Site-specific phosphorylation of platelet focal adhesion kinase by low-density lipoprotein

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Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase implicated in signalling pathways mediated by integrins and G-protein-coupled receptors (GPCRs). Upon stimulation FAK is phosphorylated on six tyrosine residues. Here we report the site-specific phosphorylation by low-density lipoprotein (LDL), which is known to induce integrin-independent FAK phosphorylation, and compare this with the effect of thrombin, which phosphorylates FAK via integrin α IIb β 3. Stimulation with LDL reveals (i) a major role for Tyr-925 phosphorylation which surpasses the phosphorylation of the other residues, including Tyr-397, in rate and extent, (ii) α IIb β 3-independent phosphorylation of Tyr-925 and Tyr-397, and (iii) complex formation between FAK and the Src-kinase Fgr but not with c-Src. These patterns differ profoundly from those induced by thrombin. LDL-induced phosphorylation of Tyr-925 and Tyr-397 was inhibited by 60–75% by receptor-associated protein, an inhibitor of members of the LDL receptor family. Thus these findings reveal a novel mechanism of FAK phosphorylation by signalling cascades involving a member of the LDL receptor family.

Key words: focal adhesion kinase (FAK), Fgr, LRP8, Src.

INTRODUCTION

Focal adhesion kinase (FAK) is a non-receptor protein-tyrosine kinase implicated in signalling pathways involved in cell motility, proliferation and apoptosis [1]. FAK is present in mesenchymal cells, neuronal cells, platelets, lymphocytes and monocytes. It consists of a central catalytic domain flanked by large N- and C-terminal regions. The C-terminal region is the site of the focal adhesion targeting sequence, which mediates the localization of FAK to focal adhesions [2]. After cell activation, FAK translocates to the cytoskeleton at focal adhesions, where it serves as a docking site for signalling proteins. FAK is activated by tyrosine phosphorylation in response to integrin clustering induced by cell adhesion or antibody cross-linking [3–5]. A second mechanism of FAK activation is via G-protein-coupled receptor (GPCR) occupancy by ligands such as bombesin or lysophosphatidic acid [6,7].

Upon stimulation, FAK is phosphorylated on six tyrosine residues. In integrin-mediated FAK phosphorylation, Tyr-397 is the primary target [8]. Phosphorylation of Tyr-397 triggers the phosphorylation of the other tyrosine residues, thereby initiating downstream signalling cascades. The initial phosphorylation of FAK at Tyr-397 is thought to create a high-affinity binding site for SH2 domains, enabling formation of a signalling complex between FAK and members of the Src-family kinases [8]. The inhibitor PP-2 markedly reduces the phosphorylation of Tyr-397, indicating that further phosphorylation of this site depends on Src kinases [9]. Formation of a FAK–Src complex leads to phosphorylation of both proteins, thereby initiating downstream signalling pathways [10].

Tyr-397 is also the primary target in GPCR-mediated FAK phosphorylation. Here, the phosphorylation of Tyr-397 is unaffected by the inhibitor PP-2, indicating that, in integrin-independent FAK phosphorylation, kinases other than Src control the phosphorylation of Tyr-397 [9]. Common to both

pathways is the finding that the initial phosphorylation of Tyr-397 is followed by phosphorylation of the other residues (Tyr-407, Tyr-576, Tyr-577, Tyr-861 and Tyr-925) in a Src-dependent manner.

Phosphorylation of the different Tyr residues is crucial for further downstream signalling. Phosphorylated Tyr-397 is the docking site for the p85 regulatory subunit of phosphoinositide 3-kinase and for phospholipase C γ [11,12]. Tyr-576 and Tyr-577 are located in the central catalytic domain and their phosphorylation is part of an autoactivation loop resulting in maximal FAK activation [13]. Tyr-925 is located in the focal adhesion targeting domain and its phosphorylation is followed by binding of the adaptor protein Grb2, which contributes to the adhesion-induced activation of the Ras/extracellular signal-regulated protein kinase (ERK1/2) pathway [14,14a]. The functions of Tyr-407 and Tyr-861 are still elusive.

