Phosphorylation of paxillin at threonine 538 by PKCδ regulates LFA1-mediated adhesion of lymphoid cells

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Accepted 22 February 2010
Journal of Cell Science 123, 1567-1577

doi:10.1242/jcs.060996

Summary

We investigated the PKCδ-mediated phosphorylation of paxillin within its LIM4 domain and the involvement of this phosphorylation in activation of LFA-1 integrins of the Baf3 pro-B lymphocytic cell line. Using phosphorylated-threonine-specific antibodies, phosphorylated amino acid analysis and paxillin phosphorylation mutants, we demonstrated that TPA, the pharmacological analog of the endogenous second messenger diacyl glycerol, stimulates paxillin phosphorylation at threonine 538 (T538). The TPA-responsive PKC isoform PKCδ directly binds paxillin in a yeast two-hybrid assay and phosphorylates paxillin at T538 in vitro and also co-immunoprecipitates with paxillin and mediates phosphorylation of this residue in vivo. Recombinant wild-type paxillin, its phospho-inhibitory T538A or phospho-mimetic T538E mutants were expressed in the cells simultaneously with siRNA silencing of the endogenous paxillin. These experiments suggest that phosphorylation of paxillin T538 contributes to dissolution of the actin cytoskeleton, redistribution of LFA-1 integrins and an increase in their affinity. We also show that phosphorylation of T538 is involved in the activation of LFA-1 integrins by TPA.

Key words: Paxillin, Threonine phosphorylation, Lymphocytes, LFA-1

Introduction

Paxillin is an adapter protein that recruits and assembles several cytoskeletal and signaling molecules to regulate cell motility, proliferation and oncogenic transformation. It has a major role in the dynamic process of actin rearrangement that is required for assembly of integrin complexes and for cell motility. The C-terminal half of paxillin contains four LIM domains. Phosphorylation of T403 and S457/481 within paxillin LIM2 and LIM3 domains facilitates paxillin recruitment to focal adhesions (Brown et al., 1998). LIM3 and LIM4 domains are involved in binding with the tyrosine phosphatase PTP-PEST (Brown et al., 1998) and microtubules (Cote et al., 1999).

The lymphocyte-function-associated antigen 1 (LFA-1; αLβ2 integrin) is selectively expressed on leukocytes and mediates interaction with its ligand, intracellular adhesion molecule-1 (ICAM-1) (for a review, see Mor et al., 2007). Leukocytes circulating in the bloodstream express an inactive LFA-1 receptor that is unable to bind its ligand. LFA-1 activation occurs during lymphocyte maturation, during the immune response and during lymphocyte migration through tissues to sites of inflammation. Signal-transduction pathways, such as those activated by stimulation of T-cell receptors or exposure to phorbol esters, also called 'inside-out' signaling, lead to change of receptor avidity and affinity. Redistribution of LFA-1 on the cell surface, i.e., receptor clustering, effects an avidity change in the receptors (Stewart and Hogg, 1996; van Kooyk and Figdor, 2000). The lateral movement of receptors requires release from the cytoskeleton, and drugs that destabilize the actin or tubulin cytoskeleton might contribute to LFA-1 clustering and activation (Lub et al., 1997; Zhou et al., 2001). Similarly to many other receptors, LFA-1 undergoes conformational changes that are thought to account for an increase in LFA-1 affinity. Ligation of LFA-1 to its ligand at the next stage, also called 'outside-in' signaling, initiates a variety of new signaling pathways. Here, we investigate the involvement of PKCδ in the initial stage of LFA-1 receptor activation.

Results

IL-3 and TPA stimulate paxillin phosphorylation through distinct pathways

TPA, the PKC activator and a pharmacological analog of the second messenger, diacyl glycerol (DAG), induces paxillin phosphorylation and activation of LFA-1 integrins in many lymphoid cells, including Baf3. To investigate the TPA effect on paxillin phosphorylation in Baf3 cells maintained in the presence of its growth factor, IL-3, which also induces paxillin phosphorylation (Romanova et al., 2004), we first investigated whether TPA and IL-3 regulate paxillin phosphorylation through distinct pathways. Baf3 cells were deprived of IL-3, and then treated with IL-3 or TPA. Both treatments produced a mobility shift of paxillin protein (Fig. 1A), suggesting that paxillin had been phosphorylated. We next used protein phosphatases of known specificities to investigate the nature of the phosphorylation. Non-specific potato acid phosphatase, PAP, dephosphorylates phosphotyrosines as well as phosphoserines and phosphothreonines, and its use reduced the mobility shift induced by both IL-3 and TPA (Fig. 1A). The phosphotyrosine-specific phosphatase PTP, reduced the mobility shift induced by IL-3. Conversely, the serine/threonine-specific phosphatase, $PP2A_2$, inhibited the mobility shift induced by TPA.

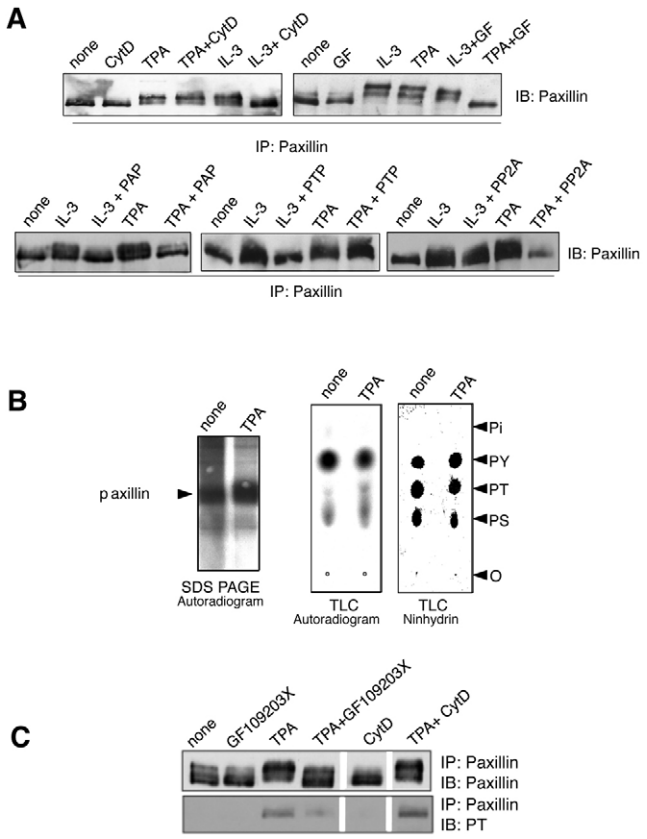

Fig. 1. TPA induces threonine phosphorylation of paxillin in the absence or presence of IL-3. (**A**) Baf3 cells were withdrawn from IL-3 for 6 hours and then treated for 10 minutes with IL-3, 100 nM TPA, 4 μM cytochalasin D or 1 μM GF109203X, as indicated. Paxillin was immunoprecipitated under denaturing conditions. In one experiment it was further treated in vitro with non-specific potato acid phosphatase (PAP), phosphotyrosine phosphatase (PTP) or phosphoprotein phosphatase 2A2 ($PP2A_2$), which specifically dephosphorylate tyrosine or serine/threonine residues, respectively. Paxillin was analyzed by western blot with anti-paxillin antibody. (**B**,**C**) Baf3 cells were maintained in IL-3. In one experiment, cells were labeled in vivo by growth in [^{32}P]orthophosphate and treated with 100 nM TPA for 15 minutes. Immunoprecipitated paxillin was resolved by SDS-PAGE electrophoresis. Hydrolyzed radiolabeled bands were analyzed on TLC plates and transferred to a membrane. The membrane was stained with ninhydrin to reveal marker phosphorylated amino acids and exposed to X-ray film to determine degree of in vivo phosphorylation. Arrowheads indicate the positions of phosphotyrosine (PY), phosphothreonine (PT), phosphoserine (PS) and the origin of TLC plate loading (O). (**C**) Cells were treated as in A. Immunoprecipitated paxillin was analyzed with anti-paxillin or anti-phosphothreonine (PT) antibodies.

We next investigated the drug sensitivity of IL-3- and TPA-induced paxillin phosphorylation. IL-3-deprived Baf3 cells were treated with the actin-disrupting drug, cytochalasin D, or the PKC-specific inhibitor GF103209X, alone or in combination with TPA or IL-3 (Fig. 1A). These results showed that the IL-3-induced phosphorylation of paxillin could be disrupted by cytochalasin D, but not by GF103209X, whereas the phosphorylation induced by TPA was inhibited by GF103209X, but not by cytochalasin D.

These data demonstrate that IL-3 stimulates paxillin phosphorylation on tyrosine residues in a manner that is dependent on an intact actin cytoskeleton and does not involve PKC. By contrast, the phosphorylation of paxillin stimulated by TPA occurs

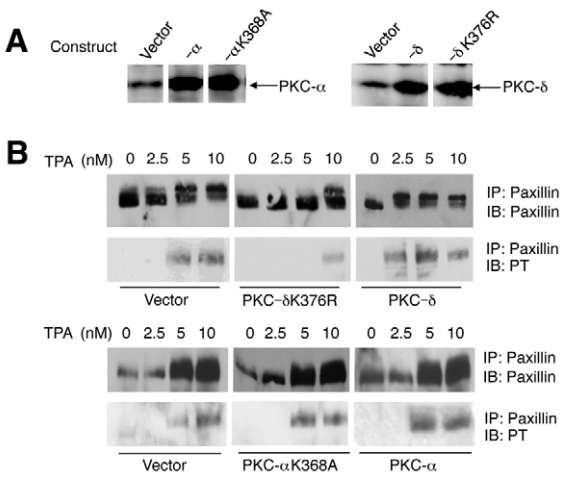

Fig. 2. PKCδ is responsible for the TPA-induced paxillin threonine phosphorylation. (**A**) Expression in Baf3 cells of recombinant wild-type murine PKCα, PKCδ or their inactive analogs, PKCαK368A and PKCδK376R was analyzed with antibodies specific for PKCα or PKCδ. (**B**) Cells were treated with the indicated concentrations of TPA for 10 minutes. Paxillin was immunoprecipitated and western blots were analyzed using anti-paxillin and anti-phosphothreonine antibodies.

on serine or threonine residues in a PKC-dependent manner and independently of an intact actin cytoskeleton. Thus, TPA stimulates paxillin phosphorylation through an IL-3-independent pathway.

