Activation-dependent Adhesion of Human Platelets to Cyr61 and Fisp12/Mouse Connective Tissue Growth Factor Is Mediated through Integrin α_{IIb}β_{3}*

(Received for publication, April 28, 1999)

Arom Jeddasayananmaë, Chih-Chiu Chen§, Maria L. Kireeva§§, Lester F. Lau§, and Stephen C.-T. Lam¶

From the Departments of §Pharmacology and ¶Molecular Genetics, University of Illinois, Chicago, Illinois 60612

Cyr61 and connective tissue growth factor (CTGF), members of a newly identified family of extracellular matrix-associated signaling molecules, are found to mediate cell adhesion, promote cell migration and enhance growth factor-induced cell proliferation in vitro, and induce angiogenesis in vivo. We previously showed that vascular endothelial cell adhesion and migration to Cyr61 and Fisp12 (mouse CTGF) are mediated through integrin α_{IIb}β_{3}. Both Cyr61 and Fisp12/mCTGF are present in normal blood vessel walls, and it has been demonstrated that CTGF is overexpressed in advanced atherosclerotic lesions. In the present study, we examined whether Cyr61 and Fisp12/mCTGF could serve as substrates for platelet adhesion. Agonist (ADP, thrombin, or U46619)-stimulated but not resting platelets adhered to both Cyr61 and Fisp12/mCTGF, and this process was completely inhibited by prostaglandin I_{2} which prevents platelet activation. The specificity of Cyr61- and Fisp12/mCTGF-mediated platelet adhesion was demonstrated by specific inhibition of this process with polyclonal anti-Cyr61 and anti-Fisp12/mCTGF antibodies, respectively. The adhesion of ADP-activated platelets to both proteins was divergent cation-dependent and was blocked by RGDS, HHLGGAKQAGDV, or echistatin, but not by RGES. Furthermore, this process was specifically inhibited by the monoclonal antibody AP-2 (anti-α_{IIb}β_{3}), but not by LM609 (anti-α_{IIa}β_{3}), indicating that the interaction is mediated through integrin α_{IIb}β_{3}. In a solid phase binding assay, activated α_{IIb}β_{3} was purified by RGD affinity chromatography, bound to immobilized Cyr61 and Fisp12/mCTGF in a dose-dependent and RGD-inhibitable manner. In contrast, unactivated α_{IIb}β_{3} failed to bind to either protein. Collectively, these findings identify Cyr61 and Fisp12/mCTGF as two novel activation-dependent adhesive ligands for the integrin α_{IIb}β_{3} on human platelets, and implicate a functional role for these proteins in hemostasis and thrombosis.

Platelet adhesion to the subendothelial matrix and platelet aggregation are key mechanisms by which platelets participate in hemostasis and thrombosis. Thus, upon vascular injury, platelets adhere to the exposed subendothelial matrix, leading to platelet aggregation caused by the binding of plasma fibrinogen or von Willebrand factor (vWF)1 to the activated platelets. Integrin α_{IIb}β_{3} is the most prominent platelet adhesion receptor, which interacts with several adhesive ligands including fibrinogen, vWF, fibronectin, and vitronectin (1–3). On resting platelets, α_{IIb}β_{3} is present in an inactive conformation incapable of binding soluble adhesive proteins. The formation of platelet agonists such as thrombin at sites of vessel injury induces platelet inside-out signaling, which leads to the binding of soluble fibrinogen and vWF to α_{IIb}β_{3}, resulting in platelet aggregation.

In normal blood vessels, the major matrix components in the subendothelium mediating platelet adhesion are thought to be vWF, fibronectin, collagen, and laminin. Initial platelet adhesion to damaged vessel walls is thought to involve both non-integrin (e.g. the GPlb-IX-V complex) and integrin adhesion receptors (e.g. α_{2}β_{1}, α_{5}β_{1}, and α_{6}β_{1}) in addition to α_{IIb}β_{3} (2, 4, 5). During the development and progression of atherosclerosis, activation of cellular components in the atherosclerotic plaques may generate as yet undefined substrates that mediate platelet adhesion to ruptured plaque lesions. In this regard, it has recently been shown that activated platelets adhere to osteopontin in atherosclerotic plaques through integrin α_{IIb}β_{3} (6). Another extracellular matrix-associated protein, connective tissue growth factor (CTGF), was found to be overexpressed in advanced atherosclerotic lesions as compared with normal blood vessels (7).