In platelets, FAK is phosphorylated following stimulation by thrombin, provided that suspensions are stirred and that they aggregate. FAK phosphorylation is markedly reduced by monoclonal antibodies against integrin α IIb β 3 and is virtually absent in α IIb β 3-deficient platelets [15]. These findings illustrate a dominant role for integrin α IIb β 3 (glycoprotein IIb/IIIa), which in activated platelet suspensions binds fibrinogen and other adhesive proteins and facilitates the fibrinogen-mediated formation of aggregates. In non-stirred suspensions a residual FAK phosphorylation remains detectable, caused probably by direct signalling by ligand-occupied thrombin receptors [protease-activated receptor (PAR) 1/3/4] in agreement with the GPCR-mediated activation observed in other cell types.

We reported earlier that low-density lipoprotein (LDL) is a potent activator of FAK in platelet suspensions and that this activation occurs in stirred as well as non-stirred suspensions [16]. An elevated level of plasma LDL is a risk factor for arterial thrombosis and patients with hypercholesterolaemia have platelets that become activated in the circulation (reviewed in [17]).

Abbreviations used: apo, apolipoprotein; FAK, focal adhesion kinase; GPCR, G-protein-coupled receptor; LDL, low-density lipoprotein; PAR, protease-activated receptor; PP-1, pyrazolopyrimidine 1; RAP, receptor-associated protein.

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LDL triggered the phosphorylation of FAK at concentrations as low as 0.5 g/l LDL (after 10 min) and as rapidly as 30 s (at 1.0 g/l LDL). LDL-induced FAK phosphorylation was independent of ligand binding to integrin $\alpha 2 \beta 1$ (one of the collagen receptors), integrin $\alpha \text{IIb} \beta 3$ and the Fc γ RIIa receptor, and proceeded normally in platelets with a deficiency of $\alpha \text{IIb} \beta 3$ present in Glanzmann thrombastenia patients [16]. The platelet LDL receptor differs from the classical apolipoprotein (apo) B/E receptor since certain antibodies against the apoB/E receptor fail to prevent the binding of LDL to platelets and the enhancement of ADP- and collagen-induced platelet aggregation observed in the presence of LDL. In addition, LDL binding to platelets is normal in individuals lacking the apoB/E receptor, a deficiency known as familial hypercholesterolaemia [18]. However, the platelet LDL receptor is likely to be a member of the LDL receptor family since an antibody against the B-site in apoB100 inhibits platelet sensitization by LDL and a peptide mimicking the B-site initiates signal transduction in platelets [19]. Thus the phosphorylation of FAK in platelets incubated with LDL may serve as an example of both a GPCR-independent as well as an integrin-independent signalling mechanism to this kinase.

In the present study we investigated the phosphorylation of FAK in platelets stimulated by LDL in further detail using site-specific antibodies that recognize the phosphorylated forms of the individual residues. The results reveal similarities and discrepancies with thrombin-induced phosphorylation of FAK and suggest that LDL activates a member of the LDL receptor family that initiates a novel mechanism of FAK phosphorylation.

MATERIALS AND METHODS

Materials

BSA (demineralized) was from Organon Technika (Eppelheim, Germany). Sepharose 2B and Protein A-Sepharose were from Amersham Biosciences (Uppsala, Sweden). Enhanced chemiluminescence (ECL) reagent was from NEN-Dupont (Boston, MA, U.S.A.). Human α -thrombin was purchased from Kordia Life Science (Leiden, The Netherlands). Precast polyacrylamide mini gels were obtained from Gradipore (Frenchs Forest, Australia).

Anti-phosphotyrosine monoclonal antibody 4G10 was from Upstate Biotechnology (Lake Placid, NY, U.S.A.), monoclonal antibody directed against total FAK was from Transduction Laboratories (Lexington, NY, U.S.A.) and polyclonal antibodies directed against total FAK, c-Fgr and Src kinases were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The site-specific antibodies directed against phosphorylated FAK were purchased from Biosource (Camarillo, CA, U.S.A.). The fibrinogen-derived peptide HHLGGAKQAGDV ($\gamma_{400-411}$) was kindly provided by the Department of Biochemistry, Utrecht University, Utrecht, The Netherlands. SDZ-GPI-562 (GPI-562) was a kind gift of Dr H.-G. Zerwes (Novartis Pharmaceuticals, Basel, Switzerland) [20]. Pyrazolopyrimidine 1 (PP-1) was obtained from Alexis Biochemicals (San Diego, CA, U.S.A.). Receptor-associated protein (RAP) was kindly provided by Dr P. J. Lenting (University Medical Center, Utrecht, The Netherlands) and was prepared as described in [20a].