TPA stimulates threonine paxillin phosphorylation in Baf3 cells maintained in IL-3

We next investigated the effect of TPA on paxillin phosphorylation in Baf3 cells growing in IL-3 by labeling paxillin in vivo with [^{32}P]orthophosphate before and after TPA exposure. An autoradiogram of an SDS-PAGE blot showed an increase in overall phosphorylation of paxillin (Fig. 1B). Autoradiograms and ninhydrin staining of hydrolyzed phosphorylated bands showed an increase of phosphorylated threonine in the TPA-treated sample (Fig. 1B). Consistent with our prior observation (Romanova et al., 2004), TPA also decreased the level of phosphorylated tyrosine. We also used phosphothreonine-specific antibodies to show that in the presence of IL-3, the TPA-induced paxillin phosphorylation occurred on threonine residues (Fig. 1C). It could be reversed with GF103209X, but not by cytochalasin D.

PKCδ is responsible for threonine phosphorylation of paxillin in response to TPA

Among the four PKC isoforms that are expressed in Baf3 cells, PKCα, PKCδ, PKCζ and PKCλ, only PKCα and PKCδ have phorbol-ester-binding sites and can be directly activated by TPA (Romanova et al., 1999; Romanova et al., 1998). To investigate which of these isoforms was responsible for the TPA-induced paxillin threonine phosphorylation, we developed Baf3 cell lines stably transfected with murine PKCα, PKCδ, their kinase-dead mutants, PKCα K368A and PKCδ K376R, or vector alone. The lowest concentration of TPA that induced paxillin phosphorylation in the parental Baf3 cells was 5 nM TPA (Fig. 2). PKCδK376R partially abolished paxillin threonine phosphorylation in response to TPA, whereas overexpressed PKCδ facilitated the effect. However, neither PKCα nor PKCα K368A affected the level of

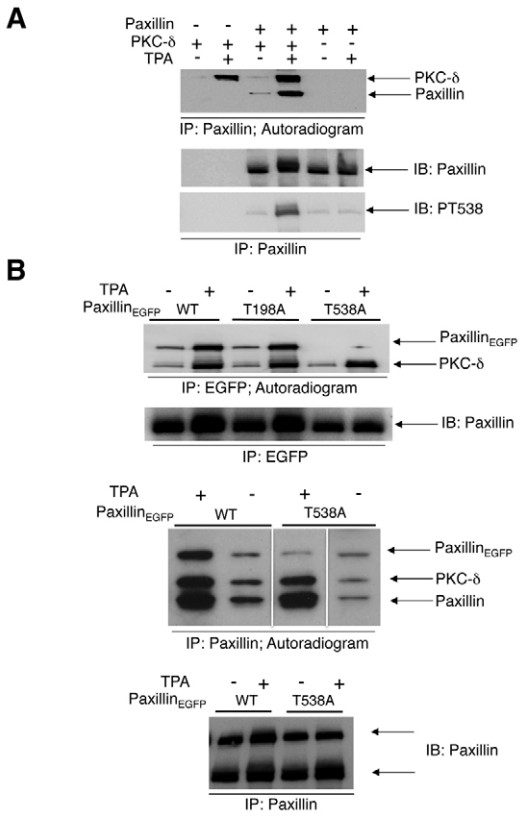

Fig. 3. PKCδ phosphorylates paxillin in vitro. We used parental Baf3 (**A**) or their derivatives stably transfected with EGFP-paxillins: WT or phosphorylation mutants, T198A or T538A. (**B**) The recombinant paxillins and/or endogenous paxillin were immunoprecipitated under denaturing conditions with antibody specific for paxillin or EGFP. In vitro phosphorylation with purified recombinant PKCδ and [γ-^{32}P]-ATP was performed in the absence or presence of 100 nM TPA. The kinase reactions were resolved by electrophoresis on 10% SDS gels, and the gels were exposed to X-ray film. Autophosphorylation of PKCδ was used as an internal control. A quarter of the reaction mixture was analyzed by western blot using antibody specific for paxillin or phosphorylated paxillin T538 (PT538). (A) Paxillin or PKCδ was not added into first two or last two lanes, respectively.

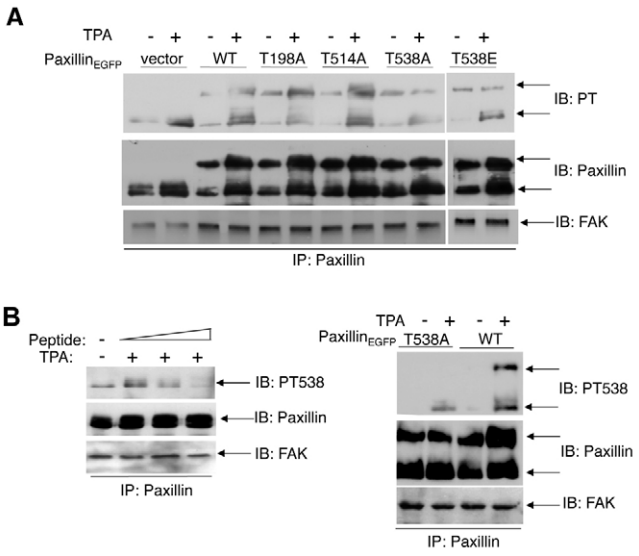

Fig. 4. TPA stimulates phosphorylation of paxillin at T538 in vivo. (**A**) Baf3 cells were stably transfected with recombinant EGFP-tagged WT paxillin, its phosphorylation mutants T198A, T514A, T538A, T538E or the vector alone. The cells were treated with 100 nM TPA for 10 minutes. Paxillin was immunoprecipitated and analyzed with antibody specific for phosphothreonine (PT), paxillin or FAK. (**B**) Immunoprecipitated paxillin was analyzed on western blots with antibody specific for T538-phosphorylated paxillin or paxillin. Phospho-mimetic T538 peptide was added to some incubation mixtures, as indicated.

paxillin threonine phosphorylation. These data implicate PKCδ in the regulation of paxillin threonine phosphorylation.

Paxillin T538 is phosphorylated by PKCδ in vitro and in response to TPA in vivo

We studied the details of paxillin phosphorylation by performing an in vitro kinase assay using recombinant mouse PKCδ. Even in the absence of TPA, PKCδ is partially active. TPA led to its further activation and to autophosphorylation that was used as an internal control in this assay (Fig. 3A). PKCδ also phosphorylated paxillin that had been immunoprecipitated from Baf3 cells under denaturing conditions. In the absence of recombinant PKCδ no notable phosphorylation of paxillin was observed, thus excluding the possibility that other TPA-inducible kinases that might have re-bound paxillin at the 'low stringency' stage of immunoprecipitation are responsible for the observed phosphorylation.

Fujii and co-workers (Fujii et al., 2004) identified amino acid motifs that were potentially suitable for phosphorylation by PKCδ in vitro. In paxillin, we recognized such sequences in the vicinity of T198, T514 and T538. Three recombinant EGFP-conjugated paxillin mutants containing alanine substitution at one of the above-mentioned threonine residues were made and stably transfected into Baf3 cells. Denaturing immunoprecipitations of the recombinant paxillin proteins alone or along with endogenous paxillin were performed with anti-EGFP or anti-paxillin antibodies, respectively, and in vitro phosphorylation by purified PKCδ was assessed (Fig. 3B). PKCδ autophosphorylated itself, as well as the endogenous paxillin, in all samples in a TPA-inducible fashion. Phosphorylation of the recombinant paxillin was observed only in the samples that contained wild-type (WT) paxillin and paxillin T198A, but not T538A paxillin, suggesting that paxillin T538 is a target of PKCδ in vitro.

We next analyzed in vivo phosphorylation of the recombinant paxillin mutants. TPA stimulated threonine phosphorylation of the endogenous paxillin in all cell lines, as was evident from mobility shifts and intensity changes of the bands visualized with the anti-phosphothreonine and anti-paxillin antibodies (Fig. 4A). Although TPA stimulated threonine phosphorylation of the recombinant WT paxillin and the T198A and T514A mutants, it did not affect threonine phosphorylation level of the T538A mutant or a phospho-mimetic mutant, with a glutamic acid substitution of the T538 (T538E). These data suggest that T538 is the residue that is phosphorylated in vivo in response to TPA. Co-immunoprecipitation with a paxillin-binding partner, FAK, whose binding is not affected by TPA (Romanova et al., 2004), demonstrated equal loading.

To further assess phosphorylation of paxillin T538, a polyclonal antibody to the phosphorylated T538 of paxillin (PT538) was developed (Fig. 4B). The TPA-induced phosphorylation of paxillin that is recognized by PT538 antibody is inhibited by the

phosphomimetic peptide in a dose-dependent manner. Moreover, phosphorylation of T538 residues was also observed in TPA-stimulated WT recombinant paxillin, but not in the T538A paxillin mutant, further confirming the antibody specificity. We used this antibody to demonstrate that, in vitro, PKCδ phosphorylates immunoprecipitated paxillin on T538 (Fig. 3A).

PKCδ binds paxillin in yeast two-hybrid assay and co-immunoprecipitates with paxillin

The direct binding of PKCδ to paxillin was demonstrated in the yeast two-hybrid system. Western blot analysis of yeast lysates showed expression of the bait, the LexA-conjugated catalytic domain of PKCδ (Fig. 5A). Screening of a mouse cDNA library resulted in selection of 24 positive clones (to be described elsewhere). Among the sequences that bound to PKCδ there was one encoding a fragment corresponding to residues 318-372 of mouse paxillin, which comprises the paxillin LIM1 domain. To test the specificity of this interaction, co-transformation of the isolated paxillin fragment with BTM-conjugated bait vectors containing fusion proteins for the PKCδ catalytic domain or unrelated proteins, BTM-Tek and BTM-laminin, was performed. Colonies growing on TL and THULL selection media, as well as the *lacZ* assay are presented (Fig. 5A). Co-expression of the bait and prey proteins was confirmed by growth on TL selection medium, and specific direct interaction of PKCδ and paxillin was confirmed by β-galactosidase-positive colonies and specific growth on THULL selection medium.

