

Integrin $\alpha_M\beta_2$ -mediated Cell Migration to Fibrinogen and Its Recognition Peptides

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Abstract

Leukocyte migration is the hallmark of inflammation, and integrin $\alpha_M\beta_2$ and its ligand fibrinogen (Fg) are key participants in this cellular response. Cells expressing wild-type or mutant $\alpha_M\beta_2$ and Fg or its derivatives have been used to dissect the molecular requirements for this receptor–ligand pair to mediate cell migration. The major conclusions are that (a) Fg, its D fragment, and its P1 and P2 $\alpha_M\beta_2$ recognition peptides support a chemotactic response; (b) when the I domain of α_L was replaced with the I domain of α_M , the chimeric receptor supported cell migration to Fg; however, the α_M subunit, containing the I domain but lacking the β_2 subunit, supported migration poorly, thus, the α_M I domain is necessary but not sufficient to support chemotaxis, and efficient migration requires the β_2 subunit and α_M I domain; and (c) in addition to supporting cell migration, P2 enhanced $\alpha_M\beta_2$ -mediated chemotaxis to Fg and the P1 peptide. This activation was associated with exposure of the activation-dependent epitope recognized by monoclonal antibody 7E3 and was observed also with human neutrophils. Taken together, these data define specific molecular requirements for $\alpha_M\beta_2$ to mediate cell migration to Fg derivatives and assign a novel proinflammatory activity to the P2 peptide.

Key words: adhesion molecules • fibrinogen • integrin • CD11b/CD18 • inflammation

Introduction

The leukocyte integrin $\alpha_M\beta_2$ (CD11b/CD18, Mac-1, CR3) and fibrinogen (Fg)¹ are key molecular participants in the immune/inflammatory response (1–4). Within the currently accepted scenario for leukocyte trafficking during the inflammatory reaction, $\alpha_M\beta_2$ is thought to play a pivotal role in the firm adhesion of leukocytes to the endothelium and in the subsequent transmigration of the adherent cells to sites of inflammation. Evidence for these functions of $\alpha_M\beta_2$ has been developed in studies with PMNs (5), monocytes (6), lymphocytes (7), and eosinophils (8). The characterization of $\alpha_M\beta_2$ -deficient mice has confirmed these findings by showing that a variety of leukocyte-dependent responses are diminished in these animals (9–11). Also, Fg is believed to play a multifaceted role in the immune and inflammatory response. For example, afibrinogenemic patients fail to de-

velop the induration associated with an inflammatory response (12), depletion of mice of Fg blunts their inflammatory response to biomaterial implants (4), and Fg-deficient mice exhibit greatly reduced joint inflammation in an antigen-induced arthritis model (13). Linking these two inflammatory/immune participants, Fg is a ligand for $\alpha_M\beta_2$ (14, 15). Deposits of Fg/fibrin are adhesive for leukocytes via $\alpha_M\beta_2$, an interaction demonstrable in numerous *in vitro* and *in vivo* studies (4, 16). Fg also has been shown to bridge $\alpha_M\beta_2$ -bearing leukocytes to intercellular adhesion molecule (ICAM)-1 on endothelial cells (17, 18). Engagement of $\alpha_M\beta_2$ by Fg induces a series of intracellular signaling events and cellular responses, which are relevant to the inflammatory response including cytokine secretion and nuclear factor κ B activation (19, 20). Most studies of Fg– $\alpha_M\beta_2$ interactions have centered on the engagement of the molecule with the receptor leading to cell adhesion (17, 21). However, little is known regarding the role of Fg in initiating the diapedesis and migration of leukocytes (18, 22).

$\alpha_M\beta_2$ is a member of the integrin family of α/β heterodimeric adhesion receptors and shares the same β_2 subunit as $\alpha_L\beta_2$ (CD11a/CD18, LFA-1), $\alpha_X\beta_2$ (CD11c/CD18, p150,95), and $\alpha_D\beta_2$ (α_D /CD18) (23–25). High af-

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¹Abbreviations used in this paper: Fg, fibrinogen; Fn, fibronectin; HPF, high power field; ICAM, intercellular adhesion molecule; MIDAS, metal ion-dependent adhesion site; NIF, neutrophil inhibitory factor; WT, wild-type.

finity binding of many protein ligands, including Fg, to $\alpha_M\beta_2$ involves a segment of ~ 200 amino acids in the α_M subunit, termed the I (or A) domain (23, 26, 27). Other I domain ligands include ICAM-1, C3bi, *Candida albicans*, and the helminth protein neutrophil inhibitory factor (NIF; references 24, 25, 28). Critical to the ligand-binding functions of the α_M I domain is the metal ion-dependent adhesion site (MIDAS). In this MIDAS motif, five noncontiguous amino acids, including the D(140), S(142), S(144), T(209), and D(242) (numbering refers to the amino acid positions in the mature α_M subunit sequence), provide coordination sites for a divalent cation and ligands bind in close proximity to this motif (29, 30). Optimal recognition of many of the α_M I domain ligands can be influenced by the activation of the $\alpha_M\beta_2$ -bearing cell, which alters receptor avidity/affinity (23, 24, 31). $\alpha_M\beta_2$ recognizes mannose and β -glucan carbohydrate structures via a lectin-like ligand-binding function. This lectin binding site is also located in the α_M subunit (32). Ligands of the lectin domain include zymosan, *Saccharomyces cerevisiae*, and nonspecific activators of cell-mediated immunity such as lentinan (32).

Fg is a dimeric molecule composed of three pairs of non-identical peptide chains. It is organized into a central E and two peripheral D domains, which give rise to E and D fragments when Fg is degraded by most proteolytic enzymes, including plasmin (33). Two specific sequences within the γ chain moiety of fragment D are recognized by $\alpha_M\beta_2$. The peptides corresponding to these sequences are designated P1 ($\gamma 190$ – 202) (34) and P2 ($\gamma 377$ – 395) (21). The P1 and P2 peptides not only inhibit $\alpha_M\beta_2$ -mediated adhesion to Fg derivatives but also support adhesion of $\alpha_M\beta_2$ -bearing cells. On a molar basis, P2 is a more potent inhibitor of $\alpha_M\beta_2$ adhesion to Fg than P1 (21). The binding sites for both the P1 and P2 peptides in $\alpha_M\beta_2$ have been mapped to the α_M I domain, and cells expressing the α_M subunit in the absence of the β_2 subunit adhere well to Fg and these recognition peptides (21).

We have previously used HEK 293 cells transfected with wild-type (WT) and mutant forms of $\alpha_M\beta_2$ to demonstrate the importance of the α_M I domain for high affinity binding of NIF (35), C3bi (36), *C. albicans* (37), and Fg (21, 38). The experiments in this study describe the utilization of these transfectants to investigate $\alpha_M\beta_2$ -dependent cell migration to Fg and its derivatives. We demonstrate that $\alpha_M\beta_2$ can, indeed, mediate directed cell migration to Fg and that the recognition peptides influence this response. Furthermore, the structural components of $\alpha_M\beta_2$ that are required for cell migration are distinct from those for cell adhesion. Also, receptor activation is shown to play a major role in regulating $\alpha_M\beta_2$ -mediated cell migration to Fg and its derivatives. Indeed, one of the recognition peptides of Fg is capable of inducing such activation not only in the $\alpha_M\beta_2$ transfectants but also in neutrophils.

