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

Tatiana V. Byzova and Edward F. Plow

Joseph J. Jacobs Center for Thrombosis and Vascular Biology, Department of Molecular Cardiology, Cleveland Clinic Foundation, Cleveland, Ohio 44195

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.

The culmination of these events is the activation of prothrombin by $\alpha_v\beta_3$ on vascular cells, suggesting that these molecules are involved in the outside-in signaling events that activate the 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 that they 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 Kreisberg, 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 an RGD recognition specificity, and αvβ3 and αIIbβ3 share many common ligands, including von Willebrand factor, fibrinogen, fibronectin, and thrombospondin (Ruoss-Lahiti, 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 (Biodesig 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). 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 α5 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 (Mazaur 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 Diagnostics 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 nonlabeled prothrombin by Factor Xa or Factor Xa/Va was the same as assessed with the Spectrozyme (American Diagnostics, 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 DME 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.
HUYVEC, HAEC, and HASMC Adhesion Assays

HUYVEC and HAEC 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 10^5 cells/ml in DME/F12, containing 1% BSA (adhesion buffer). Calcine AM (50 μg/Molecular Probes, Eugene, OR) was solubilized in 10 μl of DMSO (Sigma Chemical Co.), and then diluted with 500 μl PBS. This Calcine solution (200 μl) was added to 2 ml of cell suspension at 5 × 10^5 cells/ml. 24-well plates (Costar Corp.) were precoated with prothrombin for 1 h at 37°C (10 μg/well in 50 mM NaHCO3, 50 mM NaCl, pH 8.0) or fibrinogen (10 μg/well) and postcoated with 3% BSA for 1 h at 37°C. Cells were labeled by Calcine AM for 30 min as described above and diluted to 5 × 10^5 cells/ml in DME/F12, containing 1% BSA. For HASMC, all procedures were the same but the trypsin solution was from BioWhittaker Inc. Cells were preincubated with or without inhibitors, light-activated calphostin C (final concentration, 1 μM), bisindoylmaleimide I and V (20 nM each), calpeptin (50 μg/ml), the combination of calpain inhibitors I and II (100 μg/ml each) in the presence of additional 1 mM CaCl2, or 1 mM MnCl2, and then stimulated with PMA (200 nM) or various concentrations of ADP. The cell suspension (0.3 ml) was added to the coated wells. In some experiments, PMA-stimulated cells were treated by cytochalasin B (final concentrations, 0.1, 1, or 10 μM). At selected times (30–70 min at 37°C), wells were gently washed three times with DME/F12. Adherent cells were quantitated in a Fluorescence Multi-Well Plate Reader (PerSeptive Biosystems, Framingham, MA) and examined microscopically.

Flow Cytometry

HUYVEC, HAEC, or HASMC, harvested as described above, were resuspended at 8 × 10^6 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 and resuspended at 8 × 10^6 cells/ml in DME/F12, containing 1% BSA. Cells were labeled by Calcine AM for 30 min as described above 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 HUYVEC in Suspension

HUYVEC were diluted to 7 × 10^6/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 HUYVEC Monolayer

HUYVEC were suspended at 1 × 10^5/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 supplemented 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 by 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 αinβ3, 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 × 10^10 prothrombin molecules bound to the αvβ3-coated wells (see Fig. 1 A). In the presence of 1 mM MnCl2, ~8.4 × 10^10 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 HUYVEC 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 × 10^5 HUYVEC 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 $\alpha_v\beta_3$ 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 $\alpha_v\beta_3$ in the adhesion of the PMA-stimulated HUVEC to prothrombin, mAbs LM609 and c7E3 completely blocked adhesion, as did GRGDSP (100 $\mu$M; 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 $\alpha_v\beta_3$ in a RGD-dependent manner.