In the presence of IL-3, anti-paxillin antibody, but not non-specific mouse IgG1, co-immunoprecipitated PKCδ (Fig. 5B). Withdrawal of IL-3 or addition of GF109203X did not affect the PKCδ binding with paxillin. However, TPA resulted in a nearly complete loss of PKCδ from the complex with paxillin, and addition of GF109203X to the TPA-stimulated cells restored the complex. By contrast, TPA did not release another paxillin-binding partner, FAK. Similar results were obtained in reciprocal experiments. We also showed that PKCδ co-immunoprecipitated with FLAG-tagged WT recombinant paxillin (Fig. 5C). However, no PKCδ binding to a LIM-1 domain-deficient paxillin mutant (Wade and Vande Pol, 2006) was observed. Consistently, T538 phosphorylation was observed in WT paxillin, but not in the deletion mutant.

Paxillin also co-immunoprecipitated with PKCε-tagged kinase-dead PKCδK376R that was stably transfected into Baf3 (supplementary material Fig. S1). PKCδK376R, however, was not released from paxillin by TPA treatment, suggesting that phosphorylation of paxillin or PKCδ autophosphorylation is required for complex dissociation. Endogenous PKCδ, however, co-immunoprecipitated with recombinant T538A and T538E and was released from the complex upon addition of TPA, suggesting that PKCδ autophosphorylation is responsible for complex dissociation.

PKCδ is involved in activation of LFA-1 integrins

We assessed the possible involvement of PKCδ in activation of LFA-1 integrins, as judged by Baf3 adhesion to the immobilized LFA-1 ligand, ICAM-1. Because Baf3 cells express not only LFA-1 integrins, but also their ligand, ICAM-1, in this and further experiments, we seeded the cells at a low concentration to minimize homotypic cell aggregation. Whereas a portion of non-stimulated Baf3 cells adhered to the substrate, TPA greatly stimulated their adhesion (Fig. 6A). The TPA-induced cell adhesion could also be diminished by pre-treating the cells with the PKC inhibitor GF109203X, and was fully blocked by pretreatment with the M17/4 antibody against the αL subunit of LFA-1. The endogenous TPA analog diacyl glycerol (DAG) also stimulated phosphorylation of paxillin residue T538 (Fig. 6A, inset) and LFA-1-mediated cell adhesion in a PKC-mediated fashion. In Baf3 cells, some phosphorylation of paxillin T538 was observed even before stimulation (Fig. 6A, inset; supplementary material Fig. S2A), which was probably mediated by the pre-activated portion of PKCδ. This might contribute to the ability of cells to bind LFA-1 before addition of the activator.

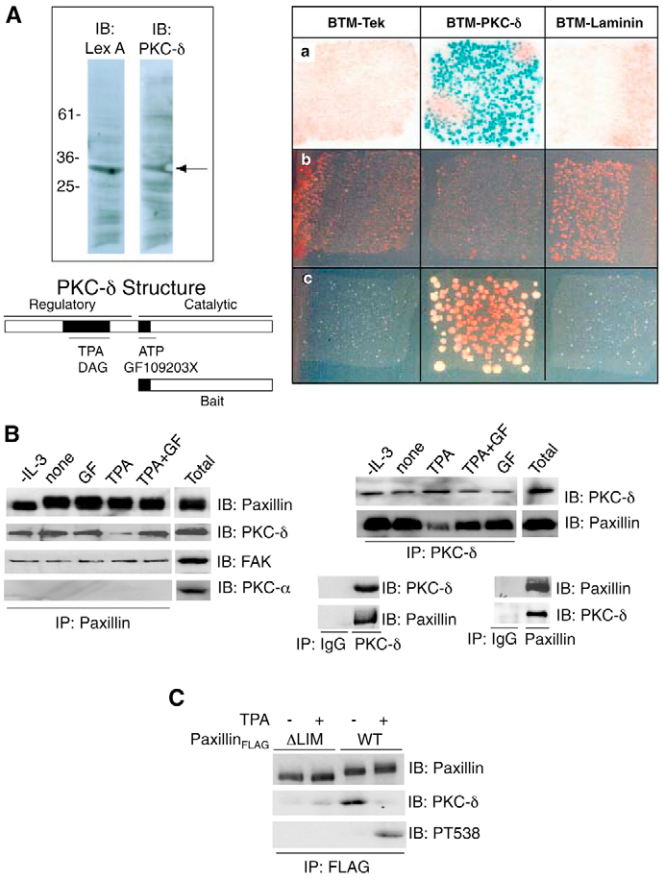

Fig. 5. PKCδ directly interacts with paxillin in a yeast two-hybrid assay and co-immunoprecipitates with paxillin. (**A**) Schematic representation of PKCδ (bottom left) showing the fragment used as bait. Binding sites for TPA and diacylglycerol (DAG) as well as for adenosine-5′-triphosphate (ATP) and GF109203X are indicated. Expression of the bait, LexA-conjugated PKCδ catalytic domain, was verified by western blot analysis (top left) of yeast lysates using monoclonal anti-PKCδ or anti-LexA antibody. (Right panel) After co-transformation of LexA-conjugated PKCδ, laminin, or Tek proteins along with VP-16-conjugated catalytic domain of PKCδ, the yeast colonies were grown on TL selection medium lacking tryptophan and leucine, or on THULL selection medium lacking also histidine, uracil and lysine and stained for β-galactosidase expression. Direct interaction was observed only when PKCδ was co-expressed with paxillin (middle column). (**B**) Baf3 cells were either withdrawn from IL-3 for 6 hours or maintained on IL-3 and treated with 100 nM TPA or 1 μM GF109203X, as indicated. Antibodies specific for paxillin, PKCδ or non-immune IgG1 were used for immunoprecipitation. Western blots were developed with antibody against paxillin, PKCδ, FAK or PKCα, as a negative control. (**C**) Cells expressing FLAG-tagged WT paxillin or its LIM1 deletion mutant were treated with TPA. After immunoprecipitation with anti-FLAG antibody, the blot was developed with antibody specific for paxillin, PKCδ or PT538. 10% of total cell lysate was loaded.

We used Baf3 cells that overexpressed PKCδ or its inactive analog, PKCδK376R, to assess the involvement of PKCδ in the TPA-induced cell adhesion. Activation of the endogenous PKCδ was followed by its downregulation by degradation 8 hours after TPA addition, whereas the recombinant isoforms, PKCδ and PKCδK376R, persisted for at least 27 hours (Fig. 6B, inset). Similarly, adhesion of the vector-expressing cells to the substrate was transient, and paralleled the pattern of PKCδ downregulation (Fig. 6B). Expression of exogenous PKCδ stimulated cell adhesion that lasted for at least 24 hours, whereas expression of exogenous PKCδK376R reduced cell adhesion at all time points, compared with vector-only controls. These data suggest that PKCδ is involved in LFA-1 activation by TPA.

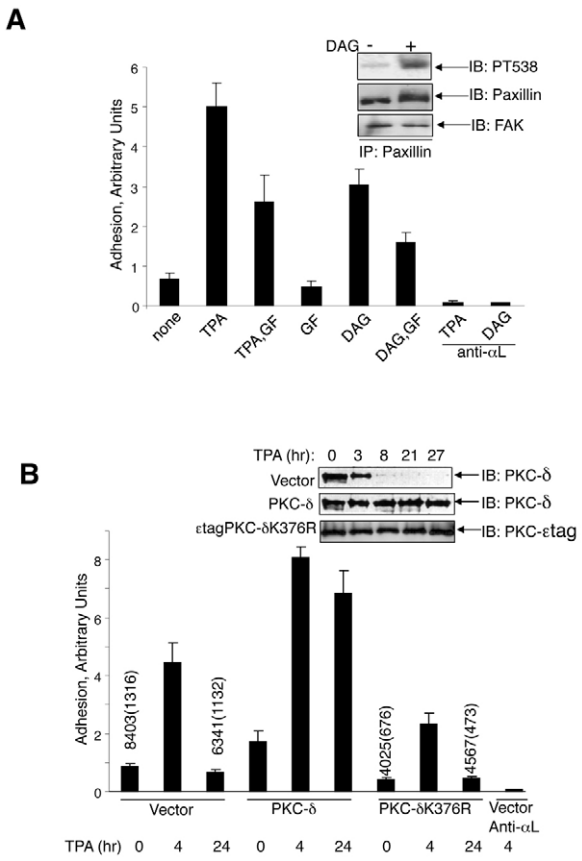

Fig. 6. TPA and its endogenous analog DAG stimulate LFA-1-mediated cell adhesion in a PKCδ-dependent manner. (**A**) Parental Baf3 cells were treated with 100 nM TPA, 1 μM DAG or 1 μM GF109203X for 15 minutes. Paxillin was immunoprecipitated and blots were developed with antibody specific to PT538 and FAK (inset). (**B**) Cells expressing PKCδ, its inactive analog, PKCδK376R, or the vector alone were treated with 100 nM TPA for the indicated periods of time. PKCδ and PKCδK376R expression were analyzed using antibodies specific for PKCδ or the PKCε-tag (inset). A portion of the cells was pretreated with a blocking antibody against the αL subunit of receptor (M17/4). Adherence to ICAM-1 substrate was analyzed. Means and s.d. from six parallel experiments are presented. The effect of PKCδ on LFA-1 adhesion was analyzed by two-factor ANOVAs, with the expression construct as a between-group factor and time of TPA treatment as a within-groups factor. A significant construct-by-time of treatment interaction (F=89.94; df=4,45; P<0.001) prompted post-hoc Newman-Keuls t-tests to determine the level of the between- and within group factors that defined the significant interactions. Compared with vector-expressing cells, PKCδ stimulated LFA-1 adhesion at all times of TPA treatment (P<0.001), whereas PKCδ K376R inhibited adhesion with a statistical significance only at 4 hours of TPA treatment (P<0.001) but not at 0 and 24 hours (P>0.24). The latter results could represent a Type II error, although, because the expression of PKCδK376R led to respective 2.0- and 1.4-fold decreases of LFA-1 adhesion at the 0 and 24 hours time points. Absolute values for means and s.d. are presented for these experimental time points.

