Phosphorylation of paxillin tyrosines 31 and 118 controls polarization and motility of lymphoid cells and is PMA-sensitive

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Summary

Tyrosine phosphorylation of paxillin regulates actin cytoskeleton-dependent changes in cell morphology and motility in adherent cells. In this report we investigated the involvement of paxillin tyrosine phosphorylation in the regulation of actin cytoskeleton-dependent polarization and motility of a non-adherent IL-3-dependent murine pre-B lymphocytic cell line Baf3. We also assessed the effect of phorbol myristate acetate (PMA), a phorbol ester analogous to those currently in clinical trials for the treatment of leukemia, on paxillin phosphorylation. Using tyrosine-to-phenylalanine phosphorylation mutants of paxillin and phosphospecific antibody we demonstrated that IL-3 stimulated phosphorylation of paxillin tyrosine residues 31 and 118, whereas the tyrosines 40 and 181 were constitutively phosphorylated. Phosphorylation of paxillin residues 31 and 118 was required for cell polarization and motility. In the presence of IL-3, PMA dramatically reduced the phosphorylation of residues 31 and 118, which was accompanied by inhibition of cell polarization and motility. This PMA effect was partially recapitulated by expression of exogenous tyrosine 31 and 118 mutants of paxillin. We also demonstrated that PMA inhibited the IL-3-induced and activation-dependent tyrosine phosphorylation of focal adhesion kinase. Thus, our results indicate that phosphorylation of paxillin tyrosine residues 31 and 118 regulates actin-dependent polarization and motility of pre-B Baf3 cells, both of which could be inhibited by PMA. They also suggest that inhibition of upstream signaling by PMA contributes to the decrease of paxillin phosphorylation and subsequent changes in cell morphology.

Key words: Paxillin, B-lymphocytes, Phorbol esters, Polarization, Motility

Introduction

A growing body of evidence has implicated tyrosine phosphorylation of paxillin in the control of actin cytoskeleton rearrangements and the resultant changes of cell morphology and motility (Petit et al., 2000; Yano et al., 2000; Nakamura et al., 2000; Iwasaki et al., 2002; Tsubouchi et al., 2002). Paxillin undergoes tyrosine phosphorylation in response to stimulation with cytokines and growth factors, including IL-3 (Sinnett-Smith et al., 1993; Rankin and Rozengurt, 1994; Seufferlein and Rozengurt, 1994; Salgia et al., 1995a; Salgia et al., 1995b). Six tyrosine residues, 31, 40, 118, 181, 434 and 488, are potential phosphorylation targets in mouse paxillin (Turner and Miller, 1994; Bellis et al., 1995; Yano et al., 2000; Nakamura et al., 2000). Residues 31 and 118 are the major tyrosine phosphorylation sites phosphorylated in response to stimulation with various growth factors in a focal adhesion kinase (FAK)-dependent manner (Bellis et al., 1995; Schaller and Parsons, 1995; Schaller and Schaefer, 2001). The principal function of phosphorylated tyrosines is thought to be the provision of binding sites for SH2-domain-containing proteins. For example, tyrosines 31, 118 and 181 of paxillin are involved in binding to CRK and CRKL proteins (Schaller and Parsons, 1995; Salgia et al., 1995c; Salgia et al., 1996). The amino acids surrounding phosphorylated paxillin tyrosine 181 and 40 constitute the binding site for phospholipase Cγ and Src, respectively (Turner and Miller, 1994; Thomas et al., 1995).

A biological role for tyrosine-phosphorylated paxillin has emerged from studies of the proteins that interact with the SH2-binding domains of paxillin. The CRKI, CRKII and CRKL adaptor proteins interact with phosphorylated tyrosine residues 31 and 118 in paxillin and bind via their SH3 domains with several signaling proteins, e.g. DOCK2, DOCK180, C3G and Sos (Gotoh et al., 1995; Feller et al., 1995; Okada et al., 1998). Although DOCK2 and DOCK180 mediate actin cytoskeleton rearrangement through Rac1 activation (Hasegawa et al., 1996; Kiyokawa et al., 1998; Fukui et al., 2001), SOS and C3G activate Ras and Rap1, respectively (Gotoh et al., 1995; Okada et al., 1998). Indeed, phosphorylation of paxillin tyrosine residues 31 and 118 was shown to be essential for actin cytoskeleton-dependent cell spreading and motility in adherent cells (Yano et al., 2000; Nakamura et al., 2000; Petit et al., 2000; Iwasaki et al., 2002). Involvement of these paxillin

tyrosine residues in actin rearrangements of non-adherent cells has never been assessed.

Maturation of lymphoid cells occurs during their migration through bone marrow and different compartments of peripheral lymphoid organs. This process is coordinated by various growth factors and cytokines, and it is associated with changes in cell motility. Acquisition of a motile phenotype is generally accompanied by cell polarization. Rearrangements of the cellular membrane, as well as redistribution of cytoskeleton, chemokine-sensing receptors and cell adhesion molecules occur during lymphocyte polarization (reviewed by del Pozo et al., 1998). Specifically, membrane ruffled protrusions are formed at the advancing front (leading edge) of the cells, where filamentous actin and chemokine receptors are concentrated, whereas the posterior lymphocyte pole (uropod) accumulates tubulin, the force-generating protein myosin II and cell adhesion molecules.

Phorbol esters are the pharmacological analogs of an endogenous protein kinase C (PKC) activator, diacyl glycerol, which is released upon stimulation of cytokine or growth factor receptors. It has been reported that phorbol esters inhibit cell proliferation and disrupt actin cytoskeleton thus producing changes in cell morphology (Goodnight et al., 1995; Romanova et al., 1999). Although several phorbol esters are undergoing clinical trials for treatment of human lymphoid malignancies (Grant et al., 1998; Han et al., 1998; Strair et al., 2002), their effect on cell morphology is unknown.

Relevant to this issue, we previously found that IL-3 was required for polarization of the IL-3-dependent murine pre-B lymphocyte line, Baf3 (Romanova et al., 1999). This polarization was crucially dependent on redistribution of actin cytoskeleton and was mediated by Rac1, but not by RhoA or cdc42. Phorbol esters disrupted Rac1-mediated polarization of Baf3 cells, in effect imitating IL-3 deprivation. Because tyrosine phosphorylation of paxillin has been shown to regulate actin cytoskeleton rearrangements in adherent cells, we suggested that it might also control the actin-dependent polarization of non-adherent cells. The purpose of the current study was to investigate the possible involvement of paxillin tyrosine phosphorylation in the regulation of actin-dependent polarization and motility of pre-B cells. We also assessed the effect of phorbol myristate acetate (PMA) on the IL-3-induced paxillin phosphorylation in Baf3.

Materials and Methods

Cell culture

The IL-3-dependent murine pre-B cell line Baf3, the IL-3-dependent murine myeloid cell line 32D, the human T cell line CCRF CEM and human Burkitt lymphoma cell line Daudi were maintained in RPMI 1640 containing 10% heat-inactivated FCS. 5% WEHI-3 supernatant was used as a source of murine IL-3. Murine and monkey fibroblast cell lines Swiss 3T3 and COS-1, respectively, were maintained in DMEM supplemented with 10% heat-inactivated FCS. To withdraw IL-3, the cells were washed three times with complete IL-3-free medium and further kept in this medium for 6 hours. Purified IL-3 at 10 ng/ml or 5% WEHI-3 supernatant was used for subsequent cell stimulation.

Reagents

The following reagents were used in the study: purified IL-3 (R&D Systems); the PKC activator PMA (Calbiochem); the PKC inhibitor GF109203X (LC Laboratories); and the actin cytoskeleton-disrupting drug, cytochalasin D (Calbiochem), non-specific potato acid phosphatase (PAP) and human recombinant protein tyrosine phosphatase (PTP) (Calbiochem) and TRITC-conjugated phalloidin (Sigma).

Expression vectors

EGFP-conjugated wild-type murine α-paxillin cDNA in pBabe-puro vector was described previously (Mazaki et al., 1998). The constructs bearing mutations of potential tyrosine phosphorylation sites to phenylalanine, i.e. Y31F, Y118F, Y31/118F, Y40/181F, Y31/40/118/181F were also described earlier (Nakamura et al., 2000). The mutants Y31/40/181/434/488F and Y40/118/181/434/488F were made using the Altered Sites II in vitro Mutagenesis System (Promega) with appropriate synthetic DNA fragments. Baf3 cells were stably transfected with these constructs using Lipofectamine Plus (Pharmacia).