Lipoprotein isolation

Lipoproteins were isolated as described in [21]. In short, fresh non-frozen plasma from four healthy subjects containing less than 100 mg of lipoprotein (a)/l was pooled and LDL (density range, 1.019–1.063 kg/l) was isolated by sequential flotation in a Beckman L-70 ultracentrifuge [22]. Centrifugations (20 h,

175 000 g, 10 °C) were carried out in the presence of NaN_3 and EDTA. The LDL preparations contained only minimal amounts of thiobarbituric acid-reacting substances ('TBARS'; 0.2 ± 0.07 nmol/mg), lipid peroxides (6.7 ± 1.9 nmol/mg) and contaminating plasma proteins (below or within reported values for native LDL). Lipoprotein (a) concentrations were below 14 mg/l. Lipoprotein (a) antibodies were obtained from Apotech (Organon Technika, Rockville, IL, U.S.A.). Lipoproteins were stored at 4 °C under nitrogen for not longer than 14 days and before each experiment were dialysed overnight against 10^4 vol. of 150 mM NaCl. ApoB100 and lipoprotein (a) concentrations were measured using the Behring Nephelometer 100. The concentration of LDL was expressed as g of apoB100 protein/l.

Platelet isolation

Freshly drawn venous blood from healthy volunteers was collected with informed consent into 0.1 vol. of 130 mM trisodium citrate. The donors claimed not to have taken any medication for 2 weeks prior to blood collection. Platelet-rich plasma was prepared by centrifugation (200 g for 15 min at 22 °C). Gel-filtered platelets were isolated by gel filtration through Sepharose 2B equilibrated in Ca^{2+} -free Tyrode's solution (137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH_2PO_4 , 1.7 mM MgCl_2 and 11.9 mM NaHCO_3 , pH 7.25) containing 0.2 % BSA and 5 mM glucose. Gel-filtered platelets were adjusted to a final count of 2×10^{11} platelets/l and incubated with LDL at 37 °C without stirring or with thrombin at 37 °C with stirring (900 rev./min).

Western blotting

Gel-filtered platelets were incubated at 37 °C with LDL or thrombin as indicated in the Figure legends. After incubation, 100 μ l aliquots were mixed with cold lysis buffer (1:10, v/v) as described before [16] and subsequently taken up in Laemmli sample buffer. Samples were heated (5 min, 100 °C) prior to SDS/PAGE (8 %) and divided into two or more samples to allow analysis of the individual phosphorylation sites together with total FAK as a control for lane loading. Proteins were transferred electrophoretically (1 h, 100 volts) to a nitrocellulose membrane in 25 mM Tris, 192 mM glycine and 20 % methanol, pH 8.3, using a mini-protean system (Bio-Rad, Richmond, CA, U.S.A.). The blots were blocked in 5 % BSA/0.05 % Tween 20 in Tris-buffered saline (TBS; pH 7.4) for 1 h at 4 °C and incubated with the site-specific phosphorylated FAK antibody (1:10 000 in 1 % BSA/0.05 % Tween in TBS, 16 h, 4 °C). After washing, the membranes were incubated with horseradish peroxidase-labelled anti-rabbit antibody (1:10 000, 1 h, 4 °C) and phosphorylated FAK tyrosine residues were visualized using the ECL reaction. The total amount of FAK was determined with a monoclonal antibody directed against FAK.

For semi-quantitative determination of the amount of site-specific phosphorylated FAK, the density of the bands was analysed using ImageQuant software (Molecular Dynamics). Phosphorylation induced by incubation with 1 g/l LDL for 10 min was set at 100 % and the other samples of the same series were expressed as a percentage of this control to enable comparison between gels.

Immunoprecipitation

Platelets were incubated with LDL as indicated. After incubation, 500 μ l aliquots were mixed with cold lysis buffer (1:10, v/v) as described above and precipitated with polyclonal anti-FAK antibody (1 μ g) and Protein A-Sepharose for 5 h (4 °C). After

Figure 1 Tyrosine phosphorylation precedes Tyr-397 phosphorylation in LDL-induced FAK phosphorylation

