Low density lipoprotein (LDL) is known to sensitize platelets to agonists via integrin mediated outside-in signaling (Hackeng, C. M., Huigeoort, M., Pladet, M. W., Nieuwenhuis, H. K., Rijn, H. J. M. v., and Ackerman, J. W. N. (1999) Arterioscler. Thromb. Vasc. Biol., in press). As outside in signaling is associated with phosphorylation of p125FAK, the effect of LDL on p125FAK phosphorylation in platelets was investigated. LDL induced p125FAK phosphorylation in a dose- and time-dependent manner. The phosphorylation was independent of ligand binding to integrin αIIbβ3 and aggregation, such as in contrast to α-thrombin-induced p125FAK phosphorylation, that critically depended on platelet aggregation. Platelets from patients with Glanzmann’s thrombastenia showed the same LDL-induced phosphorylation of p125FAK as control platelets, whereas α-thrombin completely failed to phosphorylate the kinase in the patients platelets. LDL signaling to p125FAK may play a central role in signaling pathways mediated by integrins, G-protein coupled receptors, tyrosine kinase receptors, and the v-Src and v-Crk oncoproteins (1). After cell activation, p125FAK translocates to the cytoskeleton at focal adhesions (2–4), where it serves as a docking site for signaling proteins (5, 6). The protein contains six tyrosine phosphorylation sites. Autophosphorylation of Tyr997 generates a high affinity binding site for the SH2 domain of Src family kinases. The association of Src subsequently initiates phosphorylation of Tyr407,576,577, inducing maximal kinase activity of p125FAK (7). Association of Src also leads to the phosphorylation of Tyr925, thereby creating a docking site for the adaptor protein Grb2 that is known to mediate ras activation by binding of the GDP/GTP exchange factor Sos, linking p125FAK to the Ras/MAP kinase pathway (8). The relevance of phosphorylation on Tyr925 is not entirely clear, but it could serve as another site of p125FAK interaction with Src-family kinases. Phosphorylation of p125FAK in blood platelets is different for platelets in suspension and platelets adherent to immobilized ligand. In platelet suspensions, p125FAK phosphorylation only occurred under aggregating conditions (9). An antibody against integrin αIIbβ3, that blocked fibrinogen binding and aggregation, totally abolished p125FAK phosphorylation by α-thrombin and collagen in stirred suspensions. In the absence of stirring, α-thrombin (9) or the αIIbβ3-activating antibody LIBS6 (10) failed to induce p125FAK phosphorylation. The role for αIIbβ3 in this signaling event was further supported by platelets from patients with Glanzmann’s thrombastenia, that lack αIIbβ3, in which neither α-thrombin nor collagen induced p125FAK phosphorylation in stirred suspensions (9).

Platelets adherent to immobilized ligand show αIIbβ3-dependent and -independent p125FAK phosphorylation. Platelets bound to fibrinogen show p125FAK phosphorylation via αIIbβ3. Platelets from a patient with a truncated cytoplasmic domain of the β3-subunit (11) and Chinese hamster ovary cells transfected with truncated forms of the β3-subunit (12) bound to immobilized fibrinogen but failed to induce phosphorylation of p125FAK. Expression of a constitutively active mutant of β3 together with αIIb led to a slight degree of p125FAK phosphorylation in suspended Chinese hamster ovary cells in the presence of fibrinogen. However, this phosphorylation was negligible compared with the same cells adherent to fibrinogen (13). Also αIIbβ3-independent p125FAK phosphorylation has been described. Collagen (14–16) and immunoglobulins (15) immobilized on a surface induced p125FAK phosphorylation in the presence of an anti-αIIbβ3 antibody and in Glanzmann’s platelets. Hence, in platelets p125FAK may play a central role in signal transduction after αIIbβ3 ligation or in platelet adhesion, thereby strengthening ligand-receptor interaction and coordinating further signaling.

Low density lipoprotein (LDL) is known to increase the sensitivity of human platelets to different agonists (17–20), but the intracellular mechanisms involved remain largely unknown. Among the signal transducing elements that are activated by LDL are protein kinase C (PKC) (21, 22), Ca2+ mobi-
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gate whether the phosphorylation of p125FAK by LDL involved ligand-independent signaling via \( \alpha_{IIb} \beta_{3} \), or completely bypassed the integrin, the experiments were repeated with platelets from patients with Glanzmann’s thrombastenia that are deficient in \( \alpha_{IIb} \beta_{3} \). Fig. 3B shows the phosphorylation of p125FAK by LDL in the absence and presence of \( \gamma_{400-411} \) in Glanzmann’s platelets, which was the same as seen in normal subjects. In stirred suspensions of Glanzmann’s platelets, \( \alpha \)-thrombin completely failed to induce p125FAK phosphorylation, both in the absence or presence of \( \gamma_{400-411} \). From these experiments we conclude that LDL is a unique platelet agonist, as it initiates phosphorylation of p125FAK in unstirred platelet suspensions, independent of integrin \( \alpha_{IIb} \beta_{3} \).

