The interaction of leukocyte integrin αMβ2 (CD11b/CD18, Mac-1) with fibrinogen has been implicated in the inflammatory response by contributing to leukocyte adhesion to the endothelium and subsequent transmigration. Previously, it has been demonstrated that a peptide, P1, corresponding to residues 190–202 in the γ-chain of fibrinogen, binds to αMβ2 and blocks the interaction of fibrinogen with the receptor and that Asp\(^{199}\) within P1 is important to activity. We have demonstrated, however, that a double mutation of Asp\(^{199}\), Gly\(^{200}\) to Gly-Ala in the recombinant γ-module of fibrinogen, spanning region 148–411, did not abrogate αMβ2 recognition and considered that other binding sites in the γ-module may participate in the receptor recognition. We have found that synthetic peptide P2, duplicating γ377–395, inhibited adhesion of αMβ2-transfected cells to immobilized D\(^{100}\)-fragment of fibrinogen in a dose-dependent manner. In addition, immobilized P2 directly supported efficient adhesion of the αMβ2-expressing cells, including activated and non-activated monocyteoid cells. The I domain of αMβ2 was implicated in recognition of P2, as the biotinylated recombinant αI domain specifically bound to both P2 and P1 peptides. Analysis of overlapping peptides spanning P2 demonstrated that it may contain two functional sequences: γ377–386 (P2-N) and γ383–395 (P2-C), with the latter sequence being more active. In the three-dimensional structure of the γ-module, γ190–202 and γ377–395 reside in close proximity, forming two antiparallel β strands. The juxtapositioning of these two sequences may form an unique and complex binding site for αMβ2.

The interaction of fibrinogen (Fg)\(^{1}\) with integrin αMβ2 (Mac-1, CD11b/CD18) has been directly implicated in leukocyte adhesive reactions during immune and inflammatory re-

---

*This work was supported in part by American Heart Association Grant-in-aid BG 249 from the Northeast Ohio Affiliate (to T. P. U.), a grant from the Arthritis Foundation (to L. Z.), National Institutes of Health Grant HL-54924 (to E. F. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (codes 1FIB, 1IFIC, and 1FID) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

‡ To whom correspondence should be addressed.

1 The abbreviations used are: Fg, human fibrinogen; αI domain, region of ~200 amino acids “inserted” (I domain) in the α-subunit of αMβ2; BSA, bovine serum albumin; HBSS, Hank’s balanced salt solution; mAb, monoclonal antibody; TBS, Tris-buffered saline; PVD, polyvinylpyrrolidone; NIF, neutrophil inhibitory factor; PMA, phorbol 12-myristate 13-acetate.

---

The interaction of leukocyte integrin αMβ2 within the γ-chain of Fibrinogen*

(Received for publication, January 15, 1998, and in revised form, May 13, 1998)

Tatiana P. Ugarova‡‡, Dmitry A. Solovjov‡‡, Li Zhang‡‡, Dmitry I. Loukino‡‡, Vivien C. Yee‡‡, Leonid V. Medved‡‡, and Edward F. Plow‡‡

From the ‡Joseph J. Jacobs Center for Thrombosis and Vascular Biology, Department of Molecular Cardiology, Cleveland Clinic Foundation, Cleveland, Ohio 44195 and the ¶J. Holland Laboratory, American Red Cross, Rockville, Maryland 20855

© 1998 by The American Society for Biochemistry and Molecular Biology, Inc.

http://www.jbc.org/issue/273/35/22519

This paper is available on line at http://www.jbc.org

200 amino acids “inserted” (I domain) in the α-subunit of αMβ2 mediates leukocyte adhesion to the vessel wall and subsequent transmigration in vitro and in vivo (1–3). The binding of αMβ2 to fibrinogen/fibrin also may result in adhesion of monocytes and neutrophils at sites of vascular injury, such as in atherosclerotic plaques, which are rich in Fg derivatives (4, 5). Furthermore, Fg and its derivatives directly promote the accumulation of inflammatory cells on biomaterial implants in animal models (6, 7), and depletion of Fg ablates this response (6).

An αMβ2-binding site has been previously localized within the peripheral D domain of the Fg molecule (8, 9). A low molecular weight degradation product, D\(^{30}\)-fragment, generated by plasmin proteolysis of Fg was shown to produce dose-dependent inhibition of Fg binding to stimulated monocytes and neutrophils (8). Furthermore, one of a series of overlapping peptides, spanning the constituent γ-chain of D\(^{30}\) (γ89–206), inhibited \(^{125}\)I-Fg binding to activated THP-1 cells (9). This peptide, designated P1, corresponded to γ190–202. A variant peptide in which Asp\(^{199}\) was either deleted or mutated to Glu or Asn was substantially less potent in inhibiting Fg binding to αMβ2-bearing cells (9), suggesting that this residue may play a role in the receptor recognition. In addition, when P1 was immobilized onto a surface and implanted into mice, it supported an inflammatory response (7). Nevertheless, the apparent affinity of P1 for αMβ2 was substantially lower than that of intact Fg (9) or the recombinant γ-module, which encompasses residues 148–411 of the γ-chain of Fg (10). These observations suggest that either other Fg sequences, most probably present within the γ-module, contribute to the recognition of Fg by αMβ2 or that P1 must adopt a conformation within Fg that is favorably constrained for αMβ2 recognition. Consistent with this possibility, synthetic peptides other than P1 inhibit the interaction of Fg with neutrophils (11–13). The activity of these latter peptides, however, may relate to blockade of other receptors on neutrophils (13–15), such as αMβ2 (CD11c/CD18), which recognize Fg or its derivatives generated by secreted proteolytic enzymes (14, 15).

