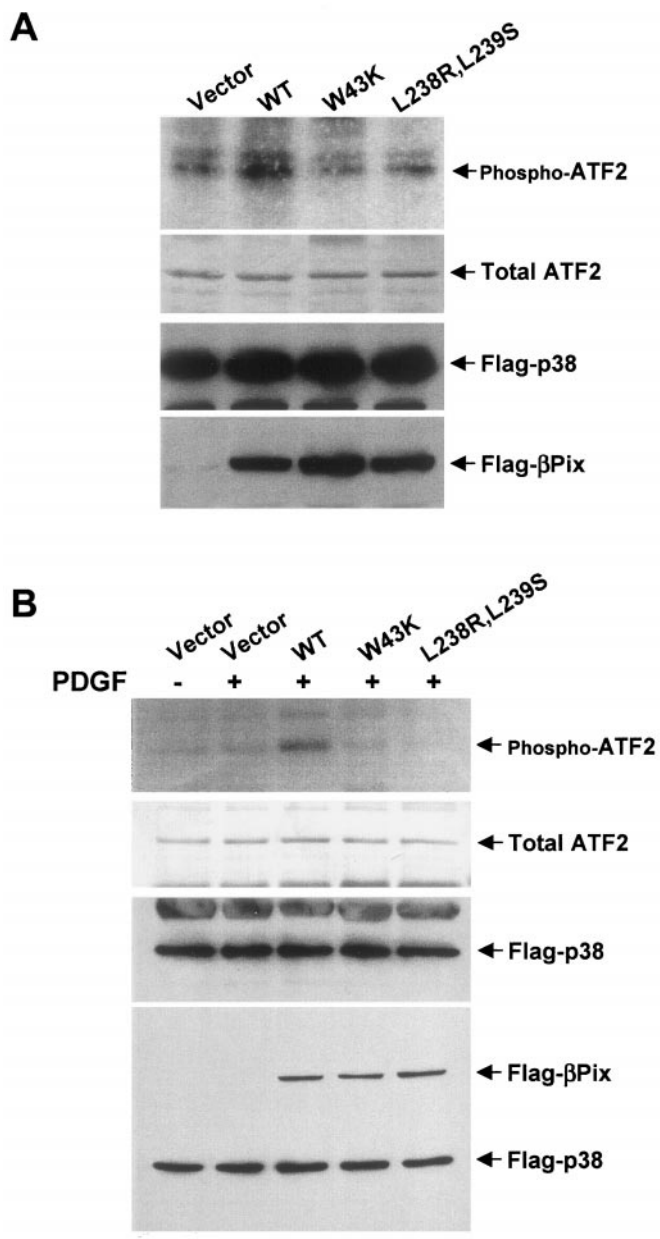


FIG. 3. **βPix SH3m (W43K) and βPix DHm (L238R,L239S) failed to activate p38 upon PDGF treatment.** NIH3T3 fibroblast cells were transfected with FLAG-tagged wild-type or mutant βPix together with FLAG-tagged p38. *A*, after 40 h of transfection, cells were lysed, and an immune complex kinase assay of p38 was performed as described in the legend to Fig. 1. *B*, after 24 h of transfection, media were changed with serum-free DMEM, and cells were cultured in serum-free media for 16 h. PDGF was treated at 20 ng/ml for 15 min, and immune complex kinase assays were performed as in *A*. The *fourth panels* show the amount of transfected FLAG-βPix and FLAG-p38 in 20 μg of total cell lysate using anti-FLAG M2 antibody. Data shown are representative of three to four independent experiments.

increased phospho-p38 signal, especially in the nucleus, compared with untransfected cells in the same field that had no or very weak signal (Fig. 2, *upper panels*). The treatment of SB203580, a p38-specific inhibitor, blocked βPix-induced p38 activation (Fig. 2, *lower panels*).