Materials and Methods

Fg and Fibronectin Peptides. Plasminogen-depleted human Fg was purified as described previously (39) or purchased from En-

zyme Research Laboratories. Fragment D₁₀₀ (M_r 100,000) was prepared by digestion of human Fg with plasmin and purified by ion-exchange chromatography on CM-Sephadex followed by gel filtration on Sephacryl S-200 (40). The Fg P1 ($\gamma 190$ – 202), P2 ($\gamma 377$ – 395), and $\gamma 400$ – 411 peptides were synthesized using an Applied Biosystems model 430 peptide synthesizer and purified by HPLC as described previously (21).

NIF, mAbs, and Reagents. NIF was a gift from Corvas International. mAbs used were as follows: OKM1 (anti-CD11b, IgG_{2b}), 44a (anti-CD11b, IgG₁), LM2/1 (anti-CD11b, IgG₁), M1/70 (anti-CD11b), IB4 (anti-CD18, IgG_{2a}), and W6/32 (anti-MHC class I, IgG₁), TS2/18 (anti- $\alpha_L\beta_2$, IgG₁), and TS1/18 (anti- β_2 , IgG₁). The hybridoma cell lines producing these mAbs were obtained from the American Type Culture Collection and purified from their conditioned media using recombinant protein G columns as described by the manufacturer (Zymed Laboratories). The anti- β_1 mAb F4611 and anti- $\alpha_V\beta_3$ mAb LM609 were purchased from Chemicon. All of these mAbs were of mouse origin, and the secondary Ab used for immunofluorescence analyses was FITC goat anti-mouse IgG (Zymed Laboratories). c7E3, the humanized chimeric Fab fragment, was provided by Drs. M. Nakada and R. Jordan of Centocor (Malvern, PA) and was labeled with FITC (Sigma-Aldrich) according to the manufacturer's instructions. β -Glucan was purchased from Molecular Probes.

Cells and Cell Lines. The HEK 293 cell lines were maintained as described previously (35) in DMEM-F12 plus 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (all from BioWhittaker). Adherent cells were removed for passage and experiments using enzyme-free cell dissociation buffer (GIBCO BRL). Construction of 293 cells stably expressing WT or mutant forms of $\alpha_M\beta_2$ and $\alpha_L\beta_2$ has been described previously (35, 41). Before use in adhesion or cell migration assays, receptor expression levels were verified to be similar by flow cytometry (FACS[®]) using a FACStar[™] instrument (Becton Dickinson). The mean fluorescent intensities for the WT and mutants used were ~ 300 when stained with an anti- α_M mAb (OKM1, 44a), the β_2 mAb TS1/18 or TS2/18 for the $\alpha_L\beta_2$ cells compared with < 25 for mock-transfected cells. U937 monocyte cells were obtained from the American Type Culture Collection and maintained in RPMI 1640 with the same supplements as for the HEK 293 cells. Human neutrophils were isolated from the peripheral blood of healthy adult volunteers using Ficoll-Paque as per the manufacturer's instructions (Amersham Pharmacia Biotech) followed by dextran sedimentation and hypotonic lysis of residual erythrocytes as described previously (42). The purity of the neutrophils used was routinely $> 96\%$.

Cell Migration Assays. Cell migration assays were performed under sterile conditions using Costar 24-well transwell plates with 8- μ m pore size uncoated polycarbonate filters (Corning, Inc.). Experiments were carried out in DMEM-F12 (BioWhittaker) or, for selected experiments, in hybridoma serum-free medium (GIBCO BRL). Lower wells contained 600 μ l medium, whereas upper wells contained a final volume of 200 μ l after the addition of cells. To begin the assay, 50 μ l of cells (5×10^5 cells) in medium was added to upper wells, and the transwells were placed in a humidified incubator at 37°C/5% CO₂. For inhibition/stimulation experiments, cells were preincubated for 30 min at 22°C with the test agents before addition to the transwells. Assays were stopped after 22 h by removing the upper wells and wiping the inside of upper wells twice with a cotton swab to remove nonmigrated cells. Quantitation of the migrated cells depended on the cells under analysis. For the HEK 293 cell lines, which remained adherent to the undersurface of the membrane,

the upper transwell assembly was immersed in 10% formalin for 1 h at 22°C. Cells were then stained for at least 1 h with Mayers hematoxylin (Richard-Allan Scientific). Migrating cells were counted using an inverted microscope with an eyepiece counting grid at 100× magnification. Data are presented as the mean cell number per high power field (HPF), a 0.1-mm² area of duplicate wells from three or more experiments with at least five random HPFs counted per well. Neutrophils did not adhere to the membrane between the chambers but rather accumulated as a suspension in the medium in the lower chamber. These cells were quantitated using the CyQuant Cell proliferation kit (Molecular Probes) according to the manufacturer's protocol. In brief, neutrophils within the medium of the lower chamber were recovered by centrifugation, and the cells were frozen for at least 3 h at -70°C. Upon thawing, the cells were resuspended in the CyQuant reagent, and the fluorescence was measured with a CytoFluor II fluorescence multiwell plate reader (PerSeptive Biosystems, Inc.) using a 485-nm excitation and a 530-nm emission filter. The data from these experiments are presented as the total number of migrated cells, determined from a standard curve developed with a known amount of CyQuant-labeled cells. Data obtained in these assays were consistent with those obtained by microscopic counting of the neutrophils. Statistical analyses of data from cell migration assays were performed using the Sigma-Plot software program (Jandel) and the Student's *t* test.

