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Integrin $\alpha_{IIb}\beta_3$-mediated pp125FAK phosphorylation and platelet spreading on fibrinogen are regulated by PI 3-kinase

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Abstract

Activation of the focal adhesion kinase pp125FAK correlates with its phosphorylation on tyrosine residues and is mediated by multiple receptor-ligand pairs. In platelets, pp125FAK phosphorylation is triggered by $\alpha_{IIb}\beta_3$ integrin or FcγRII receptor interaction with immobilized fibrinogen and IgG, respectively. In this study we used platelets as a model system to explore the role of PI 3-kinase relative to pp125FAK phosphorylation. Treatment of the platelets with two PI 3-kinase inhibitors, wortmannin and LY294002, inhibited in a dose-dependent manner $\alpha_{IIb}\beta_3$-mediated platelet spreading on fibrinogen having no effect on platelet spreading on IgG. Both inhibitors also completely abolished $\alpha_{IIb}\beta_3$-mediated pp125FAK phosphorylation but not pp72syk phosphorylation. Furthermore, FcγRII- and thrombin-induced pp125FAK phosphorylation were not affected by wortmannin and LY294002. Finally, the PI 3-kinase inhibitors' effect on $\alpha_{IIb}\beta_3$-mediated spreading and pp125FAK phosphorylation was reversed by phorbol ester treatment. These results establish that the role of PI 3-kinase relative to pp125FAK phosphorylation in platelets is receptor type-specific yet essential for $\alpha_{IIb}\beta_3$-mediated cell spreading and pp125FAK phosphorylation.

Keywords: Integrin $\alpha_{IIb}\beta_3$; PI 3-kinase; pp125FAK phosphorylation

1. Introduction

Adhesive interactions and growth factors trigger activation of the focal adhesion kinase pp125FAK in multiple cell systems. Pp125FAK is an essential signaling protein as evident from the fact that pp125FAK knockout mice die before birth [1]. However, neither the exact role of this kinase nor the pathways that are activated upstream and downstream from pp125FAK are currently fully understood [2–6].

Platelet adhesion to fibrinogen, mediated by the integrin receptor $\alpha_{IIb}\beta_3$, triggers spreading and the induction of pp125FAK tyrosine phosphorylation [7]. Both events are dependent on protein kinase C (PKC) activation as well as ADP released by the platelet itself [7,8]. Two additional platelet receptors, integrin $\alpha_2\beta_1$ and FcγRII, similarly trigger pp125FAK phosphorylation upon platelet adhesion to collagen and immunoglobulin, respectively. Integrin $\alpha_2\beta_1$ and FcγRII receptor-mediated pp125FAK phosphorylation are $\alpha_{IIb}\beta_3$-and ADP-independent, yet PKC-dependent [7,9]. These observations suggest

Abbreviations: pp125FAK, focal adhesion kinase; PtdIns, phosphoinositide; PMA, phorbol ester; PKC, protein kinase C; mAb, monoclonal antibody

