Focal adhesion kinase (pp125^{FAK}) cleavage and regulation by calpain

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Focal adhesion kinase (125 kDa form; pp125^{FAK}) is a widely expressed non-receptor tyrosine kinase that is implicated in integrin-mediated signal transduction. We have identified a novel means of pp125^{FAK} regulation in human platelets, in which this kinase undergoes sequential proteolytic modification from the native 125 kDa form to 90, 45 and 40 kDa fragments in thrombin-, collagen- and ionophore A23187-stimulated platelets. The proteolysis of pp125^{FAK} was prevented by pretreating platelets

with the calpain inhibitors calpeptin or calpain inhibitor-1, and was reproduced *in vitro* by incubating immunoprecipitated pp125^{FAK} with purified calpain. Proteolysis of pp125^{FAK} resulted in a dramatic reduction in its autokinase activity and led to its dissociation from the cytoskeletal fraction of platelets. These studies define a novel signal-terminating role for calpain, wherein proteolytic modification of pp125^{FAK} attenuates its autokinase activity and induces its subcellular relocation within the cell.

INTRODUCTION

The extracellular matrix plays a critical role in modulating the morphology, growth, migration and differentiation of cells [1,2]. The response of cells to matrix adhesive proteins appears to be mediated primarily through the integrin family of adhesion receptors. These receptors transduce signals by inducing the formation of integrin-rich cytoskeletal signalling complexes in adherent cells [3,4]. There is growing evidence that pp125^{FAK}, the 125 kDa form of focal adhesion kinase (FAK), plays an important role in regulating the assembly of these complexes, through its capacity to bind multiple cytoskeletal structural proteins and signalling enzymes [5–11].

pp125^{FAK} has a broad pattern of tissue expression and is highly conserved amongst species, suggesting that it plays a fundamental role in normal cell signalling [12–15]. Consistent with this possibility, disruption of the pp125^{FAK} gene in mice leads to a lethal phenotype in which there is a general defect in mesodermal development [16]. This defect is associated with reduced cell motility *in vitro* and an increase in focal adhesions in pp125^{FAK}-deficient cells. While a great deal of progress has been made in identifying cellular components which interact with pp125^{FAK}, there is currently limited information on the mechanism(s) regulating the enzyme activity and subcellular distribution of pp125^{FAK} within the cell.

In the present study we demonstrate a novel means of pp125^{FAK} regulation, whereby calpain-mediated cleavage of pp125^{FAK} down-regulates its autokinase activity and cytoskeletal localization within the cell.

MATERIALS AND METHODS

Reagents

All materials and antibodies were obtained from previously described sources [17,18] except for the following: anti-pp125^{FAK} monoclonal antibody 2A7 and anti-pp125^{FAK} polyclonal antibody BC3 (kindly donated by Dr. T. Parsons, University of Virginia, Charlottesville, VA, U.S.A.), and anti-pp125^{FAK} mono-

clonal antibody (Transduction Laboratories). Purified μ -calpain and calpain inhibitor-I were obtained from Calbiochem-Behring Corp. Calpeptin was purchased from Biomol Research Laboratories.

Platelet preparation and subcellular fractionation

Human platelets were washed as previously described [17], and finally resuspended in modified Tyrode's buffer (10 mM Hepes, 12 mM NaHCO₃, pH 7.5, 137 mM NaCl, 2.7 mM KCl, 1 mM CaCl₂ and 5.5 mM glucose). Washed platelets (1×10^9 /ml) were preincubated with calpain inhibitor-I (5–20 μ M), calpeptin (20–100 μ g/ml) or 0.3 % DMSO for 5 min at room temperature. Platelets were activated by the addition of collagen (10 μ g/ml), or ionophore A23187 (0.01–1 μ M), while stirring at room temperature in the presence of fibrinogen (0.3 mg/ml). When thrombin (1 unit/ml) was the agonist, assays were performed under identical conditions except that fibrinogen was omitted from the reaction mixture. Following activation, platelets were lysed and fractionated into Triton X-100-soluble and -insoluble (cytoskeletal) fractions as previously described [17]. The cytoskeletal pellet was extracted with radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris/HCl, pH 7.2, 1 % Triton X-100, 1 % sodium deoxycholate, 0.1 % SDS, 158 mM NaCl, 2 mM EDTA, 1 mM PMSF, 20 μ M calpain inhibitor-I, 1 mM Na₃VO₄ and 2 mM benzamidine). RIPA lysates were clarified by centrifugation at 15000 g for 10 min at 4 °C. In some experiments, whole-cell lysates were prepared by lysing platelets with an equal volume of 2 × RIPA buffer for 60 min at 4 °C.