Results

$\alpha_M\beta_2$ -mediated Cell Migration to Fg. To investigate the relationship between $\alpha_M\beta_2$, Fg, and cell migration, we compared the ability of transfected cells expressing $\alpha_M\beta_2$ or mock-transfected cells to migrate toward Fg in a transwell system. Migration was allowed to proceed for 22 h at 37°C, at which time the cells adherent to the underside of the filter were fixed, stained, and counted. Cell viability, as judged by trypan blue exclusion, remained high (>95%) during the course of the assays. When the cells were placed in the upper chamber and a Fg concentration of 50 $\mu\text{g/ml}$ in the lower chamber, a dramatic difference in the migration of the $\alpha_M\beta_2$ and mock-transfected cells was observed (Fig. 1 A). With the mock-transfected cells, 15 ± 3 cells migrated per HPF, whereas 396 ± 58 of the $\alpha_M\beta_2$ -transfected cells migrated at the same time point. Background migration for each cell type was measured using medium alone in lower wells. Although the number of $\alpha_M\beta_2$ -expressing cells recovered on the lower surface of the filter with only buffer present in the lower chamber was slightly higher (59 ± 9 cells/HPF) than for mock-transfected cells (15 ± 3 cells/HPF), the migration of the $\alpha_M\beta_2$ cells toward Fg was nearly sevenfold more than this background migration. Over the course of five experiments, the increase in migration of the $\alpha_M\beta_2$ -transfected cells to Fg versus buffer was $671 \pm 15\%$. Nevertheless, the mock-transfected cells were able to migrate as demonstrated when fibronectin (Fn) was placed in the lower wells. This migration of the mock-transfected cells to Fn was similar in extent to that of the $\alpha_M\beta_2$ -transfected cells to Fg and was inhibited by an mAb (F4611) to the integrin β_1 subunit (20 $\mu\text{g/ml}$), which had no effect on $\alpha_M\beta_2$ migration to Fg (see Fig. 1 A). These experiments were conducted in protein-free

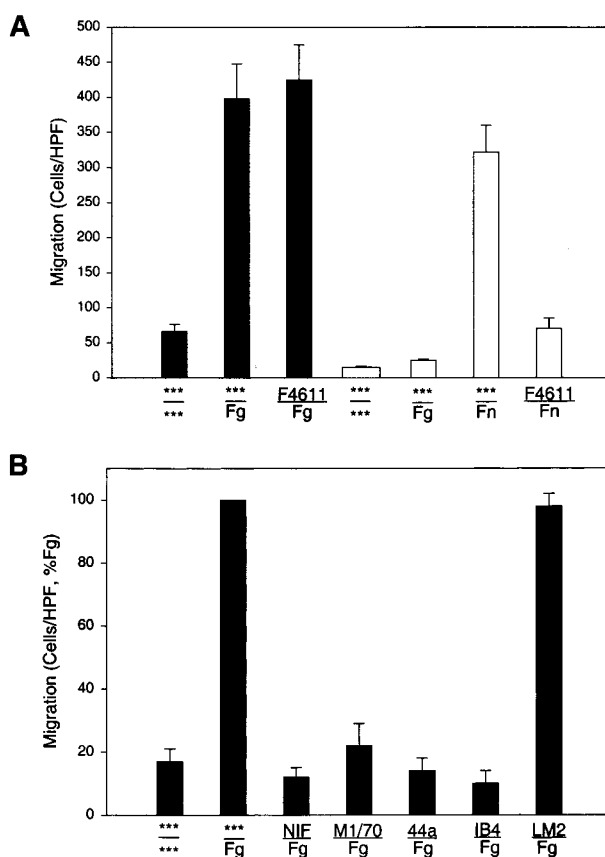


Figure 1. Migration of $\alpha_M\beta_2$ WT transfectants but not mock transfectants to Fg. Human 293 cells (5×10^5 /well) expressing $\alpha_M\beta_2$ (WT, black bars) or mock-transfected cells containing only the neomycin resistance plasmid (white bars) were assessed for their ability to migrate to Fg (50 $\mu\text{g/ml}$, 150 nM), Fn (50 $\mu\text{g/ml}$) or medium alone placed in the lower wells of transwell plates. Some cells were pretreated with 20 $\mu\text{g/ml}$ anti- β_1 blocking mAb F4611 (A) or 100 nM NIF; M1/70 and 44a, 20 $\mu\text{g/ml}$ α_M -blocking mAb; IB4, 20 $\mu\text{g/ml}$ β_2 -blocking mAb; or LM2/1, 20 $\mu\text{g/ml}$ α_M -nonblocking mAb (B) for 30 min before addition to the upper wells. Migration was assessed for 22 h at 37°C and migrated cells were fixed, stained, and counted. Migration data are expressed as mean cells/HPF \pm SD for five random fields per well (A) or as a percentage of the migration of the WT cells to Fg (B) with duplicate wells in each experiment from three or more experiments. The x-axis indicates the addition to the upper wells containing cells over the addition to the lower wells. ***Medium alone.

DMEM-F12, but similar results also were obtained in hybridoma serum-free medium.

Direct evidence that the migration of the transfected cells to Fg was dependent on $\alpha_M\beta_2$ was sought. Several specific inhibitors of $\alpha_M\beta_2$ function were tested for their ability to inhibit the migration of the $\alpha_M\beta_2$ transfectants to Fg (Fig. 1 B). The test inhibitors were preincubated with the $\alpha_M\beta_2$ transfectants for 30 min before their addition to the upper chamber of the transwell system with 50 $\mu\text{g/ml}$ Fg in the lower chamber. NIF, a high affinity ligand for the α_M I domain, eliminated migration of the $\alpha_M\beta_2$ cells to Fg. The α_M I domain-specific mAb 44a also eliminated migration of the $\alpha_M\beta_2$ transfectants to Fg, while a second α_M I-domain specific mAb M1/70 inhibited migration by 80%.

These results are consistent with the effects of these I domain mAbs on $\alpha_M\beta_2$ -mediated adhesion to a variety of ligands including Fg (26). In contrast, a nonblocking α_M I domain-specific mAb LM2/1 (26) had no effect on migration of the $\alpha_M\beta_2$ transfectants. The β_2 -specific mAb IB4 also eliminated migration of the $\alpha_M\beta_2$ transfectants to Fg. Taken together, these results demonstrate that migration of the transfected cells to Fg is $\alpha_M\beta_2$ dependent, and agents that block $\alpha_M\beta_2$ -mediated cell adhesion to Fg also block its capacity to mediate cell migration to this ligand.

Fg as a Migratory Stimulus. In the above analyses, the single concentration of 50 $\mu\text{g/ml}$ Fg was used as a migratory stimulus. As shown in Fig. 2, the extent of migration of the $\alpha_M\beta_2$ -transfected cells was dependent on the available Fg concentration. Concentrations of Fg as low as 1 $\mu\text{g/ml}$ induced a measurable increase in cell migration. Maximum migration was observed at a concentration of 50 $\mu\text{g/ml}$. At a still higher dose of Fg, migration of the $\alpha_M\beta_2$ transfectants decreased markedly, a pattern often encountered in such assays (43).