CTGF belongs to an emerging family of conserved and modular proteins with diverse biological functions (8, 9). Six members of this protein family, including CTGF and Cyr61, have been described to date. Both Cyr61 and Fisp12, the mouse ortholog of CTGF, were identified as products of immediately genes transcriptionally induced in fibroblasts in response to serum growth factors (10–13). Upon synthesis, both proteins are secreted and become associated with the cell surface and the extracellular matrix (14, 15). Both Cyr61 and Fisp12/mCTGF have been shown to mediate adhesion and to promote migration in vascular endothelial cells (16–18). Although neither protein alone induces mitogenesis in vascular endothelial cells, both are able to augment growth factor-induced DNA synthesis (15, 16). Furthermore, Fisp12/mCTGF can promote vascular endothelial cell survival under conditions that induces apoptosis (17). All of these activities are pro-angiogenic; indeed, both Cyr61 and Fisp12/mCTGF were found to induce angiogenesis in vivo in corneal micropocket assays (17, 18). While the

* This work was supported by National Institutes of Health Grants HL41793 (to S. C.-T. L.) and CA46565 and CA80080 (to L. F. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Present address: Laboratory of Molecular Mechanisms of Transcription, NCI, National Institutes of Health, Frederick, MD 21702.

‖ To whom correspondence should be addressed: Dept. of Pharmacology (M/C 888), University of Illinois, 835 S. Wolcott Ave., Chicago, IL 60612. Tel: 312-413-5928; Fax: 312-996-1225; E-mail: slam@uic.edu.

1 The abbreviations used are: vWF, von Willebrand factor; CTGF, connective tissue growth factor; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody; PGI_{2}, prostaglandin I_{2}.
mechanism through which Cyr61 and Fisp12/mCTGF induce angiogenesis in vivo is not known, biochemical and functional evidence indicates that the integrin αIβ3 serves as a receptor on endothelial cells for Cyr61 and Fisp12/mCTGF mediating cell adhesion and migration (17–19).

Both Cyr61 and CTGF proteins are present in normal and diseased blood vessel walls (7, 20). Based on the similarity of ligand recognition specificity between integrins αIβ3 and αIIBβ3, we postulate that Cyr61 and Fisp12/mCTGF may serve as adhesive substrates for the platelet integrin αIIBβ3. In the present study, we show that both Cyr61 and Fisp12/mCTGF support the adhesion of platelets in an activation-dependent manner. Furthermore, active but not inactive αIIBβ3 binds directly to purified Cyr61 or Fisp12/mCTGF in a solid-phase binding assay. Thus, these studies identify two novel activation-dependent adhesive ligands for human platelets, and implicate a functional role for these proteins in hemostasis and thrombosis.

MATERIALS AND METHODS

Antibodies, Peptides, and Reagents—The anti-β3 monoclonal antibody, 3C12 (21), was provided by Dr. Mark H. Ginsberg of the Scripps Research Institute, La Jolla, CA, and was radiiodinated with carrier-free Na125I (Amersham Pharmacia Biotech) using the IODO-BEADS iodination reagent (Pierce) to a specific activity of approximately 2 μCi/μg. The monoclonal antibodies AP-2 (22) and LM609 (23) were generous gifts of Dr. T. J. Kunicki and Dr. D. A. Cheresh, respectively, of the Scripps Research Institute, La Jolla, CA. Polyclonal anti-Cyr61 and anti-Fisp12/mCTGF antibodies were raised in rabbits as described previously (15), and purified by chromatography on protein A-Sepharose.

Peptide sequences are represented by the single-letter amino acid codes (24). The fibrinogen γ chain dodecapeptide H3γ, with the sequence HHLGGAKQAGDV was purchased from Research Genetics Inc. RGDS and RGES peptides were purchased from Peninsula Laboratories. Echistatin was purchased from Sigma, and fibrinogen was obtained from KabiVitrum, Inc.

Protein Purifications—Recombinant Cyr61 and Fisp12/mCTGF, synthesized in a baculovirus expression system using Sf9 insect cells, were purified from serum-free conditioned media by chromatography on Sepharose S as described (15, 16). SDS-PAGE analysis of purified proteins reacted specifically with their cognate antibodies (15). Activated αIIBβ3 was purified by RGD affinity chromatography as described (25). Briefly, outdated human platelets were isolated by differential centrifugation and solubilized in lysis buffer (10 mM HEPES, pH 7.4, 0.15 M NaCl, containing 1 mM CaCl2, 1 mM MgCl2, 100 μM leupeptin, 100 μM E-64, 1 mM benzamidine, 1 mM PMSF, 1 mM DTT, and 50 mM octyl glucoside). The octyl glucoside extract was incubated with 1 ml of GRDGSK-coupled Sepharose 4B overnight at 4 °C. After washing with 15 ml of column buffer (same as lysis buffer except it contained 25 mM octyl glucoside), bound αIIBβ3 was eluted with 1.7 mM H3γ (2 ml) in column buffer. The H3γ eluate was applied to a Sephacryl S-300 High Resolution column (1.5 × 95 cm), and αIIBβ3 was eluted with 10 mM HEPES, pH 7.4, 0.15 M NaCl, 1 mM CaCl2, 1 mM MgCl2, and 25 mM octyl glucoside.