pp125^{FAK} immunoprecipitation and autokinase assays

Triton X-100-insoluble (cytoskeletal) fractions were extracted with RIPA buffer prior to incubation with 1–2 μ g of anti-pp125^{FAK} polyclonal antibody (BC3), anti-pp125^{FAK} monoclonal antibody (Transduction Laboratories), or the anti-pp125^{FAK} monoclonal antibody 2A7. These antibodies were incubated in

Abbreviations used: FAK, focal adhesion kinase; pp125^{FAK}, 125 kDa form of FAK; RIPA, radioimmunoprecipitation assay.

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the presence of rabbit anti-mouse immunoglobulin (1 $\mu\text{g/ml}$) and 30 μl of Protein A–Sepharose slurry (50 %) for 2 h at 4 °C. Immunoprecipitates were washed three times with RIPA buffer at 4 °C. Autokinase assays on the immunoprecipitated pp125^{FAK} were performed as previously described [9].

***In vitro* proteolysis of pp125^{FAK}**

pp125^{FAK} was immunoprecipitated from unstimulated platelet lysates and incubated with 20 μl of proteolysis reaction mixture [50 mM Tris/HCl, pH 7.4, 30 mM NaCl, 10 mM CaCl₂ and various concentrations of μ -calpain (0.5, 2.5, 5.0 or 10.0 $\mu\text{g/ml}$)]. Control reactions were performed with 10 $\mu\text{g/ml}$ μ -calpain, in the presence of 2 mM EDTA instead of CaCl₂. After 20 min at 30 °C the reaction was terminated by the addition of 8 μl of 5 × SDS-reducing buffer (350 mM Tris/HCl, pH 6.8, 15 % SDS, 10 % glycerol, 3.6 M β -mercaptoethanol and 0.01 % Bromophenol Blue), and immediately boiled for 5 min. Proteins were separated by SDS/PAGE (7.5 % gel) and analysed by immunoblotting with the anti-pp125^{FAK} BC3 antibody.

Determination of total and specific autokinase activity of pp125^{FAK}

The total autokinase activity of pp125^{FAK} in resting and thrombin-stimulated platelets was determined by summation of the autokinase activities in the Triton X-100-soluble and cytoskeletal fractions. Autokinase activity was quantified by performing densitometry on the *in vitro* kinase autoradiograms. The specific activity of each kinase was determined by comparing the total amount of enzyme activity in each subcellular fraction with the total amount of pp125^{FAK} protein in the same fraction. Quantification of protein was determined by performing densitometry on pp125^{FAK} immunoblots. The autokinase activity of pp125^{FAK} in the Triton X-100-soluble fraction of resting platelets was arbitrarily designated as 1.0 unit of activity.

Miscellaneous methods

SDS/PAGE and immunoblot analysis were performed according to the methods of Laemmli [19] and Towbin et al. [20] respectively. Protein concentrations were determined using the Bio-Rad protein assay, with BSA as a standard. Fibrinogen was purified from fresh frozen human plasma as described previously [21].

RESULTS

Tyrosine phosphorylation status and enzyme activity of pp125^{FAK} in specific subcellular fractions of resting and thrombin-stimulated platelets

Previous studies in human platelets have demonstrated that integrins play a critical role in regulating the subcellular distribution, kinase activity and tyrosine phosphorylation status of pp125^{FAK} [9,22–25]. Although an increase in pp125^{FAK} tyrosine phosphorylation and enzyme activity is well documented in thrombin-stimulated platelets, there is currently no information on differences in pp125^{FAK} specific activity and tyrosine phosphorylation within distinct subcellular compartments of the platelet. In our initial experiments, we confirmed the redistribution of pp125^{FAK} from the Triton X-100-soluble to the cytoskeletal fraction of thrombin-stimulated platelets (Figures 1a and 1b) [26]. We investigated the tyrosine phosphorylation status of pp125^{FAK} within these subcellular compartments by immunoprecipitating the kinase from each of these extracts at the indicated time points, and performing anti-phosphotyrosine immunoblot analysis on the immunoprecipitated kinase. Con-

sistent with previous reports, our studies did not detect any tyrosine phosphorylation of pp125^{FAK} in resting platelets [26]. Following thrombin-induced platelet aggregation, the cytoskeleton-associated form of pp125^{FAK} was tyrosine phosphorylated in a time-dependent manner, with maximal phosphorylation observed after 5 min of platelet stimulation (Figure 1d). In contrast, no detectable phosphorylation of the Triton X-100-soluble form of pp125^{FAK} was observed at any of the time points examined (Figure 1c).