(A, left) Platelets were incubated with LDL (1 g/l, 37 °C) for the indicated time periods. Site-specific FAK phosphorylation was analysed by SDS/PAGE and Western blotting using a site-specific antibody directed against phosphorylated FAK followed by ECL. The upper panel shows a Western blot of LDL-induced Tyr-397 phosphorylation, the middle panel LDL-induced Tyr-925 phosphorylation. The lower panel shows total FAK detected with a monoclonal antibody against FAK as a control for lane loading. (A, right) Semi-quantification of the blots was performed based on ImageQuant; data ($P < 0.05$ for 0.5–5 min) are expressed as the percentage of the intensity at 10 min incubation with LDL (100%, □). (B) Platelets were incubated with LDL (1 g/l, 37 °C) for the indicated time periods and phosphorylation of Tyr-861 and Tyr-577 was detected. Data ($P > 0.05$) are expressed as the percentage of the intensity at 10 min incubation with LDL (100%, □). (C) Phosphorylation of Tyr-397, Tyr-925 and Tyr-577 in platelets stimulated with thrombin (1 unit/ml, 37 °C, stirring at 900 rev./min). Data are expressed as a percentage of the intensity of 2 min stimulation with thrombin. All data are means $\pm$ S.D., $n \geq 3$.

washing with lysis buffer, samples were taken up in Laemmli sample buffer. Samples were heated (5 min, 100 °C) prior to the Western-blotting procedure. The blots were blocked in 5% BSA/0.05% Tween 20 in TBS (1 h, 4 °C) and incubated with the polyclonal Src antibody (1:1000 in 1% BSA/0.05% Tween in TBS, 16 h, 4 °C), which recognizes Fgr, Fyn, c-Src and Yes, or the polyclonal Fgr antibody, which specifically recognizes Fgr. After washing, the membranes were incubated with peroxidase-linked Protein A (1:10000, 1 h, 4 °C) and co-precipitation of Src family tyrosine kinases was visualized using the ECL reaction. As a control for equal lane loading, blots were reprobed with a monoclonal antibody directed against FAK.

Statistics

Data are expressed as means $\pm$ S.D. with number of observations (n), and were analysed using the Student's t test. Differences were considered significant at $P < 0.05$.

RESULTS

Tyr-925 phosphorylation precedes Tyr-397 phosphorylation in LDL-induced FAK phosphorylation

In a first series of experiments, the site-specific phosphorylation of Tyr residues of FAK was investigated in platelets incubated

Figure 2 Dose-response relationship of LDL-induced site-specific FAK phosphorylation

Platelets were incubated with the indicated concentrations of LDL (10 min, 37 °C) and samples were split for detection of phosphorylation of Tyr-925, Tyr-397, Tyr-577 (**A**), Tyr-407 and Tyr-861 (**B**) using site-specific antibodies directed against phosphorylated FAK or an antibody against total FAK for detection of equal lane loading. Data (means $\pm$ S.D., $n \geq 3$) are expressed as percentages of the intensity found at 10 min incubation with 1 g/l LDL (100%, $\square$).

with LDL. Stimulation with 1 g/l LDL induced a rapid phosphorylation of Tyr-397, which reached more than 90 % of maximal activation after 10 min (Figure 1A) or more (results not shown). LDL also triggered the phosphorylation of Tyr-925 but this was faster than that of Tyr-397 and 90 % complete after 1 min (Figure 1A). Tyr-577, located in the autoactivation loop, and Tyr-861 were phosphorylated with approximately the same rapidity, showing maximal phosphorylation after 5–10 min (Figure 1B). This was about the same rate as the phosphorylation of Tyr-397 and slower than the LDL-induced phosphorylation of Tyr-925. Phosphorylation of Tyr-397 and Tyr-925 was also measured in stirred platelet suspensions stimulated with thrombin. Here the phosphorylation of the two residues occurred almost simultaneously with a slightly faster phosphorylation of Tyr-397. Also, Tyr-577 was phosphorylated at approximately the same speed (Figure 1C).

Theoretically, the different phosphorylation kinetics of Tyr-397 and Tyr-925 in LDL-treated platelets might be due to differences in dose-response relationships, for instance as a result of heterogeneity of receptors or further signalling cascades to FAK. Experiments were therefore repeated with different concentrations of LDL to find the optimal concentration for Tyr-397 and Tyr-925 phosphorylation. As illustrated in Figure 2(A), both residues showed the same dose-response curves with optimal

activation at 0.1 g/l LDL or more. Thus at a saturating concentration of 1 g/l, LDL induced a faster phosphorylation of Tyr-925 than of Tyr-397, a difference not seen with thrombin. These findings point to a major difference between FAK phosphorylation by LDL and by thrombin.