Unactivated αIIBβ3 was isolated by the method of Fitzgerald et al. (26) with slight modifications. The flow-through fraction of the GRDGSK-Sepharose column was applied onto a concanavalin A-Sepharose 4B column (1 × 20 cm). Unbound proteins were washed with 50 ml of column buffer, and bound αIIBβ3 was then eluted with 100 mM mannose dissolved in column buffer. Fractions containing αIIBβ3 were further purified on a Sephacryl S-300 High Resolution column as described above.

Protein concentrations were determined using the BCA protein assay (Pierce) with bovine serum albumin (BSA) as the standard. In some experiments, to ensure that equal concentrations of activated and unactivated αIIBβ3 were used, the purified receptor preparations were subjected to SDS-PAGE and densitometric scanning of the silver-stained protein bands was performed.

Platelet Isolation and Adhesion Assay—Venous blood was drawn from healthy donors and collected into acid-citrate-dextrose. Washed platelets were prepared by differential centrifugation as described (27) and finally resuspended in HEPES-Tyrode’s buffer (5 mM HEPES, pH 7.35, 1 mM MgCl2, 1 mM CaCl2, 135 mM NaCl, 2.7 mM KCl, 11.9 mM NaHCO3, 1 mg/ml dextrose, and 3.5 mg/ml BSA). The platelet count was adjusted to 3 × 1011 platelets/ml.

Microtiter wells (Immulon 2 Removawell strips, Dynex Technologies, Inc.) were coated with Cyr61, Fisp12/mCTGF, or fibrinogen (25 μg/ml) overnight at 22 °C and then blocked with BSA at 37 °C for h. Washed platelets were added to the wells (100 μl/well) in the presence of absence of platelet agonists and incubated at 37 °C for 30 min. The wells were washed with HEPES-Tyrode’s buffer and adherent platelets were detected with 125I-t-mAb15, an anti-β3 monoclonal antibody. Binding of the labeled antibody (50 nm, 50 μl/well) proceeded for 1 h at 22 °C. After extensive washing with HEPES-Tyrode’s buffer, bound radioactivity was determined by γ-counting. In inhibition studies, washed platelets were preincubated with blocking peptides or antibodies at 37 °C for 15 min prior to addition to micortiter wells. In experiments to examine the effect of divalent cation chelation, EDTA (5 mM) was added to suspensions of washed platelets and preincubated at 37 °C for 15 min.

As indicated, adherent platelets were also detected by the acid phosphatase assay (28). Briefly, following the adhesion and washing procedures described above, the substrate solution (0.1 mM sodium acetate, pH 5.0, 20 mM p-nitrophenyl phosphate, and 0.1% Triton X-100; 150 μl/well) was added and incubated for 2 h at 37 °C. The reaction was stopped by the addition of 20 μl 2 N NaOH, and absorbance at 405 nm was measured.

RESULTS

Activation-dependent Adhesion of Human Platelets to Cyr61 and Fisp12/mCTGF—Recently, we reported that vascular endothelial cells adhere to Fisp12/mCTGF and Cyr61 through interaction with integrin αIβ3 (17, 19). In this study, we investigated whether these proteins could also support the adhesion of blood platelets. Micortiter wells were coated with purified recombinant Fisp12/mCTGF or Cyr61, and the adhesion of isolated platelets to these proteins was detected with 125I-t-mAb15, an anti-β3 monoclonal antibody. It is noteworthy that this antibody binds to both activated and unactivated αIIBβ3 indifferently. As controls, fibrinogen- and BSA-coated wells were also used. Initially, we compared the adhesion of unactivated versus activated platelets to immobilized Fisp12/mCTGF and Cyr61. To ensure that the platelets were not activated during the washing procedures, PGL2 (100 nM), which inhibits activation by raising platelet Campbell levels, was added to the platelet suspensions. Fig. 1 shows that unactivated platelets failed to adhere to either protein. However, activation of platelets with 0.1 unit/ml thrombin (panel A), 500 nM U46619 (panel B), or 10 μM ADP (panel C) caused a dramatic increase in platelet adhesion to both asp12/mCTGF- and Cyr61-coated wells. To confirm that the adhesion process is activation-dependent, PGL2 (100 nM) was added with the agonists to prevent platelet activation. Under these conditions, platelet adhesion to both Fisp12/mCTGF and Cyr61 was significantly inhibited. For comparison, platelet adhesion to fibrinogen-coated wells was assessed. While unactivated platelets were capable of adhering to immobilized fibrinogen at a low level as previously reported (29–31), platelet adhesion to Cyr61 and Fisp12/mCTGF appeared to be absolutely dependent on cellular activation (Fig. 1). Following platelet activation with strong agonists such as thrombin and U46619, platelet adhesion to Cyr61 and Fisp12/mCTGF was comparable to fibrinogen. However, the weaker agonist ADP caused a lesser response. Since ADP does not induce secretion of α-granule proteins from washed human platelets and does not induce platelet aggregation in the absence of exogenous fibrinogen (32), we therefore used...
ADP to induce platelet adhesion in later experiments.