We next generated two mutants of βPix, βPix SH3m (W43K) and βPix DHm (L238R,L239S), whose SH3 domain and Dbl-homology domain were changed, respectively. Coimmunoprecipitation assay and glutathione *S*-transferase-pulldown assay showed that βPix SH3m cannot bind to PAK (data not shown). βPix DHm has been reported to have no exchange activity,

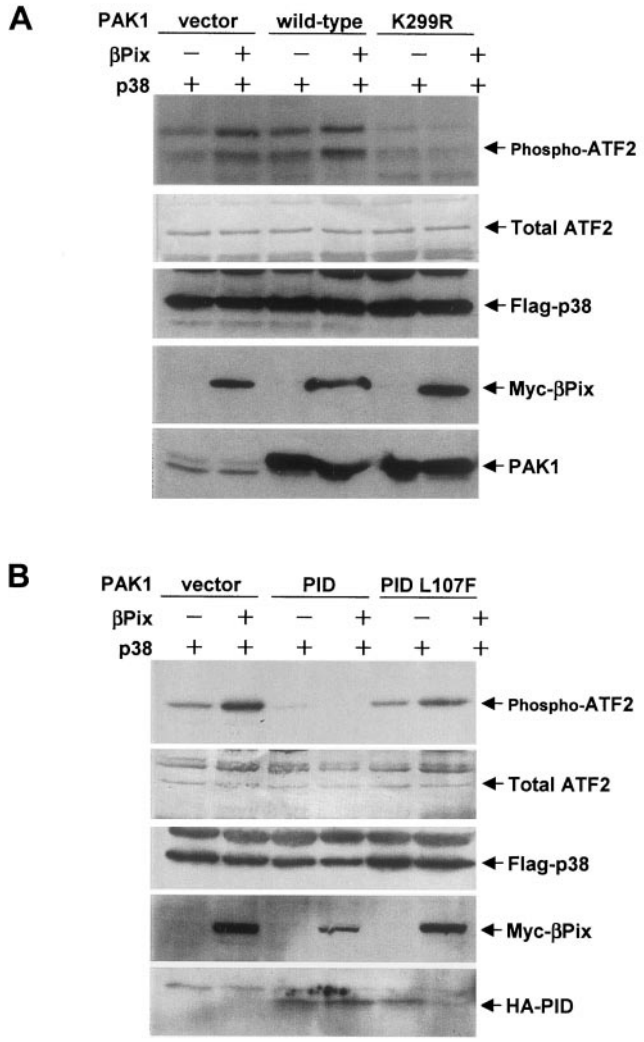


FIG. 4. **PAK1 kinase activity was essential for βPix-induced p38 activity.** NIH3T3 fibroblast cells were transfected with wild-type or K299R mutant of HA-tagged PAK1 (*A*) or wild-type or L107F mutant of HA-tagged PID (PAK1$^{83-149}$) (*B*) together with Myc-tagged βPix and FLAG-tagged p38. Immune complex kinase assays were done in the p38 immunoprecipitates as described in the legend to Fig. 1. Transfected Myc-βPix (*fourth panels* in *A* and *B*), PAK1 (*fifth panel* in *A*), and HA-PID (*fifth panel* in *B*) in 20 μg of total cell lysate were probed with anti-Myc 9E10, anti-PAK1, and anti-HA antibodies, respectively. Data shown are representative of three independent experiments.

