Cleavage of osteopontin by thrombin has been reported to enhance cell adhesion. We asked whether thrombin could regulate the αβ3-mediated adhesion of platelets and B lymphocytes to this substrate. Although there was no difference in the extent or the avidity of thrombin- and ADP-stimulated platelet adhesion to intact or thrombin-cleaved human osteopontin, both the extent and avidity of phorbol ester-stimulated B cell adhesion to thrombin-cleaved osteopontin was significantly increased. Thus, these data suggest that the ability of αβ3 to recognize osteopontin can be differentially regulated in a cell-specific manner. To localize the αβ3 binding site on osteopontin, we measured cell adhesion to the two thrombin cleavage products of osteopontin and to a series of nested RGD-containing osteopontin peptides cross-linked to albumin. Whereas ADP-stimulated platelets adhered to the amino-terminal but not the carboxyl-terminal osteopontin fragment and to the osteopontin peptide RGDSVVGLR, phorbol ester-stimulated B cells did not adhere to this peptide, although they did so in the presence of 1 mM Mn2+. Thus, our data confirm that thrombin cleavage enhances the accessibility of the binding motif for αβ3 on osteopontin, but this enhancement is also a function of the activation state of αβ3. Moreover, they indicate that the sequence RGDSVVGLR contains sufficient information to specify activation-dependent αβ3-mediated platelet and lymphocyte adhesion.

The initial event in the formation of a hemostatic platelet plug or a platelet thrombus is platelet adhesion to components of the subendothelial matrix of a damaged blood vessel (1). The acidic phosphorylated glycoprotein osteopontin (OPN) is a component of the subendothelial matrix of blood vessels involved in atherosclerosis, in which it surrounds areas of dystrophic calcification. We reported that platelets and B lymphocytes adhere to OPN-coated surfaces and that their adhesion is mediated by the integrin αβ3 (2). Unlike other cells that adhere to OPN, however, the adhesion of platelets and B cells to OPN requires agonist stimulation (2), indicating that the activation state of αβ3 on platelets and lymphocytes, like that of the homologous platelet integrin αIIbβ3, is regulated by cellular agonists.

The most potent physiologic platelet agonist is thrombin (3). It is noteworthy that OPN contains a potential thrombin cleavage site six amino acids downstream from an RGDG motif (4) and that thrombin cleavage has been reported to enhance the ability of OPN to support the attachment and spreading of a number of cultured cells in vitro (5). We postulated that not only might thrombin regulate the activation state of platelet αβ3, but it might also regulate the ability of OPN to serve as a substrate for platelet adhesion. To address this possibility, we cleaved recombinant human OPN with thrombin and compared the ability of the cleaved protein and the isolated cleavage products to support platelet adhesion. In addition, because agonist stimulation is also required to induce B cell adhesion to OPN, we examined the effect of thrombin cleavage on B cell adhesion.

EXPERIMENTAL PROCEDURES

Bacterial Expression of Recombinant Human OPN—Recombinant human OPN was synthesized as a histidine-tagged fusion protein using the pET system (Novagen) as described previously (2). Briefly, recombinant OPN was synthesized as insoluble inclusion bodies in Escherichia coli BL21(DE3)pLysS, solubilized using 6 M guanidine-HCl, and isolated by metal-chelate affinity chromatography on a Ni2+-NTA resin (His-Bind resin, Novagen). Following elution from the resin using 20 mM Tris-HCl buffer, pH 7.9, containing 0.5 M NaCl and 500 mM imidazole, the recombinant OPN was renatured by dialysis against phosphate-buffered saline, pH 7.4. The apparent molecular weight of the recombinant protein determined by SDS-polyacrylamide gel electrophoresis was 58,000. Its weight determined by electrospray mass spectrometry was 35,518, consistent with the calculated mass of the full-length osteopontin amino acid backbone (4) plus the poly-histidine tag and Factor Xa cleavage site contributed by pET16b.