In the presence of Mn$^{2+}$, a cation that stimulates the ligand binding function of $\alpha_v\beta_3$ (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 $\alpha_v\beta_3$ in the interaction (Figs. 2 D and 3 B). No additive effect on HUVEC adhesion was observed when PMA and Mn$^{2+}$ were used together (not shown); however, we did find that after 6–7 passages, HUVEC required both PMA and Mn$^{2+}$ 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 $\alpha_v\beta_3$, 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 $\alpha_v\beta_3$ (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 Ig. We also found that Mn$^{2+}$ supported $\alpha_v\beta_3$-mediated adhesion of HASMC to prothrombin (not shown). Furthermore, with HASMC, a physiologically relevant ag-

Figure 1. $^{125}$I-prothrombin binding to purified $\alpha_v\beta_3$. (A) Saturation isotherms of $^{125}$I-prothrombin binding (150 min at 37°C) to purified and immobilized $\alpha_v\beta_3$. Specific binding (■) was derived by subtracting the nonspecific binding (○), the residual binding in the presence of a 50-fold excess of nonlabeled prothrombin, from the total binding (●), no inhibitor present. Data are derived in the presence of 1 mM Ca$^{2+}$; (B) $^{125}$I-prothrombin binding to immobilized $\alpha_v\beta_3$ in the presence of 1 mM Ca$^{2+}$ (black bars), 1 mM Mn$^{2+}$ (gray bar), and 10 mM EDTA (open bar). Wells were preincubated with mAb c7E3 or LM609 (20 $\mu$g/ml each); or GRGDSP (100 $\mu$M and 100 $\mu$M) and $^{125}$I-prothrombin was added at a concentration of 30 $\mu$g/ml. The total binding (not corrected for nonspecific background) of prothrombin to $\alpha_v\beta_3$ in the presence of 1 mM Ca$^{2+}$ without inhibitors was assigned a value of 100%. The data shown are means and SD of triplicates in one experiment and are representative of three separate experiments.
Calcein, and diluted to the 5 × 10^6 cells per ml in DME containing 0.2% BSA and 1 mM CaCl_2. In A and B, HUVEC were stimulated with 200 nM PMA or 1 mM Mn^{2+}, respectively. Inhibitors used were: mAb LM609 or mAb c7E3 (20 μg/ml each), GRGDSP peptide (100 μg), or polyclonal antibodies to prothrombin (anti-prothrombin). In C, HAEC were stimulated with PMA. In addition to the inhibitors used in A and B, soluble prothrombin was used at a concentration of 300 μg/ml. After 50 min, adhesion was measured. Adhesion in the presence of 1 mM CaCl_2 and 1 PMA (A and C) or in the presence of 1 mM MnCl_2 (B) and in the absence of inhibitors was assigned a value of 100%. The data shown are means and SD of quadruplicates in one experiment and are representative of seven separate experiments.

**Figure 3.** Endothelial cell adhesion to prothrombin requires stimulation. HUVEC (A and B) or HAEC were harvested, labeled by Calcein, and diluted to the 5 × 10^6 cells per ml in DME containing 0.2% BSA and 1 mM CaCl_2. In A and B, HUVEC were stimulated with 200 nM PMA or 1 mM Mn^{2+}, respectively. Inhibitors used were: mAb LM609 or mAb c7E3 (20 μg/ml each), GRGDSP peptide (100 μg), or polyclonal antibodies to prothrombin (anti-prothrombin). In C, HAEC were stimulated with PMA. In addition to the inhibitors used in A and B, soluble prothrombin was used at a concentration of 300 μg/ml. After 50 min, adhesion was measured. Adhesion in the presence of 1 mM CaCl_2 and 1 PMA (A and C) or in the presence of 1 mM MnCl_2 (B) and in the absence of inhibitors was assigned a value of 100%. The data shown are means and SD from three experiments.

**Figure 4.** PMA-stimulated adhesion of HASMC to prothrombin is α_3β_3-dependent. HASMC were harvested, labeled by Calcein, diluted to the 5 × 10^6 cells per ml in DME containing 1% BSA and 1 mM CaCl_2. HASMC were stimulated with either 200 nM PMA (black bars) or ADP (striped bars) at the indicated concentrations. mAb LM609 at a concentration of 20 μg/ml was included as indicated. After 40 min, adhesion was measured. Adhesion in the presence of PMA without inhibitors present was assigned a value of 100%. Nonspecific adhesion to BSA-coated wells was subtracted. The data shown are means and SD from three experiments.