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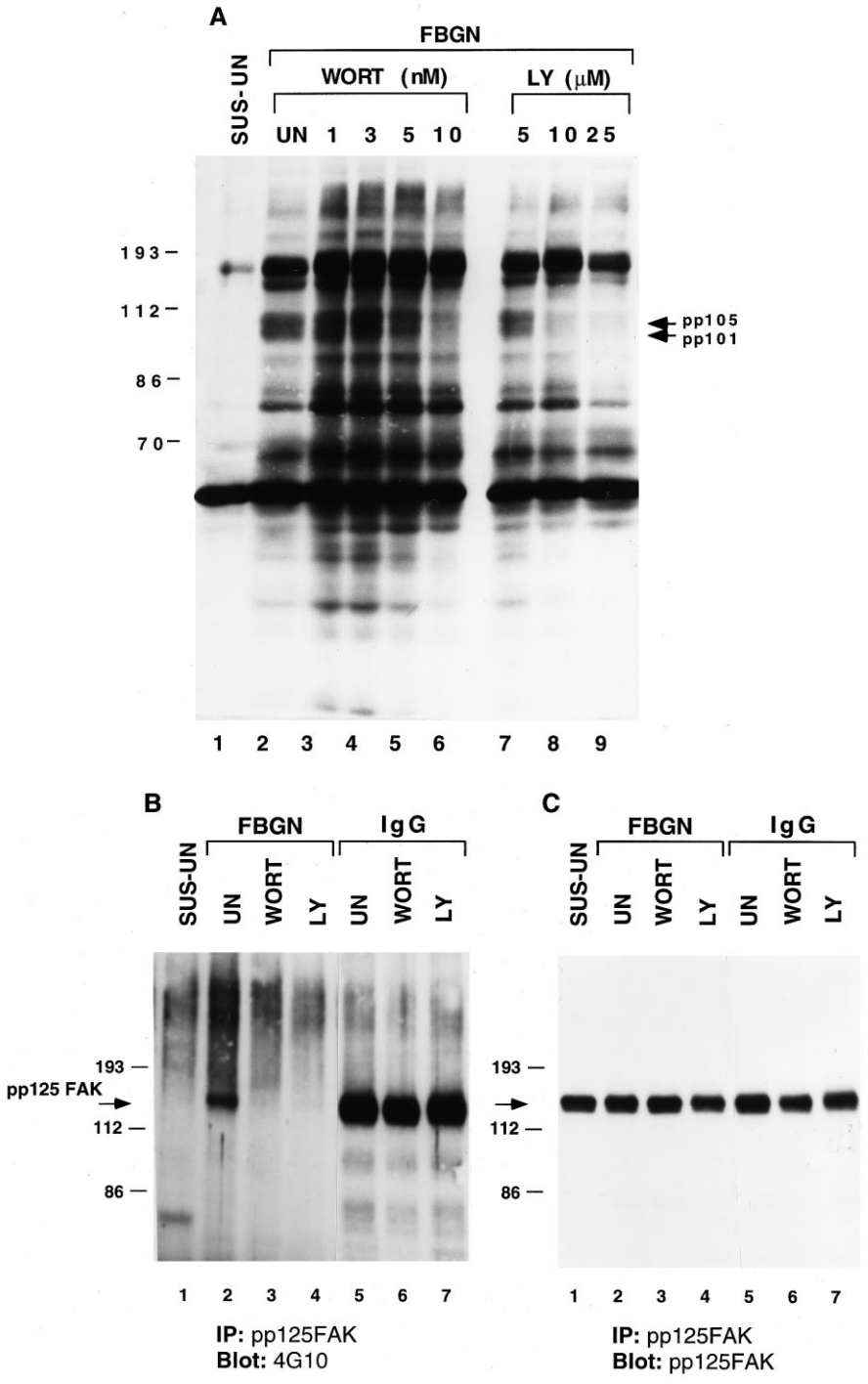

that alternative pathways may link the signaling adhesion receptor to pp125FAK phosphorylation.

A correlation between integrin $\alpha_{IIb}\beta_3$ and PI 3-kinase activation was also noted in platelets. Immobilized fibrinogen [7,10] and an antibody that binds to the β subunit of $\alpha_{IIb}\beta_3$ (anti-LIBS6) [11] trigger 'outside-in' activation of $\alpha_{IIb}\beta_3$. Both stimuli lead to the generation of PtdIns(3,4)P2, one of the phosphoinositides (PtdIns) produced by PI 3-kinase [12,13]. PtdIns(3,4)P2 was not detected in thrombin-stimulated Glanzmann's thrombasthenic platelets which are $\alpha_{IIb}\beta_3$-deficient [14]. Similarly, the RGDS tetra-