Cleavage of OPN by Thrombin and Purification of the Cleavage Products—Recombinant OPN was incubated with 0.32 unit of human thrombin (a gift of Dr. Lawrence F. Brass) per μg of OPN at 37 °C for 30 min. Thrombin activity was then quenched using a 1.3-fold excess of hirudin (Sigma). Complete OPN cleavage was verified by electrophoresis of the products of the cleavage reaction on a 0.1% SDS, 4–20% gradient polyacrylamide gel. As reported previously (5), thrombin cleaves OPN into two fragments with apparent molecular weights of 32,000 and 30,000 (Fig. 1). OPN cleavage was detectable following incubation with as little thrombin as 0.1 unit/ml, but complete cleavage required thrombin concentrations of at least 10 units/ml.

The OPN cleavage products were purified using reverse phase HPLC (series 1100 model, Hewlett Packard) on an analytical C4 column (Vydac). A linear AB gradient of 0.6% B/min at 1 ml/min was used in which solvent A was 0.1% aqueous trifluoroacetic acid and solvent B was 0.1% trifluoroacetic acid in 90% acetonitrile. Individual products were identified using SDS-polyacrylamide gel electrophoresis, amino acid analysis, and laser desorption mass spectrometry.
Platelet and Lymphocyte Adhesion to Osteopontin

RESULTS

Effect of Thrombin Cleavage on Platelet Adhesion to OPN—To test whether thrombin cleavage affects platelet adhesion to OPN-coated surfaces, we coated the wells of microtiter plates with equal quantities of intact OPN and thrombin-cleaved OPN and compared the adhesion of unstimulated and agonist-stimulated platelets to each substrate. As shown in Fig. 2A, whereas unstimulated platelets were unable to adhere to either intact or thrombin-cleaved OPN, equal numbers of thrombin-stimulated platelets readily adhered to each substrate. Nonetheless, it is possible that although the number of adherent platelets was not increased, thrombin cleavage may have increased the avidity of platelet adhesion instead. To test this possibility, we compared the ability of the tetrapeptide RGDS to inhibit platelet adhesion to each substrate, reasoning that there is a direct relationship between the IC_{50} of RGDS inhibition and the avidity of cell adhesion. However, we found no significant difference in the IC_{50} for RGDS (39.7 ± 11.9 and 34.6 ± 9.6 μM; n = 4; p = 0.75).

Thrombin is the most potent physiologic platelet agonist (3), and it is conceivable that differences in the ability of intact and thrombin-cleaved OPN to support platelet adhesion might be apparent with a weaker agonist. To test this possibility, we repeated the experiments using ADP as the platelet agonist. However, as shown in Fig. 2B, there was again no difference in the number of platelets adherent to either substrate (n = 7; p = 0.06), nor was there a significant difference in the IC_{50} for RGDS inhibition (13.2 ± 0.9 and 14.9 ± 1.1 μM; n = 3; p = 0.29). On the other hand, the platelets in these experiments were maximally stimulated by 10 μM ADP. To determine whether an effect of OPN cleavage might be observed at lower ADP concentrations, we measured platelet adhesion as a function of ADP concentration. As shown in Fig. 2C, there was no difference in platelet adhesion to either substrate as the concentration of ADP was varied from 0.2 to 20 μM. Thus, in contrast to the behavior of a number of epithelial and mesenchymal cells (5), we found that thrombin cleavage increased neither the number of platelets that adhere to OPN nor the avidity of their adhesion.

Identification of the OPN Fragment that Mediates Platelet Adhesion—To determine which of the OPN fragments generated by thrombin cleavage supports platelet adhesion, the fragments were purified using reverse phase HPLC and examined by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 3A, the purified fragments had apparent molecular weights of 32,000 and 30,000, whereas MALDI-TOF spectroscopy indicated molecular weights of 18,663 and 16,828, values corresponding to the calculated masses of the amino- and carboxyl-thrombin cleavage fragments of OPN, respectively. Amino acid analysis of the purified fragments confirmed their identity. The purified frag-

FIG. 1. Cleavage of OPN by thrombin. Recombinant human OPN was incubated with the indicated concentrations of human α-thrombin at 37 °C for 40 min as described under “Experimental Procedures.” The extent of OPN cleavage was then determined by electrophoresis of the cleavage products on a 0.1% SDS, 4–20% gradient polyacrylamide gel.