Tyr-577 is one of the two sites in the autoactivation loop that when phosphorylated mediates the further intermolecular phosphorylation of FAK at Tyr-397 [23]. To investigate the contribution of this feedback loop, dose-response studies were performed with different concentrations of LDL and the phosphorylation of Tyr-577 was analysed. As also shown in Figure 2(A), an LDL concentration as low as 0.1 g/l already induced maximal phosphorylation of Tyr-577. This is in the range of the dose-response relationships observed for the phosphorylation of the Tyr-397 and Tyr-925 residues. Thus the phosphorylation of Tyr-397, Tyr-577 and Tyr-925 in LDL-treated platelets appears closely connected. In contrast, a 10-fold higher LDL concentration was required for maximal phosphorylation of Tyr-407 and Tyr-861 (Figure 2B). These findings point to the presence of two groups of Tyr residues differing in responsiveness to LDL with Tyr-397, Tyr-577 and Tyr-925 being maximally phosphorylated at low LDL concentrations and Tyr-407 and Tyr-861 requiring a 10-fold higher concentration of LDL for maximal phosphorylation.

Figure 3 LDL and thrombin induce distinct site-specific phosphorylation of FAK

(A) Platelets were incubated with LDL (1 g/l, 10 min, 37 °C) or thrombin (1 unit/ml, 2 min, 37 °C, 900 rev./min) and samples were divided for detection of equal lane loading and the site-specific phosphorylation of Tyr-397, Tyr-407, Tyr-577, Tyr-861 and Tyr-925. The control (C) for equal lane loading was performed with a monoclonal antibody against total FAK. (B) The phosphorylation patterns induced by LDL and by thrombin were compared in concurrent experiments, analysed on single blots and expressed as a percentage of LDL-induced Tyr phosphorylation related to thrombin-induced Tyr phosphorylation, which is set at 100% (means $\pm$ S.D., $n = 4$).

To further clarify the differences in site-specific FAK phosphorylation by LDL and thrombin, platelet suspensions were divided into two parts and stimulated concurrently with optimal concentrations of LDL or thrombin to achieve maximal phosphorylation of Tyr residues. Then samples were collected and analysed on the same gel. As illustrated in Figure 3, the phosphorylation of Tyr-925 induced by LDL was weaker than that induced by thrombin, reaching $49 \pm 15\%$ ($n = 4$) of the

phosphorylation induced by thrombin. Similar comparisons for the other phosphorylation sites revealed that LDL induced about 10% phosphorylation of Tyr-397, Tyr-407, Tyr-577 and Tyr-861 compared with the respective site-specific phosphorylations induced by thrombin.

Together these findings indicate that the site-specific phosphorylation of FAK by LDL reveals a dominant role for Tyr-925 that is faster and about 5-fold higher than the phosphorylation of the other Tyr residues. The data also show that the phosphorylation of FAK by LDL is incomplete, reaching about 50% of the Tyr-925 phosphorylation by thrombin and about 10% of the phosphorylation of the other Tyr residues.

Role of Src in LDL-induced phosphorylation of Tyr-397 and Tyr-925

Salazar and Rozengurt [9] reported that integrin-mediated but not GPCR-mediated Tyr-397 phosphorylation depends on Src kinase family members. To clarify the contribution of these kinases in LDL-induced FAK phosphorylation, experiments were repeated in the presence of the inhibitor PP-1. Pretreatment with PP-1 induced a decrease in Tyr-phosphorylated sites, showing 75% (Tyr-397) to 80% (Tyr-925) inhibition at 1 μ M PP-1 (Figure 4A). Thus in this respect LDL-induced phosphorylation of these residues showed the properties of integrin-mediated FAK phosphorylation. PP-1 also inhibited the thrombin-induced phosphorylation of these residues but the inhibition was incomplete, leaving about 50% of the phosphorylation of Tyr-397 and Tyr-925 undisturbed (Figure 4B). Possibly, this PP-1-insensitive phosphorylation reflected the integrin-independent signalling pathway initiated by thrombin receptors (PAR 1/3/4).

To investigate whether the contribution of Src family kinases in FAK phosphorylation by LDL was accompanied by association of these proteins, immunoprecipitation studies were carried out with an anti-FAK antibody followed by Western blotting with an antibody against Src family members. Co-precipitation between FAK and a Src member was observed after 1 and 10 min stimulation with LDL (Figure 4C). The protein that co-immunoprecipitated with FAK had a molecular mass of approx. 55 kDa, indicating that it was not c-Src (60 kDa). This difference was confirmed in immunoprecipitates of thrombin-treated platelets showing co-precipitation of FAK with two bands, a similar 55 kDa protein and, in addition, a 60 kDa protein probably representing c-Src. Thus LDL-induced FAK phosphorylation appears to involve a specific member of the Src family kinases of 55 kDa. The involvement of Fgr was confirmed by reprobing with a specific Fgr antibody (results not shown). A time course of LDL-induced complex formation between FAK and Fgr revealed a rapid association after 0.17–0.5 min followed by dissociation 10 min later (Figure 4D). Thus FAK and Fgr associate in a period in which the different tyrosine residues of FAK are phosphorylated.

