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 $\alpha_M$ was replaced with the I domain of $\alpha_M$, the chimeric receptor supported cell migration to Fg; however, the $\alpha_M$ subunit, containing the I domain but lacking the $\beta_2$ subunit, supported migration poorly, thus, the $\alpha_M$I domain is necessary but not sufficient to support chemotaxis, and efficient migration requires the $\beta_2$ subunit and $\alpha_M$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) 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 develop 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 inflammatoryimmune 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 $\kappa B$ 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 $\alpha/\beta$ heterodimeric adhesion receptors and shares the same $\beta_2$ subunit as $\alpha_4\beta_2$ (CD11a/CD18, LFA-1), $\alpha_5\beta_2$ (CD11c/CD18, p150,95), and $\alpha_{10}\beta_2$ ($\alpha_\gamma$/CD18) (23–25). High af-
finity binding of many protein ligands, including Fg, to αmβ2 involves a segment of ~200 amino acids in the αM 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 αM 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 αM 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 αM I domain ligands can be influenced by the activation of the αMβ2-bearing cell, which alters receptor avidity/affinity (23, 24, 31). αMβ2 recognizes mannose and β-glucan carbohydrate structures via a lectin-like ligand–binding function. This lectin binding site is also located in the αM subunit (32). Ligands of the lectin domain include zymosan, Saccharomyces cerevisiae, and nonspecific activators of cell–mediated immunity such as lentilin (32).

Fg is a dimeric molecule composed of three pairs of nonidentical 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 αMβ2. The peptides corresponding to these sequences are designated P1 (γ190–202) (34) and P2 (γ377–395) (21). The P1 and P2 peptides not only inhibit αMβ2-mediated adhesion to Fg derivatives but also support adhesion of αMβ2-bearing cells. On a molar basis, P2 is a more potent inhibitor of αMβ2 adhesion to Fg than P1 (21). The binding sites for both the P1 and P2 peptides in αMβ2 have been mapped to the αM I domain, and cells expressing the αM subunit in the absence of the β2 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 αMβ2 to demonstrate the importance of the αM 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 αMβ2-dependent cell migration to Fg and its derivatives. We demonstrate that αMβ2 can, indeed, mediate directed cell migration to Fg and that the recognition peptides influence this response. Furthermore, the structural components of αMβ2 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 αMβ2-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 αMβ2 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 Enzyme Research Laboratories. Fragment D100 (M, 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 (γ190–202), P2 (γ377–395), and γ400–411 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 Corvus International. mAbs used were as follows: OKM1 (anti-CD11b, IgG2a), 44a (anti-CD11b, IgG1), LM2/1 (anti-CD11b, IgG1), M1/70 (anti-CD11b), IB4 (anti-CD18, IgG2b), and W6/32 (anti-MHC class I, IgG1), TS2/18 (anti-αMβ2, IgG1), and TS1/18 (anti-β2, 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-β2 mAb F4611 and anti-αMβ2 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). cF3e3, 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. β2–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 αMβ2 and αMβ2 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-αM mAb (OKM1, 44a), the β2 mAb TS1/18 or TS2/18 for the αMβ2 cells compared with <25 for mock-transfected cells. U937 monocyteid 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 Fi-coll-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-μm 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 μl medium, whereas upper wells contained a final volume of 200 μl after the addition of cells. To begin the assay, 50 μl of cells (5 × 104 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 underside 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 100X 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 multowell plate reader (Per Septive 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 SigmaPlot software program (Jandel) and the Student’s t test.