**Binding of Prothrombin to Endothelial Cells**

We next sought to assess whether α_3β_3, 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 Ca^{2+} and Mg^{2+} (DME/F12 without additional cations) or in the presence of additional 1 mM Mn^{2+}. 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 Mn^{2+} cause a dramatic increase of 125I-prothrombin binding to HUVEC. Saturation of binding was apparent in the presence of Mn^{2+} 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 Mn^{2+}, compared with 18,600 ± 2,050 molecules per cell in the presence of Ca^{2+}. Thus, although Ca^{2+} did support limited specific binding of prothrombin to α_3β_3 (inhibitable by nonlabeled prothrombin, c7E3 and LM609), Mn^{2+} enhanced this interaction by ~50-fold.

To investigate whether 125I-prothrombin binding in the presence of Mn^{2+} was attributable to α_3β_3, 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 Mn^{2+} and the inhibition profile of 125I-prothrombin bind-
A

\[ \text{No inhibitors} \quad \text{LM609} \quad \text{c7E3} \quad \text{GRGDSP} \]

B

\[ \text{Mn}^{2+} \quad \text{Ca}^{2+} \]

DME/F12 media, and cell-bound radioactivity was measured after 60 min incubation. Nonspecific 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 quadruplicates in one experiment and are representative of three separate experiments.

The results are summarized in Table I. Previous studies have established that fibrinogen binds poorly to purified \( \alpha_3 \beta_1 \) in the presence of \( \text{Ca}^{2+} \) (Smith et al., 1994; Suehiro et al., 1996), and fibrinogen, even in concentrations as high as 500 \( \mu \text{g/ml} \), was a poor inhibitor of prothrombin binding, producing only 11% inhibition in 1 mM \( \text{Ca}^{2+} \). In the presence of \( \text{Mn}^{2+} \), fibrinogen was a more effective inhibitor, producing 40% inhibition at 500 \( \mu \text{g/ml} \). Nevertheless, substantial binding of prothrombin was still observed. The data obtained for \( ^{125} \text{I-prothrombin} \) binding to HUVEC were consistent with those obtained in the previous studies. Competition between Prothrombin and Fibrinogen for \( \alpha_3 \beta_1 \)

The capacity of a major \( \alpha_3 \beta_1 \) ligand, fibrinogen, to compete with prothrombin for binding to the receptor was assessed. These analyses were conducted with both purified \( \alpha_1 \beta_3 \) 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 \( \alpha_3 \beta_1 \) in the presence of \( \text{Ca}^{2+} \) (Smith et al., 1994; Suehiro et al., 1996), and fibrinogen, even in concentrations as high as 500 \( \mu \text{g/ml} \), was a poor inhibitor of prothrombin binding, producing only 11% inhibition in 1 mM \( \text{Ca}^{2+} \). In the presence of \( \text{Mn}^{2+} \), fibrinogen was a more effective inhibitor, producing 40% inhibition at 500 \( \mu \text{g/ml} \). Nevertheless, substantial binding of prothrombin was still observed. The data obtained for \( ^{125} \text{I-prothrombin} \) binding to HUVEC were consistent with those obtained in the previous studies. Competition between Prothrombin and Fibrinogen for \( \alpha_3 \beta_1 \)

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![Graph A](image1.png)

![Graph B](image2.png)

**Figure 6.** \( ^{125} \text{I-prothrombin} \) binding to a HUVEC monolayer. Confluent cell monolayers were incubated with 50 \( \mu \text{g/ml} \) \( ^{125} \text{I-prothrombin} \) in DME/F12-1% BSA in the presence or absence of 200 nM PMA (solid bars) or 0.5 mM MnCl\(_2\) (gray bars). Cells were preincubated with c7E3 (30 \( \mu \text{g/ml} \)) or a cyclic RGD peptide (10 \( \mu \text{M} \)) 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 \(\alpha\)\(\beta\)_3.

