Activation of $\alpha_v\beta_3$ on Vascular Cells Controls Recognition of Prothrombin

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Abstract. Regulation of vascular homeostasis depends upon collaboration between cells of the vessel wall and blood coagulation system. A direct interaction between integrin $\alpha_v\beta_3$ on endothelial cells and smooth muscle cells and prothrombin, the pivotal proenzyme of the blood coagulation system, is demonstrated and activation of the integrin is required for receptor engagement. Evidence that prothrombin is a ligand for $\alpha_v\beta_3$ on these cells include: (a) prothrombin binds to purified $\alpha_v\beta_3$ via a RGD recognition specificity; (b) prothrombin supports $\alpha_v\beta_3$-mediated adhesion of stimulated endothelial cells and smooth muscle cells; and (c) endothelial cells, either in suspension and in a monolayer, recognize soluble prothrombin via $\alpha_v\beta_3$. $\alpha_v\beta_3$-mediated cell adhesion to prothrombin, but not to fibrinogen, required activation of the receptor. Thus, the functionality of the $\alpha_v\beta_3$ receptor is ligand defined, and prothrombin and fibrinogen represent activation-dependent and activation-independent ligands.

Activation of $\alpha_v\beta_3$ could be induced not only by model agonists, PMA and Mn$^{2+}$, but also by a physiologically relevant agonist, ADP. Inhibition of protein kinase C and calpain prevented activation of $\alpha_v\beta_3$ on vascular cells, suggesting that these molecules are involved in the inside-out signaling events that activate the integrin.

The capacity of $\alpha_v\beta_3$ to interact with prothrombin may play a significant role in the maintenance of hemostasis; and, at a general level, ligand selection by $\alpha_v\beta_3$ may be controlled by the activation state of this integrin.

Key words: integrins • endothelial cells • smooth muscle cells • cell adhesion • ligands

The adhesive properties of vascular cells and the interaction of these cells with the blood coagulation system are intimately linked to the maintenance of vascular homeostasis. Whereas the endothelial cell lining of blood vessels is usually nonthrombogenic, vascular injury or the local generation of chemokines changes the surface properties such that the endothelial cells can initiate and efficiently propagate blood coagulation (Scarpati and Sadler, 1989; Stern et al., 1991; Bombeli et al., 1997). The culmination of these events is the activation of prothrombin to thrombin at the endothelial cell surface (Sueishi et al., 1995). In concert with changes in procoagulant activity, the adhesive properties of the endothelial cells are often altered. Expression and activation of a variety of adhesion receptors occur at the surface of stimulated endothelial cells (Pober and Cotran, 1990). Such changes are not restricted to endothelial cells; vascular smooth muscle cells also respond to injury and stimulation by changing their adhesive properties, such as the endothelial cells. Two changes are required for the cell to become migratory, and by expressing procoagulant activity on their cell surface (Taubman, 1993; Sueishi et al., 1995). Thus, the adhesive properties of vascular cells and their capacity to support prothrombin activation are intimately interwoven.

Recently, we have identified a previously unrecognized linkage between the major circulating cellular participant in thrombus formation, the platelet, its adhesive properties and thrombin generation by demonstrating that prothrombin serves as a ligand for the major integrin on the platelet surface, $\alpha_{IIb}\beta_3$ (Byzova and Plow, 1997). Prothrombin binds to $\alpha_{IIb}\beta_3$ on resting platelets in a specific, saturable, and divalent cation-dependent manner. This interaction accelerates prothrombin activation to thrombin, but thrombin itself does not bind to the receptor. Recognition of prothrombin by $\alpha_{IIb}\beta_3$ is mediated by an Arg-Gly-Asp (RGD)$^1$ recognition specificity; RGD-containing pep-

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1. Abbreviations used in this paper: HAEC, human aortic endothelial cells; HASMC, human aortic smooth muscle cells; HUVEC, human umbilical vein endothelial cells; PKC, protein kinase C; RGD, Arg-Gly-Asp.
tides, which inhibit the binding of many ligands to αIIbβ3, also block prothrombin binding to the receptor. αvβ3 contains the same β subunit and an homologous α subunit as αIIbβ3 and plays a prominent role in vascular cell adhesion and migration. Cultured human umbilical vein endothelial cells (HUVEC) express αvβ3 as a major cell surface molecule (Cheresh, 1987) on both their luminal and basolateral surfaces (Conforti et al., 1992) and in endothelial cell-cell contacts (Glass and Krebsberg, 1993), and nonactivated SMC derived from large vessels also express αvβ3 (Brown et al., 1994). The binding of many ligands to αvβ3 is mediated by a RGD recognition specificity, and αvβ3 and αIIbβ3 share many common ligands, including von Willebrand factor, fibrinogen, fibronectin, and thrombospondin (Ruoslathi, 1996; Yamada, 1991).

In this study, we have sought to determine whether prothrombin can serve as a ligand for αvβ3 on vascular endothelial cells and smooth muscle cells. Our consideration of this possibility also was stimulated by the work of Bar-Shavit et al. (1991, 1993), who reported that cleavage products of thrombin or denatured thrombin, but not native thrombin, supported the adhesion of HUVEC in a RGD-dependent manner. In addition, recent studies showing prothrombin is deposited in the vessel wall at sites of injury (Hatton et al., 1995) and is synthesized by smooth muscle cells (McBane et al., 1997) adds further biological relevance to the role of prothrombin as a potential αvβ3 ligand. Here, we demonstrate that prothrombin can serve not only as an adhesive ligand for αvβ3, but also as a soluble ligand for the receptor. However, the interactions of prothrombin with αvβ3 and αIIbβ3 are fundamentally different: engagement of prothrombin by αvβ3 on vascular cells requires receptor activation, whereas its binding to αIIbβ3 on platelets does not (Byzova and Plow, 1997). Additionally, we also identify specific intracellular signaling molecules, which are involved in modulating αvβ3 to a prothrombin-competent binding state. Moreover, we show directly that αvβ3 discriminates between activation-dependent and activation-independent ligands, and prothrombin serves as the prototype of an activation-dependent ligand for αvβ3 on vascular cells.