Phosphorylation of paxillin T538 is involved in activation of LFA-1 integrins

We next investigated the involvement of paxillin phosphorylation at T538 in LFA-1-mediated adhesion. The recombinant WT, T538A and T538E EGFP paxillin proteins were expressed in Baf3 (Fig. 4B and Fig. 7B). Flow cytometric analysis showed expression of equal amounts of LFA-1 αL subunit in these cell lines (Fig. 7A). In all derivative cell lines, knockdown of approximately 95% of the endogenous mouse paxillin was achieved with siRNA specific for murine paxillin, but not with scrambled siRNA or siRNA against mouse PKCδ (Fig. 7B). The latter siRNA led to silencing of up to 90% of endogenous PKCδ. In the cells with silenced PKCδ, phosphorylation of T538 was not observed.

Transfection of either siRNA did not notably affect cell viability (not shown). In the absence or presence of TPA, we observed the following pattern of LFA-1 activation. The T538A or T538E mutants decreased or increased LFA-1-mediated adhesion, respectively, in comparison to WT recombinant paxillin, (Fig. 7C), suggesting an involvement of T538 in LFA-1 activation. Moreover, inhibition of T538 phosphorylation, achieved either by PKCδ silencing or by replacement of endogenous (silenced) paxillin with T538A, inhibited LFA-1-mediated adhesion to the same extent as paxillin silencing. This suggests that paxillin regulates LFA-1 activity primarily through phosphorylation of its T538. Finally, none of these treatments completely abolished LFA-1 activity, whereas a blocking antibody against the LFA-1 αL subunit did (Fig. 7C). This result suggests that LFA-1 receptors are regulated by TPA through paxillin-dependent and -independent pathways. In fact, TPA increased adhesion five- to sixfold when cells expressed WT recombinant paxillin or just endogenous paxillin (scrambled siRNA). However, TPA increased adhesion only 2- to 3.5-fold when cells had their endogenous paxillin replaced with T538E or T538A, or when cells had been depleted of endogenous paxillin or PKCδ with the appropriate siRNA (Fig. 7C). In other words, when TPA does not lead to phosphorylation of paxillin T538, only partial LFA-1 activation is observed, which is presumably mediated by a paxillin-independent pathway.

In summary our results suggest that: (1) phosphorylation of T538 is involved in LFA-1 activation; (2) paxillin regulates LFA-1 activation through phosphorylation of T538; and (3) TPA regulates LFA-1 in a paxillin-dependent and -independent fashion.

Phosphorylation of paxillin T538 is involved in regulation of actin cytoskeleton and cell morphology

IL-3 is required for an elongated morphology of Baf3 cells, which is generated by ruffled protrusions assembled at their leading edges and uropods at their trailing sides (Romanova et al., 1999; Romanova et al., 2004). The effect of IL-3 is mediated by paxillin phosphorylation on Y31 and Y118. We reported that the actin cytoskeleton is a major structural element that supports this cell shape and that TPA-induced activation of PKCδ depolymerizes actin and causes rounding of Baf3 cells.

Here, we assessed the involvement of paxillin phosphorylation at T538 in regulation of Baf3 cell morphology. The endogenous mouse paxillin was silenced in Baf3 derivatives that expressed

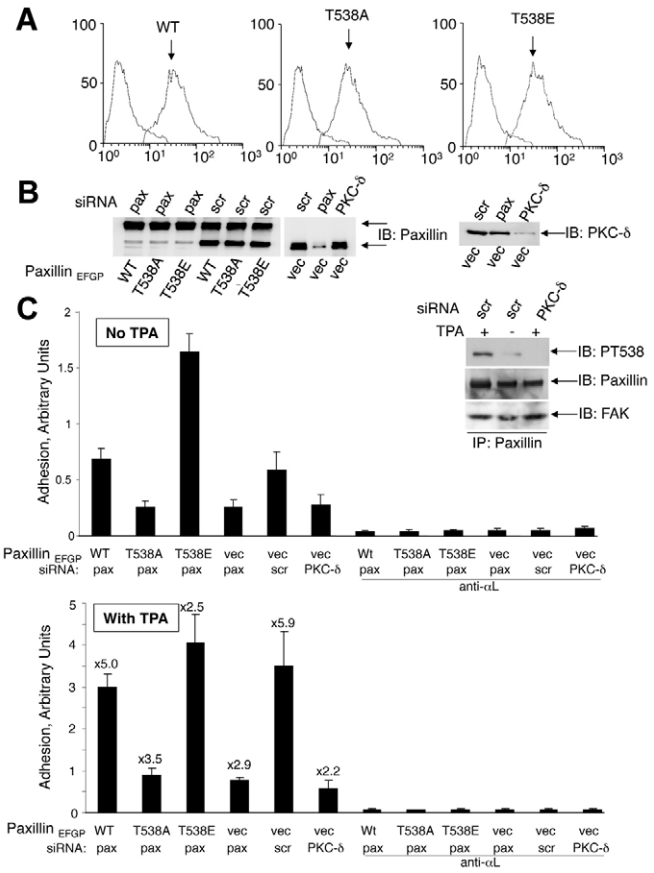

Fig. 7. Involvement of T538 paxillin phosphorylation in LFA1-mediated cell adhesion. (**A**) Expression of LFA-1 receptor in Baf3 cells transfected with the recombinant paxillin proteins was determined by FACS analysis. Cells were stained with monoclonal antibody (I21/7) against the αL subunit of LFA-1 integrin (arrow) or mouse IgG, which was followed by incubation with TRITC-conjugated anti-mouse F(ab′)2 fragments. (**B**) Cells expressing WT recombinant human paxillin, T538A, T538E, or the vector, were treated with siRNA specific for endogenous mouse paxillin (pax), PKCδ or scrambled (scr) siRNA. Expression of paxillin or PKCδ was analyzed with the appropriate antibody after 12 hours of silencing. After TPA addition to PKCδ-silenced cells, phosphorylation of immunoprecipitated paxillin T538 was analyzed. (**C**) Some cells were pre-incubated with a blocking antibody (M17/4) to the αL subunit of LFA-1 receptor. Adhesion to immobilized ICAM-1 was measured in cells either untreated or treated with TPA. Means and s.d. from eight experiments are presented. Analysis was performed by two-factor ANOVAs, with the expression construct/siRNA as a between group factor and TPA treatment as a within-groups factor. A significant construct-by-time of treatment interaction (F=84.6; df=11,168; P<0.001) was followed by post-hoc Newman-Keuls t-tests to determine the level of the between- and within group factors that defined the significant interactions. In the absence or presence of TPA there were statistically significant differences in LFA-1 mediated adhesion in WT recombinant paxillin-expressing cells and T538A or T538E (P<0.01). There was no significant difference in the effect of endogenous paxillin replacement with T538A, silencing of PKCδ or paxillin on LFA-1 adhesion (P>0.08).

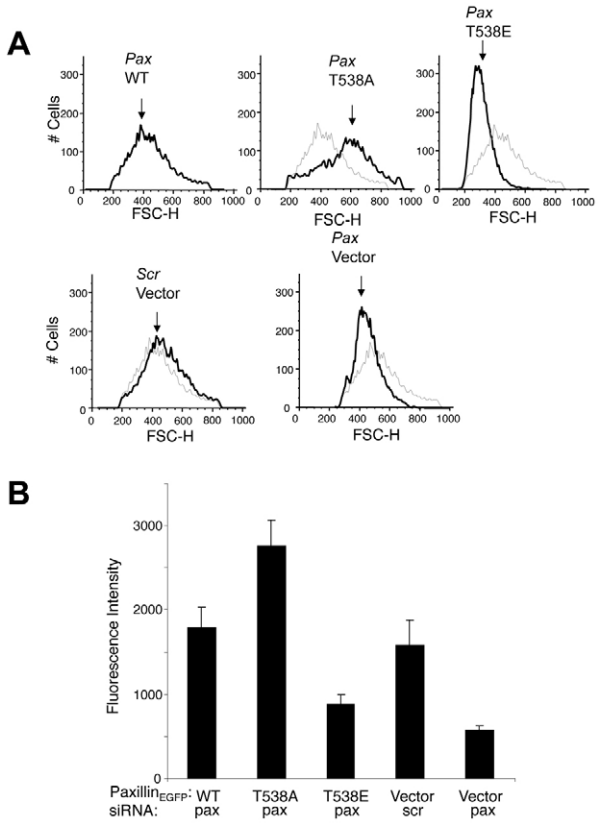

Fig. 8. Involvement of paxillin phosphorylation at T538 in regulation of morphology and actin cytoskeleton of Baf3 cells. In Baf3 cells expressing recombinant EGFP-paxillin proteins (WT, T538A, T538E or the vector alone) endogenous paxillin was silenced using siRNA. (**A**) Variations in cell shape were analyzed by flow cytometry and appear as a broad spectrum of forward light scatter (FSC-H). FACS profile of the cells expressing WT recombinant paxillin is superimposed on the other FACS patterns. (**B**) The cells were stained with TRITC-conjugated phalloidin. A quantitative measurement of cell staining was performed by flow cytometry and presented as means and s.d. from five experiments. Effect of paxillin phosphorylation constructs and siRNA on actin was analyzed by ANOVAs. Post-hoc Newman-Keuls t-tests following the significant constructs effect (F=72.0; df=4,20; P<0.001) revealed significant (P<0.001) differences between WT recombinant paxillin-expressing cells and T538E and T538A mutants.

recombinant paxillin proteins or the vector. To quantitatively measure the relative presence of elongated and round cells in each culture we performed a forward light scatter (FSC-H) flow cytometry analysis of each cell population (Fig. 8A), as described earlier (Romanova et al., 2004). When compared with the FACS profiles of the elongated cells expressing WT recombinant paxillin, expression of the T538E led to cell rounding, i.e. shifting of the spectrum to the left, whereas expression of T538A promoted cell elongation, thus shifting the spectrum to the right. The cells treated with scrambled siRNA and, therefore, expressing only endogenous paxillin, had a phenotype that was similar to that of cells expressing WT recombinant paxillin. Paxillin silencing in vector-expressing cells rounded the cells, shifting the spectrum to the left (Fig. 8A).