The enzyme activity of pp125^{FAK} in the Triton X-100-soluble and cytoskeletal fractions of resting and thrombin-stimulated platelets was compared as described in the Materials and methods section. An approximate 3-fold increase in total pp125^{FAK} activity was observed following thrombin stimulation (Figure 1e). This increase was primarily due to a 20–25-fold increase in cytoskeletal-associated pp125^{FAK} activity. When the specific autokinase activity of pp125^{FAK} was examined in the Triton X-100-soluble and cytoskeletal fractions of the platelets, it was evident that the enzyme not only translocated from the cytosol to the cytoskeleton, but also became activated 4-fold relative to the Triton X-100-soluble enzyme (Figure 1f).

We examined a range of agonists for their ability to stimulate the cytoskeletal association of pp125^{FAK} by performing autokinase assays and immunoblot analysis on the cytoskeletal extracts from activated platelets. Significant pp125^{FAK} translocation was observed in response to ADP/adrenaline (10 μM each), collagen (10 $\mu\text{g/ml}$) and ionophore A23187 (0.1 μM). As observed with thrombin-stimulated platelets, the cytoskeletal translocation of pp125^{FAK} occurred well after the formation of large platelet aggregates and was completely dependent on platelet aggregation (results not shown).

Proteolysis of pp125^{FAK} in ionophore A23187-stimulated platelets

During the course of studying pp125^{FAK} translocation in ionophore A23187-stimulated platelets, we consistently observed an alteration in the electrophoretic mobility of pp125^{FAK}, from 125 to 90 kDa, when higher concentrations of this agonist were used (1–2 μM). Time-course experiments revealed that pp125^{FAK} was rapidly proteolysed in A23187 (1 μM)-activated platelets (Figure 2a), with approx. 40 % of the native molecule cleaved within the first 60 s of platelet stimulation (Figure 2b). Later time points revealed that the 90 kDa fragment was further proteolysed to a 40/45 kDa doublet (Figure 2a). Adequate detection of these smaller fragments required overexposure of the immunoblots following development with enhanced chemiluminescence. The reason for this weaker chemiluminescence signal from the smaller fragments is unclear, but may reflect rapid proteolysis of these lower-molecular-mass forms or reduced recognition by the anti-pp125^{FAK} antibody. Previous studies have demonstrated the proteolysis of the non-receptor tyrosine kinase pp60^{c-src} and protein tyrosine phosphatase 1B by calpain in ionophore A23187-stimulated platelets [27,28]. Consistent with a role for this protease in pp125^{FAK} proteolysis, pretreatment of platelets with the calpain inhibitors calpeptin or calpain inhibitor-I completely abolished pp125^{FAK} proteolysis (Figure 2a). Time-course studies of pp125^{FAK}, protein tyrosine phosphatase 1B and pp60^{c-src} proteolysis in ionophore A23187-stimulated platelets revealed similar rates of pp125^{FAK} and protein tyrosine phosphatase 1B cleavage by calpain; however, pp60^{c-src} cleavage was significantly slower, with 50 % proteolysis within 2 min and 90 % proteolysis after 5 min of platelet stimulation (results not shown).

To investigate the potential physiological significance of pp125^{FAK} proteolysis, we examined the time course of pp125^{FAK} proteolysis in collagen- and thrombin-stimulated platelets. As

Figure 1 Subcellular distribution, tyrosine phosphorylation status and autokinase activity of pp125^{FAK} in thrombin-stimulated platelets

(a)–(d) Washed platelets were activated with thrombin (1 unit/ml) while stirring for the following times: lanes 1, 0 s; lanes 2, 15 s; lanes 3, 45 s; lanes 4, 2 min; lanes 5, 5 min. Platelets were then lysed with Triton X-100 lysis buffer and fractionated into Triton X-100-soluble (a and c) and cytoskeletal (b and d) extracts. pp125^{FAK} was immunoprecipitated from each extract and divided into two portions; one was assayed for pp125^{FAK} autokinase activity (a and b), and the other was subjected to immunoblot analysis with the anti-phosphotyrosine antibody PY20 (c and d). These results are from one experiment, representative of three. For the experiments in (e) and (f), platelets were stirred in the presence of buffer (Control) or thrombin (1 unit/ml) for 5 min. Total autokinase activity (e) and specific activity of pp125^{FAK} (f) in each of the subcellular extracts was determined as described in the Materials and methods section. Fractions: ts, Triton X-100-soluble; csk, cytoskeletal. Results represent the means $\pm$ S.E.M from five separate experiments.