**Materials and Methods**

**Reagents**

Human prothrombin purchased from Alexis Corp. (San Diego, CA) and Enzyme Research Corp. (South Bend, IN) was >99% pure as assessed by SDS-PAGE (Laemmli, 1970). The preparations used contained only one major Coomassie blue staining band, and this protein reacted with mAb to prothrombin (Biodesign International, Kennebunk, ME) in Western blots. Humanized mAb c7E3 was from Centocor (Malvern, PA); αvβ3 specific mAb LM609 (Cheresh, 1987) was from Chemicon (Temecula, CA). FITC-goat anti-mouse IgG was purchased from Zymed Laboratories (South San Francisco, CA); polyclonal affinity-purified antibodies against prothrombin were from Haematologic Technologies Inc. (Essex Junction, VT); BSA (fraction V, crystalline), calphostin C, bisindolylmaleimide I and V, and calpeptin were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). Calpain inhibitors I and II were from Boehringer Mannheim (Mannheim, Germany). PMA, protease inhibitors (leupeptin, pepstatin, PMSF), adenosine 5'-diphosphate sodium salt (ADP) and cytochalasin B were from Sigma Chemical Co. (St. Louis, MO).

**Purification of Proteins**

Fibrinogen was purified from fresh human plasma by differential ethanol precipitation (Plow et al., 1984). αvβ3 was purified from detergent extracts of human placental tissues by affinity chromatography using a KG-GRGDS-Sepharose column followed by elution with 20 mM EDTA as described previously with minor modifications (Pytel et al., 1986; Smith et al., 1990b). The preparations used exhibited only two major bands by SDS-PAGE and protein staining with Coomassie Brilliant Blue, which corresponded to the αv and β3 subunits, and was judged as being >95% pure. When immobilized in wells, αvβ3 preparation reacted with LM609, an mAb specific for αvβ3 (Cheresh, 1987), and did not react with CRC64, an mAb specific for αIIbβ3 (Mazurow et al., 1996), in an ELISA format.

**Radioiodination**

Na125I (specific activity = 15–17 mCi 125I/mg of iodine) from Nycomed Amersham Inc. (Princeton, NJ) was used for radioiodination. Prothrombin was radiolabeled using a modified chloramine-T method (Plow et al., 1984). The labeled prothrombin was indistinguishable from the unlabeled form upon SDS-PAGE under reducing and nonreducing conditions. When activated with Factor Xa + Va (5 mg/ml each; American Diagnostica Inc., Greenwich, CT), all of the radiolabeled prothrombin could be converted to thrombin within 30 min as assessed by gel analysis. Furthermore, the rate of activation of labeled and unlabeled prothrombin by Factor Xa or Factor Xa/Va was the same as assessed with the Spectrozyme (American Diagnostica, Inc.) thrombin substrate (Byzova and Plow, 1997). Radioiodinated prothrombin was stored at 4°C and used within 3–4 d of labeling.

**Solid-Phase Ligand Binding Assays**

The binding of prothrombin to immobilized αvβ3 was performed as described (Charo et al., 1991; Byzova and Plow, 1997) with minor modifications. αvβ3 (280 μg/ml) was diluted 1:70 in a buffer containing 10 mM Tris, 150 mM NaCl, pH 7.4 (Buffer A), and immobilized onto 96-well microtiter plates (Costar Corp., Cambridge, MA) at 400 ng per well for overnight at 4°C. The plates were then washed and post-coated with 40 mg/ml BSA overnight at 4°C or 1 h at 37°C. The functional activity of the immobilized αvβ3 was assessed relative to 125I-fibrinogen binding to the same receptor preparations (Suehiro et al., 1996). 125I-prothrombin was added in Buffer A, containing 2 mg/ml BSA and the selected divalent cations. After incubation for selected times (75–120 min) at 37°C, wells were washed 4–5 times with Buffer A, and bound prothrombin was quantitated by counting the bound radioactivity in a γ-counter. In some experiments, αvβ3-coated wells were preincubated for 20 min with mAbs or peptides before addition of 125I-prothrombin. When fibrinogen was used as a competitor, H-D-Phe-Pro-Arg-chloromethylketone (Bachem, Torrance, CA) was included at a final concentration of 30 μg/ml. Nonspecific binding was measured in the presence of a 50-fold excess of unlabeled prothrombin. Data were determined as the means of triplicate or quadruplicate measurements at each experimental point.

**Cell Culture**

Primary cultures of HUVEC, human aortic smooth muscle cells (HASMC), and human aortic endothelial cells (HAEC) were provided by Drs. Paul DiCorleto and Donald Jacobsen (Cleveland Clinic Foundation, OH). HUVEC were grown to preconfluence in 162-cm² plastic flasks (Costar Corp.) in DMEM/F12 (BioWhittaker Inc., Walkersville, MD) supplemented with 15% FBS (BioWhittaker Inc.), 150 μg/ml endothelial growth factor (Clonetics Corporation, San Diego, CA), and 90 μg/ml heparin (Sigma Chemical Co., St. Louis, MO; D’Souza et al., 1996). The cells were used within the second to fourth passage. HAEC were grown to preconfluence in 162-cm² plastic flasks (Corning Costar Corp.), coated with 0.1% gelatin (Sigma Chemical Co.), in DMEM/F12 (BioWhittaker Inc.) supplemented with 15% FBS (BioWhittaker Inc.), 150 μg/ml endothelial growth factor (Clonetics), and 90 μg/ml heparin (Sigma Chemical Co.) and used within the third to fifth passage. HASMC were grown in 162-cm² plastic flasks (Costar Corp.) in DMEM Medium/F12 (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FBS (GIBCO BRL), 75 μg/ml endothelial growth factor (Clonetics), and 45 μg/ml heparin (Sigma Chemical Co.), and used within the fourth to seventh passage. αvβ3 expression was verified by flow cytometry (as described below) and only αvβ3-positive cultures were used in adhesion assays. αvβ3-negative cultures did not demonstrate an agonist-induced increase in adhesion to prothrombin.