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Measurement of Platelet and Lymphocyte Adhesion to OPN and OPN-related Peptides—96-well flat-bottomed microtiter plates (Immulon 2 Dynatech) were coated with 5 μg/ml of recombinant intact OPN, thrombin-cleaved OPN, BSA, or various concentrations of peptide-BSA complexes, each dissolved in 50 mM NaHCO_{3} buffer, pH 8.0, containing 150 mM NaCl, 2.7 mM KCl, 5.6 mM glucose, 1 mM CaCl_{2}, 3.3 mM NaH_{2}PO_{4}, and 0.35 mM/ml BSA were added to the protein-coated wells in the absence or presence of a platelet agonist. Following an incubation for 30 min at 37 °C without agitation, the plates were washed four times with 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, and the number of adherent platelets was measured using a colorimetric assay, as described previously (6).

The phorbol myristate acetate (PMA)-stimulated adhesion of GM1500 B lymphocytes to OPN was measured as described previously (7). Briefly, 1.5 × 10^6 lymphocytes, metabolically labeled with [35S]methionine and suspended in 100 μl of 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, 0.5 mM CaCl_{2}, 0.1% glucose, and 1% BSA, were stimulated with 200 ng/ml PMA and added to the wells of the protein-coated microtiter plates. Following an incubation at 37 °C for 30 min, the plates were washed four times with the lymphocyte suspension buffer, and adherent cells were dissolved using 2% SDS. The SDS solutions were then counted for 35S in a liquid scintillation counter.

The statistical significance of differences in platelet or lymphocyte adhesion was determined using the Student’s t test for two samples assuming equal variance (Microsoft Excel 98 for the Macintosh).

Spectroscopic Examination of OPN-related Peptides—CD spectra were recorded with a model 62 DS spectropolarimeter (Aiviv, Lakewood, NJ), using an 1 mm path length quartz cell. The cell holder was temperature controlled at 25 °C. The bandwidth was 1 nm, with a scan step of 1 nm and an averaging time of 10 s per point. The buffer was 5 mM HEPES, pH 7.4, containing 1 mM CaCl_{2}. The concentrations of peptide stock solutions were determined by the absorbance at 280 nm in H_{2}O using an extinction coefficient of 1490 cm^{-1} m^{-1} per tyrosine. Ellipticity is reported as mean residue molar ellipticity. One-dimensional proton NMR spectra were acquired on a Bruker AMX 500 operating at a 500.13 MHz proton frequency. Peptides were studied at 1 mM in a 50 mM deuterated sodium acetate buffer, pH 5.5.

Synthesis and Characterization of OPN-related Peptides—Peptides overlapping the RGDS motif in the amino-terminal OPN cleavage fragment were synthesized using Fmoc (N-[9-(9-fluorenylethoxycarbonyl)] chemistry on an Applied Biosystems A433 peptide synthesizer. An Arg-preloaded Wang resin with a 0.33 mmol/g substitution level was used. After completion of the amino acid sequence, the peptideyl resins were treated for 2 h at room temperature with a cleavage mixture consisting of 90% trifluoroacetic acid, 5% thioanisole, 3% ethanedithiol, and 2% anisole. The crude peptides were then purified by HPLC (Dynaxam, Rainin) using a preparative C18 column (Vydac) and a 0.6%/min acetonitrile gradient at a 15 ml/min flow rate. Fractions containing pure peptide were pooled and lyophilized. The peptides were determined to be homogeneous by analytical HPLC, and their calculated molecular weights were confirmed by MALDI-TOF spectrometry.

Cross-linking OPN-related Peptides to Albumin—Nested OPN peptides were also synthesized that contained the amino acid sequence GCCGG at their amino-terminal ends. The cysteine thiol was used as a handle to attach the peptides to thiopyridone-activated bovine serum albumin. Bovine serum albumin (Sigma) dissolved in phosphate-buffered saline, pH 7.4, containing 1 mM EDTA at a concentration of 2 mg/ml was reacted with a 10-fold molar excess of the heterobifunctional cross-linker sulfosuccinimidyl-6-aminohexylthiopyridone/tiol (Pierce). The reaction was stopped after 2 h by chromatography through a Sephadex G-25 M PD-10 column (Amersham Pharmacia Biotech) equilibrated with the same buffer. Peptides at 0.2 mg/ml were subsequently incubated with the activated BSA for 48 h at 4 °C. The stoichiometry of cross-linking was calculated after spectrophotometrically measuring the release of the cross-linking byproduct pyridine-2-thione at 343 nm (ε = 8080 ± 300 m M^{-1} cm^{-1}). The molar ratio of each OPN peptide to BSA was approximately 3, as determined using two separate preparations of activated BSA.

