RESEARCH COMMUNICATION

Platelet adhesion to collagen via the $\alpha_2\beta_1$ integrin under arterial flow conditions causes rapid tyrosine phosphorylation of pp125^{FAK}

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Adhesion of human platelets to collagen under arterial flow conditions mediated by the $\alpha_2\beta_1$ integrin increased tyrosine phosphorylation of several proteins, one of which was the focal adhesion tyrosine kinase, pp125^{FAK}. Tyrosine phosphorylation of pp125^{FAK} did not occur in non-adherent flowing platelets or in platelets attached to poly(L-lysine). Neither adhesion nor tyrosine phosphorylation was affected by pretreatment of platelets with GRGDSP peptide or by anti-$\alpha_{IIb}\beta_3$ monoclonal antibody P2. Adherent platelets retained their discoid shape, suggesting that induction of pp125^{FAK} precedes platelet spreading. The tyrosine kinase inhibitor erbstatin decreased tyrosine phosphorylation in non-stimulated platelets and blocked platelet adhesion. These results suggest that pp125^{FAK} plays an important role in platelet adhesion to collagen via the $\alpha_2\beta_1$ integrin.

INTRODUCTION

Signal transduction events induced by cell adhesion to extracellular matrix proteins are beginning to be understood and suggest the involvement of integrins [1–3]. Tyrosine phosphorylation of a number of proteins occurs in fibroblasts adhering to fibronectin [4], during adhesion to fibrinogen (for a cell line expressing the $\alpha_{IIb}\beta_3$ integrin) [5] and when β_1 integrins are aggregated by anti-integrin antibodies on the surface of human epidermal carcinoma cells [6]. Blood platelets contain high levels of tyrosine kinases [7,8] and activation by various agonists leads to tyrosine phosphorylation of many proteins [9–11]. Roles are unclear, but phosphorylation is correlated with secretion [11], aggregation [12,13] and phosphoinositide hydrolysis [14,15]. It is generally believed that ligand binding to the $\alpha_{IIb}\beta_3$ integrin [12,13], to glycoprotein IV (GPIV) [16] and possibly to the $\alpha_2\beta_1$ integrin (collagen receptor) [17] helps initiate tyrosine phosphorylation in platelets. Recently, Schaller et al. [18] identified a 125 kDa protein as a novel cytosolic tyrosine kinase which is associated with focal adhesions (pp125^{FAK}), is a substrate for src kinases and has been proposed to be directly involved in integrin-mediated signal transduction [19–22]. In platelets, tyrosine phosphorylation of pp125^{FAK} has recently been demonstrated during thrombin- or collagen-induced aggregation mediated by the $\alpha_{IIb}\beta_3$ integrin [21,23].

We have described a novel system to study platelet adhesion to collagen under arterial flow conditions, in the absence of secretion and aggregation [24]. Using RGD-containing peptides and a number of antibodies raised against platelet membrane glycoproteins proposed as collagen receptors, we have shown that in plasma-free media containing Mg^{2+} this process is mediated by the $\alpha_2\beta_1$ integrin and is extremely fast, with exponential half-times as short as 0.2 s [24]. In the present study, we have focused on $\alpha_2\beta_1$-mediated signalling during rapid platelet adhesion to collagen by investigating early changes in phosphotyrosine-containing proteins and activation of the protein tyrosine kinase pp125^{FAK}.