To further substantiate the activation-dependent adhesion of platelets to these proteins, we performed an independent assay to quantitate the relative numbers of adherent platelets. This assay measured the acid phosphatase activity of adherent platelets. In Fig. 1 (C and D), both 125I-mAb15 binding and acid phosphatase assays were used to assess the adhesion of ADP-stimulated platelets to fibrinogen, Fisp12/mCTGF, and Cyr61, and similar results were obtained. Since the amounts of bound 125I-mAb15 were directly proportional to the numbers of integrin αIIbβ3 on adherent platelets, we used this method for quantitative studies hereafter.

Fig. 2 (A and B) shows that the adhesion of ADP-activated platelets to Fisp12/mCTGF and Cyr61 was dose-dependent and saturable. Again, in the presence of PGL2, unactivated platelets adhered poorly to both proteins even at high coating concentrations. The specificity of the adhesion process was characterized in inhibition studies using anti-peptide polyclonal antibodies raised against the central variable regions of Fisp12/mCTGF and Cyr61. On immunoblots, anti-Fisp12/mCTGF and anti-Cyr61 reacted specifically with Fisp12/mCTGF and Cyr61, respectively, and no cross-reactivity was observed (15). As shown in Fig. 3, anti-Fisp12/mCTGF inhibited platelet adhesion to Fisp12/mCTGF but not to Cyr61, and likewise, anti-Cyr61 inhibited Cyr61-mediated platelet adhesion but not that mediated by Fisp12/mCTGF. In specificity controls, no inhibition was observed with normal rabbit IgG. Additionally, neither anti-Fisp12/mCTGF nor anti-Cyr61 inhibited platelet adhesion to fibrinogen-coated wells. Thus, these findings indicated that the abilities of Fisp12/mCTGF and Cyr61 to mediate platelet adhesion are intrinsic properties of these proteins.

Identification of αIIbβ3 as the Receptor Mediating Platelet Adhesion to Fisp12/mCTGF and Cyr61—Upon platelet activation, the ligand binding affinities of integrin αIIbβ3 and αvβ3 are up-regulated (1, 6). To determine whether these integrin receptors mediate platelet adhesion to Fisp12/mCTGF and Cyr61, we tested the inhibitory effect of peptide antagonists and the divalent cation chelator EDTA. Fig. 4A shows that preincubation of platelets with EDTA at 37 °C completely abolished platelet adhesion to both proteins indicating that the adhesion process is divalent cation-dependent, consistent with the involvement of an integrin receptor. The major platelet integrin, αIIbβ3, is sensitive to inhibition by RGD-containing peptides and a dodecapeptide (H12) derived from the fibrinogen γ chain (33–35). As shown in Fig. 4A, the adhesion of ADP-activated platelets to Cyr61 and Fisp12/mCTGF was specifically inhibited by RGDS but not by RGES. Likewise, the RGD-containing snake venom peptide echistatin (36) also completely blocked platelet adhesion to both proteins. It has been shown that the dodecapeptide H12 preferentially interacts with integrin αIIbβ3 as compared with integrin αvβ3 (37, 38). Thus, the observation that H12 inhibited platelet adhesion to Cyr61 and Fisp12/mCTGF (Fig. 4A) suggest that this process is mediated by αIIbβ3 rather than αvβ3. Indeed, while the complex-specific monoclonal antibody AP-2 (anti-αIIbβ3) completely blocked the adhesion of ADP-activated platelets to Fisp12/mCTGF and Cyr61, no inhibition was observed with LM609 (anti-αvβ3) or with normal mouse IgG (Fig. 4B). In control samples, the adhesion of ADP-activated platelets to fibrinogen was also completely inhibited by EDTA, RGDS, echistatin, H12, or AP-2, but not by RGES or LM609 (data not shown). Taken together, these results indicate that platelet adhesion to these proteins is mediated through interaction with activated integrin αIIbβ3.