Role of integrin α IIb β 3 in the phosphorylation of Tyr-397 and Tyr-925

We have shown previously that LDL-induced FAK phosphorylation was unchanged in the presence of inhibitors of integrin α 2 β 1 and α IIb β 3 and also occurred normally in platelets deficient in α IIb β 3 [16]. To confirm this observation for the phosphorylation of Tyr-397 and Tyr-925, platelets were incubated with fibrinogen- γ -chain-derived dodecapeptide ($\gamma_{400-411}$) and

Figure 4 LDL-induced Tyr-397 and Tyr-925 phosphorylation depends on Src family tyrosine kinases

Platelets were incubated with the Src family tyrosine kinases inhibitor PP-1 (1, 5 and 10 μM , 15 min) prior to incubation with (A) LDL (1 g/l, 10 min, 37 °C) or (B) thrombin (1 unit/ml, 2 min, 37 °C, stirring at 900 rev./min). Tyr-397 and Tyr-925 phosphorylation was detected (means $\pm$ S.D., $n = 4$). (C) Complex formation between FAK and Src family tyrosine kinases was measured by incubating platelets with LDL (1 g/l, 1 and 10 min, 37 °C) or thrombin (1 unit/ml, 2 min, 37 °C, stirring at 900 rev./min) followed by immunoprecipitation (IP) with a polyclonal antibody against FAK and Western blotting (WB) with an antibody against Src family members. As a control for equal lane loading blots were reprobed with a monoclonal antibody against total FAK (lower panel). (D) Platelets were incubated with LDL (1 g/l, 10 min, 37 °C) for the indicated time periods and complex formation between FAK and Fgr was analysed with a specific antibody against Fgr.

the inhibitor GPI-562, which are both potent blockers of fibrinogen binding to the activated integrin [20]. Both inhibitors failed to affect the phosphorylation of Tyr-397 and Tyr-925 in platelets treated with LDL in accordance with the previous data based on total FAK phosphorylation (Figure 5A). In contrast, $\gamma_{400-411}$ and GPI-562 reduced the phosphorylation of these residues by 50–60 % when platelets were stimulated with thrombin (Figure 5B). This result is consistent with integrin-dependent and integrin-independent signalling pathways, both induced by thrombin receptors.

RAP inhibits LDL-induced Tyr phosphorylation

Recently, a member of the LDL receptor family named LRP8 was identified on platelets [24]. Ligand binding to LRP8 is known to be inhibited by the 39 kDa RAP. To investigate whether FAK activation by LDL was mediated by LRP8, studies were repeated in the presence of this inhibitor. Pretreatment with increasing concentrations of RAP for 10 min induced a dose-dependent inhibition of the phosphorylation of Tyr-397 and Tyr-925. There was no significant difference in the degree of inhi-

Figure 5 LDL-induced Tyr-397 and Tyr-925 phosphorylation is independent of integrin α IIb β 3

Platelets were incubated with the fibrinogen $\gamma_{400-411}$ peptide (γ -pept; 100 μ M, 2 min) or the α IIb β 3 blocker GPI-562 (10 nM, 1 min) prior to incubation with (A) LDL (1 g/l, 10 min, 37 °C) or (B) thrombin (thr; 1 unit/ml, 2 min, 37 °C, 900 rev./min). Tyr-397 and Tyr-925 phosphorylation was detected as described (means $\pm$ S.D., $n = 4$).

bition between the two tyrosine residues (Figure 6A). No such inhibition was found in thrombin-treated platelets (Figure 6B). Also, the formation of a complex between FAK and Fgr was reduced in the presence of RAP, illustrating the importance of the association between these proteins for the phosphorylation of Tyr-397 and Tyr-925 (Figures 6C and 6D). These data suggest that LDL initiates site-specific phosphorylation of FAK, at least in part via activation of a member of the LDL receptor family.