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HUVECs were washed three times with PBS and harvested by gentle trypsinization (0.25 mg/ml trypsin, 0.01% EDTA solution; Clonetics). Cells were collected into a tube containing trypsin neutralizing solution (Clonetics) and immediately centrifuged at 500 g for 10 min. The cells were resuspended in 105 cells/ml in DMEM/F12, containing 1% BSA (adhesion buffer). Cells were labeled by Calcein AM (50 μg; Molecular Probes, Eugene, OR) and examined microscopically. Reader (PerSeptive Biosystems, Framingham, MA) and examined microscopically.

Adherent cells were quantitated in a Fluorescence Multi-Well Plate Reader (PerSeptive Biosystems, Framingham, MA) and examined microscopically.

Flow Cytometry

HUVEC, HAEC, or HASMC, harvested as described above, were suspended at 8 × 104 cells/ml in adhesion buffer and incubated with LM609 (10 μg/ml) or with control mouse IgG for 60 min at 37°C. The cells were washed by centrifugation in DME/F12, 1% BSA, incubated with FITC goat anti–mouse IgG on ice for 20 min, and then analyzed by flow cytometry. Flow cytometry was performed using a FACScan® instrument; 10,000 events were recorded, and the data were analyzed using the CellQuest software program (version 1.2).

Binding of 125I-Prothrombin to HUVEC in Suspension

HUVEC were diluted to 7 × 105/ml in DME/F12, with or without 0.5 mM MnCl2. The cells were preincubated with mAb LM609 (20 μg/ml), nonimmune immunoglobulins (20 μg/ml), c7E3 (20 μg/ml), or fibrinogen (100 and 500 μg/ml) for 5 min, and 125I-prothrombin was then added at selected concentrations. Cells were activated with PMA at 200 nM as specified. After 75 min at 37°C, cell-bound ligand was separated by centrifugation through 20% sucrose for 2.5 min at 22°C in Beckman microfuge, and the cell-bound radioactivity was measured in a gamma-counter. Data were determined with quadruplicate measurements at each experimental point.

Binding of 125I-Prothrombin to the HUVEC Monolayer

HUVEC were examined at 1 × 105/ml in DME/F12, containing 1% BSA and seeded into 24-well plates, precoated by 0.1% gelatin. After 4 h incubation, nonadherent cells were removed, and the media was changed to DME/F12 for overnight. 7 h before the experiment, the media was changed for DME/F12 containing 1% BSA and no serum. Cells were preincubated with c7E3 (30 μg/ml) or GRGDSP peptide (100 μM) or without inhibitors for 10 min and then treated by PMA at 200 nM or 0.5 mM MnCl2, as indicated. 125I-prothrombin was then added at concentration of 50 μg/ml. After 70 min at 37°C, wells were washed three times with PBS, and bound radioactivity solubilized in 0.3 ml 1 N NaOH, and measured in a γ-counter. Quadruplicate measurements were made at each experimental point.

Results

125I-Prothrombin Binding to Purified αvβ3

As an initial analysis, we sought to determine whether purified αvβ3 could bind prothrombin. The αvβ3 used was isolated from human placenta by affinity chromatography on a RGD column and contained no detected α6β3, as assessed immunochemically. The functional activity of the isolated αvβ3 was evaluated with the receptor immobilized onto microtiter plates and using fibrinogen as a well-characterized αvβ3 ligand (e.g., Smith and Cheresh, 1990; Smith et al., 1990a). The binding of fibrinogen was supported by Mn2+ and was inhibited by Ca2+, consistent with the data of Smith et al. (1994); and this interaction was completely inhibited by RGD-containing peptides and the αvβ3-specific mAb, LM609.

With evidence of receptor purity and function, the binding of prothrombin to αvβ3 was assessed. Increasing concentrations of 125I-prothrombin were added to wells coated with αvβ3. As shown in Fig. 1 A, prothrombin bound in concentration-dependent manner, and this interaction was inhibited by 50-fold excess of nonlabeled ligand. The concentration of 125I-prothrombin required for half-maximal binding was <25 μg/ml. At saturation, 13.6 × 1010 prothrombin molecules bound to the αvβ3-coated wells (see Fig. 1 A). In the presence of 1 mM MnCl2, ~8.4 × 1010 fibrinogen molecules were maximally bound. Thus, the stoichiometry of binding of the two ligands to the receptor was similar. The specificity of prothrombin binding to αvβ3 is documented in Fig. 1 B. Typical of the binding of adhesive ligands to αvβ3, the interaction of prothrombin with the receptor was cation dependent: Ca2+ and Mn2+ supported binding, and EDTA inhibited the interaction. Two different mAbs reactive with αvβ3, LM609 and c7E3, also inhibited prothrombin binding to the receptor (Fig. 1 B) whereas nonimmune IgG had no effect. Although both mAbs were effective inhibitors of 125I-prothrombin binding to αvβ3, c7E3, even at higher concentrations, tended to be slightly less inhibitory than LM609, which may reflect the difference in the specificity of these mAbs (Cheresh, 1987; Jordan et al., 1997). The RGD-containing peptide, GRGDSP, produced dose dependent inhibition and, at a high concentration of 100 μM, was as effective as the mAbs and EDTA in inhibiting the interaction. Taken together, this inhibitory profile demonstrates that prothrombin can bind to αvβ3 via a RGD recognition specificity, which typifies the recognition of adhesive ligands by this integrin.