Direct Binding of Activated Integrin αIIbβ3 to Fisp12/mCTGF and Cyr61—To address whether integrin αIIbβ3 binds
Platelet Adhesion to Cyr61 and CTGF through Integrin \( \alpha_{IIb} \beta_3 \)

**Fig. 2. Dose-dependent adhesion of ADP-activated platelets to Fisp12/mCTGF and Cyr61.** Washed platelets, incubated with 10 \( \mu \)M ADP or 100 \( \mu \)M PGI\(_2\), were added to wells coated with the indicated concentrations of Fisp12/mCTGF (panel A) or Cyr61 (panel B). After incubation at 37°C for 30 min, adherent platelets were detected with \(^{125}\text{I}-\text{mAb15}\). Data shown are means of triplicate determinations, and error bars represent standard deviations.

**Fig. 3. Inhibition of platelet adhesion to Fisp12/mCTGF and Cyr61 by anti-Fisp12/mCTGF or anti-Cyr61 antibodies.** Microtiter wells coated with 25 \( \mu \)g/ml fibrinogen, Fisp12/mCTGF, or Cyr61 were preincubated with 1 mg/ml rabbit IgG, anti-Fisp12/mCTGF, or anti-Cyr61 at room temperature for 1 h. After three washes with Tyrode’s buffer, ADP-activated platelets were added. Platelet adhesion proceeded for 30 min at 37°C, and adherent cells were detected with \(^{125}\text{I}-\text{mAb15}\). Percentage of inhibition was calculated relative to the adhesion of platelets to control wells incubated without antibodies. Data shown are means of triplicate determinations, and error bars represent standard deviations.

directly to Fisp12/mCTGF and Cyr61, we performed a solid-phase binding assay to detect the receptor-ligand interaction. In these experiments, activated and unactivated \( \alpha_{IIb} \beta_3 \) were purified from platelet lysates as described under “Materials and Methods,” and the binding of purified \( \alpha_{IIb} \beta_3 \) to Cyr61 or Fisp12/mCTGF immobilized onto microtiter wells was detected with \(^{125}\text{I}-\text{mAb15}\).

Both activated and unactivated \( \alpha_{IIb} \beta_3 \) were indistinguishable on SDS-PAGE analysis as detected by silver staining (Fig. 5A). However, as reported previously (39), activated \( \alpha_{IIb} \beta_3 \) but not the unactivated receptor, was capable of binding to immobilized fibrinogen. Likewise, we observed higher binding of activated versus unactivated \( \alpha_{IIb} \beta_3 \) to Fisp12/mCTGF and Cyr61 (Fig. 5B). In contrast, similar background bindings of activated and unactivated \( \alpha_{IIb} \beta_3 \) to control wells coated with BSA were observed. Thus, these data are consistent with the observation that activated but not unactivated platelets adhered to Cyr61 and Fisp12/mCTGF.

To further characterize the interaction of \( \alpha_{IIb} \beta_3 \) with Fisp12/mCTGF and Cyr61, we performed binding isotherms with varying concentrations of RGD affinity-purified \( \alpha_{IIb} \beta_3 \). Fig. 6 shows that the dose-dependent binding of activated \( \alpha_{IIb} \beta_3 \) to Fisp12/mCTGF and Cyr61 was saturable, with half-saturation occurring at 15 nM and 25 nM \( \alpha_{IIb} \beta_3 \), respectively. Again, no significant binding of \( \alpha_{IIb} \beta_3 \) to control BSA-coated wells was observed. To demonstrate the specificity of the interaction, inhibition studies were performed. As expected, the binding of activated \( \alpha_{IIb} \beta_3 \) to Fisp12/mCTGF and Cyr61 was specifically blocked by RGDS but not by RGES (Fig. 7). Furthermore, echistatin and the H\(_{12}\) peptide also effectively inhibited \( \alpha_{IIb} \beta_3 \) binding to these proteins. These findings are consistent with results obtained in the platelet adhesion assay. Collectively, these functional and biochemical data demonstrate that activated integrin \( \alpha_{IIb} \beta_3 \) is the receptor mediating activation-dependent platelet adhesion to Cyr61 and Fisp12/mCTGF.

**DISCUSSION**

The major findings in this study are: 1) human platelets adhere to two novel angiogenic inducers, Cyr61 and Fisp12/mCTGF, in an activation-dependent manner; and 2) platelet adhesion to Cyr61 and Fisp12/mCTGF is mediated through the integrin \( \alpha_{IIb} \beta_3 \), which serves as a cell surface receptor for these proteins. These results establish Cyr61 and Fisp12/mCTGF as two additional adhesive ligands for integrin \( \alpha_{IIb} \beta_3 \) on activated platelets. Since both proteins are matrix-associated molecules synthesized by endothelial cells and smooth muscle cells of vessel walls (7, 20), these findings suggest a physiological role for Cyr61 and Fisp12/mCTGF in hemostasis and thrombosis.