MATERIALS AND METHODS

Materials

Affinity-purified rabbit polyclonal serum specific for phosphotyrosine residues and anti-pp125^{FAK} monoclonal antibody (mAb) 2A7 [25] were kindly donated by Dr. J. T. Parsons (University of Virginia, Charlottesville, VA, U.S.A.). Prostacyclin (PGI_2), aspirin, indomethacin, apyrase (grade VII; from potato), bovine thrombin, hirudin, BSA (fatty acid free), Sepharose 4B-200, and poly(L-lysine) were obtained from Sigma (St. Louis, MO, U.S.A.). CNBr was from Aldrich (Milwaukee, WI, U.S.A.). GRGDSP peptide (purity 70 %) was from Peninsula Laboratories (Belmont, CA, U.S.A.). The anti-$\alpha_{IIb}\beta_3$ mAb P2 was purchased from Amac (Westbrook, ME, U.S.A.). The antibody raised against membrane $\alpha_2\beta_1$ (6F1) [26] was a gift from Dr. Barry S. Coller (State University of New York, Stony Brook, NY, U.S.A.). Type-I collagen for the adhesion assays was from rat skin and was kindly donated by Dr. Gary Balian (University of Virginia, Charlottesville, VA, U.S.A.). Fibrillar collagen from equine tendon was used for the platelet aggregation studies (Hormon Chemie, Munich, Germany). Tyrosine kinase inhibitors [genistein (4′,5,7-trihydroxyisoflavone), tyrphostin 47 and erbstatin] were from Biomol (Plymouth Meeting, PA, U.S.A.).

Preparation of washed platelets

Human platelet-rich plasma was prepared as described [27]. Platelets were pelleted by centrifugation at 620 *g* for 20 min in the presence of PGI_2 (0.3 μg/ml), indomethacin (1 μg/ml) and apyrase (7.5 units/ml ADPase activity), and washed in acid-citrate dextrose (ACD; 85 mM sodium citrate/71 mM citric acid/67 mM dextrose) containing 0.3 % BSA and apyrase (7.5 units/ml) before resuspension in Tyrode's medium buffered with Hepes (10 mM, pH 7.4) containing 2 mM Mg^{2+}, 0.1 % BSA, 7.5 units/ml apyrase and 0.01 unit/ml hirudin. To minimize collagen-induced generation of thromboxane A_2, platelets were treated with 100 μM aspirin for 20 min at 37 °C and, when appropriate,

Abbreviations used: pp125^{FAK}, focal adhesion tyrosine kinase; GPIV, glycoprotein IV; PGI_2, prostacyclin; mAb, monoclonal antibody; PKC, protein kinase C.

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pre-incubated with 500 μM GRGDSP peptide, anti-$\alpha_{IIb}\beta_3$ mAb P2 (10 μg/ml) and tyrosine kinase inhibitors for 10 min at 37 °C. To dissociate $\alpha_{IIb}\beta_3$, platelets were treated with 10 mM EGTA for 40 min at 37 °C, washed and resuspended in the Tyrode's buffer containing Mg^{2+}.

Adhesion assay

The continuous-flow adhesion approach was essentially as described previously [24]. CNBr-activated Sepharose 4B beads were coated either with native soluble type-I collagen from rat skin or with poly(L-lysine). Washed platelets and isotonic saline were pumped through a microcolumn of the beads at 3.4 μl/s, giving a shear rate at the bead surface of 1700 s^{-1} [24]. Adhesion is determined by counting single platelets in the suspension before and after exposure to the beads and is expressed as the percentage of platelets bound to collagen.

To assess the changes in platelet phosphotyrosine proteins during adhesion, we used 50 μl of collagen- or poly(L-lysine)-coated Sepharose beads. We usually pumped 250 μl of cell suspension (4×10^8 platelets/ml) for about 2.5 min, corresponding to an average adhesion time of 1.25 min.

Aggregation

Washed-platelet suspensions were warmed for 10 min at 37 °C before activation with either thrombin (2.5 units/ml), fibrillar collagen (50 μg/ml) or with no additions. The platelets were stirred at about 1000 rev./min and after maximal aggregation was obtained (1.5 min for thrombin or 3 min for collagen), lysis buffer was added to stop the reaction [1% (v/v) Triton X-100, 0.25% sodium deoxycholate, 5 mM EGTA, 5 mM EDTA, 100 μM Na_3VO_3, 50 mM NaF, 1 mM phenylmethanesulphonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 0.05 M Tris/HCl, pH 7.5; final concentrations]. Aggregation was assessed by following the decrease in the level of single platelets [27]. In the case of thrombin, a 10-fold excess of hirudin was used to neutralize the thrombin before treating with lysis buffer.