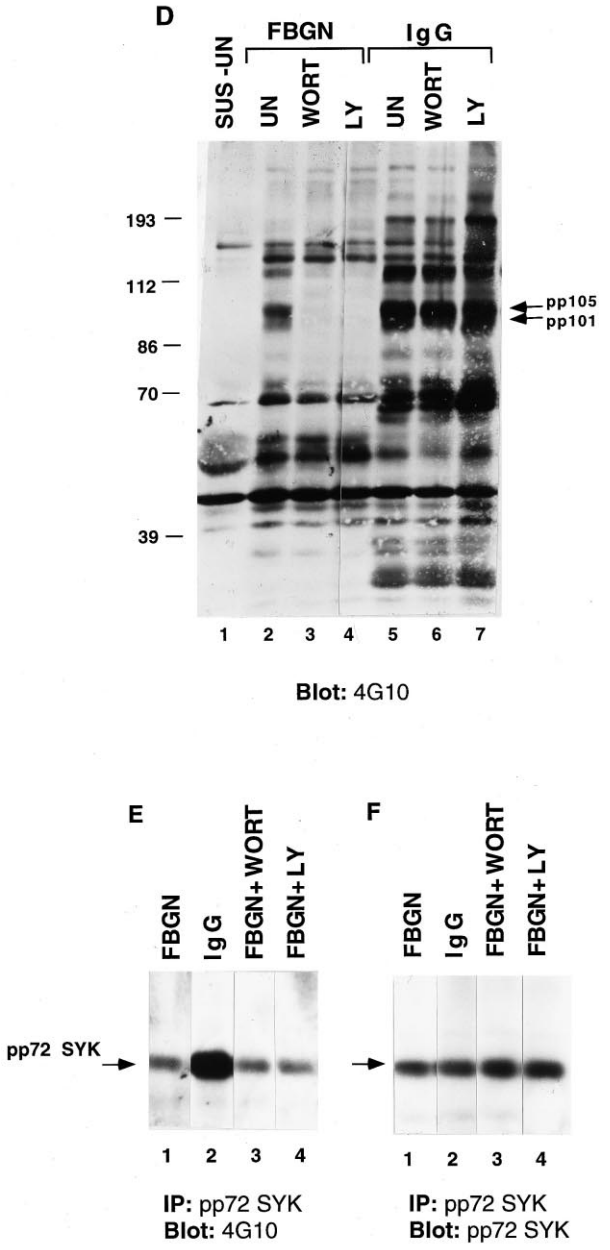

Fig. 1. Effect of PI 3-kinase inhibitors on fibrinogen- and IgG-induced protein tyrosine phosphorylation. (A) Platelets were kept in suspension (SUS-UN; lane 1) or were added to fibrinogen- (FBGN; lanes 2–9) coated plates. Prior to exposure to fibrinogen, the platelets were untreated (UN; lane 2) or treated for 10 min with 1, 3, 5 or 10 nM wortmannin (WORT; lanes 3, 4, 5, and 6, respectively) or with 5, 10 or 25 μM LY294002 (LY; lanes 7, 8, and 9, respectively). The platelets were lysed in sample buffer and equal amounts of protein were immunoblotted and probed mAb 4G10. (B–D) Platelets were kept in suspension (SUS-UN; lane 1) or were added to fibrinogen- (FBGN; lanes 2–4) or immunoglobulin- (IgG; lanes 5–7) coated plates. Prior to exposure to the protein-coated surfaces, the platelets were untreated (UN; lanes 2 and 5) or treated for 10 min with wortmannin (WORT; 10 nM) (lanes 3 and 6) or LY294002 (LY; 25 μM) (lanes 4 and 7). (B and C) Platelets were lysed in RIPA buffer. Lysates containing 200–300 μg protein were immunoprecipitated with a polyclonal antiserum to pp125FAK. The immunoprecipitates were Western blotted and probed with mAb 4G10 (B) or pp125FAK (C). (D) Platelets were lysed in sample buffer and equal amounts of protein were immunoblotted and probed with mAb 4G10. (E and F) Platelets were untreated and adhered to fibrinogen (FBGN; lanes 1, 3 and 4) or IgG (lane 2), or were treated for 10 min with 30 nM wortmannin (WORT; lane 3) or 25 μM LY294002 (LY; lane 4) prior to adherence to fibrinogen. Platelets were lysed in RIPA, and lysates containing an equal protein concentration were immunoprecipitated with a polyclonal antiserum to pp72syk. The immunoprecipitates were Western blotted and probed with mAb 4G10 (E) or pp72syk (F).