Characterization of Signaling Pathways Involved in LDL-induced p125FAK Phosphorylation—To investigate whether LDL-induced p125FAK phosphorylation involved the same signaling mechanisms as seen in surface-activated platelets, incubations were performed with agents that interfere with signal processing at the level of surface receptors (Fig. 4A) or intracellular signaling routes (Fig. 4B). As already shown in Fig. 3A, \( \gamma_{400-411} \) did not affect p125FAK phosphorylation. Earlier work has shown that collagen-induced p125FAK phosphorylation was mediated by integrin \( \alpha_{IIb} \beta_{3} \), and was inhibited by the antibody 6F1 against this integrin (16). p125FAK phosphorylation by immobilized IgG was inhibited by anti FcγRII antibody IV-3 (15). None of these antibodies affected LDL-induced...
p125FAK phosphorylation. Also inhibition of thromboxane A2
(TxA2)-formation by indomethacin had no effect. As it was
reported that p125FAK phosphorylation is regulated via PKC in
platelets adherent to immobilized fibrinogen (35) or collagen
(15), the studies were repeated in the presence of the PKC
inhibitor GF109203X, but p125FAK phosphorylation remained
inhibitor GF109203X, but p125FAK phosphorylation remained
(41). As activated platelets release LPA in vitro (42) and possibly
in vivo (43) and aggregation. This is in sharp contrast to
phosphorylation by LDL remained to be elucidated. A rise in cAMP leads to activation of protein kinase A, that in turn can activate vasodilator-stimulated protein (VASP), a 50-kDa protein that localizes to focal adhesions and regulates actin dynamics (47). Phosphorylation of VASP correlates with a decrease in α1β3 activation and aggregation (48). Thus, CAMP might inhibit LDL signaling to p125FAK via VASP by preventing cytoskeleton rearrangements.

It has been reported that phosphorylation of p125FAK on
Tyr(407,576,577), inducing maximal kinase activity of the protein
(7), but the importance of p125FAK activity remains unclear.
P125FAK knockout mice were not viable, and embryonic cells of
these mice had a reduced mobility. Surprisingly, the number of
focal adhesions in these mice was increased. From these observations, it was proposed that p125FAK regulates focal contact turnover (49), rather than their formation.

Two major signaling mechanisms downstream of p125FAK
are: (i) p130CAS associates to one of the two C-terminal proline-rich regions of p125FAK. This association results in phosphorylation of p130CAS and subsequent binding of Crk via its SH2

LDL activates p125FAK in platelets

The present findings show that LDL triggers the phosphorylation of p125FAK in platelet suspensions independent of integrin α1β3 and aggregation. This is in sharp contrast to phosphorylation by α-thrombin, collagen, and costimulation of epinephrine with ADP (9) or LILBS6 Fab fragments (33) in cell suspension, which requires ligand binding to α1β3 and platelet-platelet contact. The requirement for ligand-induced outside-in signaling is illustrated by the absence of p125FAK phosphorylation under conditions that prevent platelet spreading (10, 11) (reviewed in Ref. 5). LDL induces p125FAK phosphorylation within seconds after stimulation, reaching a maximum at physiological concentrations of LDL (0.26–1.23 g of apoB100/liter) (44). LDL signaling to p125FAK is not dependent on TxA2 formation or activation of PKC, p38MAPK, and ERK1/2. These observations indicate that p125FAK phosphorylation occurs directly downstream of the LDL receptor.