Immunoprecipitation and western blots

Immunoprecipitation from ~5×10^6 cells was performed in buffer A (10 mM Tris HCl, pH 7.4; 0.5 mM EDTA; 0.5 mM EGTA; 0.5% Triton X-100; 0.5% NP40; 1 μg/ml aprotinin; 1 μg/ml leupeptin; 100 mM PMSF; 1 mM sodium fluoride and 0.2 mM sodium orthovanadate). GammaBindRG Sepharose® (Pharmacia) beads were used to collect immunoprecipitates. For disruption of protein interactions the cell lysates were initially diluted in buffer A with the addition of 0.1% SDS (denaturing conditions). Immunoprecipitation was then performed as above after further dilution of the cell lysates with 10 volumes of buffer A.

Western blot analysis was performed as described (Romanova et al., 1996). 10% and 7% PAGE gels were used for analysis of endogenous and EGFP-tagged paxillins, respectively. We used non-immune IgG1 (Sigma); monoclonal anti-paxillin antibody (Transduction Laboratories); polyclonal anti-GFP antibody (Clontech); monoclonal anti-phosphotyrosine 4G10 (UBI) and PY20 (Transduction Laboratories) antibodies; monoclonal anti-FAK antibody (Transduction Laboratories); polyclonal anti-FAK antibody (Santa, Cruz); polyclonal FAK phosphorylation site-specific anti-pY397 antibody (Biosource International); polyclonal paxillin phosphorylation site-specific anti-pY31 and anti-pY118 antibodies (Biosource International) and goat anti-rabbit or anti-mouse IgG1 conjugated with horseradish peroxidase (Amersham) as secondary antibodies. Western blots were developed with ECL reagents (Pierce).

Phosphatase treatment

The cells were lysed in 100 μl Buffer A by addition of 1% SDS, and then further diluted with Buffer A to 1 ml (‘denaturing’ conditions). After paxillin immunoprecipitation, the beads were washed three times with the reaction buffer. Phosphatase treatment was performed for 30 minutes at 37°C. Treatment with PAP was performed in buffer containing 50 mM Tris HCl (pH 7.4), 2 mM EDTA, 2 mM $MgCl_2$, 150 mM NaCl, 1 mM PMSF and 0.016 units/μl of PAP. PTP treatment was performed in buffer containing 25 mM imidazole, 50 mM sodium chloride, 2.5 mM EDTA, 5 mM DTT, 0.1 mg/ml BSA (pH 7) with addition of 50 units PTP. The levels of paxillin phosphorylation were analyzed by western blotting with anti-paxillin antibodies.

Analysis of cell polarization

Analysis of cell polarization was performed as described (Romanova et al., 1999). Briefly, cells were kept in log phase growth for at least 2 days. Then 1×10^6 cells were fixed in 1% paraformaldehyde at 37°C for 2 hours. Cell morphology was evaluated using forward scatter on

a Becton Dickinson Immunocytometry Systems FACScan. Round cells produced a sharp peak whereas polarized cells produced a broad, shallow peak to the right of the round cells. Statistical analysis of FACS profile changes was performed using the non-parametric Kolmogorov-Smirnov test.

Analysis of cell motility

Spontaneous motility was assessed using a modification of the Boyden chamber technique. Briefly, 5×10^4 non-adherent cells (murine myeloid 32D, human T CCRF-CEM, human Burkitt lymphoma Daudi cells) or 1.5×10^4 adherent cells (fibroblast cell lines mouse Swiss3T3 and monkey COS-1) were suspended in complete medium in the upper compartments of a 96-well chemotaxis chamber (Neuro Probe, Inc.). 10 ng/ml IL-3, 100 nM PMA or 4 μM cytochalasin D were added to the cells as indicated. The lower compartments were filled with the same medium. The two compartments were separated with 5-μm-pore polycarbonate filters. The cells were allowed to migrate through the filters for 3 hours at 37°C and 5% CO_2. Non-adherent cells that had completely traversed the filter were recovered from the bottom chamber at the end of the experiment and counted after staining with trypan blue. Adherent cells were removed from the bottom part of the filter with 2 mM EDTA in PBS and counted. The statistical relevance of motility changes was analyzed by parametric *t*-test.

Immunocytochemistry and fluorescence microscopy

The cells were washed twice with PBS and resuspended in PBS at 1×10^6/ml. 200 cells were cytospun onto microscope slides. The slides were air-dried, and mounted with cover slips using SlowFade™ (Molecular Probes). Consecutive staining with monoclonal anti-paxillin antibody (Transduction Laboratories) and fluorescein-conjugated goat anti-mouse secondary antibody (Molecular Probes) were used to detect paxillin in wild-type Baf3 cells. Confocal fluorescent images were collected with a BioRad MRC 1024 confocal scan head mounted on a Nikon Optiphot microscope with a ×60 planapochromat lens. A krypton-argon gas laser provided excitation at 488 nm and an emission filter of 522 nm was used for collecting green fluorescence. DIC (differential interference contrast, i.e. Nomarski optics) images of the same cells were collected using a transmitted light detector.

Results

PMA inhibits the IL-3-induced tyrosine phosphorylation of paxillin

Here we show that paxillin had a low phosphotyrosine content when Baf3 cells were deprived of IL-3. Addition of IL-3 stimulated tyrosine phosphorylation of paxillin (Fig. 1). This phosphorylation was not inhibited by the specific PKC inhibitor GF109203X. In contrast, treatment with the actin-disrupting drug, cytochalasin D or the specific PKC activator, PMA, markedly diminished paxillin tyrosine phosphorylation. The PMA effect was reversed by GF209103X. Inhibition of paxillin tyrosine phosphorylation could also be achieved by addition of the PMA analog diacylglycerol, an endogenous PKC activator (data not shown). Note that some retardation of paxillin mobility can be observed in PMA-treated cells as a result of paxillin threonine phosphorylation, which will be described elsewhere.

Thus, we demonstrated that IL-3 stimulated paxillin tyrosine phosphorylation in a PKC-independent fashion. This phosphorylation requires the integrity of the actin cytoskeleton and PKC activation by PMA inhibits the IL-3-induced tyrosine phosphorylation of paxillin.

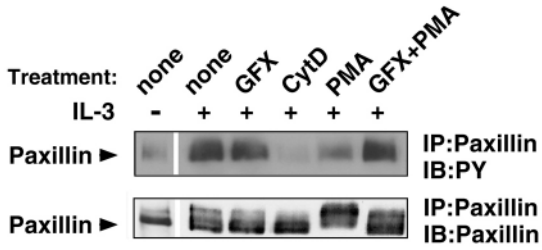

Fig. 1. Effects of IL-3 and PMA on paxillin phosphorylation. Baf3 cells were deprived of IL-3 for 6 hours or grown on 10 ng/ml IL-3, and aliquots were treated 30 minutes with 4 μM cytochalasin D, 1 μM GF109203X, with 100 nM PMA alone or with 1 μM GF109203X and 100 nM PMA. Paxillin was immunoprecipitated (IP:Paxillin) and analyzed on western blot with a mixture of anti-phosphotyrosine antibodies 4G10 and PY20 (IB:PY) or with anti-paxillin (IB:Paxillin) antibody.

IL-3 stimulated phosphorylation of paxillin tyrosines 31 and 118

We further studied the effect of IL-3 on tyrosine phosphorylation of paxillin. We did not detect a notable amount of β-paxillin in Baf3 cells, suggesting that α-paxillin is the major isoform expressed in these cells (K.-O.C., unpublished observations). Baf3 cells were stably transfected with constructs that expressed enhanced green fluorescent protein (EGFP), EGFP-conjugated wild-type mouse α-paxillin or α-paxillin bearing tyrosine-to-phenylalanine (Y→F) mutations at positions 31, 118, 31/118, 40/181, 31/40/118/181, 31/40/181/434/488, or 40/118/181/434/488. Similar to the effect on endogenous paxillin, IL-3 stimulated tyrosine phosphorylation of the recombinant wild-type paxillin (Fig. 2A). Simultaneous mutations of four paxillin tyrosines 31/40/118/181 completely inhibited the IL-3-induced paxillin tyrosine phosphorylation. Paxillin tyrosine phosphorylation mutant 31/118 was weakly, but constitutively, phosphorylated irrespective of the presence of IL-3, presumably on tyrosines 40 and/or 181. Phosphorylation of the mutants 40/181, 31/40/181/434/488 and 40/118/181/434/488 was almost undetectable in the absence of IL-3, but their phosphorylation was induced upon IL-3 addition, presumably on tyrosines 31 and/or 118. The mutants 31 and 118 were poorly phosphorylated in the absence of IL-3, and IL-3 further stimulated their phosphorylation. Thus, we concluded that tyrosines 31, 40, 118 and 181 are the major paxillin phosphorylation sites in IL-3-stimulated Baf3 cells. Although tyrosines 40 and 181 are constitutively phosphorylated, phosphorylation of tyrosines 31 and 118 is regulated by IL-3.