Scanning electron microscopy analysis

Platelets were pumped through 50 μl of collagen-coated Sepharose beads at 37 °C for a mean adhesion time of 1.25 min, fixed with 1% glutaraldehyde, treated as described previously [28] and examined in a scanning electron microscope (JSM-6400, JEOL).

Detection of phosphotyrosine-containing proteins by immunoblotting

Platelets attached to collagen, to poly(L-lysine) or in the column effluents were solubilized in lysis buffer, followed by addition of Laemmli buffer [containing 4% (w/v) SDS and 5% (v/v) 2-mercaptoethanol]. Proteins from approx. 1.1×10^7 platelets per lane (22 μg) were separated by SDS/PAGE on 8% (w/v) polyacrylamide gels and transblotted to nitrocellulose filters. The blots were incubated in 0.05 M Tris/HCl-buffered saline (TBS; pH 7.5) containing 0.05% Tween 20 (TBS-T) and 5% (w/v) albumin, washed and exposed to anti-phosphotyrosine antiserum, before incubation with alkaline phosphatase-conjugated secondary antibody (Vector Laboratories, Burlingame, CA, U.S.A.) or ^{125}I-labelled goat anti-(rabbit IgG) antibody (0.25 μCi/ml; ICN Biomedicals, Irvine, CA, U.S.A.).

Immunoprecipitation

Platelets attached to poly(L-lysine)- or collagen-coated Sepharose beads were solubilized with lysis buffer at 4 °C and cell debris removed by centrifugation at 10000 *g* for 30 min at 4 °C. Lysates from 5×10^8 cells were then incubated for 2 h at 4 °C with 10 μg of anti-pp125^{FAK} mAb 2A7 or a control mouse IgG_1 antibody, followed by the addition of rabbit anti-(mouse IgG) antibody bound to Protein A–Sepharose. The immune precipitates were washed six times with lysis buffer, solubilized in Laemmli sample buffer and boiled for 10 min before separation by SDS/PAGE on 8% (w/v) polyacrylamide gels and immunoblotting.

RESULTS AND DISCUSSION

Adhesion-induced changes in tyrosine phosphorylation via the $\alpha_2\beta_1$ integrin

Although several receptors have been proposed for platelet adhesion to collagen, including the two integrins $\alpha_2\beta_1$ (GPIa/IIa) and $\alpha_{IIb}\beta_3$ (GPIIb/IIIa), as well as GPIb/IX and GPIV [24,29], strong evidence suggests that the $\alpha_2\beta_1$ integrin is the primary receptor. This evidence includes powerful inhibition of adhesion by specific antibodies and indicates that incorporation of the purified receptor into liposomes allows adhesion to collagen in a Mg^{2+}-dependent manner [24,30,31]. Using a variety of antibodies raised against the proposed collagen receptors, we have shown that the $\alpha_2\beta_1$ integrin is the dominant receptor for rapid adhesion of non-activated platelets to collagen under arterial flow conditions in a plasma-free medium containing Mg^{2+} [24,32]. Our present study is focused on adhesion mediated by the $\alpha_2\beta_1$ integrin and potential signalling via tyrosine phosphorylation during early platelet adhesion.

The results presented in Figure 1(a) compare the tyrosine phosphorylation evoked during adhesion to collagen with that seen when an equivalent number of platelets were aggregated by fibrillar collagen or thrombin without addition of external fibrinogen and Ca^{2+}. Several bands were distinctive for adhesion (lane 2) versus controls (lane 1) or aggregation (lanes 3 and 4); these include proteins of 132 kDa, a triad at about 118, 116 and 114 kDa which may represent forms of pp125^{FAK} [19], 102 and 96 kDa. pp60^{src} was also increased during adhesion, as was a major band near 45 kDa. As the phosphorylation of pp125^{FAK} can occur during platelet aggregation and fibrinogen binding to $\alpha_{IIb}\beta_3$ [21], and as fibrinogen binding to $\alpha_{IIb}\beta_3$ induced by antibody stimulation initiates tyrosine phosphorylations before phosphorylation of pp125^{FAK} [23], we also tested potential contributions of the $\alpha_{IIb}\beta_3$ integrin for inducing tyrosine phosphorylation (Figure 1b). Treatment with EGTA was used to dissociate the $\alpha_{IIb}\beta_3$ integrin (lane 3), exposure to GRGDSP to prevent fibrinogen binding (lane 4) and mAb P2 to block this receptor (lane 5). Efficiency of adhesion to collagen in the Mg^{2+}-containing medium was unaltered by these treatments (data not shown) [24], as was the general pattern of tyrosine phosphorylation (lanes 3–5), compared with control adhesion (lane 2).