αvβ3-dependent Adhesion of Activated Vascular Cells to Prothrombin

In view of recent evidence indicating that prothrombin is deposited in the vessel wall (McBane et al., 1997), we next sought to determine whether prothrombin could function as an adhesive ligand for αvβ3 in intact cells. The results of these analyses are shown microscopically in Fig. 2 and quantitatively in Fig. 3. Under conditions where prothrombin supported nonstimulated platelet adhesion, we found that nonstimulated HUVEC did not adhere to immobilized prothrombin (Figs. 2 A and 3 A). In contrast, adhesion of PMA-stimulated cells was evident within 30 min, and after 50–60 min, many of the adherent cells were spread on the prothrombin substratum (Fig. 2 B). With 2 × 105 HUVEC added to the prothrombin coated wells, the percentage of adherent cells ranged from 30 to 60% of the added cells; of the adherent cells, ~30–40% were spread within 1 h (Fig. 2 B), and this percentage increased with
longer incubation. Background cell adhesion to microtiter wells coated with BSA was not significantly affected by PMA stimulation, and the αvβ3 mAbs (LM609 and c7E3) and GRGDSP had no effect on the nonspecific adhesion of stimulated or nonstimulated cells to BSA (not shown). Verifying the role of αvβ3 in the adhesion of the PMA-stimulated HUVEC to prothrombin, mAbs LM609 and c7E3 completely blocked adhesion, as did GRGDSP (100 μM; Fig. 3 A). In contrast, neither nonimmune IgG nor a control peptide significantly affected HUVEC adhesion (Fig. 3 A). As an additional indication of specificity, antibodies to prothrombin also blocked adhesion to the immobilized substrate by >80% (Fig. 3 A, calculated by assigning the adhesion in the absence of PMA a value of 0%). This set of data demonstrates that PMA-activated HUVEC, but not resting cells, are capable of interacting with immobilized prothrombin via αvβ3 in a RGD-dependent manner.

In the presence of Mn2+, a cation that stimulates the ligand binding function of αvβ3 (Smith et al., 1994), as well as many other integrins, (Mould et al., 1995; Suehiro et al., 1997), adhesion of the cells to prothrombin was observed without the requirement of an additional stimulus (Figs. 2 C and 3 B). This adhesion also was completely inhibited by GRGDSP, LM609, and c7E3, supporting the essential role for αvβ3 in the interaction (Figs. 2 D and 3 B). No additive effect on HUVEC adhesion was observed when PMA and Mn2+ were used together (not shown); however, we did find that after 6–7 passages, HUVEC required both PMA and Mn2+ to adhere to prothrombin. In Fig. 3 C, evidence is provided that adhesion to prothrombin also is observed with endothelial cells of a different origin. HAEC adhered to prothrombin in a similar manner as HUVEC, i.e., adhesion was stimulated by PMA and inhibited by LM609. Furthermore, as shown in Fig. 3 C, soluble prothrombin inhibited adhesion to the immobilized ligand. The later observation suggests that the soluble form of prothrombin is recognized by αvβ3, and surface denaturation is not required for prothrombin to become a ligand for the receptor. This interpretation is supported by subsequent binding studies using soluble prothrombin as a ligand for αvβ3 (see below).

The capacity of prothrombin to support cell adhesion was not restricted to endothelial cells. Upon PMA stimulation, the adherence of a second type of vascular cell, HASMC, to prothrombin increased dramatically. The increased adhesion induced by PMA was 80% (calculated by assigning the adhesion in the absence of PMA a value of 0%) inhibited by mAb LM609 (Fig. 4) but not by nonimmune IgG. We also found that Mn2+ supported αvβ3-mediated adhesion of HASMC to prothrombin (not shown). Furthermore, with HASMC, a physiologically relevant ag-
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onist (Boarder and Hourani, 1998; Murthy and Makhlof, 1998), ADP, was shown to induce adhesion to prothrombin. ADP increased adhesion in a dose-dependent manner; and, at higher concentrations (200–2,000 mM), adhesion was as extensive as that induced by PMA. The adhesion induced by ADP was αβ3-mediated as LM609 was an effective inhibitor. We also observed that ADP induced adhesion of HUVEC to prothrombin (not shown) although higher concentrations of the agonist were required to obtain a comparable effect. Taken together, these data indicate that prothrombin serves as an adhesive ligand for αβ3 on HUVEC, HAEC, and HASMC.

Binding of Prothrombin to Endothelial Cells

We next sought to assess whether αβ3 on vascular cells is capable of recognizing not only immobilized but also soluble prothrombin and how cellular activation might influence this interaction. 125I-prothrombin was incubated with HUVEC in a single cell suspension in the presence of Ca2+ and Mg2+ (DME/F12 without additional cations) or in the presence of additional 1 mM Mn2+. Results of a typical experiment are shown in Fig. 5A, which illustrates the influence of increasing concentrations of added 125I-prothrombin on specific prothrombin binding to HUVEC under the two divalent cation conditions. The nonspecific binding was determined in the presence of 50-fold molar excess of unlabeled prothrombin and corresponded to 10–15% of the total binding at the concentrations of 125I-prothrombin added. From the data presented in Fig. 5A, it is evident that the addition of Mn2+ cause a dramatic increase of 125I-prothrombin binding to HUVEC. Saturation of binding was apparent in the presence of Mn2+ at prothrombin concentrations above 50 μg/ml, which corresponds to half the prothrombin concentration in plasma. At this saturating concentration, 958,000 ± 108,500 prothrombin molecules bound per cell in the presence of Mn2+, compared with 18,600 ± 2,050 molecules per cell in the presence of Ca2+. Thus, although Ca2+ did support limited specific binding of prothrombin to αβ3 (inhibitable by nonlabeled prothrombin, c7E3 and LM609), Mn2+ enhanced this interaction by ~50-fold.