We also showed that the IL-3 or PMA-induced paxillin mobility shifts that look like an increase in paxillin level, could be removed in vitro with non-specific potato acid phosphatase (PAP) or human protein tyrosine phosphatase (PTP) that specifically dephosphorylates tyrosine (Fig. 2B). These data suggest that retardation of paxillin mobility resulted from paxillin phosphorylation not altered paxillin levels.

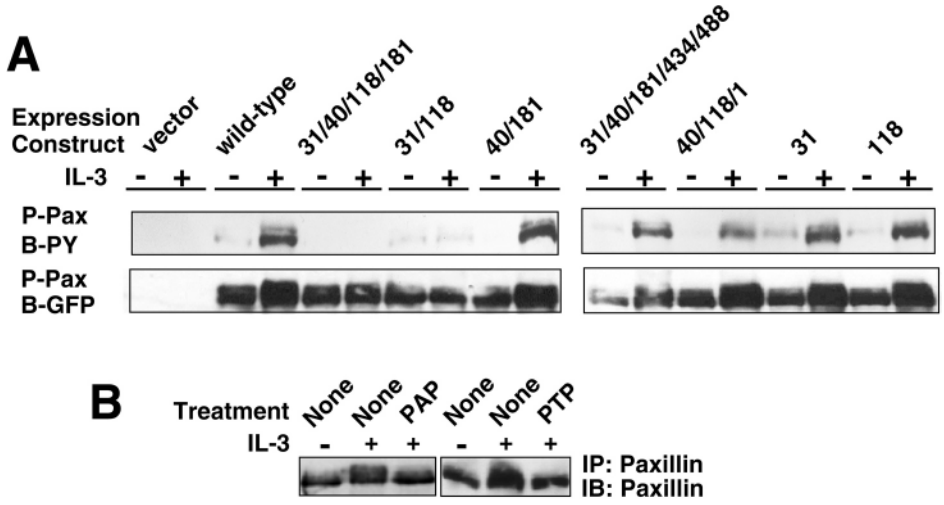

Fig. 2. IL-3 stimulates phosphorylation of paxillin tyrosine residues 31 and 118. (A) Baf3 cells were stably transfected with EGFP (vector) or EGFP-conjugated wild-type α-paxillin or the α-paxillin Y→F tyrosine phosphorylation mutants 31, 118, 31/118, 40/181, 31/40/118/181, 31/40/181/434/488 and 40/118/181/434/488. After six hours of IL-3 withdrawal, 10 ng/ml IL-3 was resupplied for 10 minutes. Immunoprecipitated paxillin (IP-Pax) was analyzed on western blots with a mixture of anti-phosphotyrosine antibodies 4G10 and PY20 (IB-PY) or anti-GFP antibody (IB-GFP). (B) Immunoprecipitated paxillin (IP:Paxillin) was treated in vitro with non-specific potato acid phosphatase (PAP) or Protein Tyrosine Phosphatase (PTP), which specifically dephosphorylates tyrosine residues and was then analyzed on western blots using anti-paxillin antibody (IB:Paxillin).

PMA inhibited phosphorylation of paxillin tyrosine residues 31 and 118

We further investigated the effect of PMA on IL-3-induced paxillin tyrosine phosphorylation in Baf3 cells that expressed EGFP, EGFP-conjugated wild-type α-paxillin or the Y→F α-paxillin mutants (Fig. 3). Similar to the effect on endogenous paxillin, PMA inhibited tyrosine phosphorylation of the recombinant wild-type paxillin. The 31/40/118/181 mutant was not tyrosine phosphorylated. The mutant 31/118 was poorly phosphorylated and this phosphorylation was not affected by PMA. On the other hand, the mutants 40/181, 31/40/181/434/488 and 40/118/181/434/488 were tyrosine phosphorylated in the presence of IL-3, and their phosphorylation was diminished by PMA. Consistent with these results, PMA efficiently inhibited tyrosine phosphorylation of the 31 and 118 mutants. Thus, we concluded that PMA inhibited phosphorylation of paxillin tyrosines 31 and 118 without affecting phosphorylation of the residues 40 and 181.

We also investigated the effects of IL-3 and PMA on phosphorylation of the endogenous paxillin using antibodies specific for phosphorylated tyrosine residues 31 or 118 of paxillin (Fig. 4). We found that IL-3 stimulated phosphorylation of both tyrosine residues 31 and 118. PMA addition to the IL-3-stimulated cells inhibited phosphorylation of these residues. To demonstrate equal levels of protein loading the same blot was developed with polyclonal antibodies that recognized the paxillin-binding protein vinculin, which binds paxillin irrespectively of its phosphorylation status (Turner et al., 1990). We concluded that phosphorylation of tyrosine residues 31 and 118 in endogenous paxillin is regulated by IL-3 and PMA.

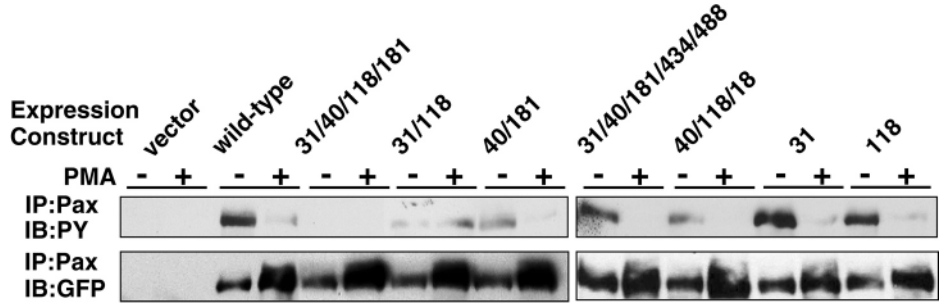

Fig. 3. PMA inhibits phosphorylation of paxillin tyrosine residues 31 and 118. Baf3 cells were stably transfected with EGFP (vector) or EGFP-conjugated wild-type α-paxillin or the α-paxillin Y→F tyrosine phosphorylation mutants 31, 118, 31/118, 40/181, 31/40/118/181, 31/40/181/434/488 and 40/118/181/434/488. Cells were grown on 10 ng/ml IL-3 and aliquots were treated with 100 nM PMA for 30 minutes. After immunoprecipitation with anti-paxillin antibody (IP:Pax), lysates were analyzed on western blots with a mixture of anti-phosphotyrosine antibodies 4G10 and PY20 (IB:PY) or with anti-GFP (IB:GFP) antibody.

PMA inhibited tyrosine phosphorylation of FAK and the proteins that co-immunoprecipitated with FAK

Inhibition of the IL-3-induced paxillin tyrosine phosphorylation in response to PMA may result from activation of phosphatases that directly dephosphorylate paxillin or inhibition of upstream kinases. Members of the FAK family interact with paxillin and stimulate its tyrosine phosphorylation at residues 31 and 118 (Schaller and Parsons, 1995; Richardson et al., 1997). Because PMA inhibited phosphorylation of these residues, we hypothesized that PMA might interfere in FAK signaling. We showed that tyrosine phosphorylation of FAK that was immunoprecipitated from Baf3 cells under denaturing conditions was stimulated by IL-3. In the presence of IL-3 this phosphorylation was inhibited by cytochalasin D or PMA (Fig. 5A). FAK activation is accompanied by its autophosphorylation at tyrosine residue Y397. Using phosphospecific antibody we demonstrated that IL-3 stimulated FAK phosphorylation at tyrosine residue 397, but PMA inhibited it (Fig. 5A).