To investigate whether the observed $\alpha_M\beta_2$ cell migration to Fg was due to chemokinesis or chemotaxis, a checkerboard analysis was conducted. Varying concentrations of Fg were placed either in the upper wells alone, the lower wells alone, or both wells simultaneously (Table I). Data are presented as the percentage of migration of the $\alpha_M\beta_2$ transfectants to the optimal (50 $\mu\text{g/ml}$) concentration of Fg in the lower well (% WT). With equal concentrations of Fg in the upper and lower wells, migration of the $\alpha_M\beta_2$ -transfected cells was only 24%, a value not significantly different ($P > 0.05$) from the 15% migration with no Fg present. With 50 $\mu\text{g/ml}$ in the upper well with the cells and none in the lower chamber, 32% of the maximal migration was observed, suggesting that Fg may cause a modest increase in cell motility. The chemotactic influence of Fg is supported by the inhibition of migration to a constant concentration

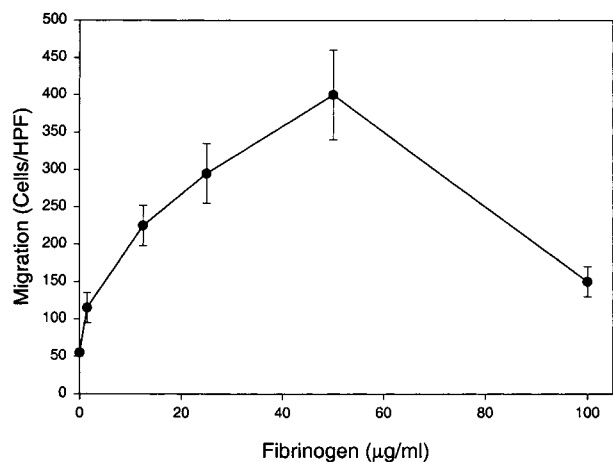


Figure 2. Migration of $\alpha_M\beta_2$ transfectants to Fg. 293 cells (5×10^5 /well) expressing $\alpha_M\beta_2$ (WT cells) were assessed for their ability to migrate to various concentrations of Fg or medium alone (0 $\mu\text{g/ml}$). Migration data are expressed as mean cells/HPF \pm SD for five random fields per well with duplicate wells in each experiment from three or more experiments.

of 50 $\mu\text{g/ml}$ Fg in the lower well as increasing concentrations of Fg were placed in the upper well: 1 $\mu\text{g/ml}$ (0% inhibition), 10 $\mu\text{g/ml}$ (35% inhibition), 50 $\mu\text{g/ml}$ (76% inhibition). These analyses indicate that the migration of the $\alpha_M\beta_2$ transfectants to Fg is largely directional and, therefore, chemotactic.

Structural Requirements within $\alpha_M\beta_2$ for Cell Migration to Fg. To investigate the role of the various domains of $\alpha_M\beta_2$ in Fg-induced cell migration, three different cell lines expressing various forms of the receptors were examined (Fig. 3 A). The three transfectants tested were HEK 293 cells expressing $\alpha_L\beta_2$ (LFA-1), only the α_M and not the β_2 subunit, and the L/M mutant, an $\alpha_L\beta_2$ heterodimer in which the α_L I domain was switched to the α_M I domain. All three cell lines have been characterized previously for their ability to support adhesion to $\alpha_M\beta_2$ ligands (21, 35). All three were determined to express similar levels of receptor or the α_M subunit as the WT $\alpha_M\beta_2$ -expressing cells by FACS[®] analyses. As shown in Fig. 3 A, the $\alpha_L\beta_2$ -expressing cells demonstrated no specific migration to Fg relative to their background migration to medium alone. The α_M alone cells exhibited a weak migratory response to Fg relative to its medium control although this level of migration was not significantly different from the background migration of the WT transfectants to medium. These data demonstrate a requirement for the β_2 chain of the intact heterodimer for efficient migration to Fg. Of particular note, the L/M cells exhibited the same extent of migration to Fg as the WT $\alpha_M\beta_2$ cells, indicating the α_M I domain is sufficient to confer the migratory phenotype to the $\alpha_L\beta_2$ heterodimer.

We have described previously a series of homologue scanning mutants in which the crystal structures of I domains were used as a guide to replace individual small secondary structural elements in the α_M I domain with the corresponding segment of the α_L I domain in the context of the $\alpha_M\beta_2$ heterodimer expressed in the same HEK 293 cells (35). Because of the extensive sequence and structural similarity between the two I domains, such swaps should not

Table I. Fg Checkerboard Analysis

Fg concentration (lower well)	Fn concentration (upper well)			
	50 $\mu\text{g/ml}$	10 $\mu\text{g/ml}$	1 $\mu\text{g/ml}$	Medium only
Medium	32	18	12	16
1 $\mu\text{g/ml}$	25	18	15	29
10 $\mu\text{g/ml}$	26	29	26	58
50 $\mu\text{g/ml}$	24	65	101	100

Migration of $\alpha_M\beta_2$ WT 293 transfectant cells was assessed as in the legend to Fig. 1 A to different concentrations of Fg placed either in the upper or lower transwells. Data are means of cells per HPF for duplicate wells from three or more experiments expressed as percentage of WT (migration to 50 $\mu\text{g/ml}$ Fg, lower well).

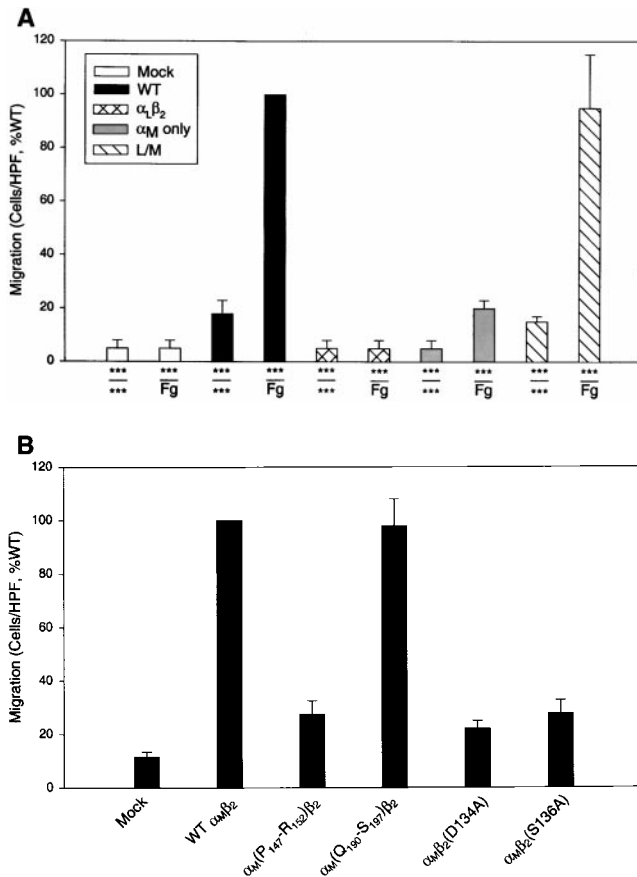


Figure 3. Migration of $\alpha_M\beta_2$ WT and mutant cell lines to Fg. In A, the cell lines are either mock-transfected or transfected cells expressing either $\alpha_M\beta_2$ WT, $\alpha_L\beta_2$, α_M only (expressing the α_M but not β_2 subunit), or L/M chimeric (the α_M I domain switched into $\alpha_L\beta_2$ receptors). In B, the cell lines are $\alpha_M(P147-R152)\beta_2$, in which residues P(147)-R(152) of the α_M I domain have been switched to the corresponding residues of the α_L I domain; $\alpha_M(Q190-S197)\beta_2$, in which residues Q(190)-S(197) have been switched to the corresponding residues of the α_L I domain; and point mutations of D(134) and S(136) to A in the β_2 subunit. Details of these mutations are described in reference 35. Migration of each cell type is to 50 $\mu\text{g/ml}$ Fg. Data are means of cells per HPF with duplicate wells in each experiment from three or more experiments ND expressed as the percentage of WT \pm SD.