observed in ionophore A23187-stimulated platelets, pp125^{FAK} was proteolysed to 90, 45 and 40 kDa forms in both collagen-stimulated (Figure 2c) and thrombin-stimulated (results not shown) platelets. pp125^{FAK} proteolysis to the 90 kDa form was observed within 2 min of platelet stimulation by collagen and was maximal by 10 min (Figure 2d). In contrast, both the 45 and 40 kDa proteolytic fragments were not evident until 5 min after platelet activation and were maximal by 30 min. These time-course experiments suggest that pp125^{FAK} is sequentially proteolysed to 90, 45 and 40 kDa forms by activated calpain.

To confirm that calpain was capable of proteolysing pp125^{FAK} directly, rather than through the activation of other platelet proteases, we examined the ability of purified μ -calpain (0.5–10 μ g/ml), which is the most abundant isoform of calpain in platelets [29], to proteolyse immunoprecipitated pp125^{FAK}. As demonstrated in Figure 2(e), the addition of increasing amounts of μ -calpain to immunoprecipitated pp125^{FAK} resulted in the dose-dependent proteolysis of pp125^{FAK} to a 90 kDa polypeptide fragment, similar in size to the proteolytic product observed in collagen-, thrombin- and ionophore A23187-stimulated platelets. To exclude the possibility that pp125^{FAK} proteolysis was due to

a non-calcium-dependent protease contaminating the purified calpain preparation, the reaction was also performed in the presence of 2 mM EDTA. Under these conditions no pp125^{FAK} proteolysis was observed (Figure 2e, lane 6). With the highest concentration of μ -calpain (10 μ g/ml), the 90 kDa proteolytic fragment appeared to be further proteolysed (Figure 2e, lane 5); however, unlike the situation in intact platelets, fragments of 40 and 45 kDa were not identified. The lack of detection of these smaller fragments in this system may reflect their rapid proteolysis *in vitro* or, alternatively, the sensitivity of our assay system may not be sufficient for their detection, as we were unable to achieve similar degrees of overexposure of these immunoblots compared with the immunoblots performed on total cell lysates.

Effect of calpain-mediated proteolysis on total pp125^{FAK} autokinase activity

The effect of the calpain-catalysed proteolysis of pp125^{FAK} on its autokinase activity was initially examined in ionophore A23187-stimulated platelets. Stimulation of platelets for 5 min resulted in greater than 90 % conversion of native pp125^{FAK} into the 90, 45

Figure 2 pp125^{FAK} proteolysis by calpain

(a)–(d) Time course of pp125^{FAK} cleavage in ionophore- and collagen-stimulated platelets. Washed platelets were stimulated with either ionophore A23187 (1 μ M) (a, b) or collagen (10 μ g/ml) (c, d) for the indicated times, in the presence of 0.3% DMSO or calpeptin (100 μ g/ml), while stirring. Platelets were lysed with RIPA buffer and the total cell lysates were subjected to immunoblot analysis using an anti-pp125^{FAK} monoclonal antibody (from Transduction Laboratories) (a and c). The rate of pp125^{FAK} proteolysis was quantified by performing densitometry on the pp125^{FAK} immunoblots (b and d). Note that the immunoblot of the smaller 40/45 kDa forms of FAK was overexposed to demonstrate their presence. These results are from one experiment, representative of three. (e) *In vitro* cleavage of pp125^{FAK} by purified calpain. pp125^{FAK} was immunoprecipitated from total platelet lysates and incubated with increasing concentrations of μ -calpain (0.5–10 μ g/ml) for 20 min at 30 °C. The reactions were terminated and the proteins subjected to immunoblot analysis using the anti-pp125^{FAK} polyclonal antibody BC3. Lanes represent immunoprecipitated pp125^{FAK} incubated with: control buffer (lane 1); 0.5 μ g/ml (lane 2), 2.5 μ g/ml (lane 3), 5 μ g/ml (lane 4) or 10 μ g/ml (lane 5) μ -calpain; or 10 μ g/ml μ -calpain in the presence of 2 mM EDTA (lane 6). The arrowhead indicates the position of the proteolysed fragment from pp125^{FAK}.