Fig. 8B presents a quantitative measurement of actin-polymerization state in cells stained with fluorescently labeled phalloidin that binds to polymerized F-actin. Consistent with the effect on cell morphology, expression of T538E led to actin depolymerization, whereas expression of T538A stabilized actin, in comparison to WT paxillin-expressing cells. Treatment of vector-expressing cells with scrambled siRNA had no significant effect on the state of actin, whereas siRNA depletion of endogenous paxillin depolymerized the actin.

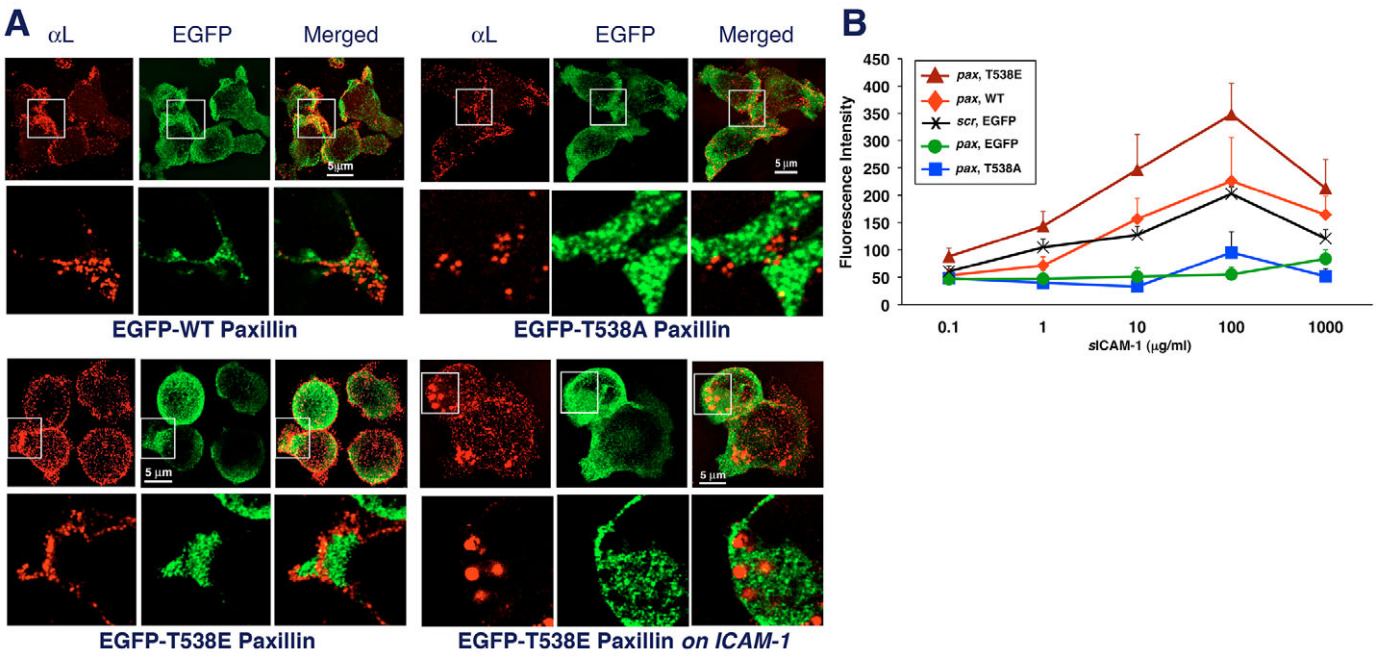

Fig. 9. Involvement of paxillin phosphorylation at T538 in redistribution of LFA-1 integrins and regulation of their affinity. In Baf3 cells that express WT EGFP-recombinant paxillin, T538A or T538E, the endogenous paxillin was silenced with siRNA. (A) Cells were labeled with antibody (I21/7) against the αL subunit of LFA-1 (red). Intracellular distribution of EGFP-conjugated recombinant paxillins and LFA-1 were analyzed by fluorescent microscopy. The LFA-1 clusters are shown in the upper panels as de-convolved projection images. The boxed regions are enlarged to show the localization pattern of the receptors and the recombinant paxillins (bottom panels). (B) Binding of soluble mouse ICAM-1-Fc to the cells expressing the indicated recombinant constructs. Soluble ICAM-1-Fc fusion protein at concentrations from 0.1 to 1000 μg/ml was incubated with the cells. Binding of TRITC-conjugated goat anti-Fc antiserum was analyzed on FACScan. Experiments were run in five replicates. Effects of various constructs on LFA-1 activation were analyzed by ANOVAs, with the expression construct/siRNA as a between-group factor and the soluble ICAM-1-Fc concentration as a within-groups factor. A significant construct-by-concentration interaction (F=8.11; df=16,100; P<0.001) prompted post-hoc Newman-Keuls t-tests to determine the level of the between-group and within-group factors that defined the significant interactions. At concentrations of 10 μg/ml and 100 μg/ml, ligand binding to the cells expressing T538E and T538 A differs from the WT (P<0.01), but no statistical differences were observed in the affinity of the cells with silenced paxillin, PKCδ and T538A (P>0.97).

It might appear surprising, at first, that both paxillin silencing and replacement of endogeneous paxillin with T538E dissolved actin and rounded the cells, but produced the opposite effects on LFA-1 – inactivating or activating integrins, respectively. However, these treatments affected actin and LFA-1 differently, in paxillin-independent and -dependent fashions. Whereas paxillin silencing abolishes IL-3-mediated paxillin downstream signaling, activation of PKCδ T538 pathways interferes with it, bringing restructuring of actin rather than its collapse (see next section).

Thus, our data suggest that phosphorylation of paxillin T538 leads to depolymerization of the actin cytoskeleton and activation of LFA-1 integrins, whereas dephosphorylation of this residue stabilizes polymerized actin and inactivates LFA-1.

Phosphorylation of paxillin T538 regulates distribution of LFA-1 integrins and their affinity

Polymerization status of the actin cytoskeleton regulates the distribution of LFA-1 integrins on the cell surface (avidity) that is often accompanied by a change in their affinity (Cambi et al., 2006; Stewart et al., 1996). Here, we investigated how recombinant paxillin proteins affect LFA-1 avidity and affinity in Baf3 cells. The endogenous mouse paxillin was replaced by recombinant EGFP-paxillin, and the αL subunits of LFA-1 integrins were stained (Fig. 9A). As expected, the cells that expressed WT recombinant paxillin or T538A had an elongated shape. Both these sets of cells showed 100-200 nM clusters of LFA-1 molecules, located primarily at the leading edges and the uropods. This is consistent with LFA-1 distribution in elongated motile lymphocytes (Viola and Gupta, 2007). The T538E mutation, however, led simultaneously to cell rounding and to a uniform distribution of LFA-1 clusters.

Remarkably, paxillin and LFA-1 integrins never co-localized. Paxillin formed ‘pockets’ in which LFA-1 receptors reside. This might be consistent with the effect of paxillin on the actin cytoskeleton that serves as a meshwork to confine the receptors.

When cells were placed on to ICAM-1-coated slides, a significant number of cells that expressed T538E paxillin adhered to the slides, whereas this ability gradually decreased in WT and T538A cells (see Fig. 7C for quantitative measurements). The ability of cells to adhere to the ligand-coated slides without TPA stimulation suggests that LFA-1 receptors are in a ‘pre-activated’ state. Binding to the ligand activates new outside-in signaling cascades leading to a further fusion of LFA-1 integrins into micrometer-sized clusters. Paxillin, however, still forms the ‘pockets’ for the receptors.

We next measured LFA-1 affinity by the binding of soluble ICAM-1-Fc. Consistent with earlier results, expression of T538A or T538E led to a decreased or increased LFA-1 affinity, respectively, compared with WT recombinant paxillin (Fig. 9B). We have also shown that phosphorylation of paxillin T538 correlated with activation LFA-1 in other lymphoid cells (supplementary material Fig. S2A). As in Baf3, phosphorylation of T538 can be induced by TPA in the myeloid cell line 32D, and in primary human blood lymphocytes (PBLs), whereas in the CEM

T-cell line and in the Daudi Burkitt lymphoma cell line, T538 is constitutively phosphorylated. Consistently, LFA-1-mediated adhesion can be induced by TPA in 32D and PBL, but not in CEM and Daudi. In fact, previous studies have shown that LFA-1 integrins in CEM are locked in an inactive state (Mobley et al., 1994) and that expression of LFA-1 in Daudi cells is extremely low (Neeson et al., 2000; Wang et al., 1990).

The recombinant paxillin mutants, WT, T538A and T538E, or the vector, were expressed in PBLs. After FACS gating of the EGFP-positive cells, binding to soluble ICAM1-Fc was assessed. When compared with WT recombinant paxillin, T538E stimulated, and T538A inhibited, soluble ICAM1 binding, suggesting an involvement of paxillin phosphorylation at T538 in LFA-1 regulation in PBLs (supplementary material Fig. S2B).

Discussion

The principal message of our results is that phorbol-ester-activated PKCδ binds paxillin in vitro and in vivo and phosphorylates paxillin within its LIM4 domain at T538. This phosphorylation leads to depolymerization of the actin cytoskeleton and to the redistribution and activation of LFA-1 receptors.

We first demonstrated that PKCδ is responsible for threonine phosphorylation of paxillin in vivo and this isoform phosphorylates paxillin in vitro. Identification of peptides that might be suitable for in vitro phosphorylation by PKCδ was described earlier (Fujii et al., 2004). Based on this analysis, we identified three residues within human paxillin, T198, T514 and T538, which could be phosphorylated by PKCδ. However, theoretical analysis of the flanking residues suggested that none would be excellent in vitro substrates for this isoform. The authors did note however, that peptides might acquire new properties in vivo when incorporated into a large protein and into protein complexes, which could affect PKCδ preference for them as substrates. Experimentally, we found that T538 of paxillin could be directly phosphorylated by PKCδ in vitro and in response to TPA in vivo. Brown and co-authors, however, did not observe phosphorylation of T538 in CHO.K1 cells when they studied the TPA-induced phosphorylation of individual LIM domains of paxillin, even though the sequence surrounding this residue is conserved between chicken, mouse and human paxillin (Brown et al., 1998; Brown and Turner, 1999). This is not surprising, given the design of their experiment in which isolated LIM domains were expressed in cells to assess kinase binding and phosphorylation. Such a design might have precluded them from observing PKCδ-related effects, which seem to involve both LIM1 and LIM4 domains for binding and phosphorylation, respectively. Mass spectrometry analysis, however, demonstrated that the site corresponding to T538 of human paxillin is phosphorylated in vivo (Webb et al., 2005).