perturb overall conformation. These mutant cell lines have been used to map the binding sites for several $\alpha_M\beta_2$ ligands (35–38). To further dissect the role of individual segments of the I domain in $\alpha_M\beta_2$ -mediated cell migration to Fg, two representative mutant receptors were tested for their ability to support the migratory response. Mutant P(147)-R(152), a mutant in which the segment between these residues on the MIDAS face of the I domain was switched to the corresponding residues in α_L , failed to migrate to Fg (Fig. 3 B). This mutant also fails to recognize NIF, iC3b, and *C. albicans* (35–37), indicative of the key role of this segment in ligand recognition by $\alpha_M\beta_2$, including a role in directed cell migration. At the other extreme, mutant Q(190)-S(197) was tested. This mutant was previously shown to exhibit enhanced adhesion to Fg relative to the WT $\alpha_M\beta_2$ cells. However, as shown in Fig. 3 B, the migra-

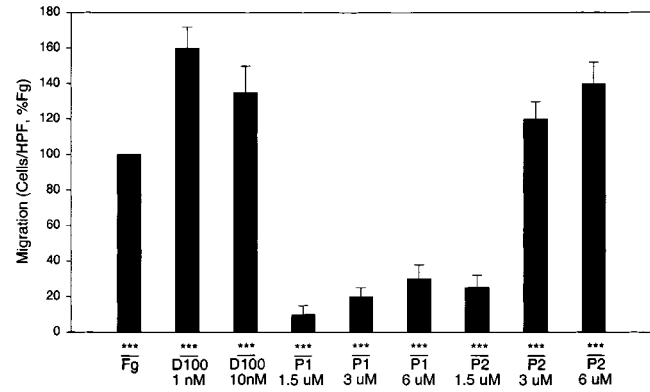


Figure 4. Migration of $\alpha_M\beta_2$ WT cells to Fg D_{100} fragment and Fg recognition peptides P1 and P2. Migration of WT cells was assessed to various concentrations of either the Fg D_{100} fragment, P1 peptide, or P2 peptide placed in the lower transwells. Data are means of cells per HPF with duplicate wells for each experiment from three or more experiments and expressed as percentage WT \pm SD.

tion of these cells was similar to that of WT cells, again providing evidence that the structural requirements for adhesion and migration are distinct. In view of the importance of the β_2 subunit in cell migration to Fg, we also tested the effect of single-point mutations of either D134 and S136 to alanines in the β_2 subunit. These mutations reside at residues which are likely to serve as cation coordination sites in a MIDAS motif in integrin β subunits (44, 45), and this MIDAS motif may be formed by an I domain-like fold within the β subunits (46, 47). Regardless of the validity of these structural speculations, these mutations are known to abolish ligand binding to multiple integrins (48, 49). As shown in Fig. 3 B, these mutations also abolished the migration of the cells to Fg.

Structural Requirements within Fg for $\alpha_M\beta_2$ -mediated Migration. Two peptide sequences, designated P1 ($\gamma 191-202$) and P2 ($\gamma 373-395$), in the γ chain of the D domain of Fg have been identified as $\alpha_M\beta_2$ recognition sites (21, 34); these peptides inhibit $\alpha_M\beta_2$ -mediated adhesion to Fg and directly support cell adhesion via $\alpha_M\beta_2$ by interacting with the I domain of the receptor (21). Accordingly, we tested the capacity of the D_{100} fragment produced by plasmin cleavage of Fg and synthetic P1 and P2 peptides to support $\alpha_M\beta_2$ -mediated cell migration. As shown in Fig. 4, the D_{100} fragment of Fg supported cell migration at very low concentrations; 1 and 10 nM concentrations of D_{100} were more effective than 150 nM (50 $\mu\text{g/ml}$) Fg. The P1 and P2 peptides also supported cell migration although higher concentrations of the recognition peptides were required. With P2, 3–6 μM peptide was as effective as Fg in supporting migration of the $\alpha_M\beta_2$ transfectants. P1 was less potent but did support a migratory response in a concentration-dependent fashion in the 1.5–6 μM range.

Stimulation of $\alpha_M\beta_2$ -mediated Cell Migration by the P2 Peptide of Fg. To further examine the migratory activity of these Fg peptides, their ability to inhibit migration to Fg and each other was assessed (Fig. 5). When placed only in the upper well together with the cells, neither of the two

peptides or the D₁₀₀ fragment stimulated spontaneous migration of the $\alpha_M\beta_2$ cells, suggesting that the induction of cell migration to these derivatives was a chemotactic response. Furthermore, when placed in equal amounts in both the upper and lower wells, P1, P2, and the D₁₀₀ fragment inhibited migration to itself, consistent with a chemotactic activity (data not shown). When testing the effects of the Fg derivatives in influencing cell migration to each other, some unexpected results were encountered. As anticipated, when the P1 peptide was added to the $\alpha_M\beta_2$ -transfected cells in the upper chamber, it did inhibit migration to P2 and Fg in the lower chamber. In contrast, when P2 was added to the cells in the upper well, it stimulated rather than inhibited migration to P1 in the lower well (Fig. 5). To further explore the stimulatory effect of P2, its influence on $\alpha_M\beta_2$ -mediated cell migration to Fg was assessed. As shown in Fig. 5, when added to the cells, P2 also stimulated migration to Fg. At a 6 μ M concentration, P2 increased migration to Fg by 50% (relative to the migration of the cells to Fg alone, 100%). In contrast, both D₁₀₀ (10 nM, 82% inhibition) and P1 (6 μ M, 77% inhibition) inhibited migration of the $\alpha_M\beta_2$ cells under the same conditions. The Fg γ 400–411 peptide, reported by other investigators to inhibit $\alpha_M\beta_2$ adhesion (50) had no effect on WT migration to Fg in concentrations as high as 50 μ M (data not shown). This stimulatory effect of P2 was similar to that induced by two known activators of $\alpha_M\beta_2$. As shown in Fig. 6, 10 nM PMA and 2 μ g/ml β -glucan were found to stimulate migration by 25 and 70%, respectively. The increases in migration induced by all three activators were statistically significant ($P < 0.05$) relative to the migration to Fg in their absence. Collectively, these data demonstrate a novel role for the P2 peptide in stimulating $\alpha_M\beta_2$ -mediated migration to Fg.