and 40 kDa proteolytic forms (Figure 3a). These conditions were ideal for examining the autokinase activity of these fragments, as their transphosphorylation by the native pp125^{FAK} enzyme is minimized. As demonstrated in Figure 3(b), intact pp125^{FAK} had at least 10 times more autokinase activity than the 90 kDa FAK fragment. In these experiments, we could not exclude the possibility that the low level of tyrosine phosphorylation of the 90 kDa fragment was due to transphosphorylation by the small amount of pp125^{FAK} remaining. Consistent with this possibility, we noted a higher level of phosphorylation of the 90 kDa fragment after 1 min of platelet stimulation, when the level of pp125^{FAK} was higher. Further proteolysis of FAK to the smaller 40/45 kDa fragments was associated with complete loss of autokinase activity (Figure 3b). Collectively, these studies indicate that the calpain-mediated cleavage of pp125^{FAK} leads to a dramatic reduction in its autokinase activity.

The physiological significance of the calpain-mediated proteolysis of pp125^{FAK} was investigated further by examining the autokinase activity of pp125^{FAK} in total cell lysates from thrombin-stimulated platelets. These studies revealed maximal pp125^{FAK} autokinase activity after 5 min of platelet stimulation by thrombin (1 unit/ml), which declined back to resting levels after 20 min (Figures 3c and 3d). This reduction in total cell

pp125^{FAK} autokinase activity correlated well with the time-dependence for pp125^{FAK} proteolysis (Figure 3e). Consistent with a role for calpain in down-regulating the autokinase activity of pp125^{FAK}, pretreating platelets with calpeptin abolished pp125^{FAK} cleavage and resulted in a sustained level of enzyme activation (Figures 3c–3e).

Effect of calpain-mediated proteolysis on the subcellular distribution of pp125^{FAK}

In further studies we examined the effects of proteolysis on the ability of pp125^{FAK} to associate with the platelet cytoskeleton. Using low concentrations of ionophore A23187 (0.01–0.1 μ M), there was a dose-dependent increase in the cytoskeletal translocation and activation of pp125^{FAK} (Figures 4a and 4b). However, with higher concentrations of ionophore A23187 (1 μ M), the calpain-induced proteolysis of pp125^{FAK} totally abolished its cytoskeletal association (Figures 4a and 4b). Time-course experiments in ionophore-stimulated platelets revealed a progressive increase in the cytoskeletal content of the 125 and 90 kDa forms of FAK within the first 2 min of platelet stimulation (Figure 4c, top panel). This initial increase in the cytoskeletal content of the

Figure 3 Effect of proteolysis on the autokinase activity of FAK

(a, b) *In vitro* autokinase activity of FAK proteolytic fragments. Washed platelets were stimulated with 1 μ M ionophore A23187 for the indicated times while stirring, in the presence of DMSO (0.3%) or calpeptin (100 μ M) (CP). Platelets were lysed with RIPA buffer and the whole-cell lysates immunoprecipitated with anti-pp125^{FAK} antibody (Transduction Laboratories). Immunoprecipitates were divided equally; one half was subjected to immunoblot analysis using the same anti-pp125^{FAK} monoclonal antibody (a), while the other half was assayed for autokinase activity (b). The total autokinase activity was quantified by performing densitometry on the autoradiographs. Note the presence of a protein band (indicated with an arrow) which migrated slightly above the 90 kDa fragment of FAK and was consistently recognized by the anti-pp125^{FAK} antibody in both resting and activated platelets. The identity of this protein is unknown; however, it should not be confused with the 90 kDa proteolytic fragment of FAK. (c, d) Effects of calpain activation on the total autokinase activity of FAK in thrombin-stimulated platelets. Washed platelets were stimulated with thrombin (1 unit/ml) for the indicated times while stirring, in the presence of 0.3% DMSO or calpeptin (100 μ g/ml). Platelets were lysed with RIPA buffer, and pp125^{FAK} was immunoprecipitated from the whole-cell lysates and assayed for autokinase activity (c). The total autokinase activity from this experiment was quantified by performing densitometry on the autokinase autoradiogram (d). These results are from one experiment, representative of four. (e) Time course of pp125^{FAK} proteolysis in thrombin-stimulated platelets. Whole-cell lysates from thrombin-stimulated platelets (1 unit/ml) were subjected to immunoblot analysis using an anti-pp125^{FAK} monoclonal antibody (Transduction Laboratories). The amount of pp125^{FAK} remaining in the whole-cell lysates was quantified by performing densitometry on pp125^{FAK} immunoblots, and expressed as a percentage of the intact pp125^{FAK} in resting platelets. These results represent means $\pm$ S.E.M. from four separate experiments.