Results

αMβ2-mediated Cell Migration to Fg. To investigate the relationship between αMβ2, Fg, and cell migration, we compared the ability of transfected cells expressing αMβ2 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 μg/ml in the lower chamber, a dramatic difference in the migration of the αMβ2 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 αMβ2-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 αMβ2-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 αMβ2 cells toward Fg was nearly sevenfold more than this background migration. Over the course of five experiments, the increase in migration of the αMβ2-transfected cells to Fg versus buffer was 671 ± 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 αMβ2-transfected cells to Fg and was inhibited by an mAb (F4611) to the integrin β1 subunit (20 μg/ml), which had no effect on αMβ2 migration to Fg (see Fig. 1 A). These experiments were conducted in protein-free 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 αMβ2 was sought. Several specific inhibitors of αMβ2 function were tested for their ability to inhibit the migration of the αMβ2 transfecteds to Fg (Fig. 1 B). The test inhibitors were preincubated with the αMβ2 transfecteds for 30 min before their addition to the upper chamber of the transwell system with 50 μg/ml Fg in the lower chamber. NIF, a high affinity ligand for the αM I domain, eliminated migration of the αMβ2 cells to Fg. The αM I-domain–specific mAb 44a also eliminated migration of the αMβ2 transfecteds to Fg, while a second αM I–domain-specific mAb M1/70 inhibited migration by 80%.
These results are consistent with the effects of these I domain mAbs on α₅β₃-mediated adhesion to a variety of ligands including Fg (26). In contrast, a nonblocking α₅ I domain–specific mAb LM2/1 (26) had no effect on migration of the α₅β₂ transfectants. The β₂-specific mAb IB4 also eliminated migration of the α₅β₂ transfectants to Fg. Taken together, these results demonstrate that migration of the transfected cells to Fg is α₅β₂ dependent, and agents that block α₅β₂-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 μg/ml Fg was used as a migratory stimulus. As shown in Fig. 2, the extent of migration of the α₅β₂-transfected cells was dependent on the available Fg concentration. Concentrations of Fg as low as 1 μg/ml induced a measurable increase in cell migration. Maximum migration was observed at a concentration of 50 μg/ml. At a still higher dose of Fg, migration of the α₅β₂ transfectants decreased markedly, a pattern often encountered in such assays (43).

To investigate whether the observed α₅β₂ 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 α₅β₂ transfectants to the optimal (50 μg/ml) concentration of Fg in the lower well (% WT). With equal concentrations of Fg in the upper and lower wells, migration of the α₅β₂-transfected cells was only 24%, a value not significantly different (P > 0.05) from the 15% migration with no Fg present. With 50 μg/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 of 50 μg/ml Fg in the lower well as increasing concentrations of Fg were placed in the upper well: 1 μg/ml (0% inhibition), 10 μg/ml (35% inhibition), 50 μg/ml (76% inhibition). These analyses indicate that the migration of the α₅β₂ transfectants to Fg is largely directional and, therefore, chemotactic.

Structural Requirements within α₅β₂ for Cell Migration to Fg. To investigate the role of the various domains of α₅β₂ 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 α₅β₂ (LFA-1), only the α₅ and not the β₂ subunit, and the L/M mutant, an α₅β₂ heterodimer in which the α₅ I domain was switched to the α₅ I domain. All three cell lines have been characterized previously for their ability to support adhesion to α₅β₂ ligands (21, 35). All three were determined to express similar levels of receptor or the α₅ subunit as the WT α₅β₂–expressing cells by FACS® analyses. As shown in Fig. 3 A, the α₅β₂–expressing cells demonstrated no specific migration to Fg relative to their background migration to medium alone. The α₅ I 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 β₃ 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 α₅β₂ cells, indicating the α₅ I domain is sufficient to confer the migratory phenotype to the α₅β₂ 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 α₅ I domain with the corresponding segment of the α₅ I domain in the context of the α₅β₂ 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

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**Table I. Fg Checkerboard Analysis**

| Fg concentration (upper well) | 50 μg/ml | 10 μg/ml | 1 μg/ml | Medium only |
|-----------------------------|---------|---------|--------|------------|
| Fn concentration (lower well) |          |         |        |            |
| Medium                      | 32      | 18      | 12     | 16         |
| 1 μg/ml                     | 25      | 18      | 15     | 29         |
| 10 μg/ml                    | 26      | 29      | 26     | 58         |
| 50 μg/ml                    | 24      | 65      | 101    | 100        |

Migration of α₅β₂ WT 293 transfected 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 μg/ml Fg, lower well).
perturb overall conformation. These mutant cell lines have been used to map the binding sites for several \( \alpha_\text{M}\beta_2 \) ligands (35–38). To further dissect the role of individual segments of the I domain in \( \alpha_\text{M}\beta_2 \)-mediated cell migration to Fg, two representative mutant receptors were tested for their ability to support the migratory response. Mutant P147-R152, a mutant in which the segment between these residues on the MIDAS face of the I domain was switched to R152, a mutant in which the segment between these residues in ligand recognition by \( \alpha_\text{M}\beta_2 \), including a role in directed cell migration. At the other extreme, mutant Q190-S197 have been switched to the corresponding residues of the I domain; and point mutations of D134 and S136 to alanines in the \( \beta_2 \) subunit. These mutations reside at residues which are likely to serve as cation coordination sites in a MIDAS motif in integrin \( \beta \) subunits (44, 45), and this MIDAS motif may be formed by an I domain–like fold within the \( \beta \) 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.