In this study, we have used 293 embryonic kidney cells stably transfected with αMβ2 (16) to analyze the molecular recognition of Fg by the receptor. These cells bind Fg exclusively via αMβ2, thereby offering an advantage over isolated leukocytes and their derivative cell lines. We identify a novel peptide, designated P2, corresponding to γ377–395 from the COOH-terminal part of the γ-chain of Fg, which is a potent inhibitor of αMβ2-mediated adhesion to Fg derivatives. In fact, on a molar basis P2 is more potent than the previously described P1 peptide. In the three-dimensional structure of the γ-module of Fg, the P1 and P2 sequences reside adjacent to one

Sponsored. The engagement of Fg by αMβ2 on activated leukocytes and by ICAM-1 on endothelial cells mediates leukocyte adhesion to the vessel wall and subsequent transmigration in vitro and in vivo (1–3). The binding of αMβ2 to fibrinogen/fibrin also may result in adhesion of monocytes and neutrophils at sites of vascular injury, such as in atherosclerotic plaques, which are rich in Fg derivatives (4, 5). Furthermore, Fg and its derivatives directly promote the accumulation of inflammatory cells on biomaterial implants in animal models (6, 7), and depletion of Fg ablates this response (6).

An αMβ2-binding site has been previously localized within the peripheral D domain of the Fg molecule (8, 9). A low molecular weight degradation product, D\(^{30}\)-fragment, generated by plasmin proteolysis of Fg was shown to produce dose-dependent inhibition of Fg binding to stimulated monocytes and neutrophils (8). Furthermore, one of a series of overlapping peptides, spanning the constituent γ-chain of D\(^{30}\) (γ89–206), inhibited \(^{125}\)I-Fg binding to activated THP-1 cells (9). This peptide, designated P1, corresponded to γ190–202. A variant peptide in which Asp\(^{199}\) was either deleted or mutated to Glu or Asn was substantially less potent in inhibiting Fg binding to αMβ2-bearing cells (9), suggesting that this residue may play a role in the receptor recognition. In addition, when P1 was immobilized onto a surface and implanted into mice, it supported an inflammatory response (7). Nevertheless, the apparent affinity of P1 for αMβ2 was substantially lower than that of intact Fg (9) or the recombinant γ-module, which encompasses residues 148–411 of the γ-chain of Fg (10). These observations suggest that either other Fg sequences, most probably present within the γ-module, contribute to the recognition of Fg by αMβ2 or that P1 must adopt a conformation within Fg that is favorably constrained for αMβ2 recognition. Consistent with this possibility, synthetic peptides other than P1 inhibit the interaction of Fg with neutrophils (11–13). The activity of these latter peptides, however, may relate to blockade of other receptors on neutrophils (13–15), such as αMβ2 (CD11c/CD18), which recognize Fg or its derivatives generated by secreted proteolytic enzymes (14, 15).

In this study, we have used 293 embryonic kidney cells stably transfected with αMβ2 (16) to analyze the molecular recognition of Fg by the receptor. These cells bind Fg exclusively via αMβ2, thereby offering an advantage over isolated leukocytes and their derivative cell lines. We identify a novel peptide, designated P2, corresponding to γ377–395 from the COOH-terminal part of the γ-chain of Fg, which is a potent inhibitor of αMβ2-mediated adhesion to Fg derivatives. In fact, on a molar basis P2 is more potent than the previously described P1 peptide. In the three-dimensional structure of the γ-module of Fg, the P1 and P2 sequences reside adjacent to one
another as part of two antiparallel β strands and may form a complex and unique binding site for α\(_{MI}\)β2.

**EXPERIMENTAL PROCEDURES**

**Peptides and Monoclonal Antibodies—**Fragment D\(_{100}\) (M, 100,000) was prepared by digestion of human Fg (Enzyme Research Laboratories, South Bend, IN) by plasmin and purified by ion-exchange chromatography on CM-Sephadex followed by gel filtration on Sephacryl S-200 (17). mAb 2G5 was described previously (18). mAb 4-2 (19) was a generous gift from Dr. B. Kudryk, New York Blood Center. mAbs OKM1 (20), 38A (21), IB4 (20), 904 (20), and w6/32 (22) were obtained from the American Tissue Culture Collection (Rockville, MD). Fluorescein isothiocyanate-conjugated goat anti-mouse Ig was from Zymed Laboratories Inc.