Identification of the OPN Fragment that Mediates Platelet Adhesion—To determine which of the OPN fragments generated by thrombin cleavage supports platelet adhesion, the fragments were purified using reverse phase HPLC and examined by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 3A, the purified fragments had apparent molecular weights of 32,000 and 30,000, whereas MALDI-TOF spectroscopy indicated molecular weights of 18,663 and 16,828, values corresponding to the calculated masses of the amino- and carboxyl-thrombin cleavage fragments of OPN, respectively. Amino acid analysis of the purified fragments confirmed their identity. The purified frag-

FIG. 1Cleavage of OPN by thrombin. Recombinant human OPN was incubated with the indicated concentrations of human α-thrombin at 37 °C for 40 min as described under “Experimental Procedures.” The extent of OPN cleavage was then determined by electrophoresis of the cleavage products on a 0.1% SDS, 4–20% gradient polyacrylamide gel.
ments were then immobilized on microtiter plates, and platelet adhesion to each was compared with platelet adhesion to intact OPN. As shown in Fig. 3B, whereas unstimulated platelets were unable to adhere to intact OPN and either the amino-terminal or carboxyl-terminal OPN fragments, ADP-stimulated platelets adhered equally well to intact OPN and to the amino-terminal fragment. Adhesion to each was reduced to baseline levels by 5 mM RGDS. Thus, these experiments indicate that agonist-stimulated platelets recognize a sequence motif located in the amino-terminal half of the OPN molecule and suggest that this motif contains the sequence RGD.

Localization of the Site on the Amino-terminal Fragment of OPN Recognized by Platelets Using Nested Peptides—To identify the sequence motif in OPN that supports platelet adhesion, we synthesized a series of nested OPN-related peptides, the carboxyl termini of which corresponded to Arg-168 at the thrombin cleavage site. However, because we were unable to consistently immobilize these peptides on polystyrene, we synthesized a second series of peptides containing the sequence Gly-Gly-Cys-Gly-Gly at their amino termini (Table I), enabling us to cross-link the peptides to BSA using the bifunctional cross-linking reagent sulfosuccinimidyl-6-[a-methyl-a(2-pyridyl)dithio)tolune hexanoate at a stoichiometry of approximately 3 molecules of peptide per molecule of albumin. We then coated the wells of microtiter plates with either the modified albumin or albumin that had been incubated with cross-linker in the absence of peptide (not shown). Platelet adhesion to these surfaces required agonist stimulation and was inhibited completely by
a series of nested OPN peptides, the carboxyl termini of which corresponded to Arg-168 at the thrombin cleavage site, was synthesized (Table I) and cross-linked to bovine serum albumin. As with platelets, there was no significant difference in lymphocyte adhesion to intact and thrombin-cleaved OPN—

![Observed data](http://www.jbc.org/)

**Fig. 4.** Platelet adhesion to RGDS-containing OPN peptides cross-linked to bovine serum albumin. A series of nested OPN peptides, the carboxyl termini of which corresponded to Arg-168 at the thrombin cleavage site, was synthesized (Table I) and cross-linked to bovine serum albumin. As with platelets, there was no significant difference in lymphocyte adhesion to intact and thrombin-cleaved OPN.

The platelets in these experiments were maximally stimulated by 10 μM ADP. To test whether differences in platelet adhesion to the three peptides might be apparent at lesser degrees of ADP stimulation, we measured adhesion as a function of ADP concentration. As shown in Fig. 5, there were no significant differences in platelet adhesion to each of the peptides as the concentration of ADP was increased from 0.2 to 10 μM. To address the possibility that differences might be seen at lower densities of immobilized peptide, the coating concentration of peptide-cross-linked albumin was varied from 0.25 to 200 μg/ml. Again, no substantial differences in platelet adhesion to the 10-, 17-, and 38-residue peptides were observed (data not shown).