125 and 90 kDa forms of FAK was transient, with a decrease in the level of cytoskeletal association of the kinase evident after 5 min of platelet stimulation (Figure 4c, top panel). These observations suggest that the sequential cleavage of pp125^{FAK} to the 40 and 45 kDa fragments ultimately leads to dissociation of the kinase from the cytoskeletal fraction of aggregated platelets (Figure 4c, bottom panel). Consistent with this possibility, pretreating platelets with calpeptin abolished the cytoskeletal dissociation of pp125^{FAK}. These observations explain why neither

the 125 nor the 90 kDa form of FAK was detected in the cytoskeletal fraction of platelets stimulated for 10 min with 1 μ M ionophore A23187. It is unlikely that the dissociation of pp125^{FAK} from the cytoskeleton is due to disassembly of the actin filament network, as the levels of a number of other cytoskeletal-associated proteins, as assessed by Coomassie Brilliant Blue staining of electrophoresed proteins, were not substantially decreased throughout the time period of these experiments (results not shown).

Figure 4 Effect of proteolysis on the association of pp125^{FAK} with the cytoskeleton

(a, b) Effect of increasing concentrations of ionophore A23187 on the cytoskeletal association of pp125^{FAK}. Washed platelets were activated with the indicated concentrations of ionophore A23187 for 10 min while stirring. The platelets were lysed and the cytoskeletal fraction analysed for (a) pp125^{FAK} autokinase activity and (b) pp125^{FAK} protein content by immunoblot analysis. (c) Time-dependence of the association of FAK with the cytoskeleton in ionophore-stimulated platelets. Washed platelets were stimulated with ionophore A23187 (1 μM) for the indicated times while stirring, in the presence of DMSO (0.3%) or calpeptin (100 μg/ml). Platelets were lysed, fractionated into Triton X-100-soluble and cytoskeletal extracts, and then subjected to immunoblot analysis using the anti-pp125^{FAK} monoclonal antibody (Transduction Laboratories). Note that the immunoblot of the smaller 40/45 kDa forms of FAK was overexposed to demonstrate their presence.

DISCUSSION

The studies reported in this paper describe an important functional relationship between two focal adhesion signalling enzymes, pp125^{FAK} and calpain. Studies in thrombin-stimulated platelets have demonstrated that both enzymes are translocated to the cytoskeletal fraction of platelets, where they are activated in an integrin $\alpha_{IIb}\beta_3$ -dependent manner [26,30,31]. Although a growing number of cytoskeletal signalling enzymes and structural proteins have been identified as calpain substrates *in vitro* and *in vivo*, the physiological significance of these proteolytic events has not been well established. Our studies indicate that the calpain-mediated proteolysis of pp125^{FAK} represents a major regulatory mechanism for this kinase. The ability of calpain to dramatically decrease the autokinase activity of pp125^{FAK} and alter its subcellular distribution suggests an important role for this protease in terminating the signalling function of pp125^{FAK}.

We have demonstrated that the total autokinase activity of pp125^{FAK} increased at least 3-fold following thrombin stimulation of platelets. This increase in total autokinase activity was due to a dramatic (20–25-fold) increase in the amount of cytoskeletal-associated pp125^{FAK} activity. Not only was pp125^{FAK} translocated to the cytoskeleton, it also became activated 4-fold relative to the Triton X-100-soluble enzyme. This increase in the activity of cytoskeletal-associated pp125^{FAK} correlated closely with its tyrosine phosphorylation. The major site of tyrosine phosphorylation of pp125^{FAK} *in vivo* is Tyr-397 [6,32]. Mutation of this site does

not abolish the kinase activity of pp125^{FAK} *in vitro*, but dramatically reduces the tyrosine phosphorylation of pp125^{FAK} cytoskeletal substrates, such as paxillin and tensin, *in vivo* [3,33]. Tyr-397 is essential for pp125^{FAK} binding to the Src-family kinases, raising the interesting possibility that complex-formation between these enzymes is essential for the phosphorylation of these target substrates [11]. Whether the tyrosine phosphorylation of pp125^{FAK} in platelets is essential for an increase in its specific activity, its cytoskeletal localization or for interacting with other SH2-containing signalling molecules, such as the p85 subunit of phosphoinositide 3-kinase, is currently unknown, and these are important areas for future study.