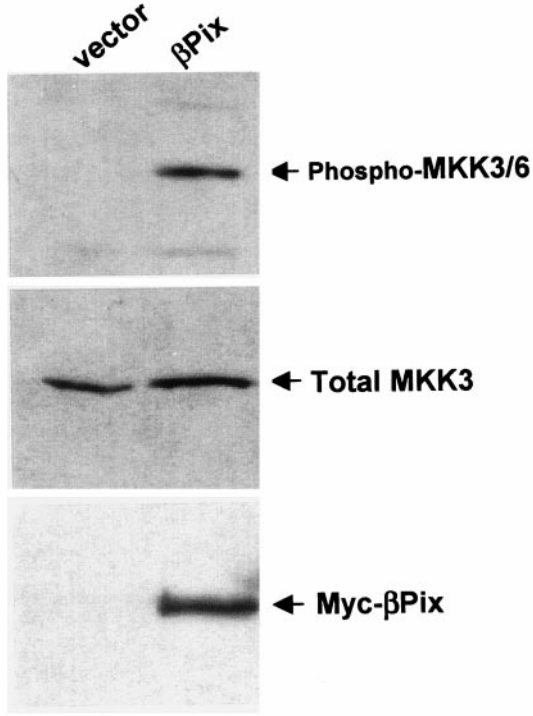
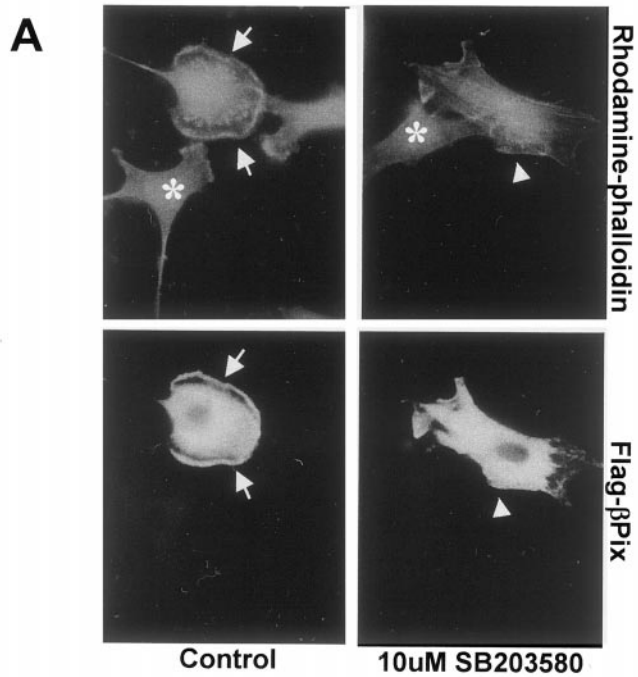


FIG. 5. **βPix overexpression enhanced MKK3/6 activation.** NIH3T3 fibroblast cells were transfected with empty vector or Myc-tagged βPix. Cell lysates were analyzed on a SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidine difluoride membrane, and immunoblotting analysis was accomplished using phospho-specific MKK3/6 (*first panel*), anti-MKK3 (*second panel*), and anti-Myc 9E10 (*third panel*) antibodies, respectively. Data shown are representative of three independent experiments.

while wild-type βPix acted as a GEF for Rac1 *in vivo* (9). Unlike wild-type βPix, overexpression of these two mutants failed to activate p38 (Fig. 3), indicating that PAK or other SH3 domain-binding proteins and GEF activity of βPix are essential for transmission of the extracellular signaling to p38 MAP kinase activation. p38 has been reported to be activated by several extracellular stimuli, such as growth factors, cytokines, and stresses (25, 26). We also examined the involvement of βPix in PDGF-induced p38 activation. In βPix-expressing cells, p38 activity was increased about four times upon PDGF treatment (Fig. 3*B*, lanes *Vector* and *WT*). However, in cells expressing βPix SH3m or βPIX DHm, PDGF-induced p38 activation was not evident (Fig. 3*B*, lanes *W43K* and *L238R,L239S*), suggesting that βPix might mediate p38 activation by PDGF *in vivo*. However, it should be noted that in either case of βPix SH3m or DHm, expression of mutant βPix still induced membrane ruffling (data not shown). The extent of ruffling was not much different from that of wild-type βPix in case of βPix SH3m and reduced by 20–30% in case of βPix DHm. In contrast, expression of the βPix SH3m-DHm double mutant failed to induce membrane ruffling and also did block PDGF-induced membrane ruffling (data not shown). Results suggest that disruptions of both PAK interaction (SH3) domain and GEF activity are necessary to block βPix-induced membrane ruffling. Blocking the function of either domain was not sufficient to inhibit membrane ruffling. This could be explained by GEF-dependent and -independent PAK activation by Pix (12) or by the role of PAK as a upstream activator of Rac (27). On the other hand, inhibition of p38 by either βPix SH3m or DHm (Fig. 3) indicates that both PAK interaction (SH3) domain and GEF activity are required for βPix-induced p38 activation. βPix-induced membrane ruffling in the case of βPix SH3m and DHm despite p38 inhibition is probably due to incomplete inhibition of p38 by either mutant. Although βPix SH3m and DHm could block p38 activation by PDGF upstream, the basal p38 activity could concert with other Rac (and/or PAK) effector(s) to induce membrane ruffling.