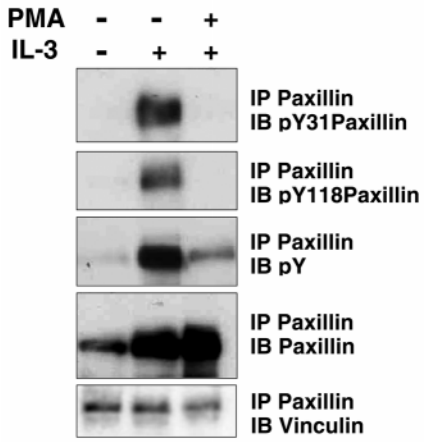

Fig. 4. Phosphorylation of paxillin tyrosine residues 31 and 118 in endogenous paxillin is stimulated by IL-3 and inhibited by PMA. After a 6-hour IL-3 deprivation in Baf3 cells, an aliquot was treated for 30 minutes with 10 ng/ml IL-3, 100 nM PMA or their combination. Paxillin was immunoprecipitated (IP Paxillin) and analyzed on western blots with anti-paxillin antibody (IB Paxillin), mixture of anti-phosphotyrosine antibodies 4G10 and PY20 (IB:PY) or antibody specific to phosphorylated paxillin tyrosine residues 31 or 118 (IB pY31Paxillin or IB pY118Paxillin, respectively). The same blot was developed with polyclonal antibodies that recognized the paxillin-binding protein vinculin (IB Vinculin) to demonstrate equal levels of protein loading.

Using non-denaturing immunoprecipitation conditions, we confirmed previous observations (Bellis et al., 1995) that the phosphorylation status of FAK does not affect its binding to paxillin (Fig. 5B). The reversed immunoprecipitation/western blot produced the same results (not shown). Also, anti-FAK antibody co-immunoprecipitated several major tyrosine-phosphorylated proteins with apparent molecular weights of 180, 120 and 60-70 kDa (Fig. 5B). The interaction was specific, since these bands were not immunoprecipitated with a pre-immune serum (IgG1). The 60-70 kDa band was recognized by anti-paxillin antibody (not shown). Treatment of Baf3 cells, growing on IL-3, with cytochalasin D or PMA decreased the intensity of four major tyrosine-phosphorylated bands. The PKC-specific inhibitor, GF109203X, reversed the effect of PMA. We concluded that PMA inhibited the IL-3-induced and activation-dependent tyrosine phosphorylation of FAK as well as several FAK-associated proteins. These results suggested that disruption of upstream signaling may contribute to the paxillin dephosphorylation in response to PMA.

Phosphorylation of paxillin tyrosine residues 31 and 118 is required for Baf3 polarization and motility

We showed earlier that IL-3 stimulated polarization of Baf3

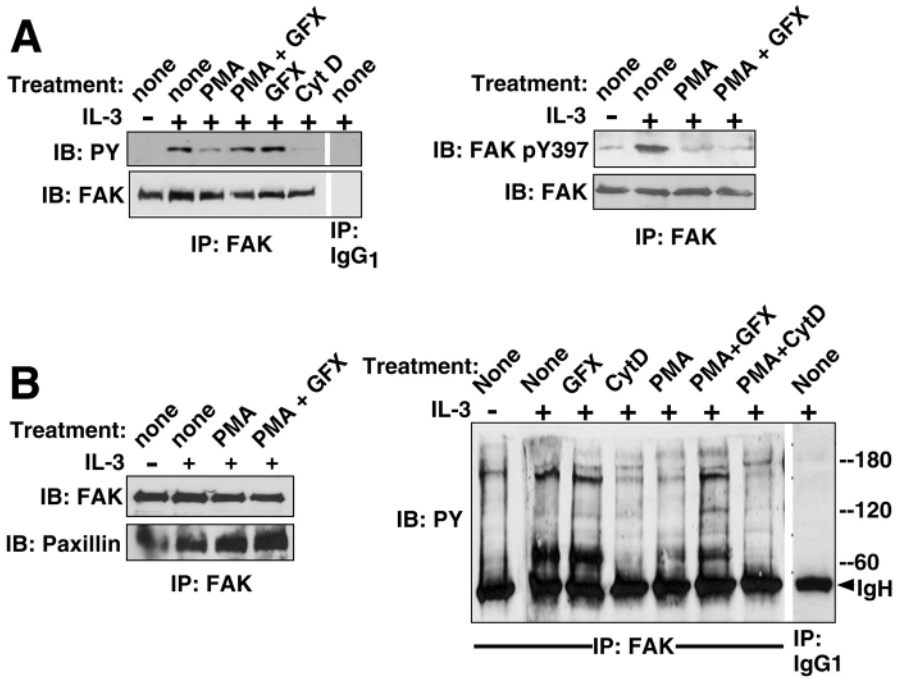

Fig. 5. PMA inhibits the IL-3-induced tyrosine phosphorylation of FAK and FAK-binding proteins. Cells were deprived of IL-3 for 6 hours or grown on IL-3 and treated with 4 μM cytochalasin D (CytD), 1μM GF109203X (GF), 100 nM PMA alone or in combination for 30 minutes. (A) After immunoprecipitation with monoclonal anti-FAK antibody (IP:FAK) or non-immune mouse IgG1 (IP:IgG1) in denaturing conditions, lysates were analyzed on western blots with a mixture of anti-phosphotyrosine 4G10 and PY20 antibody (IB:PY), a phosphospecific anti-FAK(pY397) antibody (IB:FAKpY397) or polyclonal anti-FAK antibody (IB:FAK). (B) In another series of experiments, after immunoprecipitation with anti-FAK antibody (IP:FAK) or non-immune mouse IgG1 (IP:IgG1) in non-denaturing conditions lysates were analysed on western blots with phosphotyrosine PY20 antibody (IB:PY), anti-paxillin antibody (IB:paxillin) or anti-FAK antibody (IB:FAK).

cells, which was the result of the assembly of actin containing ruffled membrane protrusions on the leading edge of the cell. PMA addition to cells growing on IL-3 ablated cell polarization, imitating the cell rounding induced by IL-3 withdrawal. These morphological changes were observed microscopically and quantitatively assessed using FACS analysis (Romanova et al., 1999) (Fig. 6A).

In the present study we investigated whether tyrosine phosphorylation of paxillin residues 31 and 118 regulated cell polarization and motility. We used stable clones of Baf3 cells that expressed EGFP or EGFP-tagged Y→F α-paxillin mutants 31, 118 and 31/118. They were expected to compete with endogenous paxillin at paxillin binding sites. Equivalent levels of exogenous paxillin expression were achieved in these clones (Fig. 2A). IL-3 stimulated polarization of Baf3 cells that expressed EGFP alone (Fig. 6A,B). Expression of exogenous EGFP-conjugated paxillin tyrosine phosphorylation mutants 31 and 118 promoted cell rounding in the presence of IL-3 and shifted FACS profiles to the left (Fig. 6B). The effect was more pronounced in the double mutant, Y31/118F. The changes of FACS profiles induced by IL-3 deprivation, PMA treatment as well as overexpression of the paxillin Y→F mutants 31, 118 or 31/118 were statistically significant with $P \leqslant 0.001$ and $D=0.56$; 0.62; 0.19; 0.18 and 0.40, respectively.

Lymphocyte polarization is essential for motility. We investigated whether tyrosine phosphorylation of paxillin residues 31 and 118 regulates spontaneous motility in Baf3 cells. The motility of the cells maintained on IL-3 was reduced upon cytochalasin D treatment ($t=9.8$, $P=0.0002$), PMA addition ($t=6.62$, $P=0.0012$) or IL-3 deprivation ($t=7.5$, $P=0.0006$) (Fig. 6C). Expression of exogenous wild-type paxillin stimulated cell motility ($t=–3.157$, $P=0.02$). In contrast, expression of paxillin tyrosine phosphorylation mutants 31, 118 and especially 31/118, reduced cell motility in the presence of IL-3 (Fig. 6C) ($t=3.37$, $P=0.0198$; $t=2.5$, $P=0.05$; $t=8.2$, $P=0.0004$). The changes in cell motility in response to drug treatment or expression of paxillin tyrosine phosphorylation mutants are statistically significant.