In yeast two-hybrid experiments the catalytic domain of PKCδ bound a fragment of paxillin (residues 318-372), which represents the paxillin LIM1 domain. PKCδ also co-immunoprecipitated with paxillin, and TPA led to a dissociation of the complex. Further experiments suggested that TPA-induced autophosphorylation of this isoform is responsible for complex dissociation.

The mechanisms that lead to LFA-1 activation in Baf3 cells have been investigated (Katagiri et al., 2000). It was shown that three independent pathways regulate activation of LFA-1 integrins in these cells. TPA induces PKC-dependent LFA-1 activation, H-Ras and Rac-1 activate the receptors through PI3-kinase, and Rap1 activates LFA-1 via a PI3-kinase-independent pathway.

We report here that in Baf3 cells, TPA-activated PKCδ is involved in activation of LFA-1 integrins through phosphorylation of paxillin T538. Moreover, the TPA-induced activation of the PKCδ T538 pathway accounts for the bulk of the effect of paxillin on LFA-1. TPA, however, also activates LFA-1 in a paxillin-independent manner. We have shown that the only other TPA-responsive PKC isoform that is expressed in Baf3 cells, PKCα, is not involved, so it is likely that paxillin-independent TPA effect is mediated by other TPA-binding proteins, such as PKDs, chimearins, rasGRPs, diacyl glycerol kinases or others (Colon-Gonzalez and Kazanietz, 2006). A certain caution should be applied to the interpretation of these results. The small fraction of endogenous PKCδ or paxillin remaining after siRNA silencing in vector-only-expressing cells might still contribute to activation of LFA-1. However, when the silenced paxillin is replaced by an abundantly expressed recombinant one, such as T538A or T538E, the effect of the remaining endogenous paxillin on LFA-1 activity can be considered negligible. The inhibition of the pathways controlled by PKCδ T538 was achieved both by silencing of PKCδ and by replacement of silenced paxillin with T538A. Both approaches produced similar levels of LFA-1 activation, without or with TPA. The overall effect of paxillin was assessed by observing the results of its silencing; cells in which paxillin was silenced retained only 5% of endogenous paxillin. Despite this, in the absence of TPA, these cells preserved approximately 50% of LFA-1 activity compared with cells carrying unsilenced endogenous paxillin (treated with scrambled siRNA), thus supporting our conclusion that factors other than paxillin contribute to LFA-1 activation.

Our data suggest that phosphorylation of paxillin T538 contributes to regulation of the actin cytoskeleton, which affects distribution and affinity of LFA-1 clusters. In T538E such ‘pre-activated’ LFA-1 clusters are capable of adherence to the ligand without further stimulation. It had been demonstrated that ‘pre-active’ LFA-1 clusters are formed before ligand binding and, therefore, were regulated by intracellular signalling, rather than by the ligand itself (Cambi et al., 2006).

Recombinant paxillins and clustered LFA-1 are never co-localized. Paxillins, rather, they form ‘pockets, in which LFA-1 clusters reside, and the phosphorylation mutants differ in the distribution of these pockets. These data suggest that paxillin constitutes, or possibly regulates, the barriers provided by cytoskeleton network for LFA-1, which would be consistent with its effect on the actin-based cytoskeleton. In fact, earlier studies demonstrated that lateral movement of transferrin receptors that, similarly to LFA-1, possess transmembrane domains, is regulated by two independent mechanisms: actin meshwork confinement and dynamic partition of the receptors (Lenne et al., 2006). Specifically, although the actin-based cytoskeleton provides the barriers responsible for the confinement of the receptors, the receptors are recruited into membrane rafts during diffusion over the cell surface. These mechanisms are independent, and disruption of one of them, using cytoskeleton-disrupting drugs or drugs that target lipid domains, leaves the other mechanism intact. Based on this observation, paxillin phosphorylation at T538 regulates LFA-1 integrins by affecting actin cytoskeleton barriers. It is possible that TPA-induced and paxillin-independent LFA-1 regulation targets membrane rafts. LFA-1 confinement to membrane rafts was shown to be essential for their activation (Cambi et al., 2006).

Integrin clusters in polarized and highly motile cells of myeloid origin are short lived, and therefore are unable to form stable adhesions. Stabilization of integrin clusters usually stimulates firm

adhesion to the substrate and immobilizes the cells (Ridley et al., 2003). Internalization of integrin clusters observed in elongated cells is crucially dependent of the activity of FAK (Chen et al., 2002), which phosphorylates paxillin at Y31 and Y118. This leads to activation of Rac-1 and Rap1 simultaneously with suppression of RhoA activity. In fact, active FAK mediates phosphorylation of paxillin Y31 and Y118, and Rac-1 activation in Baf3 cells (Romanova et al., 1999; Romanova et al., 2004) leading to a dynamic polarized cell phenotype that is unable to firmly adhere to substrate. Activity of FAK, however, requires the integrity of the actin cytoskeleton, and dissolution of actin inactivates FAK (Seufferlein and Rozengurt, 1995; Zachary et al., 1993). We reported earlier that addition of TPA to Baf3 cells depolymerizes the actin cytoskeleton, inactivates FAK and leads to dephosphorylation of paxillin Y31 and Y118, and inactivation of Rac-1 (Romanova et al., 1999; Romanova et al., 2004). This signaling cascade would lead to RhoA activation that usually mediates stable cell adhesion (Tsubouchi et al., 2002). The firm adhesion to the substrate through LFA-1 observed in Baf3 cells can be Rho mediated. In fact, LFA-1- or ICAM-1-mediated homotypic adhesion of lymphoid cells has been shown to be mediated by Rho (Tominaga et al., 1993). Similarly to TPA, the phospho-mimetic T538E depolymerizes the actin cytoskeleton and, therefore, might act through inhibition of FAK, dephosphorylation of Y31 and Y118, and inhibition of Rac-1. However, we can only speculate on how phosphorylation of T538 affects the state of actin cytoskeleton. Because tubulin binds to the LIM3 and LIM4 domains of paxillin, its phosphorylation at T538 might affect tubulin binding and polymerization. In fact, a role for paxillin in regulation of tubulin polymerization was recently reported (Efimov et al., 2008). However, we do not favor this scenario, because disruption of microtubules by colcemid brings about only minimal changes to the actin cytoskeleton and to the morphology of Baf3 cells (Romanova et al., 1999). Phosphorylation of the paxillin LIM4 domain might also affect the activity of PTP-PEST phosphatase, which binds to the same region of paxillin (Cote et al., 1999). PTP-PEST coordinates the phosphorylation and dephosphorylation state of various cytoskeleton proteins, including major regulators of actin cytoskeleton rearrangement and integrin signalling, such as FAK (Davidson and Veillette, 2001). Whether these or other mechanisms contribute to the regulation of the actin cytoskeleton by phosphorylation of paxillin T538 is a subject for future investigation.

Materials and Methods

Cell culture, reagents and expression vectors

The IL-3-dependent murine pro-B cell line Baf3, was maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, and 10 ng/ml recombinant murine IL-3 (Peprotech) or 5% WEHI-3-conditioned supernatant as a source of murine IL-3. Experiments were performed in the presence of IL-3 unless indicated otherwise. PBLs were isolated as described (Mobley, 1994).

Baf3 cells were stably transfected with εMTH-based intact PKCα or PKCδ (Mischak et al., 1993) or their inactive analogs, PKCαK368A (Rosson et al., 1997) or PKCδK376R (Romanova et al., 1999) in which the invariant lysine in the ATP-binding site had been replaced by alanine (A) or arginine (R), respectively. The εMTH vector carries a peptide tag derived from PKCε. FLAG-tagged full-length paxillin and its counterpart with deletion of the LIM1 domain (residues 326-376) were described earlier (Wade and Vande Pol, 2006). Recombinant EGFP-conjugated WT human paxillin α in the pBABEpuro expression vector was described earlier (Mazaki et al., 1998). To create the paxillin phosphorylation mutants T198A, T514A, T538A and T538E, the following mutations were made: NT 666 (A>G) and 668 (G>A) for T198A, 1614 (A>G) and 1616 (G>A) for T514A, 1686 (A>G) and 1688 (C>A) for T538A, and 1686 (A>G), 1687 (C>A) and 1688 (C>G) for T538E. These changes were inserted into key threonine residues by site-directed mutagenesis using the QuikChange II Kit from Stratagene. Mutating primers used were as follows, with the substituted nucleotides indicated in bold: T198A, 5′-AAAGCTGGGCCCCTG-**G**C**A**AAAGAGAAGCCTAAGC-3′; T514A, 5′-GGCCGCTGCATC**G**C**A**GCCA-TGGCCAAGAAG-3′; T538A, 5′-CAGCTCAACAAGGGC**G**C**A**TTCAAGGAG-CAGAACG-3′; and T538E, 5′-CAGCTCAACAAGGGC**GAG**TTCAAGGAG-CAGAACG-3′.

Endogenous murine paxillin was knocked down with siRNA [r(CGGCCUGCCACACGCUCAA)dTdT] (Qiagen) that was designed to specifically inhibit murine paxillin without affecting its human recombinant counterpart; the scrambled version of this siRNA was used as a control. PKCδ was silenced with (5′-GGCUGAGUUCUGGCUGGACTT-3′) (Qiagen) (Yoshida et al., 2003). siRNA was introduced into Baf3 cells using a standard protocol of TransPass R2 Transfection Reagent (New England BioLabs).