We also tested poly(L-lysine)-coupled Sepharose beads as a non-specific substrate for platelet adhesion (Figure 2). Adhesion to collagen (lane 2) caused similar increases in tyrosine phosphorylation as described above, while the same number of platelets adherent to poly(L-lysine) revealed a pattern (lane 3) which closely resembled non-adherent platelets emerging from the micro-adhesion column (lane 1). Consequently, collagen binding via the $\alpha_2\beta_1$ integrin is capable of signalling tyrosine phosphorylation of a number of platelet proteins. This pattern is not seen during non-specific adhesion to poly(L-lysine) or during

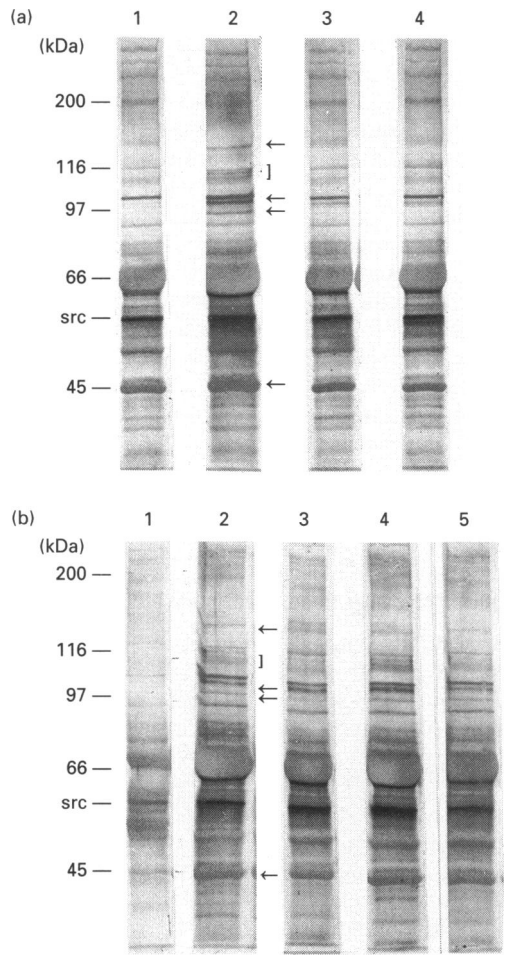

Figure 1 Tyrosine phosphorylation during platelet adhesion to collagen, aggregation, and effects of $\alpha_{IIb}\beta_3$ inhibition

Washed platelets in a Mg^{2+}-containing medium were pumped at a shear rate of about 1700 s^{-1} through collagen-coated beads in a micro-adhesion column [24], for an average time of 1.25 min at 37 °C. Proteins were analysed by SDS/PAGE on 8% polyacrylamide gels and subsequent Western blotting with an anti-phosphotyrosine antibody, followed by alkaline phosphatase visualization. (**a**) Lane 1, control static platelets; lane 2, collagen-adherent cells; lane 3, collagen-induced aggregation (3 min); lane 4, thrombin-induced aggregation (1.5 min). A separate platelet preparation was analysed for the data in (**b**). (**b**) Lane 1; control static platelets; lane 2, collagen-adherent cells; lane 3, EGTA-treated platelets; lane 4, GRGDSP-treated cells; lane 5, anti-$\alpha_{IIb}\beta_3$ mAb (P2)-treated platelets (see the Materials and methods section). Arrows identify bands in lane 2 of (**a**) which appear to be specific for adhesion and a brace indicates the bands at 118, 116 and 114 kDa.

aggregation, which involves fibrinogen binding and the $\alpha_{IIb}\beta_3$ integrin.