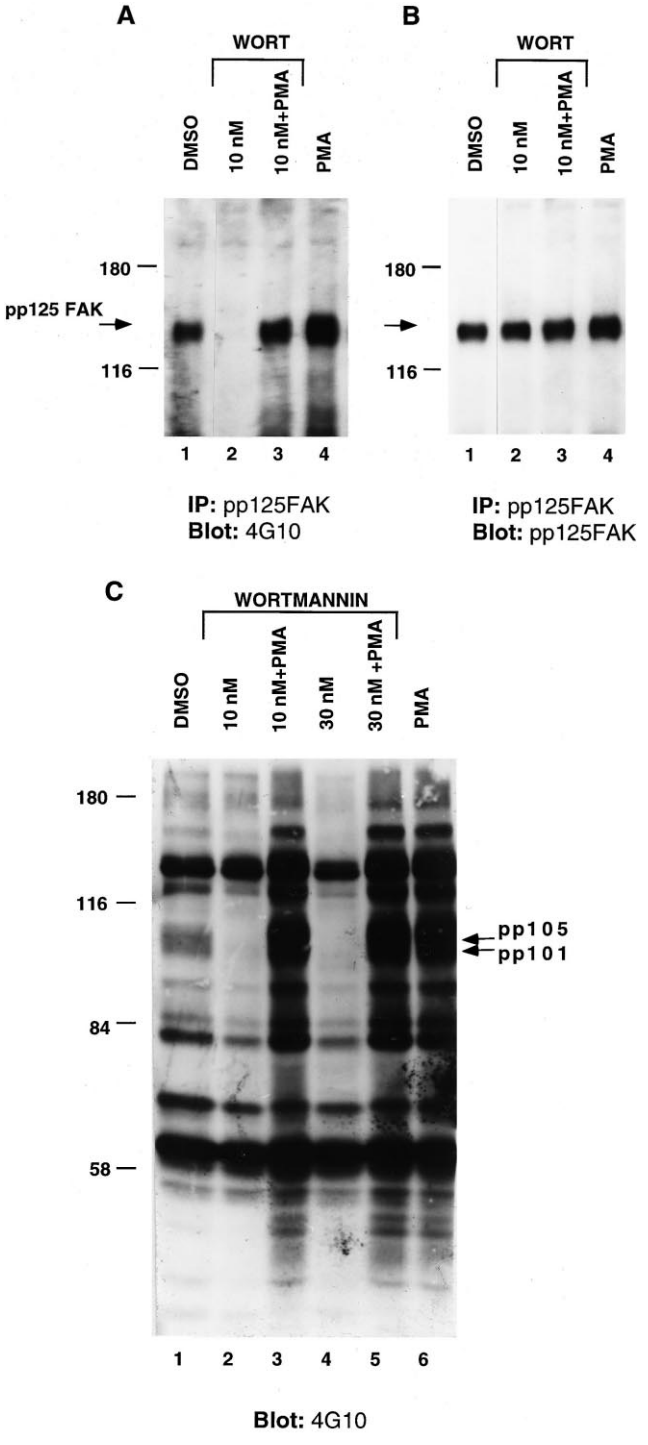

Fig. 2. Effect of wortmannin and phorbol ester co-treatment on protein tyrosine phosphorylation. (A and B) Platelets were treated without (lanes 1 and 4) or for 10 min with wortmannin (10 nM) (lanes 2 and 3). Subsequently, the platelets were treated for 10 min with DMSO (lane 1) or phorbol ester (PMA; 10 nM) (lanes 3 and 4), and were then added to the fibrinogen-coated plates for 1 h. RIPA lysates were immunoprecipitated with the polyclonal antiserum to pp125FAK. The immunoprecipitates were Western blotted and probed with mAb 4G10 (A) or pp125FAK (B). (C) Purified platelets were treated without (lanes 1 and 6) or for 10 min with 10 or 30 nM wortmannin (lanes 2–3 and 4–5, respectively). Subsequently, the platelets were treated for 10 min with DMSO (lane 1) or PMA (lanes 3, 5 and 6), and were then added to the fibrinogen-coated plates for 1 h. Platelets were lysed in sample buffer and equal amounts of total protein were immunoblotted and probed with mAb 4G10.
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peptide which blocks $\alpha_{IIb}\beta_3$ engagement prevented the accumulation of PtdIns(3,4)P2 in thrombin-stimulated platelets [14,15]. RGDS also reduced the movement of PI 3-kinase, $\alpha_{IIb}\beta_3$, and several other proteins to the detergent-insoluble fraction that forms in thrombin-stimulated platelets [16]. Collectively these data suggested that in the 'outside-in' pathway $\alpha_{IIb}\beta_3$ may at least partially regulate PI 3-kinase activation leading to PI(3,4)P2 production.