**Effect of Thrombin Cleavage on Lymphocyte Adhesion to OPN**—Because agonist-stimulated B lymphocytes also adhere to OPN-coated surfaces, we asked whether thrombin cleavage might affect B cell adhesion to this substrate. As expected, there was little adhesion of unstimulated B cells to intact OPN, whereas PMA stimulation increased adhesion ~4-fold (Fig. 6A). However, in contrast to the lack of effect of thrombin cleavage on platelet adhesion, thrombin cleavage resulted in a significant 2.1 ± 0.4-fold increase in the number of PMA-stimulated lymphocytes adherent to OPN (n = 9; p < 0.006). Moreover, as indicated by an increase in the IC₅₀ for RGDS, thrombin cleavage also increased the avidity of B cell adhesion to OPN ~6-fold (n = 8; p < 0.005).

One possible explanation for the difference in the behavior of platelets and lymphocytes toward intact and thrombin-cleaved OPN is a difference in the agonist-induced activation state of α₅β₃ on these cells. To address this possibility, we measured the adhesion of unstimulated B cells to intact and thrombin-cleaved OPN in the presence of increasing concentrations of MnCl₂ because Mn²⁺ ions are thought to modulate the activation state of integrins by affecting the conformation of their extracellular domains (8, 9). As shown in Fig. 6B, increasing the MnCl₂ concentration from 0.1 to 5 mM decreased the difference in lymphocyte adhesion to intact and thrombin-cleaved OPN such that there was no significant difference at MnCl₂ concentrations >2 mM. Thus, these results suggest that in the presence of a sufficient stimulus, α₅β₃ on lymphocytes can no longer distinguish between intact and thrombin-cleaved OPN.

**Lymphocyte Adhesion to the Nested OPN Peptides**—We next measured B cell adhesion to the nested OPN peptides cross-linked to albumin. As with platelets, there was no B cell adhesion to any of the peptides in the absence of agonist stimulation (Fig. 7A). Unlike the case with platelets, however, there was substantial PMA-stimulated adhesion to the 38-mer, but little adhesion, if any, to the 10- and 17-residue peptides. Adhesion...
Adhesion of Epstein-Barr virus-transformed GM1500 B lymphocytes to intact and thrombin-cleaved OPN. A, GM1500 B lymphocytes (1.5 × 10⁶) labeled metabolically with [³⁵S]methionine, were stimulated with 200 ng/ml PMA and added to the wells of microtiter plates coated with either intact or thrombin-cleaved OPN. Following a 30-min incubation at 37 °C, the plates were washed four times, and adherent cells were dissolved using 2% SDS. The SDS solutions were then counted for [³⁵S] in a liquid scintillation counter. The avidity of lymphocyte adhesion was determined by performing the adhesion assay in the presence of increasing concentrations of RGDS. B, concentrations of metabolically labeled GM1500 cells containing the indicated concentrations of Mn²⁺ were added to the wells of microtiter plates coated with intact or thrombin-cleaved OPN. Following a 30-min incubation, the number of adherent lymphocytes was measured as described above. The data are presented as the mean and S.E. of triplicate determinations from representative experiments. The numbers in parentheses are the ratio of lymphocyte adherence to intact versus thrombin-cleaved OPN.

To determine whether these results were a function of the activation state of α₅β₃, we measured the adhesion of unstimulated B cells to the immobilized 10-mer in the presence of increasing concentrations of Mn²⁺. As shown in Fig. 8, adhesion increased from 0.4 to 38% as the Mn²⁺ concentration increased from 0.5 to 5 mM. Adhesion of cells in the presence of Mn²⁺ was again inhibited by both LM609 and 7E3, confirming that it was mediated by α₅β₃. Thus, these experiments indicate that α₅β₃ on B cells can recognize the 10-residue peptide but suggest that its ability to do so is a function of its state of activation.