**Expression of Recombinant γ-Modules and α\(_{MI}\) Domain—**A recombinant wild-type γ-module corresponding to residues 418–441 of the human Fg γ-chain was produced in *Echerichia coli* using a pET-20b expression vector as described (18). A mutant γ-module, in which Asp\(_{199}\)-Gly\(_{200}\) were mutated to Gly-Ala, was produced using Transformertm site-directed mutagenesis kit (CLONTECH). The pET-20b construct containing DNA encoding the γ-module was modified by side-directed mutagenesis using two mutagenic primers. One primer, 5'-GTGTTTCAGAAGACTGCGECGATGATATGGCTACAAAAAC, introduced the desired mutation (the lowercase letters indicate the mutagenic bases) in a unique restriction site for NotI (underlined) to facilitate further analysis. Another primer, 5'-GGCGGCGCTGAGGACCCACCACGACCCACCAC, eliminated the unique XhoI restriction site in the polylinker region of the target plasmid to facilitate screening. The screening for the desired mutation was performed by *NotI* digestion of the plasmid DNA isolated from selected colonies of DH5α cells. The mutation was also confirmed by sequencing of the entire DNA fragment encoding the γ-module. The Bl21 Lys S. E. coli host cells were transformed with the mutant plasmid, and the mutant γ-module was prepared following the procedure previously described for the recombinant wild-type γ-module (10) with some modifications. Specifically, as revealed by SDS-polyacrylamide gel electrophoresis and Western blot analysis of the bacterial lysates, the yield of the mutant γ-module was substantially lower. As the mutant was not a dominant protein in the pellet of the lysates, two additional purification steps were introduced. First, the mutant was partially purified by size-exclusion chromatography on Superdex 75 equilibrated with 8 M urea and then refolded by the protocol described earlier (10). The remaining contaminants were subsequently removed by size-exclusion chromatography on Superdex 75 equilibrated with TBS and 1 mM Ca\(^{2+}\). The purity of the resulting mutant was verified by SDS-polyacrylamide gel electrophoresis and amino acid sequence analysis. The fluorescence-detected melting curve of the mutant (data not shown) was essentially the same as that of the wild-type protein reported previously (10), indicating that the mutant was properly folded.

Recombinant α\(_{MI}\) domain was produced and purified essentially as described earlier with minor modification (23). Briefly, the cDNA of the α\(_{MI}\) domain was amplified using the following primers: 5'-CTCCGAGGATCCCCTCAAGAGATGAGACATT and 5'-CTGGCTGACTCCCATGACTGAG and inserted in the expression vector pGEM-MI (Novagen, Madison, WI) with restriction enzymes BamHI and XhoI. To express the α\(_{MI}\) domain as a fusion protein with thioredoxin, BL21 (DE3) cells were transformed with the above vector and grown to log phase in TB medium. Protein expression was induced by addition of 0.3 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C, and the fusion protein from the inclusion bodies was dissolved in 8 M guanidine HCl. After refolding in 20 mM TBS, the fusion protein was purified using a Ni-NTA column (Qiagen, Chatsworth, CA). To obtain the wild-type α\(_{MI}\) domain, the fusion partner (thioredoxin) was removed by thrombin cleavage, and the I domain was purified using Q-Sepharose resin (Amersham Pharmacia Biotech). The α\(_{MI}\) domain was conjugated with N-hydroxysuccinimide-biotin ester (Calbiochem) as described (24).

**Peptides—**Peptides corresponding to selected sequences in Fg were synthesized using an Applied Biosystems model 430 peptide synthesizer and purified by reversed-phase high-performance liquid chromatography on a preparative C18 Vydac column using a 5–90% linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Authenticity and purity of the peptides were verified by mass spectroperty and by high performance liquid chromatography. Peptides H19 and H20, duplicating Fg sequences y340–357 and y351–370, respectively, and peptide P1-Scr (FRLWQVTGVDV), were used as controls. Selected peptides were conjugated to ovalbumin using 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide as a cross-linker (Pierce) (25), and the conjugates were stored at −70 °C until used.

For radiolabeling of peptides P2 (y377–395) and P1 (y190–202), a variant P1 peptide with KY added to the NH\(_2\) terminus of y190–202 was synthesized, whereas the naturally occurring Tyr\(_{377}\) at the NH\(_2\) terminus of P2 served as the target for iodination. These peptides were radiolabeled with Na\(^{125}\)I using a modified chloramine-T procedure (26). The radiolabeled peptides were separated from free Na\(^{125}\)I on a Sep-Pak C18 column (Waters, Milford, MA). After extensive washing with sodium phosphate buffer, pH 7.0, Na\(^{125}\)I-peptide was eluted with 60% acetonitrile in 0.1% trifluoroacetic solution, and the sample was lyophilized.

The amounts of peptides immobilized onto the microtiter plates used in cell adhesion assays were measured by two different approaches. In the first, peptides were immobilized on the plastic for 3 h at 37 °C, washed with phosphate-buffered saline and the concentration was determined by BCA method according to manufacturer’s protocol (Pierce). In the second approach, plates were coated with radiolabeled peptides, blocked with 0.5% PVD and then washed with phosphate-buffered saline. Bound peptides were solubilized in 2% SDS + 0.5 N NaOH, and radioactivity was counted in a γ counter. The amount of peptide bound to plastic wells was calculated on the basis of the amount of radioactivity recovered and the specific activity of the peptide. The efficiency of peptide immobilization on the plastic as determined by both methods was similar. At an input concentration of 0.1–40 μg/ml (0.2 μl), 10 and 8% of added P1 and P2, respectively, were immobilized.

**Cells—**The development of 293 embryonic kidney cells expressing α\(_{MI}\)β2 (or only the α\(_{MI}\) subunit (27) has been described previously. The cell lines were maintained in Dulbecco’s modified Eagle’s/F-12 medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum, 25 mM HEPES, and antibiotics. THP-1 monocyte cells were obtained from the American Tissue Culture Collection and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 25 mM HEPES, 2.5 μM 2-mercaptoethanol, penicillin, and streptomycin.