Tyrosine phosphorylation of pp125^{FAK} during platelet adhesion to collagen

The results in Figure 3(a) show that a phosphotyrosine band was detected at about 116 kDa only in platelets which had adhered to collagen both in the absence or presence of GRGDSP peptide (present to block any aggregation) (lanes 3 and 4). Unstimulated cells (lane 1) and non-specific adhesion to poly(L-lysine) (lane 2) did not show signs of tyrosine phosphorylation. To examine directly whether the band is pp125^{FAK} [18], platelet lysates were incubated with anti-pp125^{FAK} mAb 2A7, immunoprecipitated, and subsequent Western blots probed with anti-phosphotyrosine

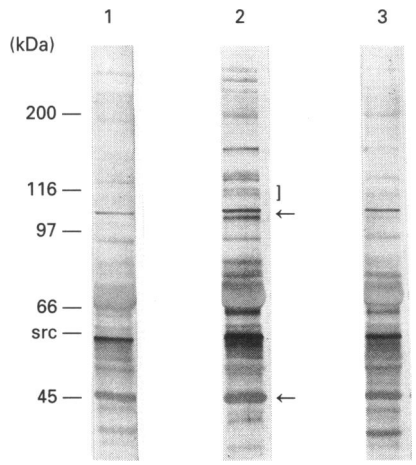

Figure 2 Tyrosine phosphorylation during collagen adhesion: effects of shear and non-specific adhesion to poly(L-lysine)

Conditions were as described in Figure 1, except for that cells were obtained from a different donor. Key to lanes: effluent platelets from the adhesion column (lane 1), collagen-adherent platelets (lane 2), and an equivalent number of platelets adherent to poly(L-lysine)-coated Sepharose beads (lane 3). Arrows indicate 96 and 45 kDa bands. The brace indicates a triad of proteins 114, 116 and 118 kDa.

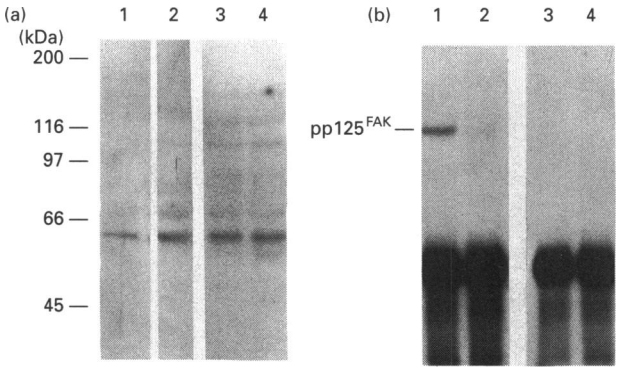

Figure 3 Presence of pp125^{FAK} in collagen-adherent platelets

(**a**) Conditions were as for Figure 1, except that detection of phosphotyrosine was by ^{125}I-IgG probing. Lane 1 represents static platelets; lane 2, non-specific adhesion to poly(L-lysine); lane 3, collagen-adherent platelets; and lane 4, same as lane 3, except for pretreatment with 0.5 mM GRGDSP peptide to prevent fibrinogen binding. (**b**) Immunoprecipitation by anti-pp125^{FAK} (lanes 1 and 2) or by a non-specific antibody (lanes 3 and 4); lanes 1 and 3 are for platelets adhered to collagen in the presence of GRGDSP, and lanes 2 and 4 are for non-specific adhesion to poly(L-lysine).

antiserum and ^{125}I-IgG as described in the Materials and methods section. Tyrosine-phosphorylated pp125^{FAK} was detected only in platelets which had adhered to the collagen-coated beads (Figure 3b, lane 1) and not in those attached to poly(L-lysine)-coated beads (lane 2). Non-adherent platelets emerging in the column effluent, which were in brief contact (1.35 s) with collagen, did not reveal tyrosine phosphorylation of pp125^{FAK} (results not shown).