Inhibition of paxillin tyrosine phosphorylation, polarization and motility by PMA was demonstrated on at least three independent single-cell-derived wild-type Baf3 clones. We used at least two independent clones that expressed the 31/118 tyrosine phosphorylation mutant to assess the involvement of paxillin tyrosine phosphorylation in Baf3 polarization and motility. Our results indicate that tyrosine phosphorylation of paxillin residues 31 and 118 is required for polarization and motility of Baf3 lymphocytes. Therefore, we suggest that inhibition of phosphorylation of these residues contributes to the cell rounding and decrease of cell motility in PMA-treated cells.

We next investigated whether tyrosine phosphorylation of paxillin residues 31 and 118 affected its intracellular distribution. Whereas EGFP was uniformly distributed throughout the cell, the recombinant EGFP-conjugated wild-type paxillin, as well as the 31/118-paxillin mutant, were concentrated primarily within ruffled protrusions and on the

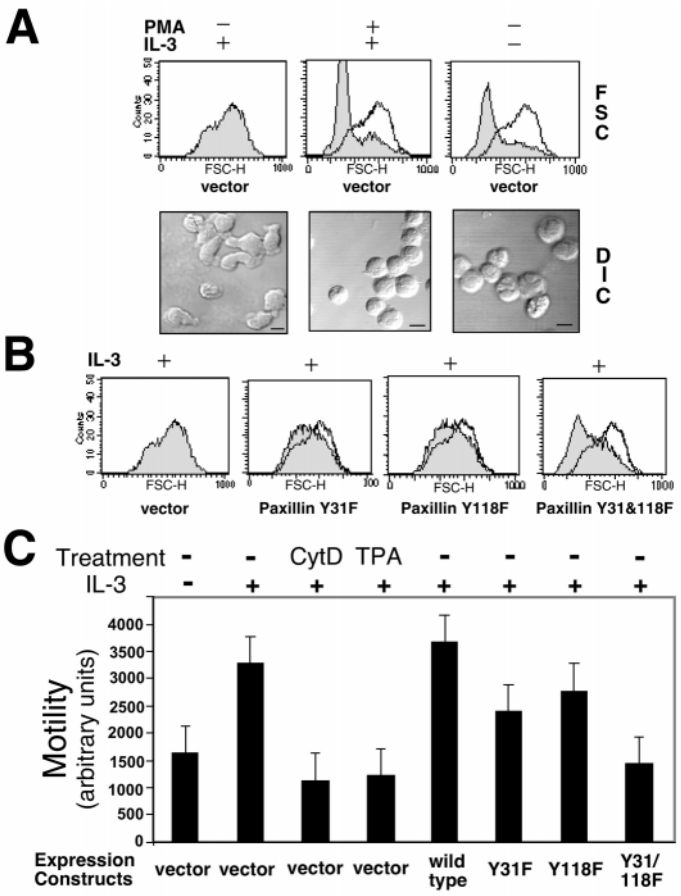

Fig. 6. IL-3-induced phosphorylation of paxillin tyrosine residues 31 and 118 is required for Baf3 cell polarization and motility, and PMA abolishes the effects of IL-3. (A) Baf3 cells expressing EGFP (vector) were deprived of IL-3 for 6 hours, maintained on 10 ng/ml IL-3, or treated with 100 nM PMA for 30 minutes in the presence of IL-3. Nomarski images (DIC) obtained by confocal microscopy are presented in the lower panel; bar, 10 μm. Quantitative analysis of cell morphology was performed by forward light scatter (FSC-H) FACS scans (upper panel). To demonstrate spectrum changes, the silhouette of the broad pattern of EGFP-expressing cells maintained on IL-3 was superimposed on the shaded patterns of exogenous paxillin-expressing cells or EGFP-expressing cells, treated as indicated. (B) Baf3 cells expressing EGFP-conjugated wild-type paxillin or Y→F tyrosine phosphorylation mutants 31, 118 or 31/118 were maintained on 10 ng/ml IL-3. FACS scan patterns of FSC-H (top panel) and Nomarski images (DIC) (bottom panel) are presented as above. The same control, i.e., FACS profiles of Baf3 cells maintained on IL-3 was used for all panels. The changes in FACS profiles induced by IL-3 deprivation, PMA treatment (A) as well as overexpression of the paxillin Y→F mutants 31, 118 or 31/118 (B) were statistically significant (Kolmogorov-Smirnov test) with $P \leqslant 0.001$ and $D=0.56$; 0.62; 0.19; 0.18 and 0.40, respectively. The figure shows that overexpression of Y→F 31/118 paxillin mutant rounds the cells in a manner resembling IL-3 deprivation or PMA addition. (C) Spontaneous cell motility was analyzed as described in the Materials and Methods. Results are expressed relative to the motility of Baf3 cells that express EGFP vector growing in IL-3. The average and s.e.m. from six experiments are presented. The motility changes induced by IL-3 deprivation ($t=7.5$, $P=0.0006$), cytochalasin D ($t=9.8$, $P=0.0002$) or PMA treatment ($t=6.62$, $P=0.0012$), as well as overexpression of wild-type paxillin ($t= –3.157$, $P=0.02$) or tyrosine phosphorylation mutants 31 ($t=3.37$, $P=0.0198$), 118 ($t=2.5$, $P=0.05$) and 31/118 ($t=8.2$, $P=0.0004$) were assessed by *t*-test and were statistically significant. Thus, expression of the Y→F 31/118 paxillin mutant inhibits cell motility in a manner similar to IL-3 deprivation or PMA treatment of Baf3 cells.

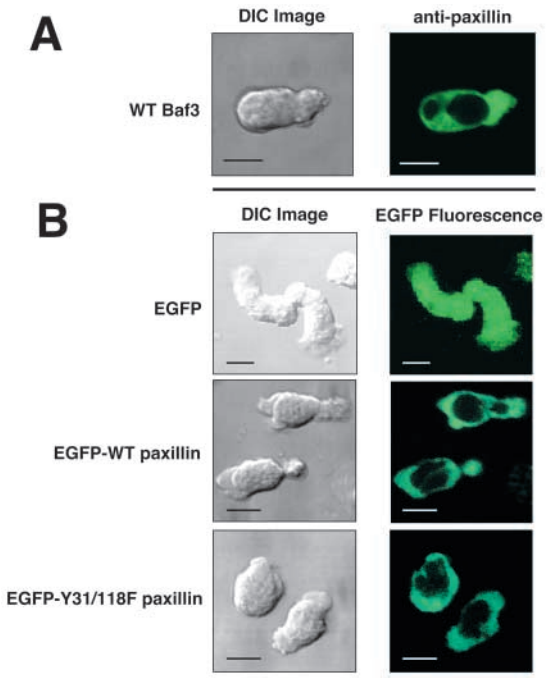

Fig. 7. Tyrosine phosphorylation of paxillin residues 31 and 118 does not affect its intracellular distribution in Baf3 cells. Parental Baf3 cells (A) and Baf3 cells expressing EGFP (vector), EGFP-conjugated wild-type paxillin or Y→F tyrosine phosphorylation mutant 31/118 (B) were maintained on 10 ng/ml IL-3. Endogenous paxillin was visualized by the consecutive staining with monoclonal anti-paxillin and fluorescein-conjugated goat anti-mouse secondary antibody (A). DIC images were collected using transmitted light; intracellular distribution of endogenous paxillin (A) and EGFP-paxillin (B) was analyzed by fluorescent confocal microscopy. Bars, 10 μm. Thus, we show similar distribution of endogenous paxillin, EGFP-conjugated wild-type paxillin and its mutants.

opposite side of the cells (Fig. 7B). Distribution of the endogenous (Fig. 7A) and EGFP-conjugated (Fig. 7B) wild-type paxillin were similar, indicating that EGFP does not interfere with paxillin intracellular distribution. These data suggested that phosphorylation of paxillin residues 31 and 118 does not control its intracellular distribution.