**Adhesion Assays—**The wells of tissue culture plates (Costar, Cambridge, MA) were coated with varying concentrations of peptides, peptide-ovalbumin conjugates, recombinant γ-modules, or D\(_{100}\) fragment for 3 h at 37 °C. The coated wells were then post-coated with 0.5% PVD for 1 h at 22 °C. α\(_{MI}\)β2 and α\(_{MI}\) expressing cells, grown in 10-cm Petri dishes for 24 h as subconfluent monolayers, were harvested with cell-dissociating buffer (Life Technologies, Inc.) for 1 min at 22 °C and washed twice in HBSS/HEPES solution containing 10 mg/ml BSA. These cells were labeled with Na\(^{125}\)I-CuO\(_4\) (0.5 μCi/ml) for 30 min at 22 °C. The cells were then washed with 0.2% PVD/10 mM PVP-10 to remove unbound label and plated on the adhesive substrates for 25 min at 37 °C in a 5% CO\(_2\), humidified atmosphere. The nonadherent cells were removed by three washes with HBSS containing 10 mg/ml BSA. The adherent cells were solubilized with 2% SDS, and 31Cr was quantitated in a β counter. In a separate experiment, the cells were incubated with mAbs OKM1, 44a, 904, and IB4 directed against the α and β subunits of α\(_{MI}\)β2 for 15 min at 22 °C and then added to the wells coated with P1 or P2.

**Solid Phase Binding Assay—**96-well plates (Costar, Cambridge, MA) were coated with 25 pmol of P2, P1 and control H19 peptides for 3 h at 37 °C and post-coated with 1% BSA for 1 h at 22 °C. 10 μg/ml biotinylated α\(_{MI}\) domain in 0.05 mM TBS supplemented with 0.1% BSA, 1 mM Ca\(^{2+}\), and 1 mM Mg\(^{2+}\) was added to the wells and incubated for 3 h at 22 °C. In parallel, the same amount of the α\(_{MI}\) domain was incubated with 1 μg/ml neutrophil inhibitory factor (NIF), 50 μg/ml P1, and P2. After washing, streptavidin conjugated to alkaline phosphatase was added and incubated for 1 h at 37 °C. The α\(_{MI}\) domain binding was detected by reaction with goat anti-mouse IgG, conjugated to alkaline-phosphatase and using p-nitrophenyl phosphate as substrate and added for 1 h at 37 °C. The α\(_{MI}\) domain binding was detected by reaction with p-nitrophenyl phosphate, measuring the absorbance at 405 nm. Alternatively, the binding of a nonlabeled P2-related peptide, y373–385, to the immobilized α\(_{MI}\) domain was detected using mAb 2G5, which recognizes a linear epitope of peptide 18. The binding of this mAb to the peptide was measured by reaction with goat anti-mouse IgG, conjugated to alkaline-phosphatase and using p-nitrophenyl phosphate for detection.

**Flow Cytometry—**Fluorescence-activated cell sorting analyses were performed to assess the expression of α\(_{MI}\) or α\(_{MI}\)β2 on the surface of transfected cells or THP-1 cells. The cells were harvested as described
RESULTS

Mutation of Asp<sup>199</sup> in the Recombinant γ-Module Does Not Abrogate Its Recognition by α<sub>Mβ2</sub>—Recently, we have shown that the recombinant γ-module (γ148–411), which preserves the conformational and functional properties of this region in native Fg and contains the previously identified α<sub>Mβ2</sub> binding site at γ190–202 (GWTVFQKRLDSV), supports adhesion of the α<sub>Mβ2</sub>-bearing cells but not mock-transfected cells (10). We further observed that the γ-module inhibited α<sub>Mβ2</sub>-mediated adhesion to the D<sub>100</sub> fragment of Fg, and its inhibitory potency was about 20-fold greater than that reported for the synthetic peptide P1 corresponding to the γ190–202. To consider whether other sequences within γ148–411 might contribute to the recognition of Fg by α<sub>Mβ2</sub>, we expressed a mutant γ-module in which Asp<sup>199</sup> was replaced by Gly-Ala. Previous studies have indicated (9) that Asp<sup>199</sup> is of major importance in the recognition of P1 by the receptor. As shown in Fig. 1, when the recombinant proteins were immobilized, the mutant γ-module supported adhesion of the α<sub>Mβ2</sub>-expressing cells as efficiently as the wild-type γ-module. Adhesion to both proteins was NIF-dependent. NIF, a specific inhibitor of this integrin (28), effectively blocked binding (data not shown). These results prompted us to search for other sequences within the γ-module that might be involved in α<sub>Mβ2</sub> recognition.

Effect of γ377–395 on α<sub>Mβ2</sub>-mediated Adhesion—The crystal structure of the Fg γ-module encompassing residues γ144–411 (29) revealed that a β strand containing portions of P1 is adjacent to a second β strand formed by residues γ380–390. This proximity led us to consider whether this second β strand contributes to α<sub>Mβ2</sub> recognition. Accordingly, we synthesized a peptide, designated P2, corresponding to γ377–395, which encompasses this β strand and the flanking residues. As shown in Fig. 2, this peptide inhibited adhesion of the α<sub>Mβ2</sub>-expressing cells to the immobilized Fg derivative, D<sub>100</sub>, in a dose-dependent manner. On a molar basis, the concentration of P1 tested (130 nM) might contribute to α<sub>Mβ2</sub> recognition. Accordingly, we synthesized a peptide, designated P2, corresponding to γ377–395, which encompasses this β strand and the flanking residues. As shown in Fig. 2, this peptide inhibited adhesion of the α<sub>Mβ2</sub>-expressing cells to D<sub>100</sub> fragment (re-
equally efficient in the supporting of αMβ2-mediated adhesion. Adhesion was concentration-dependent, and about 60–65% of added cells adhered to each peptide (maximal adhesion). Three control peptides, P1-Scr, H19, and H20, did not support significant adhesion (results for P1-Scr are shown). Furthermore, mock-transfected cells adhered poorly to P2 as well as to P1 (data not shown). The molar concentrations of each immobilized P2 and P1 required for half-maximal adhesion were 2.4 ± 0.8 and 7.2 ± 1.5 pmol (Fig. 3A). These values are based upon direct quantitation of the amount of each immobilized peptide (see “Experimental Procedures”). When P2 and P1 were conjugated to ovalbumin, the maximal adhesion to the immobilized P2-ovalbumin conjugate was about 2-fold higher than to P1-ovalbumin (data not shown). Although conjugation to protein carrier has been shown to improve the adhesive properties of some peptides (30), coupling did not significantly affect the activity of P1 and P2. It should be noted that, although P1 in solution was a relatively poor inhibitor of the αMβ2-mediated adhesion (Fig. 2), upon immobilization, it supported adhesion almost as well as immobilized P2. The low inhibitory activity of soluble P1 and its propensity to aggregate has been reported previously (9). Thus, soluble P1 may not adopt a preferred conformation for interaction with αMβ2.