Effect of tyrosine kinase inhibitors

To help evaluate the role of tyrosine phosphorylation in platelet adhesion to collagen, we tested three kinase inhibitors: genistein

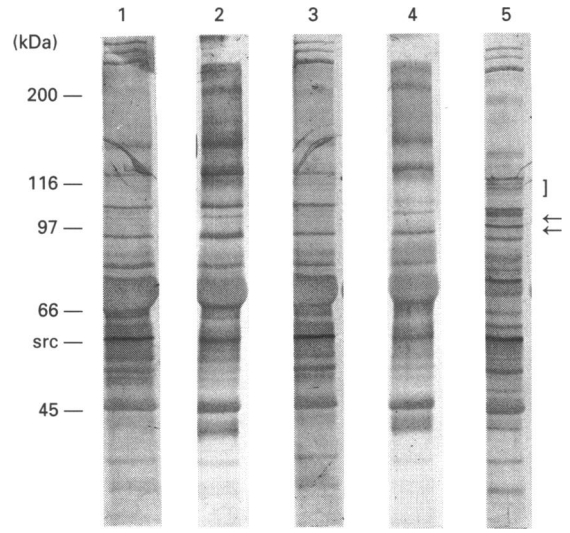

Figure 4 Effect of erbstatin on platelet tyrosine phosphorylation and adhesion

Washed platelets in Mg^{2+}-containing buffer were pretreated with 0.5 mM erbstatin for 10 min at 37 °C before being pumped through the micro-adhesion column. Samples were processed for phosphotyrosine detection with alkaline phosphatase as for Figure 1. Lane 1, control static platelets; lane 2, same with erbstatin; lane 3, control platelets in column effluent; lane 4, effluent platelets with erbstatin; lane 5, collagen-adherent cells. Arrows indicate pp102 and pp96. The brace indicates a triad of proteins 114, 116 and 118 kDa.

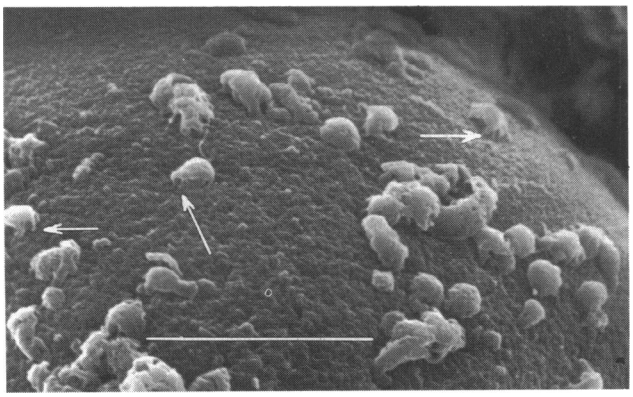

Figure 5 Scanning electron micrograph of collagen-adherent platelets

Platelets were pumped through collagen-coated Sepharose beads for an average time of 1.25 min and then fixed with glutaraldehyde before scanning electron microscopy processing (see Materials and methods section). Adhesion conditions were the same as in the phosphotyrosine experiments. The long white line represents 10 μm. Arrows indicate surface projections of attached platelets.

[33], tyrphostin 47 [34], and erbstatin [35]. Both genistein and tyrphostin 47 had no significant effect on initial rates or extent of early platelet adhesion to collagen (< 2.5 s), even at high concentrations (0.5 mM each). In contrast, erbstatin (0.5 mM) decreased long-term adhesion (1–2.5 s), although it had little effect on initial rates (< 0.75 s). Erbstatin did not cause major changes in the tyrosine phosphorylation of resting platelets (Figure 4, lanes 1 and 2) or in platelets emerging from the adhesion column (lanes 3 and 4), except for decreased levels of $pp60^{src}$ and three bands above 200 kDa. Also shown in Figure 4 (lane 5) are the adhesion-induced increases noted earlier in tyrosine phosphorylation of the 118, 116 and 114 kDa bands and strong increases at 102 and 96 kDa (Figures 1a and 1b).