Figure 3. Migration of \( \alpha_\text{M}\beta_2 \) WT and mutant cell lines to Fg. In A, the cell lines are either mock-transfected or transfected cells expressing either \( \alpha_2\beta_3 \) WT, \( \alpha_2\beta_3 \), \( \alpha_\text{M}\beta_2 \), or L/M chimeric (the \( \alpha_1 \) I domain switched into \( \alpha_2\beta_3 \)) receptors. In B, the cell lines are \( \alpha_\text{M}(\text{P}147\text{-R152})\beta_2 \), which residues P147-R152 of the \( \alpha_\text{M} \) I domain have been switched to the corresponding residues of the \( \alpha_1 \) I domain; \( \alpha_\text{M}(\text{Q190-S197})\beta_2 \), in which residues Q190-S197 have been switched to the corresponding residues of the \( \alpha_1 \) I domain; and point mutations of D134 and S136 to A in the \( \beta_2 \) subunit. Details of these mutations are described in reference 35. Migration of each cell type is to 50 \( \mu \)g/mL Fg. Data are means of cells per HPF with duplicate wells in each experiment from three or more experiments and expressed as percentage WT \( \pm \) SD.

Structural Requirements within Fg for \( \alpha_\text{M}\beta_2 \)-mediated Migration. Two peptide sequences, designated P1 (\( \gamma \)191–202) and P2 (\( \gamma \)373–395), in the \( \gamma \) chain of the D domain of Fg have been identified as \( \alpha_\text{M}\beta_2 \) recognition sites (21, 34); these peptides inhibit \( \alpha_\text{M}\beta_2 \)-mediated adhesion to Fg and directly support cell adhesion via \( \alpha_\text{M}\beta_2 \) by interacting with the I domain of the receptor (21). Accordingly, we tested the capacity of the D100 fragment produced by plasmin cleavage of Fg and synthetic P1 and P2 peptides to support \( \alpha_\text{M}\beta_2 \)-mediated cell migration. As shown in Fig. 4, the D100 fragment of Fg supported cell migration at very low concentrations; 1 and 10 nM concentrations of D100 were more effective than 150 nM (50 \( \mu \)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 \( \mu \)M peptide was as effective as Fg in supporting migration of the \( \alpha_\text{M}\beta_2 \) transfectants. P1 was less potent but did support a migratory response in a concentration-dependent fashion in the 1.5–6 \( \mu \)M range.

Stimulation of \( \alpha_\text{M}\beta_2 \)-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 D_{100} fragment stimulated spontaneous migration of the α_{M}β_{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 D_{100} 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 α_{M}β_{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 α_{M}β_{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 μM concentration, P2 increased migration to Fg by 50% (relative to the migration of the cells to Fg alone, 100%). In contrast, both D_{100} (10 nM, 82% inhibition) and P1 (6 μM, 77% inhibition) inhibited migration of the α_{M}β_{2} cells under the same conditions. The Fg γ400–411 peptide, reported by other investigators to inhibit α_{M}β_{2} adhesion (50) had no effect on WT migration to Fg in concentrations as high as 50 μM (data not shown). This stimulatory effect of P2 was similar to that induced by two known activators of α_{M}β_{2}. As shown in Fig. 6, 10 nM PMA and 2 μg/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 α_{M}β_{2}-mediated migration to Fg.

We sought to determine if the activating activity of P2 on α_{M}β_{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 α_{M}β_{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 μM concentration, the extent of cell migration to P2 was similar to that induced by 50 μg/ml Fg. Addition of P2 at this concentration (or 5 μM) to neutrophils in the upper chamber enhanced their migration to Fg by >50%. This increment was also α_{M}β_{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 ~50% (data not shown). Thus, P2 exerted its activating effect on a cell which expresses α_{M}β_{2} naturally.