We next tested the ability of P2 to support adhesion of THP-1 monocytoid cells that bear αMβ2 and are known to adhere to P1 (9). As shown in Fig. 4, P2 supported adhesion of the THP-1 cells in a concentration-dependent manner, and the extent of adhesion to P2 was greater than for P1. Adhesion to both immobilized peptides was enhanced 1.2–2-fold by PMA stimulation of the cells. Under the same conditions, with and without PMA stimulation, P1-Scr did not support cell adhesion (data not shown).
have examined the ability of each peptide in soluble form to adhesion of PMA-activated THP-1 cells, either non-activated (filled bars) or activated with 1 nm PMA (open bars), to immobilized P1 and P2 (numerical values on x axis is the amount of immobilized peptides in pmol) and post-coated with 0.5% PVD. Adhesion was performed as described for the αMβ2-expressing cells in Figs. 1–3. Results are expressed as a percentage of the added cells.

**TABLE I**

| mAbs   | αMβ2 Transfectants | THP-1 cells |
|--------|-------------------|-------------|
|        | P2     | P1     | D100   | P2     | P1     |
| OKM1   | 5 ± 2  | 0      | 0      | 4 ± 2  | 19 ± 2 |
| 44a    | 55 ± 7 | 61 ± 7 | 87 ± 8 | 40 ± 3 | 43 ± 5 |
| 904    | 21 ± 2 | 18 ± 3 | 22 ± 2 | 10 ± 1 | 14 ± 4 |
| IB4    | 50 ± 3 | 67 ± 7 | 77 ± 10| 61 ± 6 | 40 ± 2 |
| W6/32  | 0      | 23 ± 2 | 5 ± 2  | 2      | 0      |

The specificity of αMβ2-P2 interaction was substantiated further using function-blocking mAbs, which recognize αM or β2 subunits. Adhesion of αMβ2-expressing cells to three adhesive substrates, P2, P1, and D100 fragment, was reduced in a dose-dependent manner by mAb 44a to the αM subunit. At 5 μg/ml, this mAb produced 71% inhibition of adhesion to P1, 55% to P2, and 87% to D100 fragment (Table I). mAb IB4 to the β2 subunit was also inhibitory; at the 5 μg/ml concentration, it reduced adhesion of the αMβ2-expressing cells to P1, P2, and D100 by 67, 50, and 77%, respectively. In contrast, another anti-αM mAb, 904, was moderately inhibitory, and mAb OKM1 was not effective. In parallel experiments, mAbs 44a and IB4 also inhibited adhesion of PMA-activated THP-1 cells to immobilized P2 and P1 (Table I). A control mAb w6/32 against the class I major histocompatibility complex was consistently non-inhibitory.

**Cross-competition between P1 and P2 for αMβ2—**To investigate the relationship between P1 and P2 binding to αMβ2, we have examined the ability of each peptide in soluble form to inhibit adhesion of the αMβ2-bearing cells to immobilized P1 and P2. As shown in Fig. 6, both peptides were able to cross-inhibit adhesion of the αMβ2-expressing cells; i.e. P2 inhibited adhesion to immobilized P1, and P1 inhibited adhesion to immobilized P2. The concentrations of soluble peptides required for half-maximal inhibition (IC50) of adhesion were 100 μM P2 and 630 μM P1 with immobilized P2 (3.5 pmol) as the substrate. The IC50 values for P2 and P1 on immobilized P1 (5.8 pmol) were 250 and 890 μM, respectively. Thus, as observed with D100

![Fig. 4](image1.png)

**Fig. 4. Adhesion of THP-1 cells to P2 and P1.** 51Cr-labeled THP-1 cells, either non-activated (filled bars) or activated with 1 nm PMA (open bars), were added to the wells coated with different concentrations of P2 and P1 (numerical values on y axis is the amount of immobilized peptides in pmol) and post-coated with 0.5% PVD. Adhesion was performed as described for the αMβ2-expressing cells in Figs. 1–3. Results are expressed as a percentage of the added cells.

![Fig. 5](image2.png)

**Fig. 5. Inhibition of αMβ2-expressing cell adhesion to immobilized P2 and P1 by soluble P1 and P2.** Wells were coated with 0.2 ml P2 (solid lines) or P1 (dashed lines) at 0.5 μg/ml. 51Cr-labeled cells were incubated with various concentrations of P2 (closed symbols) or P1 (open symbols) for 15 min at 22 °C and then added to the coated wells, and adhesion was quantitated. Results are presented as a percentage of maximal adhesion in the absence of competing peptides. Data are from two separate adhesion assays, performed with triplicates at each experimental point, and the standard errors of the means were ±10%.