Cyr61 and Fisp12/mCTGF are members of a family of multifunctional extracellular signaling molecules (8, 9). Originally identified as products of growth factor-inducible immediate-early genes, these proteins were thought to mediate the biological responses of growth factors. Recent studies have demonstrated the roles of these proteins in cell adhesion, migration, proliferation, survival, and differentiation (16–18, 40). Furthermore, both proteins have been implicated in complex biological processes such as angiogenesis, wound healing, embryogenesis, and tumor growth (17, 18, 40–42). Members of this protein family share four conserved structural domains, which include: 1) an insulin-like growth factor-binding protein homology domain, 2) a von Willebrand factor type C domain, 3) a thrombospondin type 1 repeat homology domain, and 4) a carboxyl-terminal domain with homology to some types of collagens and mucins. Heparin-binding sequence motifs can be found in domains 3 and 4, consistent with the observation that both Cyr61 and Fisp12/mCTGF bind heparin (14, 15). The human ortholog of Fisp12, CTGF, was first identified as a mitogenic factor in the conditioned medium of human umbilical vein endothelial cells (13). However, the mechanism and the receptor mediating its mitogenic activity have not yet been elucidated.

We have previously shown that, on vascular endothelial cells, both Cyr61 and Fisp12/mCTGF interact with integrin \( \alpha_{IIb} \beta_3 \), which mediates cell adhesion and migration (17, 19). While integrin \( \alpha_{IIb} \beta_3 \) is found in a number of cell types, it is present at a very low copy number on platelets (38, 43). In contrast, a closely related integrin, \( \alpha_{IIb} \beta_3 \), is the predominant adhesion receptor on blood platelets mediating platelet adhesion and aggregation. On resting platelets, integrin \( \alpha_{IIb} \beta_3 \) is present in a low affinity state incapable of binding soluble
Platelet Adhesion to Cyr61 and CTGF through Integrin $\alpha_{IIb}\beta_3$

adhesive ligands. Activation of platelets by physiological agonists such as thrombin, ADP, and thromboxane A$_2$ up-regulates the ligand binding affinity of $\alpha_{IIb}\beta_3$ through inside-out signaling processes. In the present study, we found that unactivated platelets failed to adhere to Cyr61 and Fisp12/mCTGF, whereas platelets activated by a variety of agonists adhere strongly to both proteins. Furthermore, we conclude that Cyr61 and Fisp12/mCTGF interact with the platelet integrin $\alpha_{IIb}\beta_3$ based on the observations that RGD-containing peptides and the dodecapeptide H$_{12}$, as well as the anti-$\alpha_{IIb}\beta_3$ monoclonal antibody AP-2, blocked platelet adhesion to these proteins. Although integrin $\alpha_v\beta_3$ on endothelial cells serves as the receptor for Cyr61 and Fisp12/mCTGF, the inability of the anti-$\alpha_v\beta_3$ monoclonal antibody LM609 to inhibit platelet adhesion to these proteins may reflect the relatively low abundance of this receptor as compared with integrin $\alpha_{IIb}\beta_3$ on the platelet surface.

Solid-phase binding assays with purified integrin $\alpha_{IIb}\beta_3$ to immobilized Cyr61 and Fisp12/mCTGF confirmed that these proteins are direct ligands of this integrin. Furthermore, consistent with the platelet adhesion data, we observed that activated purified $\alpha_{IIb}\beta_3$, but not the unactivated receptor, binds directly to these proteins. Thus, both Cyr61 and Fisp12/mCTGF are ligands specific for the activated conformer of integrin $\alpha_{IIb}\beta_3$. Interestingly, neither proteins contain the RGD motif or the fibrinogen $\gamma$ chain dodecapeptide sequence recognized by integrin $\alpha_{IIb}\beta_3$. Nevertheless, both peptides were able to inhibit the interaction of $\alpha_{IIb}\beta_3$ with Cyr61 and Fisp12/mCTGF, possibly due to conformation changes induced by peptide binding to $\alpha_{IIb}\beta_3$ (21, 44). These proteins, therefore, represent the first examples of activation-dependent ligands for integrin $\alpha_{IIb}\beta_3$ that do not contain either the RGD or the fibrinogen $\gamma$ chain dodecapeptide sequence motifs.

The expression of both $cyr61$ and $fisp12/mCTGF$ is developmentally regulated in a tissue-specific and temporally restricted manner during embryogenesis (15, 20, 42). In particular, $cyr61$ is expressed in developing blood vessel walls. Both Cyr61 as well as human and mouse CTGF proteins can be colocalized with smooth muscle cells of arterial walls (7, 20). In the adult, expression of both $cyr61$ and $fisp12/mCTGF$ is induced in the granulation tissue of healing cutaneous wounds, consistent with the abilities of these proteins to promote chemotaxis and proliferation of fibroblasts and to induce angiogenesis at the site of wound repair (8, 20, 41). These expression patterns and activities implicate a role for Cyr61 and Fisp12/mCTGF in the development and maintenance of blood vessels.