Pp125FAK provides a docking site for multiple signaling components [2,3,17]. Guan et al. were the first to show that in NIH 3T3 cells the p85 subunit of PI 3-kinase was associated with pp125FAK [18]. A p85/pp125FAK complex was also immunoprecipitated from thrombin-activated platelet lysates [19]. Autophosphorylation of pp125FAK enhanced its interaction with PI 3-kinase [18] and binding of the proline-rich sequence of pp125FAK to the p85 subunit enhanced the catalytic activity of PI 3-kinase [19]. These data suggested that pp125FAK may act upstream of PI 3-kinase. Rankin et al. [20] have subsequently shown that inhibitors of PI 3-kinase prevented PDGF-stimulated, but not bombesin-, endothelin-, or phorbol ester-stimulated, pp125FAK phosphorylation in Swiss 3T3 cells, suggesting that PI 3-kinase may at times act upstream from pp125FAK. Finally, in COS 7 cells PI 3-kinase inhibition had only a modest effect on pp125FAK phosphorylation triggered by integrin-mediated cell binding to fibronectin [21]. Thus, the relationship between PI 3-kinase and pp125FAK is most likely cell-type and stimulus-specific. The goal of this study was to examine whether pp125FAK phosphorylation in platelets is dependent on PI 3-kinase activation.

2. Materials and methods

2.1. Platelet preparation

Human platelets were purified from freshly drawn blood by gel filtration as previously described [7]. For adhesion studies, polystyrene plates were coated with fibrinogen (100 μg/ml; from Sigma) or IgG (50 μg/ml; from Sigma) as previously described [7]. Platelet morphology was examined by light microscopy.

To examine the inhibitors' effect, platelets were pre-incubated for 10 min with either wortmannin or LY294002 (2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one) in 0.5% Me_2SO4 (DMSO). Both inhibitors were purchased from Biomol. As a control, platelets were treated with 0.5% DMSO alone for 10 min. Where indicated, the platelets were treated for 5 min with phorbol ester (10 nM; from Sigma) or for 10 min with mAb 7E3 (10 μg/ml) kindly provided by Dr. Barry Coller (Mount Sinai School of Medicine, New York).

2.2. Immunoprecipitation and immunoblotting

Suspended and adherent platelets were lysed for 20 min in RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 158 mM NaCl, 10 mM Tris-HCl, pH 7.2, 1 mM sodium EGTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium vanadate) to immunoprecipitate pp125FAK and p72syk. The pp125FAK and p72syk antiserum were purchased from Santa Cruz (FAK C-20, cat. # sc-558 and Syk(LR) cat. # sc-573). The lysates were clarified by centrifugation at $15\,000 \times g$ for 10 min at 4°C, and were subsequently analyzed for protein content using the Pierce BCA reagents. Equal amounts of protein were then subjected to immunoprecipitation. The lysates were first pre-cleared for 1 h with protein A/G plus-agarose (from Santa Cruz) and then incubated with the antiserum to pp125FAK or p72syk for at least 2 h. The immune complexes were precipitated with protein A/G plus-agarose. The immunoprecipitates prepared for Western blot analysis were washed four times with RIPA buffer and eluted in Laemmli sample buffer.

Total cell lysates were prepared by lysis of the platelets in sample buffer (66 mM Tris-HCl, pH 7.4, 2% SDS) at 90°C. The samples were subsequently processed as previously described [9]. Lysates containing equal amounts of protein were subjected to immunoblotting analysis with mAb 4G10 (from Upstate Biotechnology) or the antiserum to pp125FAK. Immunoreactivity was detected using the ECL kit from Amersham and autoradiography.