PMA effect on paxillin phosphorylation and migration of non-adherent and adherent cells

In addition to Baf3, we also investigated the effect of PMA on paxillin phosphorylation (Fig. 8A) and migration (Fig. 8B) of several hemopoietic non-adherent cell lines: the IL-3-dependent murine myeloid 32D, human T CCRF-CEM, human Burkitt lymphoma Daudi; as well as adherent mouse and monkey fibroblast cell lines, Swiss3T3 and COS-1, respectively. We found that paxillin was tyrosine phosphorylated in all cell lines when they were maintained on complete medium. Addition of PMA inhibited paxillin tyrosine phosphorylation and motility of 32D and CCRF CEM cells. Motility inhibition of 32D (t=9.2, P=0.0003) and CCRF CEM (t=8.4, P=0.0004) was considered to be significant. PMA did not inhibit paxillin tyrosine phosphorylation and motility of lymphoma Daudi cell line (t=1.1, P=0.33) or fibroblast cell lines Swiss 3T3 (t=–4.16, P=0.0088) and COS-1 (t=–0.49, P=0.64). We concluded that the PMA effects described in this paper for Baf3 cells can also be applied to other hematopoietic cells.

Discussion

IL-3 induces phosphorylation of paxillin residues 31 and 118

Using anti-phosphotyrosine antibody, antibodies specific for paxillin phosphorylated on tyrosine residues 31 or 118 and paxillin tyrosine phosphorylation mutants (Y→F) we demonstrated that paxillin tyrosine residues 434 and 488 were not phosphorylated either in the absence or the presence of IL-3. Conversely, tyrosine residues 40 and 181 were constitutively phosphorylated whether IL-3 was present or not. Phosphorylation of tyrosine residues 31 and 118, however, was fully dependent upon the presence of IL-3.

Our results are consistent with observations in other systems, for example, paxillin tyrosine residues 434 and 488 are not phosphorylated in TGFβ1-stimulated normal murine mammary NmuMG cells (Nakamura et al., 2000). The

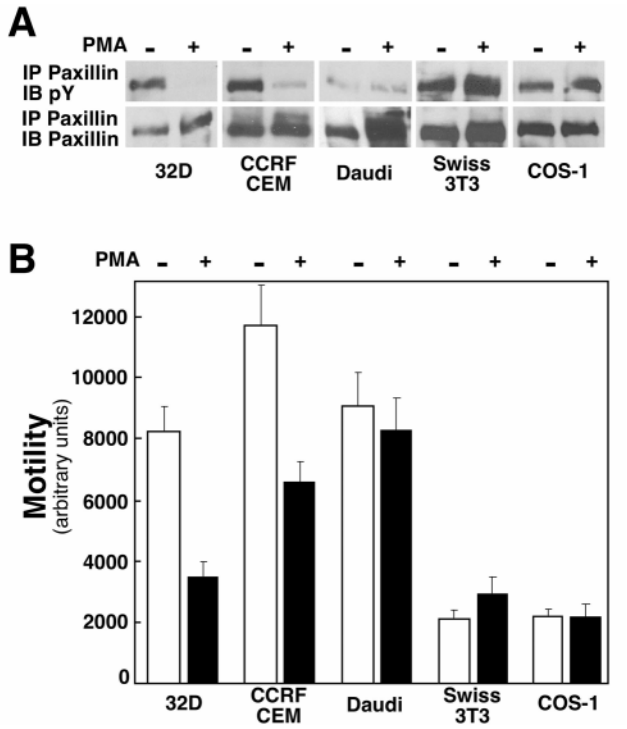

Fig. 8. Effect of PMA on tyrosine phosphorylation and motility of several non-adherent (32D, CCRF CEM, Daudi) and adherent (Swiss 3T3, COS-1) cell lines maintained in complete medium. (A) After a 15-minute treatment with 100 nM PMA, paxillin was immunoprecipitated and analyzed by western blotting with a mixture of anti-PY20 and 4G10 antibody (IB pY) or with anti-paxillin antibody (IB Paxillin). (B) Spontaneous cell motility was analyzed as described in the Materials and Methods. The mean and s.e.m. from six experiments are presented. PMA inhibited the motility of 32D cell line (t=9.2, P=0.0003) and CCRF CEM cell line (t=8.4, P=0.0004), increased the motility of Swiss 3T3 (t=–4.16, P=0.0088) but did not significantly affect the motility of Daudi (t=1.1, P=0.33) or Cos1 cells (t=–0.49, P=0.64).

phosphorylation status of residues 40 and 181, however, largely depends on the cell type. For instance, phosphorylation of both of these tyrosine residues could be induced by TGFβ1 in NmuMG cells (Nakamura et al., 2000), whereas tyrosine 40, but not tyrosine 181, was phosphorylated upon adherence of primary chicken embryo fibroblasts to fibronectin (Schaller and Schaefer, 2001). Similar to our results, it is reported that in rat ascites hepatoma MM1 cells phosphorylation of tyrosine 181 is constitutive and not augmented by fibronectin and lysophosphatidic acid stimulation (Iwasaki et al., 2002). Our data complement earlier observations demonstrating that tyrosines 31 and 118 were the major phosphorylation sites during adhesion of primary chicken embryo fibroblasts to fibronectin, Nara Bladder Tumor (NBTII) cells to collagen and rat ascites hepatoma MM1 cells to fibronectin in the presence of lysophosphatidic acid (Petit et al., 2000; Schaller and Schaefer, 2001; Iwasaki et al., 2002). The same tyrosine residues were phosphorylated in response to stimulation of rat smooth muscle A7r5 cells with PDGF, rat GN4 cells with angiotensin II and normal murine mammary NmuMG cells with TGFβ1 (Nakamura et al., 2000; Schaller and Schaefer, 2001).

Tyrosine phosphorylation of paxillin residues 31 and 118 is required for lymphocyte polarization and motility

Lymphocyte polarization is a critical prerequisite for cell motility. Similar to the events described for other lymphoid cells, polarization in Baf3 cells was accompanied by assembly of membrane-ruffled protrusions at the anterior cell pole where a mesh of actin filaments is concentrated (Romanova et al., 1999). Experiments with cytoskeleton-inhibiting drugs also showed that redistribution of actin was a driving force of cell polarization (Romanova et al., 1999). Although it has been demonstrated that phosphorylation of paxillin residues 31 and 118 controls formation of the mesh of actin filaments at the periphery of migrating murine mammary NmuMG cells (Nakamura et al., 2000), the involvement of paxillin in actin-dependent polarization of lymphoid cells has never been studied. Here we showed that the IL-3-induced phosphorylation of paxillin tyrosine residues 31 and 118 regulates Baf3 polarization and motility, presumably through redistribution of actin cytoskeleton.

We also showed that wild-type paxillin phosphorylated on tyrosine residues 31 and 118, as well as the 31/118 paxillin tyrosine phosphorylation mutant, were similarly distributed within the cells, suggesting that residues 31 and 118 do not control paxillin targeting within Baf3 cells. In a related finding it is reported that these paxillin residues did not regulate paxillin targeting to focal adhesion contacts in NBTII cells (Petit et al., 2000). On the contrary, phosphorylation of paxillin threonine 403 and serine 481, which can be induced by phorbol esters, was responsible for paxillin targeting to focal adhesions (Brown et al., 1998).

PMA inhibits phosphorylation of paxillin tyrosine residues 31 and 118, cell polarization and motility

In the presence of IL-3 PMA reduced phosphorylation of paxillin tyrosine residues 31and 118, disrupted polarized cell phenotype and inhibited motility. Because phosphorylation of paxillin residues 31 and 118 was crucial for cell polarization and motility we suggested that PMA interference in paxillin tyrosine phosphorylation contributed to the observed cell morphological changes. The described PMA effect was not restricted to the one cell line, but can be demonstrated in other hematopoietic cell types.

The steady-state level of protein tyrosine phosphorylation is controlled by the coordinated action of protein tyrosine kinases and tyrosine phosphatases. Therefore, activation of phosphatases that directly dephosphorylate paxillin or inhibition of upstream kinases may account for the inhibition of paxillin tyrosine phosphorylation.

We demonstrated that the activation-dependent tyrosine phosphorylation of FAK in PMA-treated cells is diminished. Consistent with our results, it has been reported that phorbol esters inhibited FAK or Src activity. For instance, PMA inhibits CD3/CD28-induced FAK and PYK2 activity in phorbol ester-treated T cells (Rankin and Rozengurt, 1994; Tsuchida et al., 2000) as well as in Swiss fibroblasts treated with PDGF, a growth factor that involves PKC in its signaling (Zang et al., 1997). Regulation of Src activity by phorbol esters is complex. One of the PMA-activated PKC isoforms, PKC-δ, directly binds Src, phosphorylates its serine residues and stimulates its activity (Li et al., 1994; Zang et al., 1997). However, inhibition of Src activity could occur after a concomitant activation of PKC-δ and ligation of IgE receptor (Song et al., 1998).