Platelets play an essential role in hemostasis, the arrest of blood flow from injured vessels. The presence of Cyr61 and CTGF in arterial vessel walls suggests that platelet adhesion to...
these proteins may contribute to the stability of the hemostatic plug. The initial adhesion of unactivated platelets to injured blood vessels is thought to be due to the interaction of the GP Ib-IX-V complex with vWf on the exposed subendothelium (4, 5). Such interaction has been shown to activate the platelet integrin αIIbβ3, thus allowing αIIbβ3 to bind to other adhesive ligands including soluble fibrinogen (31, 45). Our present finding that activated αIIbβ3 also binds immobilized Cyr61 and Fisp12/mCTGF suggest that these proteins may contribute to the tight adhesion of platelets to the subendothelial matrix following the initial GP Ib-IX-V interaction with vWf. Furthermore, the generation of thrombin and other platelet agonists would activate circulating platelets, thus allowing their interaction with Cyr61 and CTGF in the injured vessel walls.

CTGF has been shown to be overexpressed in advanced atherosclerotic lesions (7). Specifically, Northern blot analysis shows that the level of CTGF mRNA was expressed 50–100-fold higher in atherosclerotic blood vessels as compared with normal arteries. In advanced atherosclerotic lesions, CTGF protein was highly expressed in vascular smooth muscle cells as well as in endothelial cells at the luminal sites of the vessels and in the vasa vasmorum of the plaque lesions. CTGF may have multiple roles in the pathogenesis of atherosclerosis. First, it may act in concert with other growth factors and cytokines to promote cell migration and proliferation. Second, since it is an angiogenic factor, it would likely induce neovascularization of the fibrous plaques. Third, it may also be involved in the formation of occlusive thrombi since retraction or removal of endothelial cells of atherosclerotic plaques would expose CTGF in the underlying subendothelial matrix to which activated platelets could adhere. In this regard, our findings that activated αIIbβ3 mediates platelet adhesion to CTGF may have important implications in the pathogenesis of acute arterial occlusion resulting from ruptures or fissures of atherosclerotic plaques. Thus, the potential role of CTGF and Cyr61, and perhaps other members of this protein family, in hemostasis and thrombosis as manifested by platelet adhesive functions merits further investigation.