Genistein and tyrphostins are relatively specific for tyrosine kinases [33,34], whereas erbstatin inhibits protein kinase C (PKC) and cyclic nucleotide-dependent kinases [36]. PKC is also involved in adhesion, as phorbol esters stimulate and PKC inhibitors reduce the formation of focal adhesions [37]. The relative roles of these and other kinases during adhesion are unknown, and more specific kinase inhibitors or probes would be useful. Recently, we have found that platelet adhesion to collagen mediated by the $\alpha_2\beta_1$ receptor lowered levels of cyclic AMP and increased cyclic GMP levels in the adherent cells [32]. In epithelial cells, a membrane-associated tyrosine phosphatase is activated by the cyclic AMP-dependent protein kinase [38]. This suggests that decreased cyclic AMP levels in adherent platelets might stimulate tyrosine phosphorylation by decreasing the activity of tyrosine phosphatases.

Scanning electron microscopy of adherent platelets

Platelets were generally discoid, as noted before [24], and minimally activated compared with ADP- or thrombin-treated cells [28]. However, many adherent platelets possessed small projections on their lower surface (Figure 5, arrows). The absence of significant shape changes suggests that tyrosine phosphorylation of $pp125^{FAK}$ occurs before spreading, as these morphological studies were done at the same time (1.25 min) as the immunoprecipitation experiments (Figure 3). The report by Kornberg et al. [19] on human epidermal carcinoma (KB) cells adhering to fibronectin, collagen type IV, or to laminin, also suggests that phosphorylation of $pp125^{FAK}$ precedes cell spreading.

Concluding remarks

The results of our research show that platelet adhesion to collagen via the $\alpha_2\beta_1$ integrin under arterial flow conditions caused the tyrosine phosphorylation of a number of proteins (Figures 1–3), including $pp125^{FAK}$. These findings support the suggestion that signal transduction via integrins involves activation of $pp125^{FAK}$ and probably other kinases, as an early step in cell adhesion to the extracellular matrix [22]. Several other reports are relevant. Kornberg et al. [19] show that adhesion of KB cells via several β_1-containing integrins can activate $pp125^{FAK}$. The fibrinogen-binding integrin $\alpha_{IIb}\beta_3$ has also been proposed as a major mediator of platelet tyrosine phosphorylation, including $pp125^{FAK}$, induced by collagen (Lipfert et al. [21]; Huang et al. [23]). These studies, performed under test-tube stirring conditions, concluded that binding to the $\alpha_2\beta_1$ receptor was not sufficient for $pp125^{FAK}$ activation, in contrast with our results under arterial flow conditions. Recently, Hamawy et al. [39] found that adhesion of basophilic leukaemia cells to fibronectin was essential for activation of $pp125^{FAK}$, identified as a 115 kDa phosphotyrosine protein, close to the apparent molecular mass we found in platelets (Figure 3).

These studies all emphasize the critical involvement of tyrosine phosphorylation after integrin activation by a range of stimuli and in varied cell types. For platelet adhesion to collagen via the $\alpha_2\beta_1$ integrin, it is clear that $pp125^{FAK}$ and a number of other proteins are phosphorylated; however, the sequences and specific functions of these proteins are not known. The high speed of platelet adhesion to collagen under flow conditions, which can be nearly complete within 1 s [24], requires understanding of the

involvement of tyrosine phosphorylation during these very early events. Phosphorylation of $pp60^{src}$ also occurs (Figure 1), and it is possible that activation of this important tyrosine kinase and others are prerequisites for $pp125^{FAK}$ phosphorylation and the formation of stable focal adhesion points, as recently proposed by Guan and Shalloway [22].

Since submission of our manuscript, Haimovich et al. [40] have reported that tyrosine phosphorylation of $pp125^{FAK}$ occurred in spread platelets after adhesion for 1 h to a collagen matrix under static conditions.

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