Rho family GTPases regulate JNK and p38, as appeared in the previous reports that the dominant-negative form of Cdc42 or Rac blocked p38 activity and transfection of active Cdc42 or Rac caused a large enhancement of p38 activity (16–18). To elucidate the hierarchy of Cdc42/Rac and βPix, the effect of dominant-negative (T17N) Cdc42 or Rac1 on the p38 activation by βPix were examined. T17N mutants of Cdc42 and Rac1 completely inhibited βPix-enhanced p38 activation as expected (data not shown), indicating that Cdc42 and Rac1 act downstream of βPix, possibly being activated by the GEF activity of βPix (9).

The PAK family of protein kinases has been suggested as a main target of the Cdc42/Rac1, and Pix tightly binds to a



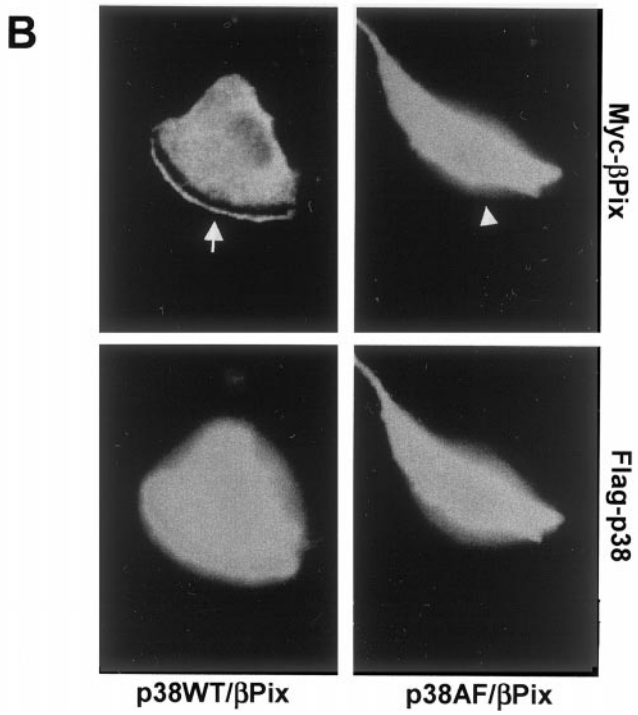


FIG. 6. **Inhibition of p38 by SB203580 blocked βPix-induced membrane ruffling.** *A*, NIH3T3 fibroblast cells were transiently transfected with FLAG-tagged βPix. At 40 h after transfection, cells were treated without (*Control*) or with 10 μM SB203580 for 1 h and then double-immunofluorescence staining for F-actin and FLAG-βPix was carried out using rhodamine-phalloidin (*upper two panels*) and anti-FLAG M2 (*lower two panels*) antibodies, respectively. *B*, cells were transfected with Myc-tagged βPix together with FLAG-tagged p38 wild-type (*p38WT/βPIX*) or p38 kinase-inactive mutant (*p38AF/βPIX*). At 40 h after transfection, double-immunofluorescence staining for Myc-βPix and FLAG-p38 was carried out using anti-βPix antiserum (30,000:1 dilution, *upper two panels*) and anti-FLAG M2 (*lower two panels*) antibody, respectively. *Arrows* indicate membrane ruffles, and *arrowheads* show the cell that expressed FLAG (in *A*) or Myc (in *B*)-tagged βPix but failed to make membrane ruffles. *, untransfected cells. For each panel, cells are representative of three independent experiments.