REFERENCES

1. Shattil, S. J., Kashiwagi, H., and Pampori, N. (1998) Blood 91, 2645–2657
2. Xu, A., and Ginsberg, M. H. (1997) Thromb. Haemostasis 78, 96–100
3. Liang, Y., Plow, E. F., and Topol, E. J. (1996) N. Engl. J. Med. 332, 1133–1139
4. Xu, A., and Ginsberg, M. H. (1999) Adv. Mol. Cell Biol. 26, 269–301
5. Clemenson, K. J. (1997) Thromb. Haemostasis 78, 266–270
6. Bennett, J. S., Chan, C., Vilaire, G., Measa, S. A., and DeGrado, W. F. (1997) J. Biol. Chem. 272, 8137–8140
7. Oemer, B. S., Werner, A., Garnier, J.-M., Do, D.-D., Godoy, N., Nauck, M., Marx, W., Rupp, J., Pech, M., and Luscher, T. F. (1997) Circulation 95, 831–839
8. Lau, L. F., and Lam, S. C.-T. (1999) Exp. Cell Res. 248, 44–57
9. Briggstock, D. R. (1999) Endocr. Rev. 20, 189–206
10. Lau, L. F., and Nathans, D. (1998) EMBO J. 17, 3145–3151
11. O’Brien, T. P., Yang, G. P., Sanders, L., and Lau, L. F. (1990) Mol. Cell. Biol. 10, 3569–3577
12. Ryseck, R. P., Macdonald-Bravo, H., Mattei, M.-G., and Bravo, R. (1991) Cell Growth Differ. 2, 225–233
13. Bradham, D. M., Igarashi, A., Potter, R. L., and Grotendorst, G. R. (1991) J. Cell Biol. 114, 1285–1294
14. Yang, G. P., and Lau, L. F. (1991) Cell Growth Differ. 2, 351–357
15. Kireeva, M. L., Latinkic´, B. V., Kolesnikova, T. V., Chen, C.-C., Yang, G. P., Ahler, A. S., and Lau, L. F. (1997) Exp. Cell Res. 233, 63–77
16. Kireeva, M. L., Mo, F.-E., Yang, G. P., and Lau, L. F. (1996) Mol. Cell. Biol. 16, 1326–1334
17. Babir, A. M., Chen, C.-C., and Lau, L. F. (1999) Mol. Cell. Biol. 19, 2958–2966
18. Babir, A. M., Kireeva, M. L., Kolesnikova, T. V., and Lau, L. F. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 6355–6360
19. Kireeva, M. L., Lam, S. C.-T., and Lau, L. F. (1998) J. Biol. Chem. 273, 3090–3096
20. Latinkic´, B. V. (1994) Regulation of Expression of Growth Factor-inducible Immediate Early Genes Cyr61 and Fisp92. Ph.D. thesis, University of Illinois, Chicago, IL
21. Frelinger, A. L., III, Cohen, I., Plow, E. F., Smith, M. A., Roberts, J., Lam, S. C.-T., and Ginsberg, M. H. (1996) J. Biol. Chem. 271, 6346–6352
22. Pidard, D., Montgomery, R. R., Bennett, J. S., and Kunicki, T. J. (1983) J. Biol. Chem. 258, 12592–12597
23. Cheresh, D. A., and Spiro, R. C. (1987) J. Biol. Chem. 262, 17703–17711
24. IUPAC-IUB (1968) J. Biol. Chem. 243, 3557–3559
25. Knezevic, I., Leisner, T. M., and Lam, S. C.-T. (1996) J. Biol. Chem. 271, 16416–16421
26. Fitzgerald, I. A., Leung, B., and Phillips, D. R. (1985) Anal. Biochem. 151, 169–177
27. Kinlough-Rathbone, R. L., Mustard, J. F., Packham, M. A., Perry, D. W., Reimers, H.-J., and Cazenave, J.-P. (1977) Thromb. Haemostasis 2, 281–288
28. Connolly, D. T., Knight, M. B., Harakas, N. K., Wittwer, A. J., and Feder, J. (1986) Anal. Biochem. 152, 136–140
29. Coller, B. S. (1980) Blood 55, 169–178
30. Savage, B., and Ruggieri, Z. M. (1991) J. Biol. Chem. 266, 11227–11233
31. Savage, B., Shattil, S. J., and Ruggieri, Z. M. (1992) J. Biol. Chem. 267, 11306–11309
32. Packham, M. A., Bryant, N. L., Guccione, M. A., Kinlough-Rathbone, R. L., and Mustard, J. F. (1989) Thromb. Haemostasis 62, 968–976
33. Plow, E. F., Pierschbacher, M. D., Ruoslahti, E., Marguerie, G., and Ginsberg, M. H. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 8057–8061
34. Kloczewiak, M., Timmons, S., Lukas, T. J., and Hawiger, J. (1984) Biochemistry 23, 1767–1774
35. Lam, S. C.-T., Plow, E. F., Smith, M. A., Andreiuss, A., Rytkova, J., Marku, G., and Ginsberg, M. H. (1987) J. Biol. Chem. 262, 947–950
36. Gan, Z. R., Pierschbacher, M. D., Ruoslahti, E., Marguerie, G. A., and Ginsberg, M. H. (1987) J. Biol. Chem. 262, 947–950
37. Cheresh, D. A., Berliner, S. A., Vicente, V., and Ruggieri, Z. M. (1989) Cell 58,
Platelet Adhesion to Cyr61 and CTGF through Integrin $\alpha_{IIb}\beta_3$

38. Lam, S. C.-T., Plow, E. F., D’Souza, S. E., Cheresh, D. A., Frelinger, A. L., III, and Ginsberg, M. H. (1989) *J. Biol. Chem.* **264**, 3742–3749

39. Kouns, W. C., Hadvary, P., Haering, P., and Steiner, B. (1992) *J. Biol. Chem.* **267**, 18844–18851

40. Wong, M., Kireeva, M. L., Kolesnikova, T. V., and Lau, L. F. (1997) *Dev. Biol.* **192**, 492–508

41. Igarashi, A., Okochi, H., Bradham, D. M., and Grotendorst, G. R. (1993) *Mol. Biol. Cell* **4**, 637–645

42. O’Brien, T. P., and Lau, L. F. (1992) *Cell Growth Differ.* **3**, 645–654

43. Coller, B. S., Cheresh, D. A., Asch, E., and Seligsohn, U. (1991) *Blood* **77**, 75–83

44. Parise, L. V., Helgerson, S. L., Steiner, B., Nannizzi, L., and Phillips, D. R. (1987) *J. Biol. Chem.* **262**, 12597–12602

45. De Marco, L., Girolami, A., Russell, S., and Ruggeri, Z. M. (1985) *J. Clin. Invest.* **75**, 1198–1203