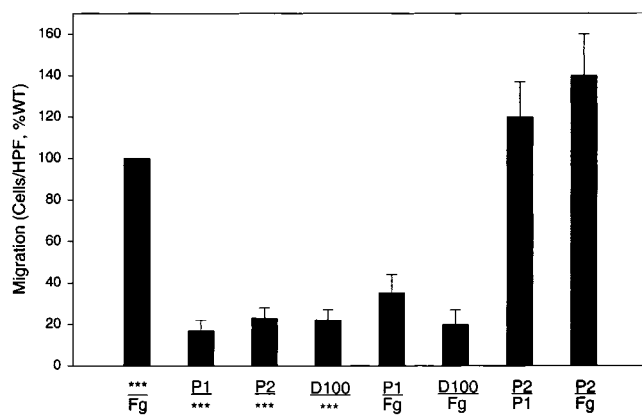


Figure 5. Migration of $\alpha_M\beta_2$ WT cells to Fg is inhibited by P1 but stimulated by P2. Migration of $\alpha_M\beta_2$ WT cells was assessed to various combinations of optimal concentrations of 50 μ g/ml Fg, 6 μ M P1 peptide, 6 μ M P2 peptide, or 10 nM D₁₀₀ fragment in the upper or lower transwells as indicated. Fg or peptides added to the upper well were preincubated with cells for 30 min before addition of the entire mixture to the transwell. Data are means of cells per HPF with duplicate wells for each experiment from three or more experiments and expressed as percentage of WT \pm SD.

We sought to determine if the activating activity of P2 on $\alpha_M\beta_2$ occurs with cells other than the HEK 293 transfectants. For this purpose, we tested the effects of P2 on migration of peripheral blood neutrophils to Fg. As shown in Fig. 7, neutrophils exhibited a substantial migratory response to Fg, which was $\alpha_M\beta_2$ dependent as indicated by the inhibitory effect of mAb 44a. The neutrophils also migrated to P2. When added to the lower wells at a 10 μ M concentration, the extent of cell migration to P2 was similar to that induced by 50 μ g/ml Fg. Addition of P2 at this concentration (or 5 μ M) to neutrophils in the upper chamber enhanced their migration to Fg by $>50\%$. This increment was also $\alpha_M\beta_2$ dependent as the migration was fully inhibited by mAb 44a. Under these conditions, addition of the same concentration P1 to the upper chamber inhibited the migration of the neutrophils to Fg by $\sim 50\%$ (data not shown). Thus, P2 exerted its activating effect on a cell which expresses $\alpha_M\beta_2$ naturally.

P2 Activation of $\alpha_M\beta_2$ Is Associated with Exposure of the 7E3 Epitope. To further investigate the mechanism of P2 stimulation of $\alpha_M\beta_2$ -mediated migration to Fg, we conducted blocking studies with the chimeric Fab fragment (abciximab) of mAb 7E3. This mAb was originally developed against integrin $\alpha_{IIb}\beta_3$ (51) but has subsequently been shown to interact with $\alpha_V\beta_3$ and $\alpha_M\beta_2$ (for a review, see reference 52). The epitope in $\alpha_M\beta_2$ resides in the I domain and appears to require activation of the receptor for expression (53, 54). Although this mAb blocks Fg binding to the activated $\alpha_M\beta_2$ on leukocytes, it does not react with our nonstimulated WT $\alpha_M\beta_2$ transfectants as assessed by FACS[®] analyses (data not shown). Based on these characteristics of the 7E3 epitope, we hypothesized that c7E3 might exhibit a differential effect on nonstimulated and P2-stimulated migration of the $\alpha_M\beta_2$ transfectants to Fg. To maximize the stimulatory effect of P2, a lower concentration of 10 μ g/ml Fg was employed in the transwell migra-

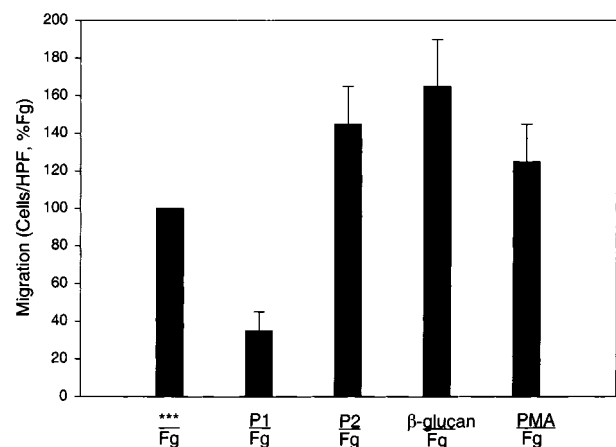


Figure 6. Stimulation of $\alpha_M\beta_2$ WT cell migration to Fg. Migration of $\alpha_M\beta_2$ WT cells to 50 μ g/ml Fg was assessed in the presence of various reagents after preincubation for 30 min. Reagents are Fg recognition peptides P1 (6 μ M) or P2 (6 μ M), 2 μ g/ml β -glucan, and 10 nM PMA. Data are means of cells per HPF with duplicate wells for each experiment from three or more experiments and expressed as percentage of WT \pm SD.

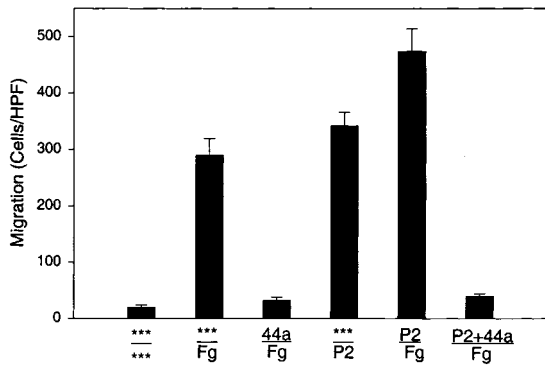


Figure 7. Stimulation of neutrophil migration to Fg by P2. Isolated human neutrophils were placed in the upper wells and 20 $\mu\text{g/ml}$ Fg or 10 μM P2 in the lower wells, and migration was measured after 24 h. In the experiments in which P2 or mAb 44a were used in the upper chambers, these were preincubated with the neutrophils for 30 min before addition of the cells to the upper wells. Migration data are expressed as mean cells per HPF \pm SD for five random fields per well with duplicate wells in each experiment from three or more experiments.

tion assay. As shown in Fig. 8 A, 40 $\mu\text{g/ml}$ c7E3 had no inhibitory effect on $\alpha_{\text{M}}\beta_2$ -mediated migration to Fg alone. When 6 μM P2 was added to the cells, migration was stimulated ~ 2.5 -fold. However, when c7E3 and P2 were added to the cells in the upper well, the increment in cell migration was inhibited. A similar inhibitory effect of c7E3 on the migration of the $\alpha_{\text{M}}\beta_2$ -transfected cells stimulated by β -glucan was also observed (90% inhibition; Fig. 8 A). To rule out possible recognition of $\alpha_{\text{V}}\beta_3$ as the basis of the observed effects of c7E3, a potent $\alpha_{\text{V}}\beta_3$ -blocking mAb, 20 $\mu\text{g/ml}$ LM609, was substituted for c7E3 and found to have no effect on P2-stimulated (or baseline, data not shown), $\alpha_{\text{M}}\beta_2$ -mediated cell migration to Fg. The data in Fig. 8 A indicate that the P2 induces, not suppresses, expression of the c7E3 epitope. We also considered whether P2 influences c7E3 reactivity with its epitope. This question was addressed with platelets. 10 μM P2 did not inhibit the binding of c7E3 to platelets as assessed by FACS[®].