To investigate whether 125I-prothrombin binding in the presence of Mn2+ was attributable to αβ3, binding studies were performed in the presence of mAbs LM609 and c7E3 and GRGDSP. As shown in Fig. 5B, LM609 inhibited 125I-prothrombin binding to HUVEC by 75–80% and to c7E3 by ~50%. At the same concentration, nonimmune IgG had no effect. The extent of inhibition by GRGDSP was similar to that produced by c7E3. The stimulatory effect of Mn2+ and the inhibition profile of 125I-prothrombin bind-
microscopically, but did elute 96% of the bound 125I-prothrombin. This treatment did not disrupt the monolayer as assessed. These analyses were conducted with both purified prothrombin and with HUVEC in suspension, and the experiments were performed under different divalent cation conditions. The results are summarized in Table I. Previous studies have established that fibrinogen binds poorly to purified αβ3 in the presence of Ca2+ (Smith et al., 1994; Suehiro et al., 1996), and fibrinogen, even in concentrations as high as 500 μg/ml, was a poor inhibitor of prothrombin binding, producing only 11% inhibition in 1 mM Ca2+. In the presence of Mn2+, fibrinogen was a more effective inhibitor, producing 40% inhibition at 500 μg/ml. Nevertheless, substantial binding of prothrombin was still observed. The data obtained for 125I-prothrombin binding to HUVEC were consistent with those obtained in the pu-

**Figure 5.** HUVEC bind soluble 125I-prothrombin. (A) HUVEC in suspension were incubated with increasing concentrations of 125I-prothrombin in DME/F12 in the presence of 0.5 mM MnCl2 or 1 mM CaCl2 for 60 min, and cell-bound radioactivity was quantitated as described. Non-specific binding was measured in the presence of 50-fold excess of nonlabeled prothrombin and subtracted to yield the specific binding data shown. Values represent the means and SD of four determinations. (B) Specificity of prothrombin binding to HUVEC. 125I-prothrombin (50 μg/ml) was incubated with HUVEC (8 × 10^5 cells/ml) in the presence of mAb LM609, mAb c7E3 (20 μg/ml each; a similar concentration of nonimmune IgG was without effect), or 100 μM GRGDSP. MnCl2 (1 mM) was added to the media, and cell-bound radioactivity was measured after 60 min incubation. Non-specific binding was measured in the presence of 50-fold excess of nonlabeled prothrombin and subtracted. The data shown are means and SD of quadruplicates in one experiment and are representative of three separate experiments.

Competition between Prothrombin and Fibrinogen for αβ3

The capacity of a major αβ3 ligand, fibrinogen, to compete with prothrombin for binding to the receptor was assed. These analyses were conducted with both purified αβ3 and with HUVEC in suspension, and the results confirm the role of αβ3 in interaction of HUVEC with soluble prothrombin.

Next, we assessed whether 125I-prothrombin is capable of interacting with HUVEC in a monolayer. 125I-prothrombin at 50 μg/ml was added to a confluent and intact HUVEC monolayer in the presence of PMA, 0.5 mM Mn2+ or no addition. After 70 min, the cells were rapidly washed and HUVEC-associated radioactivity was extracted and counted. The αβ3-mediated component of prothrombin binding accounted for 194,000 ± 5,780 molecules/cell. A contribution of αβ3 to prothrombin binding also was demonstrable in the presence of 0.5 mM Mn2+ (Fig. 6). It should be noted that substantial specific binding of 125I-prothrombin (inhibited by excess nonlabeled prothrombin) to the untreated HUVEC monolayer was observed (not shown), but this interaction was unaffected by mAb LM609, i.e., the binding was not αβ3-mediated. PMA-stimulation substantially increased the 125I-prothrombin binding, and this increment was abrogated by LM609 as well as c7E3 (Fig. 6). Thus, αβ3 can recognize prothrombin, either as an adhesive substrate or as a soluble ligand, provided the HUVEC had been exposed to PMA or Mn2+. To determine whether 125I-prothrombin binding to the HUVEC monolayer resulted in internalization of ligand, we tested whether EDTA could elute bound prothrombin from the cells. 125I-prothrombin was bound to the cells for 70 min and then HUVEC were washed 4–5 times with phosphate buffer, containing 10 mM EDTA. This treatment did not disrupt the monolayer as assessed microscopically, but did elute 96% of the bound 125I-prothrombin, indicating that the reaction was reversible and that prothrombin was not internalized under the conditions used.

**Figure 6.** 125I-prothrombin binding to a HUVEC monolayer. Confluent cell monolayers were incubated with 50 μg/ml 125I-prothrombin in DME/F12-1% BSA in the presence or absence of 200 nM PMA (solid bars) or 0.5 mM MnCl2 (gray bars). Cells were preincubated with c7E3 (30 μg/ml) or a cyclic RGD peptide (10 μM) or without inhibitors for 10 min. After 70 min at 37°C, wells were washed three times with PBS, and the cells were solubilized in 1 N NaOH. Prothrombin binding to nonstimulated endothelial cells was subtracted from the total binding, and the difference is displayed. Values are means and SD of quadruplicates from one of five experiments with similar results.
rified system. Specifically, in the absence of Mn$^{2+}$, fibrinogen was a poor inhibitor of $^{125}$I-prothrombin binding. Inhibition was more extensive in the presence of 1 mM Mn$^{2+}$, but substantial prothrombin binding was still observed, even at the higher fibrinogen concentration. Thus, under some, but not all conditions, fibrinogen competes but does not appear to be a particularly effective inhibitor of $^{125}$I-prothrombin binding to $\alpha_\text{V}\beta_3$.