Spectroscopic Examination of the OPN-related Peptides—To investigate structural features of the OPN peptides that might account for the data shown in Figs. 7 and 8, we used CD and NMR spectroscopy. As shown in Fig. 9A, CD spectra of the 10-, 17-, and 38-residue peptides did not reveal strong negative peaks at 208 and 222 nm, implying that none of the peptides contained substantial stretches of α-helix. It is noteworthy, however, that as the length of the peptides increased, the magnitude of the n−π* transition near 220 nm also increased. This suggests that the longer peptides may have some increased conformational order. To further characterize the conformation of the 38-mer, we recorded its one-dimensional NMR spectrum. As shown in Fig. 9B, the methyl region of the spectrum (0–1.5 ppm) revealed 12 resolved resonances, compared with the theoretical number of 20, suggesting that the peptide may have a partially folded structure in solution. However, two-dimensional NOESY spectra failed to show any long-range NOEs that would be indicative of a well defined tertiary structure (data not shown). Thus, the β structure in the 38-mer is only marginally stable, and the peptide appears to also adopt a population of unfolded conformations in solution.
DISCUSSION
A number of integrins, including αvβ1 (10), αvβ3 (11, 12), αvβ5 (11, 13), αβ1 (14), αβ1 (15), and αvβ3 (16, 17), support cell adhesion to OPN. In most instances, these integrins bind constitutively to OPN, and this interaction requires the presence of an RGD motif located in the amino half of the OPN molecule (18). The RGD sequence in OPN is located in proximity to an Arg-Ser sequence that is susceptible to cleavage by thrombin. Senger et al. (5) reported that the adhesion, spreading, and migration of a variety of cultured cells on surfaces coated with OPN was increased by 2–5.5-fold following OPN cleavage by thrombin. In an extreme example of this phenomenon, HT1080 fibrosarcoma cells were unable to adhere to intact OPN, but readily attached following thrombin cleavage (5). Similarly, Smith et al. (16) reported that αvβ3-mediated adhesion to OPN requires thrombin cleavage. Because the adhesion of the cultured cells to OPN was also RGD-dependent, these data suggest that thrombin cleavage makes the RGD motif more accessible (5, 19).

In contrast to cultured epithelial and mesenchymal cells, the adhesion of platelets and B lymphocytes to intact OPN requires agonist stimulation (2). Because OPN in plasma can be cleaved by thrombin during blood coagulation (20), we postulated that thrombin might convert OPN into a more efficient adhesive substrate, perhaps enabling it to bypass the requirement for cell stimulation. However, we found that although platelets and B cells readily adhere to surfaces coated with thrombin-cleaved OPN, their adhesion remained agonist-dependent. Nevertheless, we did find striking differences in the ability of platelets and B cells to interact with the intact or thrombin-cleaved protein. Regardless of the agonist used, there was no difference in the ability of platelets to bind to either form of the protein, whereas both the extent and the avidity of phorbol ester-stimulated B cell adhesion to OPN were increased by thrombin cleavage. Thus, these data indicate that for some cells of hematopoietic origin, thrombin cleavage makes OPN a more efficient adhesion substrate. Our data also suggest that the ability of αvβ3 to recognize OPN can be differentially regulated and that this regulation is cell type-specific.

The prototypic example of regulated integrin activity is platelet αIIbβ3. αIIbβ3 is unable to bind soluble ligands such as fibrinogen in the absence of platelet stimulation, but its affinity for fibrinogen increases rapidly following platelet exposure to agonists such as ADP (21). Stimulation by stronger agonists, such as thrombin, however, is necessary to enable αIIbβ3 to bind soluble fibronectin (10), indicating that the ability of αIIbβ3 to discriminate among ligands is regulated by the strength of the platelet stimulus. Unlike αIIbβ3, the integrin αvβ3 binds constitutively to VCAM-1 and to the alternatively spliced fibronectin CS1 domain. Nevertheless, its ability to bind to these ligands can be modulated at the cellular level. For example, Masumoto and Hemler (22) and Yednock et al. (23) found substantial variability in the ability of αvβ3 to interact with VCAM-1 or CS1 among a number of leukocyte cell lines. However, the variability could be largely eliminated by increasing the affinity of αvβ3 for these ligands with Mn2+ or β1 integrin-activating mAbs (8, 9), implying that αvβ3 can display a range of cell-specific activation states. Similarly, Garcia et al. used a spinning disc device to apply hydrodynamic forces to adherent...
cells and found that although the interaction of αb3 with fibronectin was constitutive, the force required to break αb3-fibronectin bonds was sensitive to the activation state of αb3 (24).