**Identification of the αMβ2 Binding Site within P2—**To begin to define the minimal recognition sequence(s) within P2 for αMβ2, three overlapping peptides from within this segment, γ377–391, γ377–386 (P2-N), and γ383–395 (P2-C) were synthesized (Fig. 6A). All three peptides inhibited adhesion of the αMβ2-bearing cells to D100 fragment with IC50 values of 24, 46, and 1.5 μM for γ377–391, γ377–386, and γ383–395, respectively (Fig. 6B). Thus, on the molar basis, the inhibitory activity of γ383–395 (P2-C) was only 2-fold less than parental P2 (IC50 = 0.7 μM). Truncation of the COOH-terminal part of P2 yielded a peptide, γ377–391, which was 34-fold less active than P2; and further truncation, γ377–386, resulted in still further loss of inhibitory activity. However, whereas γ377–386 (P2-N) was 65-fold less active than P2, its activity was still significant compared with control peptides H19, H20, and P1-Ser.

The ability of these peptides to directly support adhesion of the αMβ2-expressing cells was also tested. All three truncated peptides supported significant adhesion, although adhesion-promoting activity of γ383–395 (P2-C), γ377–391, and γ377–386 (P2-N) was 1.2-, 1.5-, and 3.3-fold lower than P2, respectively (Fig. 6C). Thus, the inhibitory and adhesion-promoting activity of three peptides can be placed in the following order: γ383–395 > γ377–391 > γ377–386. As two overlapping peptides, γ377–391 and γ383–395, differ significantly in activity, the most active determinant of adhesive function must reside in the extreme COOH-terminal part of P2.

To explore further the contribution of the P2-C portion of P2 in αMβ2 recognition, we employed mAb 4-2, directed against the γ392–406 (19). Preliminary enzyme-linked immunosorbent assay experiments (data not shown) indicated that mAb 4-2 interacted with immobilized P2 and P2-C, suggesting that at least part of its epitope resides in the P2-C. As shown in Fig. 7, mAb 4-2 inhibited adhesion of the αMβ2-expressing cells to as the immobilized substrate (Fig. 2), soluble P1 was a weaker inhibitor of cell adhesion than soluble P2. The concentrations of peptides required to obtain 50% inhibition were significantly higher on immobilized peptides than on D100. This difference was not due to variations in the amounts of immobilized peptides as the amounts of all three ligands adsorbed onto the surface were similar. The competition between peptides suggests that they can interact with sterically or allosterically overlapping sites within αMβ2.
studies and approaches have implicated the I domain of the α₂ integrin (18), did not affect adhesion. A synthetic peptide corresponding to this sequence, designated P2, efficiently inhibited adhesion of the α₂β₃-transfected cells and THP-1 monocytoid cells to the D₁₀₀ fragment of Fg, directly supported adhesion of the α₂M-transfectants in a dose-dependent manner. However, the maximal level of adhesion of the α₂M-expressing cells to the peptides was about 2-fold lower than that of the α₂β₂-bearing cells (33 ± 3% and 33 ± 13% maximal adhesion for the α₂M cells to P2 and P1, respectively, versus 63 ± 5% maximal adhesion to either peptide for the α₂Mβ₂-bearing cells).

**DISCUSSION**

In this study, we identified a novel sequence within the COOH-terminal part of the Fg γ-chain, γ377–385, which is recognized by leukocyte integrin α₂β₂. A synthetic peptide corresponding to this sequence, designated P2, efficiently inhibited adhesion of the α₂β₂-transfected cells and THP-1 monocytoid cells to the D₁₀₀ fragment of Fg, directly supported adhesion of these cells, and interacted with the α₂I domain of α₂Mβ₂. Thus, the P2 sequence defines a new recognition specificity for α₂Mβ₂ and may mediate the interaction of Fg derivatives with this receptor.

That recognition sites, in addition to the previously identified γ190–202 sequence, are involved in the interaction of Fg
trations of the derivative P2 peptide, 

After washing, mAb 2G5 (5 μg/ml) was added to the wells coated with 25 pmol of P2 and P1 in the absence (control) or in the presence of 1 μg/ml NIF, 50 μg/ml P2 or P1, and incubated for 3 h at 22 °C. After washing, the bound αM domain was detected using streptavidin conjugated to alkaline phosphatase and p-nitrophenyl phosphate for disclosure. Results are the mean ± S.E. of three independent determinations. A, assessment of P2 binding to the αM domain with mAb 2G5. Different concentrations of the derivative P2 peptide, γ373–385, were incubated with recombinant αM domain coated onto microtiter plates for 2 h at 22 °C. After washing, mAb 2G5 (5 μg/ml) was added to the wells and incubated for an additional 1.5 h. The binding of mAb 2G5 then was detected with a secondary goat anti-mouse IgG conjugated to alkaline phosphatase with subsequent development of the reaction with p-nitrophenyl phosphate.

part of the γ-chain but folds back and inserts into the middle subdomain of the γ-module, next to the P1 β strand. Thus, although γ190–202 and γ380–390 are separated by 178 residues in terms of linear amino acid sequence, the specific folding of the γ-module brings these two αMβ2 recognition sequences into close proximity.