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

We sought to understand the mechanism by which \(\alpha\)\(\beta\)_3 became competent to bind prothrombin in the presence of agonist. FACS analysis was used to determine whether the expression levels of \(\alpha\)\(\beta\)_3 on HUVEC and HASMC is altered by PMA stimulation. This analysis confirmed high levels of their expression but not altered the expression level of \(\alpha\)\(\beta\)_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 adhesion to prothrombin (see Fig. 8 A). Similar results were obtained with a second PKC inhibitor, bisindolylmaleimide I, but not with the low affinity control inhibitor, bisindolylmaleimide V (Toullec et al., 1991). At the concentration used, calphostin C and bisindolylmaleimide I did not affect cell viability, as assessed by trypan blue staining, even with extended incubation times. These results indicate that the effect of PMA on increased adhesion depends upon PKC. Of note, treatment with calphostin C also abolished attachment and spreading of HUVEC on prothrombin in the presence of Mn\(^{2+}\) (see Fig. 8 A). This observation suggests that, like PMA, the induction of prothrombin binding to \(\alpha\)\(\beta\)_3 on HUVEC by Mn\(^{2+}\) requires inside-out signaling events.

As a next step, we investigated the role of another potential candidate in PMA-induced cell adhesion to prothrombin, calpain. Numerous activities have been ascribed to this neutral calcium-dependent protease, including the capacity to cleave the cytoplasmic tail of \(\beta\)_3 subunit (Du et al., 1995) and to regulate cell migration (Huttenlocher et al., 1997). HUVEC, pretreated with membrane permeable calpain inhibitor, calpeptin (Tsujimaka et al., 1988), were unable to adhere to prothrombin either after PMA stimulation or in the presence of Mn\(^{2+}\) (Fig. 7 A). The combination of calpain inhibitors I and II also was effective in inhibiting HUVEC adhesion to prothrombin. These findings indicate that active calpain is required for modulation of \(\alpha\)\(\beta\)_3 affinity on endothelial cells induced by PMA and Mn\(^{2+}\). To determine if PMA stimulation exerted its effect on prothrombin binding by \(\alpha\)\(\beta\)_3 by influencing postligand binding events, such as integrin clustering and cell spreading, PMA-activated cells were treated with cytochalasin B, an effective inhibitor of actin cytoskeleton reorganization. Untreated HUVEC adhered and spread on prothrombin, whereas, in the presence of cytochalasin B, the cells remained adherent but entirely rounded (Fig. 8 B). This effect indicates that the cytochalasin treatment altered the cytoskeletal response of the cells. Quantitation of the number of adherent cells verified that the adhesion of PMA-stimulated HUVEC to prothrombin was not substantially changed by cytochalasin B at a concentration of 1 or 10 \(\mu\)M (Fig. 8 C). Neither of these concentrations were toxic for cells. HUVEC adhesion in the presence of

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**Table I. Effect of Fibrinogen on Prothrombin Binding to \(\alpha\)\(\beta\)_3 in a Purified System and on Endothelial Cells**

<table>
<thead>
<tr>
<th>Cation conditions</th>
<th>(1) mM Ca(^{2+})</th>
<th>(1) mM Mn(^{2+})</th>
<th>(2) mM Ca(^{2+})</th>
<th>(2) mM Mn(^{2+})</th>
<th>(2) mM Mg(^{2+})</th>
<th>(1) mM Mn(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>No competitor</td>
<td>100 ± 7.1%</td>
<td>100 ± 9%</td>
<td>100 ± 12%</td>
<td>100 ± 18%</td>
<td>90 ± 12%</td>
<td>85 ± 11%</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>89 ± 9%</td>
<td>60.7 ± 4%</td>
<td>85.4 ± 11%</td>
<td>38 ± 5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(500 µg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>107 ± 14%</td>
<td>93 ± 10.3%</td>
<td>92.7 ± 11.9%</td>
<td>76.9 ± 4%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 \(\alpha\)\(\beta\)_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 one experiment which is representative of at least five experiments.

*Experimental conditions are the same as in Fig. 1 with purified \(\alpha\)\(\beta\)_3 immobilized onto microtiter wells.