Our results indicate that the regulation of αb3 function resembles that of αvβ3. Byzova et al. (26) reached similar conclusions after studying ligand binding to αb3 on endothelial and smooth muscle cells and JY B lymphoblasts, respectively. Nevertheless, like αb3 function in leukocytes, we found substantial differences in the extent of agonist-stimulated αb3 activation in platelets and B cells. Thus, the αb3 on both ADP- and thrombin-stimulated platelets appeared to be present in a highly activated state such that it was unable to distinguish between cleaved and uncleaved OPN and was able to bind to each of the nested OPN peptides. By contrast, the interaction of αb3 on PMA-stimulated B cells with OPN was significantly augmented by thrombin cleavage, and it did not mediate cell adhesion to surfaces coated with the 10- and 17-residue peptides. Accordingly, it is possible that these peptides simply do not contain sufficient information to produce productive adhesion in the solid state; however, both readily supported αb3-mediated platelet adhesion, and the 10-mer supported B cell adhesion when the activation state of αb3 was enhanced by Mn2+.

Adhesion assays preclude direct measurements of the affinity of αb3 on platelets and lymphocytes for OPN. Thus, it is conceivable that unlike endothelial and JY cells (25, 26), agonists regulate the avidity, rather than the affinity, of αb3 on platelets and B cells for this ligand. Pampori et al. (26) were unable to detect the binding of a patch-engineered mAb containing a 50-residue RGDF-containing stretch from the adenovirus penton base (WOW-1) to thrombin-stimulated platelets, although the construct bound to antibody-activated αb3 on CHO cells and to αb3 on PMA-stimulated JY lymphoblasts. It is possible, however, that there is an insufficient amount of αb3 on platelets to detect WOW-1 binding using flow cytometry (27). Moreover, they did not detect penton base binding to thrombin-stimulated platelets, suggesting that perhaps this protein is not a ligand for αb3 on platelets. On the other hand, we found that ADP-stimulated platelet adhesion to immobilized OPN was completely inhibited by soluble OPN with an IC50 of ~9 µM (data not shown). Thus, these data suggest that agonist stimulation provided αb3 with access to soluble OPN, implying an increase in the affinity of αb3 for this ligand (8, 9).

The studies using nested peptides provide considerable information regarding the specificity of β3 integrins for their ligands. The αb3-specific adhesion of agonist-stimulated platelets to the peptide RGDSVYGLR indicates that this sequence contains sufficient information to differentiate between αb3 and αINaβ3 and to confer activation dependence to adhesion. This finding is particularly significant in view of the fact that αINaβ3 is present at 100–1000-fold excess over αb3 in human platelets (27). However, examination of the secondary structure of this peptide and of the larger 17- and 38-residue peptides by CD spectroscopy indicated that they adopt flexible, random conformations in solution, although further examination of the 38-residue peptide by one-dimensional NMR revealed some evidence for a partially folded structure. Nonetheless, the NOESY spectra for this peptide clearly showed that the peptide has no defined tertiary structure, but instead appears to adopt a condensed but nevertheless flexible conformation. It is possible to design highly rigid cyclic peptides with a very high affinity and specificity for αb3, presumably because the peptides are locked into conformations that closely match the conformation recognized by this integrin. For example, replacing a type II β turn with a type I β turn in the cyclic RGD peptide DMP728 results in a substantial decrease in the distance between the Arg and Asp side chains and a >10,000-fold increase in the selectivity of the compound for αb3 over αINaβ3 (28). On the other hand, it is clear that the ability of the 10-mer to differentiate between αb3 and αINaβ3 does not have a purely conformational basis. Rather, side chains within the carboxyl-terminal heptapeptide must account for this specificity.

Activation of αb3 to an intermediate level was sufficient to mediate lymphocyte adhesion to the 38-mer, but not to the 10- and 17-residue peptides. This difference was not due to differences in binding affinity because the three peptides had similar IC50 values for inhibiting lymphocyte adhesion to intact OPN. The mechanism responsible for this effect is under investigation, but may involve selective “outside-in” activation by the longest peptide. This effect may also be conformational because the longest peptide showed evidence of a partially organized structure that may become consolidated following binding to αb3. Alternatively, the 38-mer may contain sequence information that is specifically required for triggering tight adhesion following the initial binding event.

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