Analysis of partially overlapping peptides indicated that P2 may contain two adhesive sequences, one in its NH2-terminal part, γ377–386 (designated P2-N), and a second in its COOH-terminal region, γ383–395 (designated P2-C). These two adhesive sites differed in their adhesion-promoting and inhibitory activities; P2-N is ~65-fold less active than parental P2, and P2-C is almost as active as P2. As the simplest interpretation of these results, P2-C represents a major αMβ2 recognition site and P2-N a minor one. Indeed, at only 13 amino acids, P2-C is a relatively short and potent peptide to begin detailed structure-function analyses. Key residues involved in P2-C recognition by αMβ2 should be exposed on the surface of the γ-module of Fg. In the crystal structure of the γ-module, the side chains of Thr389, Lys380, and Phe389 are partially exposed whereas the remaining residues within the γ380–390 β strand are buried, and the extreme COOH terminus emerging from this β strand is again well exposed, including the side chains of Asn389, Arg391, and Leu392. As shown in Fig. 10A, the exposed residues might well be critical to the activity of P2-C. Despite the weak adhesive activity of P2-N, it may be premature to dismiss its role in αMβ2 recognition. As shown in Fig. 10B, the exposed residues in the NH2-terminal part of P2 are Lys380 and Lys381. These residues lie adjacent to the antiparallel β-strand containing the P1 region, which includes Arg196, Leu198, and Asp199 as its only exposed residues. The distances between Asp199 and Lys380 and between Asp199 and Lys381, as determined from the crystal structure, are 9.9 and 9.8 Å, respectively. This distance is considerably shorter than the 29 Å which separate Lys381 in P2-N from Asn389 in P2-C or 34 Å between Lys380 and Asn389.

What is the relationship among these three subsites (P1, P2-N, and P2-C)? Based on the dissection of the recognition sites for other integrin ligands, two models can be considered. In one model, the binding pocket for αMβ2 in fibrinogen may represent a wide surface that can engage multiple sequences, P1, P2-N, P2-C and/or others, simultaneously. This model would be analogous to the recognition of fibronectin by αMβ1 in
which the appropriate positioning of at least two distant short sequences, the RGDS sequence in the 10th type III repeat and the HPSRN synergistic sequence in the 9th type III repeat, are crucial for high affinity ligand binding of the receptor (33). In the second model, P1, P2-N, and P2-C may bind to the same or overlapping sites within the receptor. This model may apply to the binding of multiple fibronectin peptides to \( \alpha_6 \beta_1 \) (30, 34) or RGD and Fg \( \gamma 406-411 \) to \( \alpha_{III} \beta_3 \) (35). The binding of both P1 and P2 to the \( \alpha_6 \)I domain and their cross-inhibition of cell adhesion seem to be consistent with the latter model.

An additional level of complexity in considering the recognition of P1 and P2 by \( \alpha_5 \beta_2 \) is the exposure of these sequences on the surface of intact Fg. Although portions of these sequences are exposed in the \( \gamma \)-module, our preliminary data indicate that residues in P1, involved in the receptor recognition, are only partially available on the surface of Fg and can be further exposed by proteolysis of the parent molecule. Furthermore, the P2-C, \( \gamma 383-395 \), resides in immediate proximity and partially overlaps \( \gamma 392-406 \). This latter sequence is cryptic in intact soluble Fg based upon its lack of reactivity with mAb 4-2 (19). The epitope for this mAb becomes exposed when Fg is immobilized or proteolyzed to fragments D100 or DD-dimer. The nature of the conformational alteration which occurs upon conversion of Fg to these fragments and exposes \( \gamma 392-406 \) is not known. A potential mechanism of this transition may be associated with the positioning of the COOH-terminal part of \( \gamma \)-chain (\( \gamma 392-411 \)), which is a flexible appendage in intact Fg (29, 36).

In summary, at least two sites within Fg can be recognized by \( \alpha_5 \beta_2 \). One site resides in \( \gamma 190-202 \), and the second one is located in the \( \gamma 377-395 \). This latter site may be composed of two subsites. Although separated in linear amino acid sequence, \( \gamma 190-202 \) and \( \gamma 377-395 \) are brought into close proximity by the folding of the \( \gamma \)-module and could potentially form the recognition site for \( \alpha_5 \beta_2 \). Further studies involving mutation analyses should provide insight into the relationship between these recognition sequences and the \( \alpha_5 \beta_2 \) binding pocket in Fg. Considering the complexity of the P2 site, such analyses will be complicated. Furthermore, our preliminary data indicate that the \( \gamma 146-226 \) fragment with Asp\(^{180} \) in the P1 region mutated to Ala still supported cell adhesion,\(^3 \) consistent with the previously described reduction but not the complete loss of the adhesive activity of P1 peptides with a single substitution at this position (9). Thus, multiple mutations in P1 and both P2 regions will be required to dissect the role of these as well as other regions in adhesive functions. Such systematic studies are in progress.

Acknowledgments—We are grateful to Dr. B. Kudryk for providing mAb 4-2. We thank Dr. C. Forsyth for useful discussions while the work was in progress, Dr. K. Ingham for helpful criticism during the preparation of the manuscript, and Jane Rein for secretarial assistance.