Polyclonal antibodies to the phosphorylated T538 of human paxillin were produced in rabbits according a standard protocol (Invitrogen). For rabbit immunization the phosphorylated and non-phosphorylated peptides (CQLNKG-pT-FKEQN-AMIDE and CQLNKGTFKEQN-AMIDE), which correspond to residues 533-543 of human paxillin, were used.

The PKC activator, TPA (Calbiochem); the PKC inhibitor, GF109203X (LC Laboratories); and the actin cytoskeleton-disrupting drug, cytochalasin D (Calbiochem) were used in this study.

Statistical analysis

Data were analyzed with the statistical package Statistica (StatSoft) factorial analysis of variance (ANOVA). The expression of recombinant paxillin 'Construct'/siRNA treatment was a between group factor; within group factors varied according to individual experiments. All analyses were two-tailed. *P* values less than 0.05 defined statistical significance.

Immunoprecipitation and western blots

Cell lysates were prepared, and western blot analysis was performed as described (Romanova et al., 1999). Immunoprecipitation (IP) 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, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μM PMSF, 1 mM sodium fluoride and 0.2 mM sodium orthovanadate). For disruption of non-covalent protein-protein interactions, the cell lysates were initially suspended in buffer A that also contained 1% SDS (denaturing conditions). After a subsequent dilution with 10 volumes of buffer A, immunoprecipitation was performed as above. We used rabbit polyclonal anti-PKCδ antibodies (R&D antibodies), monoclonal anti-paxillin antibodies (Transduction Laboratories), polyclonal anti-phosphothreonine (Zymed Laboratories) antibodies and polyclonal anti-GFP antibody (Santa Cruz). Non-immune mouse IgG1 (Sigma) was used as a negative, non-specific control. Goat anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase (Amersham) was used as the secondary antibody. GammaBindRG Sepharose® (Pharmacia) beads were used to collect immunoprecipitates. Bands were visualized with ECL reagents (Pierce).

Phosphatase treatment

After paxillin was immunoprecipitated with antibody and bound to GammaBindRG Sepharose® (Pharmacia) beads, the beads were washed three times with buffer A. Treatment with phosphatase was carried out for 30 minutes at 37°C. Treatment with non-specific potato acid phosphatase (PAP, Sigma) 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 U/μl PAP. Phosphotyrosine phosphatase (PTP, Calbiochem) 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 U PTP. Treatment with the serine/threonine protein phosphatase, $PP2A_2$, was carried out in 50 mM Tris-HCl, pH 7, 10% glycerol, 1 mM benzamidine, 0.2 mM PMSF, 14 mM 2-mercaptoethanol, 0.2 mg/ml BSA and 4 μg/ml protamine sulfate with addition of 6.5 U/ml $PPA2A_2$. The levels of paxillin phosphorylation were analyzed by western blots with anti-paxillin, anti-phospho-serine and anti-phospho-threonine antibodies.

Phosphoamino acid assay

Baf3 cells were washed three times with phosphate-free RPMI 1640 (Biofluids) containing 1% dialyzed fetal bovine serum (Gibco; BRL) and seeded at a concentration of 2×10^6/ml in the same medium with addition of 30 mM caspase inhibitor, Z-VAD-fmk (Calbiochem), 50 μM β-mercaptoethanol, 1 mM sodium pyruvate, 25 μM cycloheximide (Calbiochem) and 10 ng/ml recombinant IL-3 (Peprotech). 10×10^6 cells were used for each experiment. After addition of 1 mCi/ml [^{32}P]orthophosphate (ICN), the cells were incubated for 2.5 hours at 37°C. Stimulation with 100 nM TPA was performed for the last 15 minutes, when desired. The cells were washed twice with ice-cold PBS, resuspended in 1 ml PBS, and lysed by the addition of an equal volume of buffer containing 0.5 % Triton X-100, 0.5 % NP-40, 10 mM Tris-HCl, pH 7.2, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM sodium fluoride, 0.2 mM sodium orthovanadate, 25 nM calyculin A, 100 μM AEBSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 4 μg of each calpain inhibitor I and II. Paxillin was immunoprecipitated from the lysate, as described above.

Immunoprecipitated paxillin was released from the beads and resolved by electrophoresis on 10% SDS-PAGE and electrophoretically transferred to a PVDF membrane. The membrane was exposed to X-ray film (Kodak) or to a phosphorimager

screen (Molecular Dynamics). The membrane fragments that contained the radioactively labeled bands were excised, washed twice with distilled water, and boiled for 2 hours at 110°C in 0.1 ml of 6 N HCl. After removing the membrane, the acid eluate was vacuum-dried and redissolved in 5 μl distilled water that contained 2.5 mM each of unlabeled phosphotyrosine, phosphothreonine and phosphoserine standards (Sigma). The samples were resolved by one-dimensional thin layer cellulose chromatography (EM Science, TLC plate 20×20 Cellulose) in buffer containing n-butanol: pyridine: glacial acetic acid: deionized water at the v/v ratio 1250:38:96:58. The exact location of the three phosphorylated amino acids on this chromatogram was determined by staining the chromatogram with ninhydrin.

Immunocytochemistry and fluorescence microscopy

To minimize cell aggregation, in some experiments the cells were grown at low density, 5×10^4 cells/ml. Some cells were allowed to adhere to glass microscope slides coated with ICAM-1 substrate for 10 minutes. We used 2% paraformaldehyde in PBS for cell fixation. Non-adherent cells were resuspended in PBS at 1×10^6/ml, then 50,000 cells were cytospun onto each microscope slide. We used rat monoclonal antibody (I21/7) to the αL subunit of LFA-1 receptor (Santa Cruz), Texas-Red-conjugated rabbit anti-rat IgG (Fc) antibody (Molecular Probes), and SlowFade (Molecular Probes). Stained cells were observed on a Zeiss Axiovert 100M microscope equipped with a Zeiss Plan-NeoFluor 100×/1.3 oil-immersion objective, and z-stack confocal images were obtained at 1000× magnification on a Zeiss LSM 510 scanning laser microscope. Images of LFA-1 integrin and EGFP-conjugated paxillins were deconvolved. A single plane from a *Z*-stack was chosen to show a localization pattern of the receptors and the recombinant paxillin proteins.

Flow cytometry

To assess the level of expression of LFA-1 integrins, the cells were stained for 1 hour with monoclonal antibody (I21/7, Santa Cruz) to the αL subunit of LFA-1 integrin or anti-mouse IgG in the staining buffer that contained Hank's balanced salt solution containing 3% FBS, 0.1% sodium azide and 10 mM HEPES (pH 7.4). This incubation was followed by incubation with TRITC-conjugated goat anti-mouse IgG F(ab′)2 fragments and flow cytometric analysis with FACScan (Becton Dickinson). A quantitative measurement of cell shapes in culture was performed as described (Romanova, 1999). The cells were kept in log phase growth for at least 48 hours, and then fixed in 2% paraformaldehyde at 37°C for 2 hours. This fixation preserved cell shape, which was then evaluated by FACScan (Becton Dickinson). To measure the state of actin (F-actin) polymerization cells were stained with TRITC-conjugated phalloidin, as described (Ha and Exton, 1993). Fluorescence intensity was measured using the fluorescence analyzer FLUOstar Omega multi-channel plate reader (BMG Labtech).

Soluble ICAM-1 binding

Cells were resuspended in buffer B containing 20 mM Tris-HCl, pH8.0, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, and 0.5% BSA. 50,000 cells were incubated with different concentrations (0.1-1000 μg/ml) of mouse soluble ICAM-1-Fc recombinant protein (R&D Systems) for 30 minutes at 37°C. After washing with buffer B, the cells were incubated with TRITC-conjugated secondary goat anti-mouse Fc antibody (Jackson ImmunoResearch Labs) for another 30 minutes at 37°C. After removing unbound antibody by further washing, the cells were analyzed by FACScan. Values are depicted as percentage of positive cells.

Yeast two-hybrid screening

Mouse cDNA encoding the catalytic domain of murine PKCδ (C3 to V5 regions), corresponding to the C-terminal 300 amino acids, was fused to the DNA-binding domain of a Lex-A sequence in the yeast expression vector BTM116. These hybrid bait cDNAs were transfected into *HIS3* and *lacZ* reporter genes containing yeast strain L40 and assayed for interaction with proteins encoded by a VP16 activation domain cDNA library from the E10.5 mouse embryo library. Expression of the bait fusion protein was verified by western blot analysis of yeast lysates using a PKCδ-specific (BD Bioscience Transduction Laboratories) and a LexA-specific (Clontech) antibody. Clones were selected on media lacking tryptophan, histidine, uracil, leucine and lysine (THULL) containing 20 mM 3-amino-trizole (Sigma). On the sixth day the growth of colonies were analyzed by β-galactosidase staining, resulting in isolation of 24 positive clones. VP-16 plasmids of yeast were isolated from these colonies and transformed into HB101 *E. coli*. Next, DNA was isolated and subjected to sequencing of both strands with an ABI sequencer. For testing the specificity of interaction with BTM-PKCδ, single co-transformations with isolated clones and two other irrelevant bait vectors containing fusion proteins for BTM-Tek, and BTM-Laminin were performed. The transformants were grown on TL selection medium lacking tryptophan and leucine; then the *lacZ* assay was performed. The cells were grown on THULL medium to demonstrate the specificity of interaction between the paxillin-containing clone and the baits. Only the data for paxillin and PKCδ interaction are presented here.

In vitro paxillin phosphorylation by purified PKCδ

Paxillin immunoprecipitation from parental Baf3 cells or Baf3 cells stably transfected with recombinant EGFP-conjugated paxillins was performed under denaturing conditions using anti-paxillin antibody (see above). In vitro phosphorylation with recombinant PKCδ (Mischak et al., 1991) that had been purified from baculovirus cultures was performed for 10 minutes at 30°C in the presence of 10 μM ATP and 1 μCi [γ-^{32}P]ATP, with and without 100 nM TPA. The kinase reactions were resolved on a 10% SDS gel. The gel was exposed to X-ray film.