DISCUSSION

The present report shows that LDL-induced signalling to FAK shows discrepancies as well as similarities with integrin- and GPCR-mediated FAK phosphorylation. First, LDL induces a faster and more complete phosphorylation of Tyr-925 than of Tyr-397, which contrasts with thrombin-mediated responses where the phosphorylation of these residues go hand in hand. Second, LDL-induced phosphorylation of Tyr-397 and Tyr-925 is completely inhibited by the Src-inhibitor PP-1, in common with integrin-mediated signalling to FAK but not with GPCR-mediated responses. Third, LDL-induced phosphorylation of Tyr-397 and Tyr-925 is unchanged by inhibitors of ligand binding to integrin α IIb β 3 although its sensitivity to PP-1 would suggest otherwise.

LDL induced a faster phosphorylation of Tyr-925 than of Tyr-397 whereas with thrombin the phosphorylation of the two

residues was almost equally rapid. LDL also induced a 5-fold higher phosphorylation of Tyr-925 than of Tyr-397, Tyr-407, Tyr-577 and Tyr-861 under equilibrium conditions. Compared with thrombin, the phosphorylation induced by LDL was incomplete, resulting in a 50% (Tyr-925) to 90% (Tyr-397, Tyr-407, Tyr-861) lower Tyr phosphorylation. These findings reveal major differences between the two agonists in the regulation of kinase and phosphatase activities that control the phosphorylation of FAK.

There is general consensus that Tyr-397 is a first target for FAK activation and that its phosphorylation is a prerequisite for subsequent phosphorylation of the other Tyr residues. Mutation of Tyr-397 to the non-phosphorylatable residue phenylalanine impaired FAK phosphorylation, association of Src and initiation of signal transduction in COS cells. Hence, Tyr-397 phosphorylation seems crucial for the biochemical and biological functions of FAK [8,25].

Kinetic studies in LDL-stimulated platelets showed a similar time course of Tyr-397, Tyr-577 and Tyr-861 phosphorylation and a 5–10 times faster phosphorylation of Tyr-925. Interestingly, no such discrepancy was found in thrombin-stimulated platelets where the phosphorylation of Tyr-925, Tyr-397 and Tyr-577 was equally fast. Tyr-576 and Tyr-577 are located in the activation loop and their phosphorylation is thought to contribute to the phosphorylation of Tyr-397 [23]. The much faster phosphorylation of Tyr-925 in LDL-treated platelets suggests the presence of an additional control step between Tyr-577 and Tyr-925 phosphorylation, which is dormant in thrombin-treated cells.

Figure 6 RAP inhibits LDL-induced Tyr-397 and Tyr-925 phosphorylation

Platelets were (A) incubated with RAP (0.001–10 $\mu\text{g/mL}$, 10 min, 37 °C) prior to incubation with LDL (1 g/L, 10 min, 37 °C) or (B) incubated with RAP (10 $\mu\text{g/mL}$, 10 min, 37 °C) prior to incubation with thrombin (1 unit/mL, 2 min, 37 °C, 900 rev./min). The blots were semi-quantified and the data were expressed as a percentage of Tyr-397 and Tyr-925 phosphorylation in the absence of RAP. Data are means $\pm$ S.D., $n \geq 3$ ($P > 0.05$ between Tyr-397 and Tyr-925 phosphorylation). (C, D) The results of an immunoprecipitation (IP) with anti-FAK antibody followed by Western blotting (WB) with anti-Fgr antibody (means $\pm$ S.D., $n = 3$, $P < 0.05$). As a control (c) for equal lane loading blots were reprobed with a monoclonal antibody against total FAK (lower panel).

Dose-response studies revealed two types of responsiveness to increasing concentrations of LDL. The phosphorylation of Tyr-925, Tyr-397 and Tyr-577 was maximal at about 0.1 g/L LDL but maximal phosphorylation of Tyr-407 and Tyr-861 required a 10-fold higher concentration. It is difficult to explain this difference at the level of the kinases and phosphatases that control the phosphorylation status of the different Tyr residues. Instead, it might be the result of complex regulatory mechanisms at the level of the LDL receptor with, for instance, homo- or heterotypic receptor dimerization or changes in affinity and avidity, triggering different signalling routes to FAK.