REFERENCES

1. Languino, L. R., Plescia, J., Duperray, A., Brian, A. A., Ploow, E. F., Geltosky, J. E., and Altieri, D. C. (1993) Cell 75, 1423–1434
2. Languino, L. R., Duperray, A., Joganic, K. J., Fernaro, M., Thornton, G. B., and Altieri, D. C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1505–1509
3. Sriramarao, P., Languino, L. R., and Altieri, D. C. (1996) Blood 88, 3416–3423
4. Bini, A., Fenoglio, J. J., Jr., Mesa-Tejada, R., Kudryk, B., and Kaplan, L. A. (1989) Arteriosclerosis 9, 109–121
5. Valenzuela, R., Shainoff, J. R., DiBello, P. M., Urbanic, D. A., Anderson, J. M., Matsuada, G. R., and Kudryk, B. J. (1991) Am. J. Pathol. 141, 861–880
6. Tang, L., and Eaton, J. W. (1993) J. Exp. Med. 178, 2147–2156
7. Tang, L., Ugarova, T. P., Ploow, E. F., and Eaton, J. W. (1996) J. Clin. Invest. 97, 1329–1334
8. Altieri, D. C., Agbanyo, F. R., Plescia, J., Ginsberg, M. H., Edgington, T. S., and Ploow, E. F. (1990) J. Biol. Chem. 265, 1847–1853
9. Medved, L., Litvinovich, S., Ugarova, T., Matsuka, Y., and Ingham, K. (1997) Biochemistry 36, 4685–4693
10. Arnaout, M. A. (1990) Blood 75, 1037–1050
11. Oriol, J. D., Sodeik, B., Cao, L., Leuenberger, S., Weiss, J. I., Detmers, P. A., Wright, S. D., and Silverstein, S. C. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 1044–1048
12. Gresham, H. D., Goodwin, J. F., Allen, P. M., Anderson, D. C., and Brown, E. J. (1989) J. Cell Biol. 108, 1935–1943
13. Gustafson, E. J., Lukasiewicz, H., Wachtleg, Y. T., Norton, K. J., Schmaier, A. H., Niewiarowski, S., and Colman, R. W. (1989) J. Cell Biol. 109, 377–387
14. Simon, D. I., Ezretsky, A. M., Francis, S. A., Rennke, H., and Loscalzo, J. (1993) Blood 82, 2414–2422
15. Zhang, L., and Ploow, E. F. (1996) J. Biol. Chem. 271, 18211–18216
16. Ugarova, T. P., and Budzynski, A. Z. (1992) J. Biol. Chem. 267, 13687–13693
17. Zamaron, C., Ginsberg, M. H., and Ploow, E. F. (1991) J. Biol. Chem. 266, 16193–16199
18. Ploow, E. F. (1995) J. Biol. Chem. 270, 271–272
19. Wright, S. D., Rao, P. E., Van Voorhis, W. C., Craigmyle, L. S., Iida, K., Talle, M. A., Westberg, E. F., Goldstein, G., and Silverstein, S. C. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 5699–5703
20. Dana, N., Styr, B., Griffin, J. D., Todd, R. F., III, Klemper, M. S., and Arnaout, M. A. (1986) J. Immunol. 137, 3259–3263

---

\(^2\) T. Ugarova, D. Solovjov, and E. Ploow, manuscript in preparation.

\(^3\) L. Medved and T. Ugarova, unpublished data.
Recognition Sequence in Fibrinogen for $\alpha_{\text{Mb}}$

22. Altieri, D. C., Bader, R., Mannucci, P. M., and Edgington, T. S. (1988) J. Cell Biol. 107, 1893–1900
23. Muchowski, P. J., Zhang, L., Chang, E. R., Soule, H. R., Plow, E. F., and Moyle, M. (1994) J. Biol. Chem. 269, 26419–26423
24. Shattil, S. J., Cunningham, M., and Hoxie, J. A. (1987) Blood 70, 307–315
25. Iida, J., Skubitz, A. P. N., Furcht, L. T., Wayner, E. A., and McCarthy, J. B. (1992) J. Cell Biol. 118, 431–444
26. Plow, E. F., and Ginsberg, M. H. (1981) J. Biol. Chem. 256, 9477–9482
27. Zhang, L., and Plow, E. F. (1997) J. Biol. Chem. 272, 17558–17564
28. Moyle, M., Foster, D. L., McGrath, D. E., Brown, S. M., Laroche, Y., De Meutter, J., Bogowitz, C. A., Fried, V. A., Ely, J. A., Soule, H. R., and Vlasuk, G. P. (1994) J. Biol. Chem. 269, 10008–10015
29. Yee, V. C., Pratt, K. P., Cote, H. C. F., LeTrong, I., Chung, D. W., Davie, E. W., Stenkamp, R. E., and Teller, D. C. (1997) Structure 5, 125–138
30. Humphries, M. J., Komoriya, A., Akiyama, S. K., Olden, K., and Yamada, K. M. (1987) J. Biol. Chem. 262, 6886–6892
31. Diamond, M. S., Garcia-Aguilar, J., Bickford, J. K., Corbi, A. L., and Springer, T. A. (1993) J. Cell Biol. 120, 1031–1043
32. Zhou, L., Lee, D. H., Plescia, J., Lau, C. Y., and Altieri, D. C. (1994) J. Biol. Chem. 269, 17075–17079
33. Aota, S., Nomizu, M., and Yamada, K. M. (1994) J. Biol. Chem. 269, 23756–23761
34. Mould, A. P., and Humphries, M. J. (1991) EMBO J. 10, 4089–4095
35. Plow, E. F., Marguerie, G. A., and Ginsberg, M. (1987) Biochem. Pharmacol. 36, 4035–4040
36. Spraggon, G., Everse, S. J., and Doolittle, R. F. (1997) Nature 389, 455–462
37. Kraulis, P. J. (1991) J. Appl. Crystallogr. 42, 959–960
38. Esnouf, R. M. (1997) J. Mol. Graph. Model. 15, 132–134
39. Merritt, E. A., and Murphy, M. E. P. (1994) Acta Crystallogr. D 50, 869–873