**Molecular Basis for Adhesion of Activated Vascular Cells to Prothrombin**

We sought to understand the mechanism by which $\alpha_\text{V}\beta_3$ became competent to bind prothrombin in the presence of agonist. FACS$^\text{®}$ analysis was used to determine whether the expression levels of $\alpha_\text{V}\beta_3$ on HUVEC and HASMC is altered by PMA stimulation. This analysis confirmed high expression of $\alpha_\text{V}\beta_3$ on HUVEC and lower expression on HASMC. PMA-stimulated cells were found to express $\alpha_\text{V}\beta_3$ at levels similar to that found on nontreated cells. These data indicate that short-term PMA treatment did not alter the expression level of $\alpha_\text{V}\beta_3$.

Since PMA is a potent activator of protein kinase C (PKC), the possible role of PKC in PMA-stimulated HUVEC adhesion on prothrombin was examined. Calphostin C, a specific and potent inhibitor of PKC (Kobayashi et al., 1989), completely blocked the effect of PMA on HUVEC. This analysis confirmed high expression of $\alpha_\text{V}\beta_3$ on HUVEC and lower expression on HASMC. PMA-stimulated cells were found to express $\alpha_\text{V}\beta_3$ at levels similar to that found on nontreated cells. These data indicate that short-term PMA treatment did not alter the expression level of $\alpha_\text{V}\beta_3$.

| Cation conditions | $^{125}$I-Prothrombin binding to purified $\alpha_\text{V}\beta_3$ ($\times 10^4$) | $^{125}$I-Prothrombin binding to HUVEC ($\times 10^4$) |
|------------------|---------------------------------|---------------------------------|
| No competitor    | 100 ± 7.1%                      | 100 ± 12%                       |
| Fibrinogen (500 µg/ml) | 89 ± 9%                         | 85.4 ± 11%                     |
| Fibrinogen (100 µg/ml)  | 107 ± 14%                        | 92.7 ± 11.9%                   |

In all assays, H-D-Phe-Pro-Arg-chloromethylketone was present throughout at a final concentration of 30 µg/ml. Specific prothrombin binding, inhibited by excess nonlabeled prothrombin, was assigned the value of 100% under each cation condition. With purified and immobilized $\alpha_\text{V}\beta_3$, 100% was $7.7 \times 10^{10}$ molecules/well in the presence of 1 mM Ca$^{2+}$ and $9.8 \times 10^{10}$ molecules/well in Mn$^{2+}$. In the cell experiments, 100% was 110,000 molecules/cell in the presence of 2 mM Ca$^{2+}$, 2 mM Mg$^{2+}$, and 780,000 molecules/cell in the presence of 1 mM Ca$^{2+}$, 2 mM Mg$^{2+}$, and 1 mM Mn$^{2+}$. Values are the means ± SD of quadruplicates in each experiment which is representative of at least five experiments.

$^\text{a}$Experimental conditions are the same as in Fig. 1 with purified $\alpha_\text{V}\beta_3$ immobilized onto microtiter wells.

$^\text{b}$Experimental conditions are the same as in Fig. 5 with HUVEC in suspension.

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![Figure 7](image-url)
Mn^{2+} or Mn^{2+} + PMA also was unaffected by cytochalasin B. These results suggest that PMA stimulation modulates the affinity of αvβ3 for the prothrombin ligand and that cytoskeletal reorganization is not required for recognition of prothrombin by the receptor. Of note, we found that certain concentrations of cytochalasin B stimulated cell adhesion to prothrombin in the absence of PMA or Mn^{2+}. Similar observations were reported by Qi et al. (1998), who found that cytochalasin D could activate αIIbβ3-mediated adhesion to fibrinogen.

To determine if the activation requirement for recognition of prothrombin by αvβ3 extends to other αvβ3 ligands, we assessed the effects of cell stimulation and of the inhibitors, calphostin C and calpeptin, on αvβ3-mediated HUVEC adhesion to fibrinogen. Consistent with previous reports (Cheresh, 1987; D’Souza et al., 1996; Suehiro et al., 1997), HUVEC adhere well to fibrinogen although only a portion of this adhesion was αvβ3 mediated. αvβ3-dependend adhesion was identified as that component of total cell adhesion that was sensitive to the anti-αvβ3 blocking mAbs, LM609 or c7E3 (Fig. 9A). For nonstimulated cells, αvβ3-mediated adhesion was ~37% (100% is defined as the total adhesion in the absence of PMA). Treatment with PMA caused an increase in total HUVEC adhesion, but the αvβ3-mediated portion of adhesion remained unchanged (35%). The same pattern was demonstrable in the presence of Mn^{2+}. In the experiment shown in Fig. 9B, αvβ3-mediated adhesion in the presence of Mn^{2+} was 17% of the total adhesion, and with Mn^{2+} + PMA present, 19% of the total adhesion was αvβ3 mediated (Fig. 9B). In contrast to HUVEC adhesion to prothrombin, pretreatment of HUVEC with calphostin C did not significantly decreased the number of cells adherent to fibrinogen (Fig. 9C). Furthermore, whereas pretreatment of HUVEC with calpeptin resulted in complete inhibition of cell adhesion to prothrombin, calpeptin had no effect on cell adhesion to fibrinogen (Fig. 9D). Thus, the requirements for αvβ3-mediated adhesion to prothrombin and fibrinogen are quite distinct.