LFA-1-mediated cell-adhesion assay

Purified mouse ICAM-1-Fc fusion protein (R&D Systems) was diluted in PBS to a final concentration of 12.5 μg/ml. To coat the wells in 96-well plates, 50 μl of ICAM-1 solution was added to each well and incubated at 37°C for 90 minutes and then further incubated with 1% BSA in PBS. Some wells were coated with 1% BSA as a control for non-specific cell adhesion. Baf3 cells were labeled with CellTracker Red^{TM} (Invitrogen) according to the manufacturer's protocol and then suspended in incubation medium. The cells were transferred to the wells at 5×10^4 cells/well and then incubated at 37°C for 15 minutes. Blocking monoclonal antibody, M17/4 and 6D247 against mouse or human αL subunit of LFA-1 receptor, respectively, were used. Non-adherent cells were removed by aspiration, and bound cells were quantified using a fluorescence analyzer FLUOstar Omega multi-channel plate reader (BMG Labtech).

This research was supported in part by the Intramural Research Program of the NIH, the National Cancer Institute and the National Institute of Allergy and Infectious Diseases. The authors thank Stephen Shaw for helpful discussions and pre-publication access to his PKC motif data, Harald Mischak of Hannover Medical School and Mosaiques Diagnostics & Therapeutics AG for his generous gift of purified PKCδ, and Kee-Oh Chay for sharing his data on paxillin radiolabeling. We also thank Scott vande Pol for providing the LIM-1-deficient paxillin constructs and Igor Elman for statistical analysis. Deposited in PMC for release after 12 months.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/9/1567/DC1

References

Brown, M. C. and Turner, C. E. (1999). Characterization of paxillin LIM domain-associated serine threonine kinases: activation by angiotensin II in vascular smooth muscle cells. *J. Cell Biochem.* **76**, 99-108.

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.

Cambi, A., Joosten, B., Koopman, M., de Lange, F., Beeren, I., Torensma, R., Fransen, J. A., Garcia-Parajo, M., van Leeuwen, F. N. and Figdor, C. G. (2006). Organization of the integrin LFA-1 in nanoclusters regulates its activity. *Mol. Biol. Cell* **17**, 4270-4281.

Chen, B. H., Tzen, J. T., Bresnick, A. R. and Chen, H. C. (2002). Roles of Rho-associated kinase and myosin light chain kinase in morphological and migratory defects of focal adhesion kinase-null cells. *J. Biol. Chem.* **277**, 33857-33863.

Colon-Gonzalez, F. and Kazanietz, M. G. (2006). C1 domains exposed: from diacylglycerol binding to protein-protein interactions. *Biochim. Biophys. Acta* **1761**, 827-837.

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.

Davidson, D. and Veillette, A. (2001). PTP-PEST, a scaffold protein tyrosine phosphatase, negatively regulates lymphocyte activation by targeting a unique set of substrates. *EMBO J.* **20**, 3414-3426.

Efimov, A., Schiefermeier, N., Grigoriev, I., Ohi, R., Brown, M. C., Turner, C. E., Small, J. V. and Kaverina, I. (2008). Paxillin-dependent stimulation of microtubule catastrophes at focal adhesion sites. *J. Cell Sci.* **121**, 196-204.

Fujii, K., Zhu, G., Liu, Y., Hallam, J., Chen, L., Herrero, J. and Shaw, S. (2004). Kinase peptide specificity: improved determination and relevance to protein phosphorylation. *Proc. Natl. Acad. Sci. USA* **101**, 13744-13749.

Ha, K. S. and Exton, J. H. (1993). Activation of actin polymerization by phosphatidic acid derived from phosphatidylcholine in IIC9 fibroblasts. *J. Cell Biol.* **123**, 1789-1796.

Katagiri, K., Hattori, M., Minato, N., Irie, S., Takatsu, K. and Kinashi, T. (2000). Rap1 is a potent activation signal for leukocyte function-associated antigen 1 distinct from protein kinase C and phosphatidylinositol-3-OH kinase. *Mol. Cell. Biol.* **20**, 1956-1969.

Lenne, P. F., Wawrezinieck, L., Conchonaud, F., Wurtz, O., Boned, A., Guo, X. J., Rigneault, H., He, H. T. and Marguet, D. (2006). Dynamic molecular confinement in the plasma membrane by microdomains and the cytoskeleton meshwork. *EMBO J.* **25**, 3245-3256.

Lub, M., van Kooyk, Y., van Vliet, S. J. and Figdor, C. G. (1997). Dual role of the actin cytoskeleton in regulating cell adhesion mediated by the integrin lymphocyte function-associated molecule-1. *Mol. Biol. Cell* **8**, 341-351.

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.

Mischak, H., Bodenteich, A., Kolch, W., Goodnight, J., Hofer, F. and Mushinski, J. F. (1991). Mouse protein kinase C-delta, the major isoform expressed in mouse hemopoietic cells: sequence of the cDNA, expression patterns, and characterization of the protein. *Biochemistry* **30**, 7925-7931.

Mischak, H., Pierce, J. H., Goodnight, J., Kazanietz, M. G., Blumberg, P. M. and Mushinski, J. F. (1993). Phorbol ester-induced myeloid differentiation is mediated by protein kinase C-alpha and -delta and not by protein kinase C-beta II, -epsilon, -zeta, and -eta. *J. Biol. Chem.* **268**, 20110-20115.

Mobley, J. L., Ennis, E. and Shimizu, Y. (1994). Differential activation-dependent regulation of integrin function in cultured human T-leukemic cell lines. *Blood* **83**, 1039-1050.

Mor, A., Dustin, M. L. and Philips, M. R. (2007). Small GTPases and LFA-1 reciprocally modulate adhesion and signaling. *Immunol. Rev.* **218**, 114-125.

Neeson, P. J., Thurlow, P. J. and Jamieson, G. P. (2000). Characterization of activated lymphocyte-tumor cell adhesion. *J. Leukoc. Biol.* **67**, 847-855.

Ridley, A. J., Schwartz, M. A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Borisy, G., Parsons, J. T. and Horwitz, A. R. (2003). Cell migration: integrating signals from front to back. *Science* **302**, 1704-1709.

Romanova, L. Y., Alexandrov, I. A., Nordan, R. P., Blagosklonny, M. V. and Mushinski, J. F. (1998). Cross-talk between protein kinase C-alpha (PKC-alpha) and -delta (PKC-delta): PKC-alpha elevates the PKC-delta protein level, altering its mRNA transcription and degradation. *Biochemistry* **37**, 5558-5565.

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-delta disrupts IL-3-induced membrane ruffles downstream of Rac1. *J. Cell Physiol.* **179**, 157-169.

Romanova, L. Y., Hashimoto, S., Chay, K. O., Blagosklonny, M. V., Sabe, H. and Mushinski, J. F. (2004). Phosphorylation of paxillin tyrosines 31 and 118 controls polarization and motility of lymphoid cells and is PMA-sensitive. *J. Cell Sci.* **117**, 3759-3768.

Rosson, D., O'Brien, T. G., Kampherstein, J. A., Szallasi, Z., Bogi, K., Blumberg, P. M. and Mullin, J. M. (1997). Protein kinase C-alpha activity modulates transepithelial permeability and cell junctions in the LLC-PK1 epithelial cell line. *J. Biol. Chem.* **272**, 14950-14953.

Seufferlein, T. and Rozengurt, E. (1995). Sphingosylphosphorylcholine rapidly induces tyrosine phosphorylation of p125FAK and paxillin, rearrangement of the actin cytoskeleton and focal contact assembly. Requirement of p21rho in the signaling pathway. *J. Biol. Chem.* **270**, 24343-24351.

Stewart, M. and Hogg, N. (1996). Regulation of leukocyte integrin function: affinity vs. avidity. *J. Cell Biochem.* **61**, 554-561.

Stewart, M. P., Cabanas, C. and Hogg, N. (1996). T cell adhesion to intercellular adhesion molecule-1 (ICAM-1) is controlled by cell spreading and the activation of integrin LFA-1. *J. Immunol.* **156**, 1810-1817.

Tominaga, T., Sugie, K., Hirata, M., Morii, N., Fukata, J., Uchida, A., Imura, H. and Narumiya, S. (1993). Inhibition of PMA-induced, LFA-1-dependent lymphocyte aggregation by ADP ribosylation of the small molecular weight GTP binding protein, rho. *J. Cell Biol.* **120**, 1529-1537.

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

van Kooyk, Y. and Figdor, C. G. (2000). Avidity regulation of integrins: the driving force in leukocyte adhesion. *Curr. Opin. Cell Biol.* **12**, 542-547.

Viola, A. and Gupta, N. (2007). Tether and trap: regulation of membrane-raft dynamics by actin-binding proteins. *Nat. Rev. Immunol.* **7**, 889-896.

Wade, R. and Vande Pol, S. (2006). Minimal features of paxillin that are required for the tyrosine phosphorylation of focal adhesion kinase. *Biochem. J.* **393**, 565-573.

Wang, F., Gregory, C., Sample, C., Rowe, M., Liebowitz, D., Murray, R., Rickinson, A. and Kieff, E. (1990). Epstein-Barr virus latent membrane protein (LMP1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMP1 cooperatively induce CD23. *J. Virol.* **64**, 2309-2318.

Webb, D. J., Schroeder, M. J., Brame, C. J., Whitmore, L., Shabanowitz, J., Hunt, D. F. and Horwitz, A. R. (2005). Paxillin phosphorylation sites mapped by mass spectrometry. *J. Cell Sci.* **118**, 4925-4929.

Yoshida, K., Wang, H. G., Miki, Y. and Kufe, D. (2003). Protein kinase Cdelta is responsible for constitutive and DNA damage-induced phosphorylation of Rad9. *EMBO J.* **22**, 1431-1441.

Zachary, I., Sinnett-Smith, J., Turner, C. E. and Rozengurt, E. (1993). Bombesin, vasopressin, and endothelin rapidly stimulate tyrosine phosphorylation of the focal adhesion-associated protein paxillin in Swiss 3T3 cells. *J. Biol. Chem.* **268**, 22060-22065.

Zhou, X., Li, J. and Kucik, D. F. (2001). The microtubule cytoskeleton participates in control of beta2 integrin avidity. *J. Biol. Chem.* **276**, 44762-44769.

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