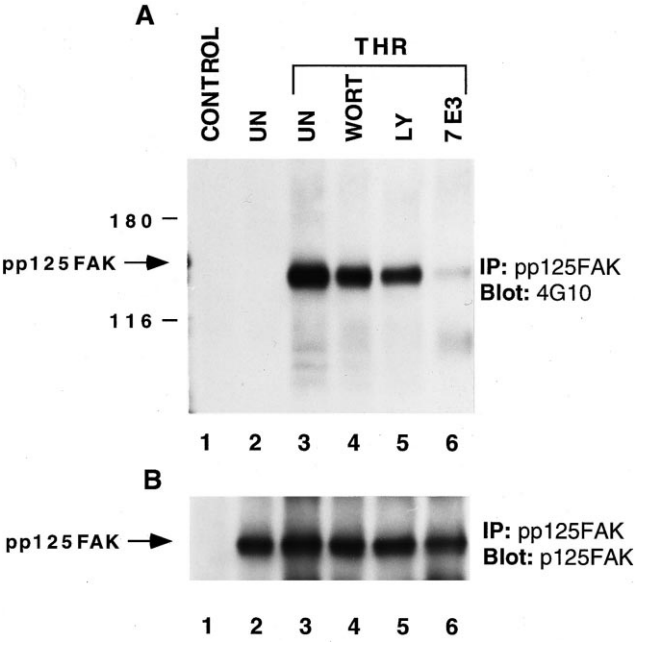

Fig. 3. Effect of PI 3-kinase inhibitors on thrombin-induced pp125FAK phosphorylation. Platelets were kept in suspension untreated (UN; lanes 2) or stirred in suspension with 1 U/ml thrombin for 10 min (lanes 3–6). The thrombin-stimulated platelets were either untreated (UN; lanes 3) or pretreated for 10 min with LY294002 (LY; 25 μM), wortmannin (WORT; 10 nM), or with mAb 7E3 (10 μg/ml) to inhibit fibrinogen-$\alpha_{IIb}\beta_3$ receptor interaction. Platelets were lysed in RIPA buffer and immunoprecipitated with a control antiserum (lanes 1) or the pp125FAK antiserum (lanes 2–6). Pp125FAK phosphorylation was examined as described in the legend to Fig. 1. The blots shown in A and B were probed with mAb 4G10 and the pp125FAK antiserum, respectively.

3. Results

3.1. PI 3-kinase inhibitors abrogate fibrinogen- but not IgG-stimulated platelet spreading and pp125FAK phosphorylation

Pp125FAK phosphorylation in platelets is closely linked to spreading as demonstrated by the ability of

several inhibitors to simultaneously prevent both events. To examine the relationship among pp125FAK phosphorylation, PI 3-kinase activation, and platelet spreading, we have treated purified platelets with the two unrelated and well characterized PI 3-kinase inhibitors, wortmannin and LY294002. Control platelets treated with 0.5% DMSO alone and inhibitor-treated platelets were added to fibrinogen- or IgG-coated plates for 1 h. Treatment of the platelets with wortmannin at a concentration equal to or greater than 10 nM, or with LY294002 at a concentration of 10 μM or higher, prevented platelet spreading on fibrinogen; a partial inhibitory effect was observed with wortmannin at 5 nM and LY294002 at 2.5 μM (data not shown). In contrast, the inhibitors had no effect on platelet spreading on IgG. We have previously shown that platelet spreading on fibrinogen correlates with the phosphorylation of two proteins that migrate with an electrophoretic mobility of 101 and 105 kDa (pp101/105), respectively [7,8]. As shown in Fig. 1A, wortmannin and LY294002 abrogated the phosphorylation of pp101/105 in a dose-dependent manner and within the same concentration range needed to inhibit spreading on fibrinogen and as demonstrated by Kovacsovics et al. [12], PI 3-kinase activation in platelets.

To examine the inhibitors' effect on the induction of pp125FAK phosphorylation, platelet lysates were immunoprecipitated with an antiserum to pp125FAK. The immunoprecipitates were analyzed by immunoblotting with the mAb against phosphotyrosine, 4G10 (Fig. 1B), and the pp125FAK antiserum (Fig. 1C). As shown in Fig. 1B, both wortmannin (Fig. 1B, lane 3) and LY294002 (Fig. 1B, lane 4) completely inhibited the fibrinogen-induced pp125FAK phosphorylation. In contrast, densitometric analysis of the data shown in Fig. 1B,C as well as data obtained in two additional experiments revealed a less than 20% reduction in the induction of pp125FAK phosphorylation following treatment of the platelets with wortmannin or LY294002 as compared to untreated platelets. Consistent with this differential effect, wortmannin and LY294002 abrogated the phosphorylation of pp101/105 triggered by fibrinogen but not by IgG (Fig. 1D).

Platelet adhesion to fibrinogen also triggers weak phosphorylation of yet another protein tyrosine kinase, p72syk [9,22]. In contrast, platelet adhesion to immobilized IgG or FcγRII receptor cross linking trigger robust activation of p72syk ([22,23] and Fig. 1E,F). Wortmannin and LY294002 did not prevent $\alpha_{IIb}\beta_3$-mediated pp72syk phosphorylation (Fig. 1E,F). Densitometric analysis of the data shown in Fig. 1E,F revealed no effect of wortmannin and a less than 15% reduction in the induction of pp72syk phosphorylation following treatment of the platelets with LY204002 as compared to untreated platelets. Consistent with data reported by others [9,24], these data demonstrated that $\alpha_{IIb}\beta_3$-mediated pp125FAK and p72syk phosphorylation are independently regulated. FcγRII-induced p72syk phosphorylation was also not affected by the PI 3-kinase inhibitors (data not shown). Taken together these data indicate that PI 3-kinase is specifically involved in the regulation of $\alpha_{IIb}\beta_3$-mediated pp125FAK phosphorylation and platelet spreading on fibrinogen.