Several phosphatases have been implicated in paxillin dephosphorylation, including PTP1B, SHP2, PTP-PEST and PTPϕ (Shen et al., 1998; Tamura et al., 1998; Arregui et al., 1998; Yu et al., 1998; Cote et al., 1999; Shen et al., 2000; Pixley et al., 2001). Phorbol esters stimulate serine phosphorylation of PTP1B in vivo. This phosphorylation, nevertheless, does not affect its phosphatase activity (Flint et al., 1993). What is more, in vivo phosphorylation of serine residues 576 and 591 by various PKC isoforms had no effect on SHP2 phosphatase activity (Strack et al., 2002). Finally, phorbol ester-induced serine phosphorylation of PTP-PEST inhibited its activity (Garton and Tonks, 1994). The effect of PMA on the activity of PTPϕ phosphatase, which reportedly may participate in paxillin dephosphorylation in non-adherent cells, has not been investigated.

PTP-PEST and PTPϕ are not expressed in Baf3 cells; SHP1 and SHP2 are expressed, but consistent with cited data their activity is not regulated by phorbol esters (unpublished data). Thus, we suggest that inhibition of upstream signaling may be involved in reduction of paxillin tyrosine phosphorylation in response to PMA. Although phorbol ester-induced activation of phosphatases that directly dephosphorylate paxillin is also possible, our data and the current literature do not support this notion.

The principal message of this report is that growth factor-induced phosphorylation of paxillin tyrosine residues 31 and 118 regulates actin-dependent polarization that is required for motility of a non-adherent transformed pre-B lymphocyte line. Phorbol esters can inhibit this phosphorylation, thus interfering with lymphocyte polarization and motility. Our findings contribute to the understanding of the endogenous mechanisms of lymphocyte polarization and motility and have potential relevance to human cancer, suggesting that cytostatic derivatives of phorbol esters may prove to be useful for inhibiting motility of transformed cells and thus interfering with their metastasis.

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References

Arregui, C. O., Balsamo, J. and Lilien, J. (1998). Impaired integrin-mediated adhesion and signaling in fibroblasts expressing a dominant-negative mutant. PTP1B *J. Cell Biol.* **143**, 861-873.

Bellis, S. L., Miller, J. T. and Turner, C. E. (1995). Characterization of tyrosine phosphorylation of paxillin in vitro by focal adhesion kinase. *J. Biol. Chem.* **270**, 17437-17441.

Brown, M. C., Perrotta, J. A. and Turner, C. E. (1998). Serine and threonine phosphorylation of the paxillin LIM domains regulates paxillin focal adhesion localization and cell adhesion to fibronectin. *Mol. Biol. Cell* **9**, 1803-1816.

Cote, J. F., Turner, C. E. and Tremblay, M. L. (1999). Intact LIM 3 and LIM 4 domains of paxillin are required for the association to a novel polyproline region (Pro 2) of protein-tyrosine phosphatase-PEST. *J. Biol. Chem*. **274**, 20550-20560.

del Pozo, M. A., Nieto, C. E., Serrador, J. M., Sancho, D., Vicente-Manzanares, M., Martinez, C. and Sanchez-Madrid, F. (1998). The two poles of the lymphocyte: specialized cell compartments for migration and recruitment. *Cell Adhes. Commun.* **6**, 125-133.

Feller, S. M., Knudsen, B. and Hanafusa, H. (1995). Cellular proteins binding to the first Src homology 3 (SH3) domain of the proto-oncogene product c-Crk indicate Crk-specific signaling pathways. *Oncogene* **10**, 1465-1473.

Flint, A. J., Gebbink, M. F., Franza, B. R. Jr, Hill, D. E. and Tonks, N. K. (1993). Multi-site phosphorylation of the protein tyrosine phosphatase. *EMBO J.* **12**, 1937-1946.

Fukui, Y., Hashimoto, O., Sanui, T., Oono, T., Koga, H., Abe, M., Inayoshi, A., Noda, M., Oike, M., Shirai, T. and Sasazuki, T. (2001). Haematopoietic cell-specific CDM family protein DOCK2 is essential for lymphocyte migration. *Nature* **412**, 826-831.

Garton, A. J. and Tonks, N. K. (1994). PTP-PEST: a protein tyrosine phosphatase regulated by serine phosphorylation. *EMBO J.* **13**, 3763-3771.

Goodnight, J. A., Mischak, H., Kolch, W. and Mushinski, J. F. (1995). Immunochemical localization of eight protein kinase C isozymes overexpressed in NIH 3T3 fibroblasts. Isoform-specific association with microfilaments, Golgi, endoplasmic reticulum, and nuclear and cell membranes. *J. Biol. Chem.* **270**, 9991-10001.

Gotoh, T., Hattori, S., Nakamura, S., Kitayama, H., Noda, M., Takai, Y., Kaibuchi, K., Matsui, H., Hatase, O. and Takahashi, H. (1995). Identification of Rap1 as a target for the Crk SH3 domain-binding guanine nucleotide-releasing factor C3G. *Mol. Cell. Biol.* **15**, 6746-6753.

Grant, S., Roberts, J., Poplin, E., Tombes, M. B., Kyle, B., Welch, D., Carr, M. and Bear, H. D. (1998). Phase Ib trial of bryostatin 1 in patients with refractory malignancies. *Clin. Cancer Res.* **4**, 611-618.

Han, Z. T., Zhu, X. X., Yang, R. Y., Sun, J. Z., Tian, G. F., Liu, X. J., Cao, G. S., Newmark, H. L., Conney, A. H. and Chang, R. L. (1998). Effect of intravenous infusions of 12-O-tetradecanoylphorbol-13-acetate (TPA) in patients with myelocytic leukemia: preliminary studies on therapeutic efficacy and toxicity. *Proc. Natl. Acad. Sci. USA* **95**, 5357-5361.

Hasegawa, H., Kiyokawa, E., Tanaka, S., Nagashima, K., Gotoh, N., Shibuya, M., Kurata, T. and Matsuda, M. (1996). DOCK180, a major CRK-binding protein, alters cell morphology upon translocation to the cell membrane. *Mol. Cell. Biol.* **16**, 1770-1776.

Iwasaki, T., Nakata, A., Mukai, M., Shinkai, K., Yano, H., Sabe, H., Schaefer, E., Tatsuta, M., Tsujimura, T., Terada, N. et al. (2002). Involvement of phosphorylation of Tyr-31 and Tyr-118 of paxillin in MM1 cancer cell migration. *Int. J. Cancer.* **97**, 330-335.

Kiyokawa, E., Hashimoto, Y., Kobayashi, S., Sugimura, H., Kurata, T. and Matsuda, M. (1998). Involvement of phosphorylation of Tyr-31 and Tyr-118 of paxillin in MM1 cancer cell migration. *Genes Dev*. **12**, 3331-3336.

Li, W., Yu, J. C., Michieli, P., Beeler, J. F., Ellmore, N., Heidaran, M. A. and Pierce, J. H. (1994). Stimulation of the platelet-derived growth factor beta receptor signaling pathway activates protein kinase C-delta. *Mol. Cell. Biol.* **14**, 6727-6735.

Mazaki, Y., Uchida, H., Hino, O., Hashimoto, S. and Sabe, H. (1998). Paxillin isoforms in mouse. Lack of the gamma isoform and developmentally specific beta isoform expression. *J. Biol. Chem.* **273**, 22435-22441.

Nakamura, K., Yano, H., Uchida, H., Hashimoto, S., Schaefer, E. and Sabe, H. (2000). Tyrosine phosphorylation of paxillin-α is involved in temporospatial regulation of paxillin-containing focal adhesion formation and F-actin organization in motile cells. *J. Biol. Chem*. **275**, 27155-27164.

Okada, S., Matsuda, M., Anafi, M., Pawson, T. and Pessin, J. E. (1998). Insulin regulates the dynamic balance between Ras and Rap1 signaling by coordinating the assembly states of the Grb2-SOS and CrkII-C3G complexes. *EMBO J.* **17**, 2554-2565.