**Discussion**

In this study, we sought to assess whether prothrombin is a ligand for αvβ3 on vascular cells. A direct interaction between prothrombin and αvβ3 on human vascular cells, endothelial cells, derived from umbilical vein and from aorta, and smooth muscle cells was demonstrable, establishing a previously unrecognized interface between the adhesive and procoagulant properties of these cells. Moreover, in characterizing this interaction, we found that activation of αvβ3 by model agonists (PMA or Mn^{2+}) or physiological agonists (ADP) is required for recognition of prothrombin, and this requirement is not necessary for fibrinogen to engage the receptor. Therefore, whereas recent studies have emphasized that αvβ3 can exist in different activation states (Pelletier et al., 1996; Bennett et al., 1997; Sadhu et al., 1998), it appears that the functionality of the receptor is defined by the ligand under analysis; and prothrombin and fibrinogen serve as prototypes of activation-dependent and activation-independent ligands for αvβ3 on vas-
monolayer, and this interaction required stimulation of the prothrombin to stimulated HUVEC in suspension and in a control. Antibodies to prothrombin also blocked this adhesion. RGD-containing peptides. As an additional specificity evidenced by its blockade by mAbs to the receptor and by thrombin was evaluated. Adhesion in the presence of 1 mM CaCl<sub>2</sub> was measured. Prothrombin was found to support attachment and spreading of stimulated HUVEC, HAEC and spiral cells. Taken together, these analyses clearly document that prothrombin, presented either in a soluble or an immobilized form, is a ligand for α<sub>V</sub>β<sub>3</sub> on vascular cells.

Prothrombin contains a RGD sequence within its catalytic domain. Analysis of the crystal structure of thrombin revealed that the RGD is involved in the formation of the active site and lies at the bottom of the S1 specificity pocket (Stubbs and Bode, 1993). This positioning is likely to preclude access of α<sub>V</sub>β<sub>3</sub> and other integrins with a RGD recognition specificity to the sequence in native thrombin. The orientation of the RGD may be different in prothrombin and permit recognition by α<sub>V</sub>β<sub>3</sub>. In the crystal structure of prothrombin 2 (Vijayalakshmi et al., 1994), a catalytically inactive intermediate generated during prothrombin activation, the RGD sequence resides in a surface-exposed configuration. As additional support for this possibility, Bar-Shavit et al. (1991, 1993) demonstrated that active thrombin was not adhesive but could be modified into a potent RGD-dependent adhesion molecule for endothelial cells. Our data showing that soluble prothrombin inhibits adhesion to the immobilized ligand suggest that the requisite sequence(s) for α<sub>V</sub>β<sub>3</sub> recognition are expressed on the surface of the native molecule. This interpretation by no means excludes the possibility that other sequences in prothrombin could mediate recognition of prothrombin by α<sub>V</sub>β<sub>3</sub>; i.e., similar to the recognition of the γ chain, rather than the RGD sequence of fibrinogen by α<sub>IIb</sub>β<sub>3</sub> (Farrell et al., 1992), even though α<sub>IIb</sub>β<sub>3</sub> has also a RGD recognition specificity. Also to be resolved is whether factor X, which also contains a RGD sequence, can interact with α<sub>V</sub>β<sub>3</sub>.

The α<sub>V</sub>β<sub>3</sub> integrin is widely expressed on vascular cells. It is present on luminal and basolateral surfaces of endothelial cells, on smooth muscle cells, and is also expressed on certain circulating blood cells (Cheresh, 1987; Savill et al., 1990; Moulder et al., 1991; Conforti et al., 1992; Brown et al., 1994). This expression profile suggests that α<sub>V</sub>β<sub>3</sub> is directly exposed to plasma proteins, including prothrombin. From our analyses of the interaction of prothrombin with α<sub>V</sub>β<sub>3</sub>, either in purified form or on cells, half-maximal binding occurred at input concentrations of ~50 μg/ml and almost 10<sup>6</sup> prothrombin molecules were bound per endothelial cell. Thus, the plasma concentration of prothrombin at ~100 μg/ml would potentially place substantial quantities of prothrombin on cell surfaces that must be nonthrombogenic to maintain hemostasis. In addition to its presentation as a soluble ligand from plasma, prothrombin may also be a relevant substrate for vascular cell adhesion. Recent studies have demonstrated that prothrombin is synthesized by smooth muscle cells (McBane et al., 1997). Furthermore, prothrombin accumulates within the vascular matrix, particularly at sites of lesion formation. High levels of prothrombin have been identified in the aortic intima after deendothelializing injury (Hatton et al., 1995) and in early atherosclerotic lesion (Smith and Staples, 1981). Based on our studies of prothrombin binding to α<sub>V</sub>β<sub>3</sub>, a potential functional consequence of prothrombin binding to α<sub>V</sub>β<sub>3</sub> would be more efficient activation to thrombin (Byzova and Plow, 1997). Also, with the deposition of prothrombin in the vessel wall under pathophysiological conditions (Hatton et al., 1995; Smith and Staples, 1981), adhesion itself may be a biologi-
cally relevant endpoint of prothrombin–αVβ3 interactions. With these potential biological ramifications, the interaction of prothrombin with this receptor requires tight regulation. Such regulation appears to be established by the activation state of αVβ3. Whether competition with other αVβ3 ligands provides an additional level of control remains to be established. In this regard, based upon their plasma levels, the two primary competitors for plasma prothrombin binding to αVβ3 are predicted to be vitronectin and fibrinogen. Denaturation is required for vitronectin to become a soluble ligand for αVβ3 (Seiffert and Smith, 1997); with fibrinogen, the role of αVβ3 in mediating its binding to HUVEC has been variable (Languino et al., 1993). In our analyses, we found that fibrinogen did inhibit 125I-prothrombin binding to HUVEC, but only under specific cation conditions, i.e., when Mn2+ was present. This result is consistent with the suppression of fibrinogen binding to purified αVβ3 that has been previously reported (Smith et al., 1994; Suehiro et al., 1996). Thus, competition between prothrombin and fibrinogen will be determined by specific microenvironmental cation conditions and the relative affinity of the two ligands for the receptor. Detailed studies are in progress to assess this latter parameter. Also, concentration is not the sole determinant of the competition between these ligands, e.g., although von Willebrand factor is present at much lower concentrations in plasma than fibrinogen, it is still a preferred substrate at high shear conditions (Savage et al., 1996). In the matrix, still other conditions will determine the importance of prothrombin, fibrinogen, vitronectin, and other αVβ3 ligands as adhesive substrates. Ultimately, the relative capacity of these various ligands to support cell migration, as well as adhesion, will be functionally important. Thus, it is uncertain whether ligand competition will play a significant role in regulating prothrombin binding to αVβ3.