3.2. *Phorbol ester reverses the inhibitory effect of wortmannin on fibrinogen-induced pp125FAK phosphorylation and platelet spreading*

PKC is an integral component of the $\alpha_{IIb}\beta_3$-mediated signaling cascade leading to pp125FAK phosphorylation [8]. These data, in conjunction with data presented in Fig. 1, raised the questions of whether there is a link between the activation of PKC and PI 3-kinase, and if so, in what sequential order these two signaling components may be linked. To search for a potential association between PKC and PI 3-kinase, wortmannin-treated platelets were subsequently treated with the PKC activator, phorbol ester. Addition of phorbol ester to platelets pretreated with either 10 nM or 30 nM wortmannin restored spreading (data not shown) and the induction of pp125FAK and pp101/105 phosphorylation (Fig. 2). In fact, treatment of the platelets with phorbol ester alone or with wortmannin followed by phorbol ester enhanced pp125FAK phosphorylation by about 2-fold relative to the phosphorylation observed in DMSO-treated platelets. These data suggested that PI 3-kinase may act upstream of PKC in the pathway leading from $\alpha_{IIb}\beta_3$ to pp125FAK phosphorylation. These data, however, do not exclude the possibility that the phorbol ester-induced PKC activation triggers a parallel signaling pathway independent of PI 3-kinase activation.

3.3. Effect of PI 3-kinase inhibitors on thrombin-induced pp125FAK phosphorylation

We next asked whether PI 3-kinase affects thrombin-induced pp125FAK activation. As a control for these studies platelets were treated with mAb 7E3, an inhibitor of fibrinogen-$\alpha_{IIb}\beta_3$ interaction and platelet aggregation [25]. Treatment of the platelets with mAb 7E3 prior to the addition of thrombin blocked aggregation and pp125FAK phosphorylation (Fig. 3 and previously demonstrated [26]). In contrast, wortmannin or LY294002 reduced the thrombin-induced pp125FAK phosphorylation by 17% and 56% respectively. Thus, PI 3-kinase appears to play a secondary role relative to the activation of pp125FAK when thrombin is the stimulating agonist.

4. Discussion

The goal of this study was to establish whether PI 3-kinase regulates signaling events that effect spreading and pp125FAK phosphorylation in platelets. Since platelets are not amenable to genetic manipulations ex vivo, the role of PI 3-kinase was investigated using two well characterized PI 3-kinase inhibitors, wortmannin and LY294002. Wortmannin is a cell-permeate fungal metabolite that inhibits PI 3-kinase by irreversibly binding to the p110 catalytic subunit of PI 3-kinase (IC_{50} 3 nM) [27] whereas LY294002 is a competitive antagonist for the ATP binding site of PI 3-kinase (IC_{50} 1.4 μM) [28]. We report that wortmannin and LY294002 completely inhibit $\alpha_{IIb}\beta_3$-mediated platelet spreading on fibrinogen and pp125FAK phosphorylation. The effect was dose-dependent; complete inhibition of pp125FAK phosphorylation was obtained with wortmannin at a concentration of 10 nM or higher and LY294002 at a concentration equal to or greater than 25 μM. Kovacsovics et al. have shown that LY294002 at a concentration of 25 μM and wortmannin at a concentration of 100 nM inhibit PI 3-kinase activation in platelets stimulated with the thrombin receptor activating peptide TRAP [12]. Integrin-stimulated PI 3-kinase activation in COS 7 cells was similarly prevented by 100 nM wortmannin and 25 μM LY294002 [21]. It is important to note, however, that even within this concentration range the inhibitory effect of wortmannin may not be limited to PI 3-kinase alone [29]. In contrast, wortmannin and LY294002 did not affect $\alpha_{IIb}\beta_3$-mediated pp72syk phosphorylation. Furthermore, FcγRII receptor-induced pp125FAK phosphorylation was not affected by the PI 3-kinase inhibitors. Based on these data we conclude that PI 3-kinase specifically regulates $\alpha_{IIb}\beta_3$-mediated platelet spreading and pp125FAK phosphorylation.