Our results are consistent with the hypothesis that pp125^{FAK} participates in the formation of integrin-rich cytoskeletal signalling complexes at 'focal contact-like' sites in aggregated platelets [4]. The localization of non-receptor tyrosine kinase at these sites is considered an important event for the tyrosine phosphorylation of a number of cytoskeletal proteins, such as paxillin and tensin. These phosphoproteins have been postulated to play an important role in the assembly of focal adhesions and in the anchorage of integrins to the contractile cytoskeleton, as a necessary event for cellular contractile processes such as fibrin clot retraction [17,34]. Our time-course studies, along with previous reports [26], indicate that pp125^{FAK} translocation and activation occur late in the platelet activation process, well after the formation of large platelet aggregates, which is consistent with a role for this kinase in the regulation of post-aggregation events such as clot retraction. The ability of calpain to proteolyse pp125^{FAK} and members of the Src-family of tyrosine kinases suggests a potentially important role for this protease in regulating the extent of clot retraction by activated platelets. Consistent with this possibility, we have recently demonstrated that the calpain-mediated proteolysis of pp125^{FAK} and pp60^{c-src} is associated with the decreased tyrosine phosphorylation of multiple cytoskeletal proteins leading to relaxed fibrin clot retraction, thus suggesting a functionally important role for these proteolytic events *in vivo* (S. M. Schoenwaelder, Y. Yuan, P. Corey, H. H. Salem and S. P. Jackson, unpublished work).

Our studies represent the first report of pp125^{FAK} proteolysis by calpain. Previous investigations in thrombin- and collagen-stimulated platelets have not detected pp125^{FAK} proteolysis, presumably because these investigators primarily used autokinase assays or anti-phosphotyrosine immunoblots to detect pp125^{FAK}, rather than direct pp125^{FAK} immunoblot analysis, and also because those studies focused on the early stages of platelet activation, in which pp125^{FAK} proteolysis is not so clearly evident [9,23,26]. In our hands, autokinase assays are an insensitive means of detecting pp125^{FAK} proteolytic fragments, as these fragments lack autophosphorylating activity. Furthermore, it is unclear whether the antibodies used in previous studies to immunoprecipitate pp125^{FAK} are able to detect the proteolytic fragments, or whether these fragments remain tyrosine phosphorylated. We are currently performing immunoprecipitation studies with the monoclonal antibody 2A7 to see if this commonly used antibody recognizes the pp125^{FAK} proteolytic products.

The observation that pp125^{FAK} is a physiological substrate for activated calpain has potentially important implications for cell adhesion processes. Gene targeting experiments have revealed an essential role for this kinase in cell migration [16]. Like pp125^{FAK}, calpain is ubiquitously expressed and highly conserved across species, suggesting a fundamental role for this protease in normal cell function. Calpain has been localized to focal adhesions in a number of adherent cell types, and a growing number of focal adhesion proteins have been identified as calpain substrates *in*

vitro or *in vivo* [35]. These include structural proteins such as talin, actin-binding protein and vinculin, as well as a variety of signalling proteins such as pp125^{FAK}, pp60^{c-src}, protein kinase C and paxillin [36,37]. Focal adhesion plaques are dynamic structures which assemble and disassemble at distinct phases of the cell cycle. It has previously been postulated that calpain activation may play a role in regulating the assembly of focal adhesions, although direct evidence for this is still lacking [35]. The ability of calpain to down-regulate the signalling function of non-receptor tyrosine kinases, such as pp125^{FAK} and pp60^{c-src}, has important implications for integrin-mediated signal transduction. These kinases have been postulated to play an important role in regulating a number of cell processes, including cell proliferation, differentiation, transformation and apoptosis. The ability of these kinases to regulate such diverse cell processes is probably a reflection of their capacity to activate multiple signalling molecules, including components of the Ras/mitogen-activated protein kinase pathway, as well as the lipid kinase phosphoinositide 3-kinase. Whether calpain-mediated proteolysis of pp125^{FAK} can modify these downstream signalling events is clearly an important issue for future research.

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