Interaction of prothrombin with αVβ3 on intact cells was not observed unless the cells were stimulated. Such activation was induced by a well-characterized, model integrin agonist, PMA. In addition, a physiological agonist, ADP (Boarder and Hourani, 1998), also activated smooth muscle cells and endothelial cells to adhere to prothrombin. ADP is a physiologically relevant agonist (Nurden et al., 1995) for activation of αIIbβ3 on platelets, and the second β3 integrin, αVβ3, also responds to this stimulus (Boarder and Hourani, 1998). Higher concentrations of ADP were required to activate αVβ3 on HUVEC than on HASMC. This may reflect the higher levels of CD39, an ecto-ADPase, on HUVEC (Marcus et al., 1997). Three potential explanations for the effects of these agonists on αVβ3 function may be considered. First, PMA stimulation could increase the number of αVβ3 receptors. However, FACS® analysis of treated and untreated HUVEC and SMC showed that expression levels of αVβ3 were not changed upon stimulation. In addition, the induction of adhesion was observed after short-term treatment by the agonists (1 h or less), a time insufficient for extensive de novo synthesis. Second, receptor clustering may enhance ligand binding to integrins (Miyamoto et al., 1995; Detmers et al., 1987; Hato et al., 1998). When PMA-stimulated cells were treated with cytochalasin B at concentrations that inhibited actin cytoskeleton rearrangements as evidenced by the abolition of cell spreading on prothrombin, cell adhesion to prothrombin was not diminished. This observation does not exclude a role of integrin clustering in αVβ3 activation. Indeed, we observed that cytochalasin B in the absence of PMA could induce cell adhesion to prothrombin. Integrin activation by cytochalasins has been observed by others (Kucik et al., 1996; Qi et al., 1998) and may arise from the increased mobility of receptors in the plane of the membrane. Therefore, αVβ3 multimerization may regulate vascular cell adhesion to prothrombin. Third, PMA stimulation may change the affinity state of αVβ3 for prothrombin. It is well established that integrins can exist in multiple conformational states (Schwartz et al., 1995; Shattil and Ginsberg, 1997), which exhibit distinct functions. Such affinity modulation is a consequence of inside-out signaling and is central to the function of αIIbβ3 on platelets (Schwartz et al., 1995). Indeed, PMA is one of the agonists that activates αIIbβ3 (Shattil and Brass, 1987). Affinity modulation also has been ascribed to αVβ3 (Altiere et al., 1988), αVβ1 (Masumoto and Hemler, 1993), αVβ1 (Faull et al., 1993), and αVβ1 (Delwel et al., 1996). The capacity of αVβ3 to exist in different functional states also has been previously demonstrated (Bennett et al., 1997) although the mechanisms underlying these functional differences were not fully resolved.

PMA induced the conversion of αVβ3 from a low- to a high-affinity/avidity state for prothrombin. The activity of this agonist suggests that PKC activation may be important in the activation of αIIbβ3 (Danilov and Juliano, 1989; Vuori and Ruoslahti, 1993). This conclusion was supported by the observation that known inhibitors of PKC, calphostin C (Kobayashi et al., 1989) and bisindolylmaleimide I (Toullec et al., 1991), abolished the effect of PMA on cell adhesion to prothrombin. Calphostin C also blocked adhesion of HUVEC to prothrombin induced by Mn2+, indicating a role of this cation in activation of PKC. Thus, the influence of Mn2+ on integrin function is not restricted to its effects on the extracellular ligand binding domains of integrins (Smith et al., 1994). In addition, a second intracellular signaling molecule, the neutral protease calpain, was implicated in the activation pathway of αVβ3 based upon the effects of the membrane permeable and highly potent calpain inhibitor, calpeptin. Calpain can influence multiple intracellular signaling pathways by cleaving any of a variety of substrates including PKC, phospholipase C, pp60 Src, as well as cytoskeletal proteins including talin and paxillin (Kishimoto et al., 1989; Suzuki et al., 1992; AI and Cohen, 1993). Indeed, it has been reported that calpain can directly cleave the cytoplasmic tail of β3-subunit (Du et al., 1995). Thus, the proteolysis of any one of many potential substrates by calpain could lead to activation of αVβ3, and careful dissection will be required to identify the requisite event(s). Whereas calpain activity and integrin function have been previously linked, to date, the effects of calpain have been assigned to post-ligand binding events, outside-in signaling (Suzuki et al., 1992; Cooray et al., 1996). Our results suggest a potential role of calpain in agonist-induced activation of integrins, inside-out signaling.

In contrast to prothrombin, HUVEC adhesion to fibrinogen occurred in the absence of added agonists, and PMA treatment of the cells did not effect αVβ3-dependent adhesion to this protein. This observation provides the first di-
rect evidence that different activation states of $\alpha_\beta_3$ can discriminate between different ligands. While we cannot presume that cultured HUVEC necessarily present $\alpha_\beta_3$ in a resting or basal state, it is clear that these cells adhere to fibronectin, with or without additional stimulation, whereas interaction with prothrombin requires additional activation of the receptor. This distinction suggest that $\alpha_\beta_3$ ligands may be classified as being activation-dependent or as activation-independent. Fibronectin is an activation-independent ligand, and prothrombin represents the activation-dependent ligands of note. Platelet adhesion to both prothrombin (Byzova and Plow, 1997) and fibronogen (Savage et al., 1995) does not require activation of $\alpha_\beta_3$, emphasizing the fine differences in the recognition specificity of these two $\alpha_\beta_3$ integrins.

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