These data support a model in which an activated state of $\alpha_{\text{M}}\beta_2$, defined by expression of the 7E3 epitope, is induced by P2 and supports enhanced migration to Fg. To assess this possibility, the reactivity of the $\alpha_{\text{M}}\beta_2$ transfectants with 7E3 was assessed by FACS[®] at various time points during their migration to Fg with or without P2 present in the upper chamber (Fig. 8 B). As noted above, the $\alpha_{\text{M}}\beta_2$ transfectants did not express the 7E3 epitope, and this lack of reactivity was not changed by addition of P2 to the cells. On the other hand, the cells developed c7E3 reactivity over time in the presence of P2. The reactivity was noted at 1 h and increased still further by 12 h (Fig. 8 B). This reactivity with c7E3 was enhanced substantially in the presence of P2.

Discussion

Leukocyte migration is the hallmark of inflammation in vivo, and $\alpha_{\text{M}}\beta_2$ and Fg have been shown to contribute to leukocyte migration in multiple systems (23, 24). This

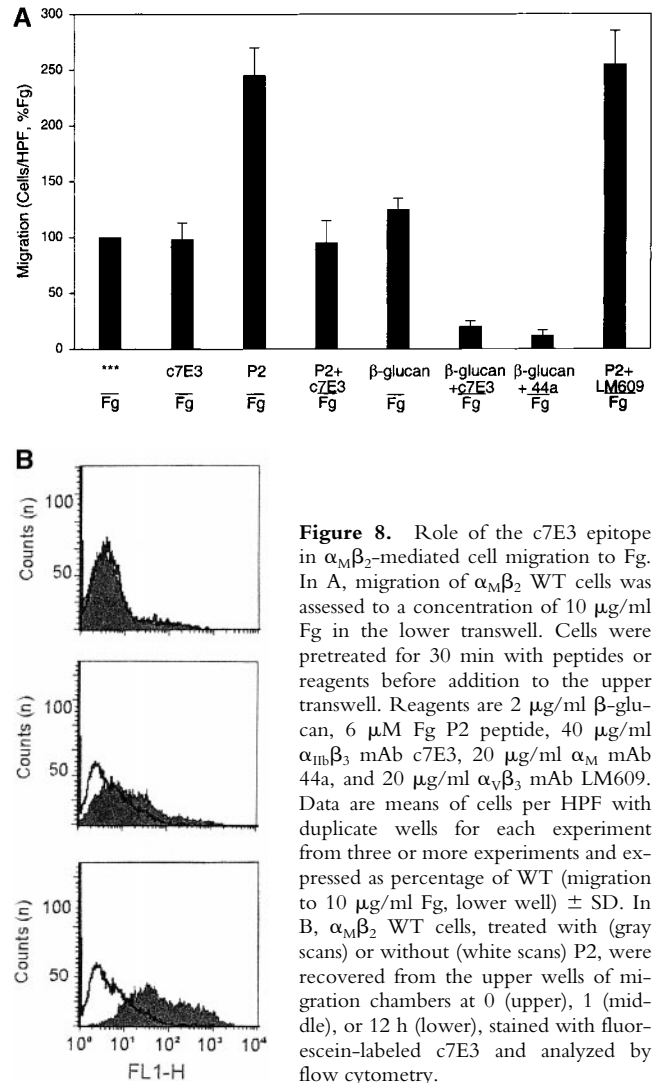


Figure 8. Role of the c7E3 epitope in $\alpha_{\text{M}}\beta_2$ -mediated cell migration to Fg. In A, migration of $\alpha_{\text{M}}\beta_2$ WT cells was assessed to a concentration of 10 $\mu\text{g/ml}$ Fg in the lower transwell. Cells were pretreated for 30 min with peptides or reagents before addition to the upper transwell. Reagents are 2 $\mu\text{g/ml}$ β -glucan, 6 μM Fg P2 peptide, 40 $\mu\text{g/ml}$ $\alpha_{\text{IIb}}\beta_3$ mAb c7E3, 20 $\mu\text{g/ml}$ α_{M} mAb 44a, and 20 $\mu\text{g/ml}$ $\alpha_{\text{V}}\beta_3$ mAb LM609. Data are means of cells per HPF with duplicate wells for each experiment from three or more experiments and expressed as percentage of WT (migration to 10 $\mu\text{g/ml}$ Fg, lower well) \pm SD. In B, $\alpha_{\text{M}}\beta_2$ WT cells, treated with (gray scans) or without (white scans) P2, were recovered from the upper wells of migration chambers at 0 (upper), 1 (middle), or 12 h (lower), stained with fluorescein-labeled c7E3 and analyzed by flow cytometry.

study has used $\alpha_{\text{M}}\beta_2$ transfectants and selected mutants to dissect the molecular requirements for $\alpha_{\text{M}}\beta_2$ -mediated cell migration to Fg and its derivatives. The major conclusions of our study are the following. (a) Fg supports a chemotactic cell migration mediated by $\alpha_{\text{M}}\beta_2$. This response is dependent on Fg concentration and occurs at low (1–50 $\mu\text{g/ml}$) Fg levels. (b) The α_{M} I domain is necessary but not sufficient to support cell migration to Fg. In contrast to cell adhesion to Fg, efficient migration requires the β_2 subunit. (c) The P1 and P2 peptides, as well as the D₁₀₀ fragment, support cell migration. Thus, the same Fg derivatives that mediate $\alpha_{\text{M}}\beta_2$ -dependent cell adhesion also support cell migration. (d) The P2 peptide stimulates $\alpha_{\text{M}}\beta_2$ -mediated cell migration to Fg and the P1 peptide, in a manner similar to other $\alpha_{\text{M}}\beta_2$ activators, β -glucan, and PMA. In addition, the activation-dependent epitope of c7E3 is induced by P2, c7E3 inhibits P2, and β -glucan stimulated $\alpha_{\text{M}}\beta_2$ -mediated migration to Fg. Many of these findings, including the activating activity of P2, are also observed in the migration of human neutrophils to Fg. Thus, these data reveal new information regarding the molecular interactions between

$\alpha_M\beta_2$ and Fg that are necessary for cell migration and define a novel mechanism whereby the P2 peptide of Fg stimulates $\alpha_M\beta_2$ -mediated chemotaxis.