Petit, V., Boyer, B., Lentz, D., Turner, C. E., Thiery, J. P. and Valles, A. M. (2000). Phosphorylation of tyrosine residues 31 and 118 on paxillin regulates cell migration through an association with CRK in NBT-II cells. *J. Cell Biol.* **148**, 957-970.

Pixley, F. J., Lee, P. S. W., Condeelis, J. S. and Stanley, E. R. (2001). Protein tyrosine phosphatase phi regulates paxillin tyrosine phosphorylation and mediates colony-stimulating factor 1-induced morphological changes in macrophages. *Mol. Cell. Biol*. **21**, 1795-1809.

Rankin, S. and Rozengurt, E. (1994). Platelet-derived growth factor modulation of focal adhesion kinase (p125FAK) paxillin tyrosine phosphorylation in Swiss 3T3 cells. Bell-shaped dose response and cross-talk with bombesin. *J. Biol. Chem.* **269**, 704-710.

Richardson, A., Malik, R. K., Hildebrand, J. D. and Parsons, J. T. (1997). Inhibition of cell spreading by expression of the C-terminal domain of focal adhesion (FAK) is rescued by coexpression of Src or catalytically inactive FAK: a role for paxillin tyrosine phosphorylation. *Mol. Cell. Biol.* **17**, 6906-6914.

Romanova, L. Y., Alexandrov, I. A., Schwab, G., Hilbert, D. M., Mushinski, J. F. and Nordan, R. P. (1996). Mechanism of apoptosis suppression by phorbol ester in IL-6-starved murine plasmacytomas: role of PKC modulation and cell cycle. *Biochemistry* **35**, 9900-9906.

Romanova, L., Y., Alexandrov, I. A., Blagosklonny, M. V., Nordan, R. P., Garfield, S., Acs, P., Nguyen, P., Trepel, J., Blumberg, P. M. and Mushinski, J. F. (1999). Regulation of actin cytoskeleton in lymphocytes: PKC-δ disrupts IL-3-induced membrane ruffles downstream of Rac1. *J. Cell. Physiol.* **179**, 157-169.

Salgia, R., Brunkhorst, B., Pisick, E., Li, J. L., Lo, S. H., Chen, L. B. and Griffin, J. D. (1995a). Increased tyrosine phosphorylation of focal adhesion proteins in myeloid cell lines expressing p210BCR/ABL. *Oncogene* **11**, 1149-1155.

Salgia, R., Li, J. L., Lo, S. H., Brunkhorst, B., Kansas, G. S., Sobhany, E. S., Sun, Y., Pisik, E., Hallek, M., Ernst, T. et al. (1995b). Molecular cloning of human paxillin, a focal adhesion protein phosphorylated by P210BCR/ABL. *J. Biol. Chem.* **270**, 5039-5047.

Salgia, R., Uemura, N. K., Okuda, J. L., Li, E., Pisick, M., Sattler, R., de Jong, B., Druker, N., Heisterkamp, L., Chen, B. et al. (1995c). CRKL links p210BCR/ABL with paxillin in chronic myelogenous leukemia cells. *J. Biol. Chem.* **270**, 29145-29150.

Salgia, R., Pisick, E., Sattler, M., Li, J. L., Uemura, N., Wong, W. K., Burky, S. A., Hirai, H., Chen, L. B. and Griffin, J. D. (1996). p130CAS forms a signaling complex with the adapter protein CRKL in hematopoietic cells transformed by the BCR/ABL oncogene. *J. Biol. Chem.* **271**, 25198-25203.

Schaller, M. D. and Parsons, J. T. (1995). pp125FAK-dependent tyrosine phosphorylation of paxillin creates a high-affinity binding site for Crk. *Mol. Cell. Biol.* **15**, 2635-2645.

Schaller, M. D. and Schaefer, E. M. (2001). Multiple stimuli induce tyrosine phosphorylation of the Crk-binding sites of paxillin. *Biochem. J.* **360**, 57-66.

Seufferlein, T. and Rozengurt, E. (1994). Structural requirements for function of the Crkl adapter protein in fibroblasts and hematopoietic cells. *J. Biol. Chem.* **269**, 9345-9351.

Shen, Y., Schneider, G., Cloutier, A., Veillette, J. F. and Schaller. M. D. (1998). Direct association of protein-tyrosine phosphatase PTP-PEST with paxillin. *J. Biol. Chem.* **273**, 6474-6481.

Shen, Y., Lyons, P., Cooley, M., Davidson, D., Veillette, A., Salgia, R., Griffin, J. D. and Schaller, M. D. (2000). The noncatalytic domain of protein-tyrosine phosphatase-PEST targets paxillin for dephosphorylation in vivo. *J. Biol. Chem.* **275**, 1405-1413.

Sinnet-Smith, J., Zachary, I., Valverde, A. M. and Rozengurt, E. (1993). Bombesin stimulation of p125 focal adhesion kinase tyrosine phosphorylation. *J. Biol. Chem.* **268**, 14261-14268.

Song, J. S., Swann, P. G., Szallasi, Z., Blank, U., Blumberg, P. M. and Rivera, J. (1998). Tyrosine phosphorylation-dependent and -independent associations of protein kinase C-delta with Src family kinases in the RBL-2H3 mast cell line: regulation of Src family kinase activity by protein kinase C-delta. *Oncogene* **16**, 3357-3368.

Strack, V., Krutzfeldt, J., Kellerer, M., Ullrich, A., Lammers, R. and

Haring, H. U. (2002). The protein-tyrosine-phosphatase SHP2 is phosphorylated on serine residues 576 and 591 by protein kinase C isoforms alpha, beta 1, beta 2, and eta. *Biochemistry* **41**, 603-608.

Strair, R. K., Schaar, D., Goodell, L., Aisner, J., Chin, K. V., Eid, J., Senzon, R., Cui, X. X., Han, Z. T., Knox, B. et al. (2002). Administration of a phorbol ester to patients with hematological malignancies: preliminary results from a phase I clinical trial of 12-O-tetradecanoylphorbol-13-acetate. *Clin. Cancer Res.* **8**, 2512-2518.

Tamura, M., Gu, J., Matsumoto, K., Aota, S., Parsons, R. and Yamada, K. M. (1998). Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. *Science* **280**, 1614-1617.

Thomas, S. M., Soriano, P. and Imamoto, A. (1995). Specific and redundant roles of Src and Fyn in organizing the cytoskeleton. *Nature* **376**, 267-271.

Tsubouchi, A., Sakakura, J., Yagi, Y., Mazaki, R., Schaefer, E., Yano, H. and Sabe, H. (2002). Localized suppression of PhoA activity by Tyr31/118-phosphorylated paxillin in cell adhesion and migration. *J. Cell Biol.* **159**, 673-683.

Tsuchida, M., Manthei, E. R., Alam, T., Knechtle, S. J. and Hamawy, M. M. (2000). Regulation of T cell receptor- and CD28-induced tyrosine phosphorylation of the focal adhesion tyrosine kinases Pyk2 and Fak by protein kinase C. A role for protein tyrosine phosphatases. *J. Biol. Chem.* **275**, 1344-1350.

Turner, C. E. and Miller, J. T. (1994). Primary sequence of paxillin contains putative SH2 and SH3 domain binding motifs and multiple LIM domains: identification of a vinculin and pp125Fak-binding region. *J. Cell Sci.* **107**, 1583-1591.

Turner, C. E., Glenney, J. R. Jr and Burridge, K. (1990). Paxillin: a new vinculin-binding protein present in focal adhesions. *J. Cell Biol*. **111**, 1059-1068.

Yano, H., Uchida, H., Iwasaki, T., Mukai, M., Akedo, H., Nakamura, K., Hashimoto, S. and Sabe. H. (2000). Paxillin alpha and Crk-associated substrate exert opposing effects on cell migration and contact inhibition of growth through tyrosine phosphorylation. *Proc. Natl. Acad. Sci. USA* **97**, 9076-9081.

Yu, D. H., Qu, C. K., Henegariu, O., Lu, X. and Feng, G. S. (1998). Protein-tyrosine phosphatase Shp-2 regulates cell spreading, migration, and focal adhesion. *J. Biol. Chem.* **273**, 21125-21131.

Zang, Q., Lu, Z., Curto, M., Barile, N., Shalloway, D. and Foster, D. A. (1997). Association between v-Src and protein kinase C delta in v-Src-transformed fibroblasts. *J. Biol. Chem.* **272**, 13275-13280.