Integrin-mediated cell adhesion initiates phosphorylation of Tyr-397 via Src family tyrosine kinases whereas GPCR-mediated phosphorylation of Tyr-397 is independent of this kinase family [9]. This report shows that LDL initiates the phosphorylation of Tyr-925 and Tyr-397 via Src tyrosine kinases but is unaffected by inhibitors of ligand binding to integrin $\alpha\text{IIb}\beta 3$. This finding accords with the normal FAK phosphorylation observed in $\alpha\text{IIb}\beta 3$ -deficient platelets [16]. FAK activation in platelets has been demonstrated upon treatment with collagen, an agonist of integrin $\alpha 2\beta 1$ and glycoprotein VI [26,27], thrombin, an inducer of platelet aggregation via integrin $\alpha\text{IIb}\beta 3$ (this study and [15]), and also with agents that cross-link Fc γ RIIa [28], indicating the presence of integrin-dependent and -independent signalling pathways to FAK.

In chicken embryo cells, FAK forms stable complexes with c-Src and Fyn via binding to their SH2 domains, thereby targeting the cytoplasmic Src members to focal adhesions [29]. When platelets are stimulated with thrombin, Src translocates from the Triton X-100-soluble fraction to the cytoskeleton-rich Triton X-100-insoluble fraction that is also enriched in FAK. The translocation is absent in $\alpha\text{IIb}\beta 3$ -deficient platelets, illustrating the importance of this integrin in the targeting of Src to focal adhesions [30]. Also in LDL-stimulated platelets FAK associates with a member of the Src family. The two proteins remain associated during the period in which the different Tyr residues are phosphorylated and thereafter dissociate. The Src family tyrosine kinases are cytosolic proteins with molecular masses ranging from 53 to 62 kDa. In platelets stimulated by LDL, FAK associates with a protein of approx. 55 kDa. The anti-Src antibody used in the precipitation experiments is directed against several members of the Src family, including Fgr (55 kDa), Fyn (59 kDa), c-Src (60 kDa) and Yes (62 kDa), but only a single band at approx. 55 kDa was detected. Reprobing with a specific antibody identifies this 55 kDa protein as the Src family member Fgr. Fgr is a negative regulator of $\beta 2$ integrin signalling and cell spreading in monocytes and has recently been identified in human platelets [31,32]. In contrast, thrombin-induced FAK phosphorylation appears to involve a more complex association with Src family members, including both Fgr and c-Src. It is

possible that these different associations between FAK and Src family members explain the different Tyr-phosphorylation patterns induced by LDL and thrombin. They may also explain differences in downstream signalling, with LDL failing to induce the activation of Ras and thrombin being a potent activator of this small GTPase [33,34].

Thrombin induced the phosphorylation of Tyr-925 and Tyr-397 in stirred platelet suspensions. This response was inhibited by about 50% in the presence of the Src inhibitor PP-1 and the α IIB/ β 3 antagonists $\gamma_{400-411}$ and GPI-562. Under the same conditions, thrombin induced the association of FAK with Fgr and c-Src. These findings suggest that the activated thrombin receptors (PAR 1/3/4 [35]) initiate two signalling pathways to FAK. First, there is the GPCR-mediated activation of FAK via Src-independent signalling steps. The low but significant FAK phosphorylation induced by thrombin in unstirred platelet suspensions might be a result of this pathway (results not shown). Second, there is the activation of integrin α IIB/ β 3 via inside-out signalling mechanisms followed by ligand binding and further outside-in signalling to FAK. This second pathway might include formation of a FAK–Fgr–c-Src complex. Thus, together with the LDL-induced formation of a FAK–Fgr complex, there appear to be at least three different mechanisms for the regulation of FAK with possibly important consequences for FAK activity and formation of focal adhesions.

So far, the identity of the platelet receptor that transmits the signalling properties of LDL to intracellular messenger systems has not been elucidated. The observation that platelets are activated by a peptide that mimics a part of apoB100, a major protein constituent of LDL, suggests that the platelet receptor has properties in common with the apoB/E receptor on fibroblasts [19]. In a recent publication Riddell et al. [24] reported the presence of a member of the LDL receptor family on platelets, named LRP8. RAP, a 39 kDa protein that consists of three homologous domains, is an antagonist for receptor–ligand interactions of LDL receptor family members. The results show that RAP interfered with the phosphorylation of Tyr-397 and Tyr-925 induced by LDL, resulting in an inhibition of 60–75%. In addition, RAP interfered with the formation of the FAK–Fgr complex. Both observations suggest that LRP8 or a closely related receptor family member serves a role in the FAK activation by LDL. This might imply that the site-specific Tyr phosphorylation induced by LDL, which differs from that induced by integrins and GPCRs, reflects a novel mechanism of FAK activation that is typical for members of the LDL receptor family.

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