**Experimental conditions are the same as in Fig. 5 with HUVEC in suspension.**

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**Figure 7.** Short-term PMA treatment does not increase \(\alpha\)\(\beta\)_3 expression. HUVEC (A and B) or HASMC (C and D) were grown and harvested as described. For each experimental point, 10\(^{6}\) cells were incubated in the presence of nonimmune IgG (I) or mAb LM609 (2) for 50 min. In B and D cells were treated by 200 nM PMA. After washing, the cells were incubated with anti-mouse IgG FITC-conjugated antibody and analyzed by flow cytometry.
Mn$^{2+}$ or Mn$^{2+}$ + PMA also was unaffected by cytochalasin B. These results suggest that PMA stimulation modulates the affinity of $\alpha_\text{V}\beta_3$ 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 $\alpha_{\text{IIb}}\beta_3$-mediated adhesion to fibrinogen.

To determine if the activation requirement for recognition of prothrombin by $\alpha_\text{V}\beta_3$ extends to other $\alpha_\text{V}\beta_3$ ligands, we assessed the effects of cell stimulation and of the inhibitors, calphostin C and calpeptin, on $\alpha_\text{V}\beta_3$-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 $\alpha_{\text{IIb}}\beta_3$ mediated. $\alpha_\text{V}\beta_3$-dependent adhesion was identified as that component of total cell adhesion that was sensitive to the anti-$\alpha_\text{IIb}\beta_3$ blocking mAbs, LM609 or c7E3 (Fig. 9A). For nonstimulated cells, $\alpha_\text{V}\beta_3$-mediated adhesion was ~37% (100% is defined as the total adhesion in the presence of PMA). Treatment with PMA caused an increase in total HUVEC adhesion, but the $\alpha_\text{V}\beta_3$-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, $\alpha_\text{V}\beta_3$-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 $\alpha_\text{V}\beta_3$ mediated (Fig. 9B). In contrast to HUVEC adhesion to prothrombin, pretreatment of HUVEC with calphostin C did not significantly decrease 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 $\alpha_\text{V}\beta_3$-mediated adhesion to prothrombin and fibrinogen are quite distinct.

**Discussion**

In this study, we sought to assess whether prothrombin is a ligand for $\alpha_\text{V}\beta_3$ on vascular cells. A direct interaction between prothrombin and $\alpha_\text{V}\beta_3$ 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 $\alpha_\text{V}\beta_3$ 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 $\alpha_\text{V}\beta_3$ 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 $\alpha_\text{V}\beta_3$ on vas-
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 α3β1 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 α3β1. 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 α3β1 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 α3β1; i.e., similar to the recognition of the γ chain, rather than the RGD sequence of fibrinogen by αMβ3 (Farrell et al., 1992), even though αMβ3 also has a RGD recognition specificity. Also to be resolved is whether factor X, which also contains a RGD sequence, can interact with α3β1.

The α3β1 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 α3β1 is directly exposed to plasma proteins, including prothrombin. From our analyses of the interaction of prothrombin with α3β1, either in purified form or on cells, half-maximal binding occurred at input concentrations of ~50 μg/ml and almost 100 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 also may 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 α3β1, a potential functional consequence of prothrombin binding to α3β1 would be its 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 (Seifert 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 αIbβ3 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 cytochalasin B 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 αIbβ3 on platelets (Schwartz et al., 1995). Indeed, PMA is one of the agonists that activates αIbβ3 (Shattil and Brass, 1987). Affinity modulation also has been ascribed to αvβ3 (Altieri et al., 1988), αvβ6 (Masumoto and Hemler, 1993), αvβ3 (Faull et al., 1993), and αvβ5 (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 αvβ3 (Danilov and Juliano, 1989; Vuori and Rusolatti, 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; Al 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 αβ can discriminate between different ligands. While we cannot presume that cultured HUVEC necessarily present αβ in a resting or basal state, it is clear that these cells adhere to fibrinogen, with or without additional stimulation, whereas interaction with prothrombin requires additional activation of the receptor. This distinction suggest that αβ ligands may be classified as being activation-dependent or as activation-independent. Fibrinogen is an activation-independent ligand, and prothrombin represents the activation-dependent ligands. Of note, platelet adhesion to both prothrombin (Byzova and Plow, 1997) and fibrinogen (Savage et al., 1995) does not require activation of αβ, emphasizing the fine differences in the recognition specificity of these two β integrins.

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