Two PI 3-kinase isotypes have been identified in platelets, a heterodimeric complex composed of a 85-kDa regulatory subunit and a 110-kDa catalytic subunit (p85/p110) and PI 3-kinase γ, which is composed of a single p110γ unit [30]. All current data indicate that the p85 subunit of PI 3-kinase binds to pp125FAK; a p85/pp125FAK complex was immunoprecipitated both from fibronectin-adherent NIH 3T3 cells and from thrombin-activated platelets [18,19]. These data suggest that p85/p110 rather than PI 3-kinase γ may associate with the $\alpha_{IIb}\beta_3$-pp125FAK pathway. However, since both PI 3-kinases are affected by wortmannin [31] additional studies are necessary to confirm this hypothesis.

The $\alpha_{IIb}\beta_3$ to PI 3-kinase-dependent step may lie upstream of PKC since phorbol ester treatment reversed the PI 3-kinase inhibitors' effect. Phorbol ester treatment similarly reversed the inhibitory effect of apyrase [8] on pp125FAK phosphorylation suggesting that PKC activation is key to $\alpha_{IIb}\beta_3$-mediated pp125FAK phosphorylation in platelets. Pp125FAK phosphorylation in fibronectin-adherent CHO cells is also PKC-dependent [32]. In vitro, phosphoinositides up-regulate the kinase activity of the novel PKC-δ, -ε, and -η isoforms, and the atypical PKC-ζ isoform [33,34]. Two of these isoforms, PKC-δ and -ζ, are expressed in platelets [35,36]. Data obtained in our laboratory suggest that platelet adhesion to fibrinogen does not trigger a significant increase in the kinase activity of PKC-δ [42]. Thus, an intermediate other than PKC-δ most likely regulates the pathway from $\alpha_{IIb}\beta_3$ to pp125FAK phosphorylation.

The ability of D3- and D4-phosphoinositides to uncap actin filaments may provide an alternative and/or additional mechanism by which these phosphoinositides could regulate cell spreading and pp125FAK phosphorylation [37,38]. Actin filament assembly is dependent on the availability of fast-growing ends, also known as barbed ends. In resting

platelets the barbed ends are blocked or 'capped' by proteins such as gelsolin, which are removed upon platelet activation. Hartwig et al. [37] showed that when added to *n*-octyl-β-glucopyranoside-permeabilized platelets, PI(4,5)P2, PI(3,4)P2 and PI(3,4,5)P3 increased the number of actin barbed ends by several-fold. Furthermore, PI(4,5)P2 and PI(3,4)P2 effectively inhibited the interaction between gelsolin and actin which could explain the correlation between the increase in D3- and D4-phosphoinositides and the number of barbed ends [37,38]. Both thrombin and phorbol ester induce the synthesis of D3 and D4 phosphoinositides in platelets. In thrombin-stimulated platelets actin filaments assembly was not affected by PI 3-kinase inhibitors, while the opposite was noted in phorbol ester-stimulated platelets [38]. Consistent with this observation, we found that PI 3-kinase inhibitors did not affect thrombin-induced pp125FAK phosphorylation. These data further indicate that both PI 3-kinase-dependent and PI 3-kinase-independent pathways lead to pp125FAK phosphorylation in platelets.

The effect of the cytoskeleton organization on pp125FAK phosphorylation has been evident since the initial observation, by now reproduced in several model systems [39,40], that inhibition of actin filament assembly by cytochalasin D prevents pp125FAK phosphorylation in platelets [7,26]. Chrzanowska-Wodnicka and Burridge [41] have subsequently shown that myosin light chain kinase inhibitors trigger loss of stress fibers, focal adhesions and a decrease in pp125FAK phosphorylation. Based on these and other data, theses authors proposed that pp125FAK phosphorylation is dependent on integrin receptor clustering which in turn is regulated by the tension produced by actin filaments [4]. The model suggests that disruption of the actin cytoskeleton affects integrin aggregation leading to an inhibition of pp125FAK phosphorylation. The opposite should hold true for activators of the cytoskeleton assembly. If D3 phosphoinositides produced as a result of $\alpha_{IIb}\beta_3$ ligation regulate the cytoskeleton organization, this model could provide an explanation for the effect of PI 3-kinase on $\alpha_{IIb}\beta_3$-mediated pp125FAK phosphorylation.

In summary, we have shown that PI 3-kinase regulates platelets spreading and pp125FAK phosphorylation mediated by $\alpha_{IIb}\beta_3$ but not by the FcγRII receptor or thrombin. Further studies are required to establish the exact mechanism by which PI 3-kinase affects platelet spreading and pp125FAK phosphorylation in platelets.

Acknowledgements

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