More than a decade ago, Fg was identified as a ligand for $\alpha_M\beta_2$ (14), and numerous subsequent studies have examined the structure function requirements for $\alpha_M\beta_2$ -mediated cell adhesion to Fg (38, 41). The capacity of this ligand–receptor interaction to mediate chemotaxis has received less attention but, in view of the propensity of Fg/fibrin to accumulate at sites of inflammation, it is of clear physiological relevance. Indeed, Fg and the P1 peptide have been shown to mediate an $\alpha_M\beta_2$ -dependent inflammatory response in vivo (53, 55). Two notable in vitro studies have also shown that Fg, as well as the D₁₀₀ fragment and P1 peptide, induce leukocyte transmigration and chemotaxis which is $\alpha_M\beta_2$ dependent (56, 57). Thus, the demonstration that $\alpha_M\beta_2$ transfectants migrate to Fg and its recognition peptides is consistent with published data on leukocytes and supports the relevance of these cells to dissect the molecular requirements for the chemotactic response. In this regard, we found that the I domain of the receptor was not sufficient in the α_M alone cells to mediate efficient migration to Fg. However, when the α_M I domain was placed in the context of another β_2 integrin, $\alpha_L\beta_2$, that does not mediate migration to Fg, chemotaxis of these L/M cells was observed. Thus, within the context of an intact heterodimer, the α_M I domain is necessary and sufficient to confer migration to Fg. Furthermore, within the α_M I domain, at least some of the sequences which are key for recognition of Fg and other $\alpha_M\beta_2$ ligands also influence $\alpha_M\beta_2$ -mediated cell migration. Thus, the P147–152 $\alpha_M\beta_2$ mutant that fails to adhere to NIF (35), iC3b (36), *C. albicans* (37), and the D₁₀₀ fragment of Fg (38) also fails to migrate to Fg. These data are consistent with results demonstrating that Fg is a ligand for $\alpha_M\beta_2$ but not $\alpha_L\beta_2$, adhesion to Fg is mediated principally via the I domain of $\alpha_M\beta_2$, and sequences on the MIDAS face of the α_M I domain are involved in the migratory and adhesive response of the receptor. However, the β_2 subunit is more influential in cell migration than in cell adhesion (21). The role of the β_2 subunit may depend on the interaction of its cytoplasmic tail with cytoskeletal elements, such as talin and paxillin, which must undergo rearrangements in order for cells to migrate (24). Such requirements for the β subunit cytoplasmic segment for migration were demonstrated with Chinese hamster ovary cells expressing integrin $\alpha_{IIb}\beta_3$ and migrating on Fg (58).

Integrin $\alpha_M\beta_2$ -mediated migration was triggered not only by intact Fg but also by its D₁₀₀ fragment and by peptides duplicating its γ 191–202 and γ 373–395 sequences. While Fg was a potent chemoattractant, the plasmin-derived D₁₀₀ fragment was active at 150-fold lower concentrations. The activity of the D₁₀₀ fragment and other degradation products may be important in the recruitment of leukocytes to inflammatory sites, where proteolysis is a major mechanism for resolution of Fg/fibrin deposits. In addition, fibrin(ogen) degradation products in blood could potentially suppress leukocyte emigration. Both the P1 and P2 peptide sequences reside in the D₁₀₀ fragment. We have

found that these sequences are poorly exposed in soluble Fg but become exposed upon its deposit or degradation (unpublished results). The combined activity and exposure of these sequences may account for the potency of the D₁₀₀ fragment as a chemoattractant. The P2 peptide was more potent than P1 in supporting and inhibiting $\alpha_M\beta_2$ -dependent cell migration. This difference in activity is consistent with the greater apparent affinity of P2 for $\alpha_M\beta_2$ (21).

When P2 was added to the $\alpha_M\beta_2$ transfectants, it enhanced cell migration. This increase appears to reflect an activation of $\alpha_M\beta_2$ by the peptide. This interpretation is supported by the observation that the epitope for c7E3, which is expressed by activated $\alpha_M\beta_2$ (53), was induced by P2, and c7E3 inhibited the increase in cell migration evoked by P2. The activation of integrins by peptide ligands was originally demonstrated with $\alpha_{IIb}\beta_3$ (59). Particularly relevant to the present observation, an $\alpha_M\beta_2$ ligand peptide derived from ICAM-2 activates $\alpha_M\beta_2$ -mediated cell migration (60). The fact that P2, but not P1, induced such activation implies that, even though both peptides bind to the α_M I domain, they must interact in a fundamentally different way to initiate a differential response in the receptor. Activation of $\alpha_M\beta_2$ has been shown to be important for optimal recognition of Fg to mediate cell adhesion (15) and migration (57). Such activation can arise from interactions within (60) or outside of the α_M I domain (61), or outside of $\alpha_M\beta_2$ altogether (PMA stimulation). β -Glucan is a ligand for the lectin-binding domain of $\alpha_M\beta_2$ (61), and its activation of the receptor has been studied extensively (62). β -Glucan activation of $\alpha_M\beta_2$ also is known to result in expression of the α_M I domain activation neoepitope (61) recognized by mAb CBRM1/5, and this mAb eliminates $\alpha_M\beta_2$ adhesion to Fg (63). Nevertheless, mAbs CBRM1/5 and c7E3 do not compete with each other for binding to activated $\alpha_M\beta_2$ (54). Our data support a model in which β -glucan and P2 stimulation results in an activated $\alpha_M\beta_2$ conformation, which expresses the c7E3 epitope. The greater inhibition by c7E3 of β -glucan- versus P2-stimulated migration suggests that the mechanism and/or extent of $\alpha_M\beta_2$ activation may be different. Also, P2 was shown to enhance neutrophil migration to Fg, emphasizing that modulation of $\alpha_M\beta_2$ function may be of physiological significance. The γ chain of Fg in which P2 resides is capable of undergoing conformational modulations, including upon ligation by integrins (64). Whether activation of the receptor via a P2-dependent mechanism is in itself of physiological importance can only be the subject of speculation at this time.

In summary, we have demonstrated that $\alpha_M\beta_2$ can mediate a chemotactic cell migration to Fg and Fg derivatives. Both the P1 and P2 recognition peptides can support this migratory response. Such migration depends on the α_M I domain and is influenced by other domains of $\alpha_M\beta_2$. While our earlier studies identified a negative role for the β_2 subunit in modulating adhesion (37), this study identifies a positive role for the β_2 subunit in influencing cell migration. Thus, the structural requirements for $\alpha_M\beta_2$ -mediated cell adhesion and migration to the same ligand, Fg, are dis-

tinct. In addition, we identify a novel proinflammatory function for the P2 sequence, as well as β -glucan, to activate $\alpha_M\beta_2$ and stimulate cell migration to Fg.

C.B. Forsyth was supported by an American Heart Association and a National Institutes of Health (NIH) Postdoctoral Fellowship. This work was supported in part by NIH grants HL54924 and HL63199 and by a grant-in-aid from the American Heart Association.

Submitted: 3 December 1999

Revised: 22 March 2001

Accepted: 9 April 2001

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