P125FAK is phosphorylated independent of α1β3 when platelets adhere to immobilized collagen via integrin α2β1 (14–16) or to immobilized IgG via the FcyRII receptor (15). It is therefore possible that the LDL particle acts as an activating surface that phosphorylates p125FAK by clustering of membrane receptors. However, antibodies against integrin α2β1 and FcyRII, known to block further signal generation to p125FAK, had no effect. Additionally, LPA did not induce p125FAK phosphorylation. Another candidate for LDL-induced p125FAK phosphorylation is the collagen receptor glycoprotein (GPVI). Collagen signaling through this glycoprotein leads to phosphorylation of p125FAK (45) and is inhibited by cAMP (46). However, GPVI-mediated p125FAK phosphorylation is not observed in Glaunzmann’s platelets or in the presence of the RGDS peptide, such in contrast to the effect of LDL. The nature of the cAMP sensitivity of p125FAK phosphorylation by LDL remains to be elucidated. A rise in cAMP leads to activation of protein kinase A, that in turn can activate vasodilator-stimulated protein (VASP), a 50-kDa protein that localizes to focal adhesions and regulates actin dynamics (47). Phosphorylation of VASP correlates with a decrease in α1β3 activation and aggregation (48). Thus, CAMP might inhibit LDL signaling to p125FAK via VASP by preventing cytoskeleton rearrangements.

It has been reported that phosphorylation of p125FAK on
Tyr907 induces Src activity, leading to phosphorylation of Tyr1057/767, inducing maximal kinase activity of the protein
(7), but the importance of p125FAK activity remains unclear.
P125FAK knockout mice were not viable, and embryonic cells of
these mice had a reduced mobility. Surprisingly, the number of
focal adhesions in these mice was increased. From these observations, it was proposed that p125FAK regulates focal contact turnover (49), rather than their formation.

Two major signaling mechanisms downstream of p125FAK
are: (i) p130CAS associates to one of the two C-terminal proline-rich regions of p125FAK. This association results in phosphorylation of p130CAS and subsequent binding of Crk via its SH2

FIG. 4. LDL-induced p125FAK phosphorylation is not affected by a wide variety of platelet inhibitors. A, platelets were incubated with vehicle, γ-irradiated LDL (200 μg, 2 min) or antibody (2 mg/liter, 30 min, 37 °C). Data were analyzed as indicated in the legend to Fig. 1B. Data were corrected for background intensities without LDL and expressed as means ± S.D., n = 3. B, platelets were incubated with vehicle, indomethacin (30 μM, 15 min), GF109203X (5 μM, 1 min), PD98059 (20 μM, 10 min), SB203580 (10 μM, 15 min), PGE2 (10 ng/ml, 2 min), iloprost (2 μg/ml, 15 min), or Bt2cAMP (250 μM, 10 min) before addition of LDL (1.0 g/liter, 10 min). Phosphotyrosine-containing p125 FAK was identified using 4G10 as a precipitating antibody. Data were analyzed as indicated in the legend to Fig. 1B. Data were corrected for background intensities without LDL and expressed as means ± S.D., n = 3. C, platelets were incubated with vehicle, LDL (1 g/liter), or LPA at the indicated concentrations and time periods. Phosphotyrosine-containing p125FAK was identified using 4G10 as a precipitating antibody.

Taken together, these data show that LDL-induced phospho-
domain (reviewed in Refs 5 and 6). Subsequently, Crk can associate with the GDP/GTP exchange factors Sos (for Ras) or C3G (for Rap1) (50). (ii) The GTPase regulator associated with FAK (Graf) associates directly to p125FAK via the proline-rich region of residues Pro757–Arg860 in an SH3 domain-dependent manner. Activation of Graf was induced by phosphorylation mediated by ERK1/2 and was shown to stimulate the GTPase activity of CDC42 and Rho, but not Ras or Rac (51). The implications of CDC42 and Rho activity for stress fiber and focal adhesion assembly (52) led to the proposition that activation of Graf might down-regulate these CDC42 and Rho-mediated cytoskeletal changes (1).

Recently, we observed that LDL induced activation of the small GTPases Rap1 and Rac in platelets.2 Activation of Rap1 and Rac critically depend on Ca2+ (53, 54), and Rac is a putative effector molecule of Rap1 (55). In turn, Rac is thought to be an upstream regulator of a member of the Rac/Rho family, CDC42. This small GTPase is involved in the rearrangement of the cytoskeleton. The strong phosphorylation of p125FAK triggered by LDL described in the present report might therefore initiate these two pathways: (i) activation of Rap1 and Rac and CDC42 via C3G and (ii) cytoskeleton regulation via Rac-activated Rho, controlled by Graf.

In conclusion, via these two separate mechanisms, LDL might be controlling cytoskeleton rearrangements, thereby targeting several signal transducing proteins to an appropriate site of action, leading to an increased sensitivity to platelet agonists such as α-thrombin and collagen.

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