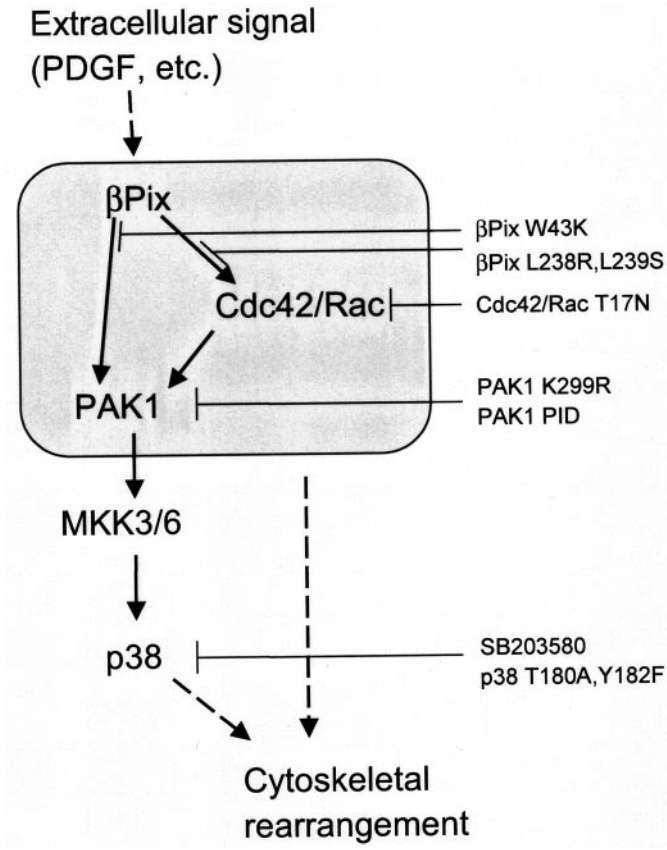


FIG. 7. **A model showing the signaling pathway involving βPix leading to p38 activation and cytoskeletal rearrangement.** Inhibitors or mutants that blocked each step are shown in the *right side* of the figure.

proline-rich motif of PAK and regulates its kinase activity (9, 12). But it is also suggested that PAK could act upstream of Rac1 mediating lamellipodia formation through interaction with Pix (27). To address the role of PAK in βPix-induced p38 activation, we utilized wild-type PAK1 or the K299R mutant of PAK1 whose kinase activity was eliminated. Immune complex kinase assay after triple transfection of FLAG-tagged p38, HA-tagged wild-type, or mutant PAK1 and Myc-tagged βPix showed that kinase-inactive PAK1 blocked p38 activation induced by βPix (Fig. 4*A*). To determine whether the blocking of βPix-induced p38 activation by kinase-inactive PAK1 was due to loss of kinase activity or due to dominant-negative effects of the expression of mutant PAK1 that could sequester interacting proteins, we examined βPix-induced p38 activation in cells expressing the PID, which can block endogenous PAK kinase activity (Fig. 4*B*). Expression of PID abolished βPix-induced p38 activation. Under the same conditions, inactive PID (PID L107F) did not block βPix-induced p38 activation. The results suggest that PAK kinase activity is essential for βPix-induced p38 activation. We next investigated the effect of βPix on MAP kinase kinases, MKK3 and MKK6, which are known to phosphorylate and activate p38 MAP kinase, using phosphorylation-specific MKK3/6 antibody. βPix overexpression led to a large enhancement of MKK3/6 activity (Fig. 5).

It has been reported that PDGF stimulation of some adherent cells leads to an induction of membrane ruffles in a Rac1 activity-dependent pathway (27). When we examined the effect of βPix overexpression on actin structure by double immunofluorescence assay, we found that actin structure modified by βPix overexpression mimicked the PDGF-induced membrane

ruffles (Fig. 6).[2] In porcine aortic endothelial cells, PDGF treatment enhanced chemotaxis in addition to ruffling, and these effects were blocked by SB203580, suggesting that PDGF-induced membrane ruffling and chemotaxis are dependent on p38 activity (25). In this regard, to test whether βPix-induced membrane ruffle is also mediated by p38 activity or not, we examined the effect of a p38-specific inhibitor, SB203580. As shown in Fig. 6*A*, under the βPix-transfected condition, control cells formed enhanced membrane ruffles, whereas 10 μM SB203580-treated cells failed to make membrane ruffles. In addition, the localization of FLAG-tagged βPix was cytoplasmic in the SB203580-treated cells, in sharp contrast with the membrane and edge-localized FLAG-βPix in control cells. We observed similar results in the cells expressing kinase-inactive p38 (T180A,Y182F) (Fig. 6*B*, *p38AF*/*βPix*).

There is now increasing evidence showing the involvement of p38 in actin cytoskeleton regulation and/or cell migration (23). PDGF activates p38 through a Ras-dependent pathway that is important for actin reorganization and cell migration in porcine aortic endothelial cells (25). Activation of p38 MAP kinase pathway by growth factors and inflammatory cytokines regulates smooth muscle cell migration (24). In this report, p38 activates MAP kinase-activated protein kinase-2 and -3 (MAPKAPK2 and -3), and these kinases phosphorylate various substrates including heat shock protein 27 (HSP27). Phosphorylated HSP27 appears to modulate the polymerization of actin and is proposed to play a role in actin cytoskeletal remodeling. In our experiments, SB203580, at the concentration physiologically blocking p38 activity, inhibited βPix-induced membrane ruffling. It seems likely that p38 has a role in mediating Pix/PAK/Cdc42/Rac signaling to the cytoskeletal rearrangement. It is reported that activation of LIM-kinase by PAK1 couples Cdc42/Rac GTPase signaling to actin cytoskeletal dynamics (28, 29). LIM-kinase is known to catalyze phosphorylation of cofilin, thereby inactivating actin depolymerizing activity and leading to accumulation of actin filaments (30, 31). We cannot rule out the possibility that βPix also acts downstream of p38, where it is proximal to actin structure regulation (Pix-Cdc42/Rac/PAK-LIM kinase-cofilin-actin) and p38 activation is a prerequisite for Pix/PAK/Cdc42/Rac action in actin regulation, rather than downstream of βPix.

In many cases, the actin reorganization and MAP kinase activation arose from the same extracellular stimuli, such as the ligands of several receptor tyrosine kinases, and were mediated by the same cytoplasmic signaling components during the signal transduction pathway (16, 19). Cdc42 and Rac have been implicated in JNK/SAPK and p38, and to a lesser degree, Erk kinase activation (16, 17, 32). We tested the involvement of βPix in the MAP kinase pathway and found that βPix activates p38 significantly (Fig. 1). It is well established that growth factor stimulation of Ras leads to the activation of the Raf/MEK/Erk pathway, and Ras is also involved in signaling to the cytoskeleton (25). In recent reports, Erk can be activated by Rho family proteins, and Cdc42/Rac selectively interacts and activates MEK kinases (32, 33). In our experiment, βPix did not activate Erk kinase activity, excluding the possibility that βPix acts upstream of the Erk-mediated mitogenic signaling pathway in NIH3T3 fibroblast cells. It is still unclear whether βPix has a role in the downstream of Ras.

Zhang *et al.* (18) proposed that Cdc42 and Rac might regulate p38 activity through the downstream effector, PAK1. In our experiment, kinase-inactive PAK1 blocked p38 activity, supporting the results of others (Refs. 17 and 18 and Fig. 4). It is possible that PAK-Pix interaction enhances both kinase activity of PAK and the GEF activity of Pix, as suggested by others (5, 9). βPix might activate PAK, MKK3/6 and p38 in two ways: by activating Cdc42/Rac1 (and effector PAK) through its GEF activity and by interacting directly with the PAK regulatory N terminus. The complexity of the signaling involving βPix might arise from the dual property of βPix, which interacts with the Cdc42/Rac downstream effector, PAK, and also acts as a GEF of Cdc42/Rac1.

We propose a model of the Pix/PAK/Rho GTPase-mediated p38 activation signaling pathway that is shown in Fig. 7. βPix might be activated by extracellular stimuli such as PDGF, then activate Cdc42 and Rac through its GEF activity. PAK can be activated by Cdc42/Rac binding and also by the interaction with βPix SH3 domain. PAK activates MAPK kinase, MKK3 and/or MKK6, leading to p38 activation. p38 activation has a role in actin cytoskeletal change, such as lamellipodia formation and membrane ruffling. It remains to be resolved what is the upstream activator of βPix. Yoshii *et al.* (15) reported that αPix is activated by interaction with phosphatidylinositol 3-kinase and we also have observed the association between βPix and phosphatidylinositol 3-kinase *in vivo*.[3] We are now investigating other upstream regulators of βPix and extracellular stimuli that activate βPix. Our present study suggests that signaling pathways through βPix, in addition to Cdc42/Rac and PAK, are orchestrated to yield a coordinated response of cytoskeletal and nuclear events to extracellular stimuli.

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