P2 Activation of α_{M}β_{2} Is Associated with Exposure of the 7E3 Epitope. To further investigate the mechanism of P2 stimulation of α_{M}β_{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 α_{IIb}β_{3} (51) but has subsequently been shown to interact with α_{M}β_{2} and α_{M}β_{3} (for a review, see reference 52). The epitope in α_{M}β_{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 α_{M}β_{2} on leukocytes, it does not react with our nonstimulated WT α_{M}β_{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 α_{M}β_{2} transfectants to Fg. To maximize the stimulatory effect of P2, a lower concentration of 10 μg/ml Fg was employed in the transwell migra-

![Figure 5](image5.png)

**Figure 5.** Migration of α_{M}β_{2} WT cells to Fg is inhibited by P1 but stimulated by P2. Migration of α_{M}β_{2} WT cells was assessed to various combinations of optimal concentrations of 50 μg/ml Fg, 6 μM P1 peptide, 6 μM P2 peptide, or 10 nM D_{100} fragment in the upper or lower transwells as indicated. Fg or peptides added to the upper well were pre-incubated 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 ± SD.

![Figure 6](image6.png)

**Figure 6.** Stimulation of α_{M}β_{2} WT cell migration to Fg. Migration of α_{M}β_{2} WT cells to 50 μg/ml Fg was assessed in the presence of various reagents after preincubation for 30 min. Reagents are Fg recognition peptides P1 (6 μM) or P2 (6 μM), 2 μg/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 ± SD.
tion assay. As shown in Fig. 8A, 40 μg/ml c7E3 had no inhibitory effect on αMβ2-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 αMβ2-transfected cells stimulated by β-glucan was also observed (90% inhibition; Fig. 8A).

To rule out possible recognition of αβ, as the basis of the observed effects of c7E3, a potent αβ-blocking mAb, 20 μg/ml LM609, was substituted for c7E3 and found to have no effect on P2-stimulated (or baseline, data not shown), αMβ2-mediated cell migration to Fg. The data in Fig. 8A 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 αMβ2, 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 αMβ2 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. 8B). As noted above, the αMβ2 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. 8B). This reactivity with c7E3 was enhanced substantially in the presence of P2.

Discussion

Leukocyte migration is the hallmark of inflammation in vivo, and αMβ2 and Fg have been shown to contribute to leukocyte migration in multiple systems (23, 24). This study has used αMβ2 transfectants and selected mutants to dissect the molecular requirements for αMβ2-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 αMβ2. This response is dependent on Fg concentration and occurs at low (1–50 μg/ml) Fg levels. (b) The αM I domain is necessary but not sufficient to support cell migration to Fg. In contrast to cell adhesion to Fg, efficient migration requires the β3 subunit. (c) The P1 and P2 peptides, as well as the D100 fragment, support cell migration. Thus, the same Fg derivatives that mediate αMβ2-dependent cell adhesion also support cell migration. (d) The P2 peptide stimulates αMβ2-mediated cell migration to Fg and the P1 peptide, in a manner similar to other αMβ2 activators, β-glucan, and PMA. In addition, the activation-dependent epitope of c7E3 is induced by P2, c7E3 inhibits P2, and β-glucan stimulated αMβ2-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...
α₃β₂ and Fg that are necessary for cell migration and define a novel mechanism whereby the P2 peptide of Fg stimulates α₃β₂-mediated chemotaxis.

More than a decade ago, Fg was identified as a ligand for α₃β₂ (14), and numerous subsequent studies have examined the structure function requirements for α₃β₂-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 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 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 α₃β₂ dependent (56, 57). Thus, the demonstration that α₃β₂ 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 α₃β₂ alone cells to mediate efficient migration to Fg. However, when the α₃M I domain was placed in the context of another β₂ integrin, α₃β₂, that does not mediate migration to Fg, chemotaxis of these L/M cells was observed. Thus, within the context of an intact heterodimer, the α₃ I domain is necessary and sufficient to confer migration to Fg. Furthermore, within the α₃ I domain, at least some of the sequences which are key for recognition of Fg and other α₃β₂ ligands also influence α₃β₂-mediated cell migration. Thus, the P147-152 α₃β₂ 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 α₃β₂, but not α₃β₂, adhesion to Fg is mediated principally via the 1 domain of α₃β₂, and sequences on the MIDAS face of the α₃ I domain are involved in the migratory and adhesive response of the receptor. However, the β₂ subunit is more influential in cell migration than in cell adhesion (21). The role of the β₂ 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 α₃β₃ and migrating on Fg (58).

Integrin α₃β₂-mediated migration was triggered not only by intact Fg but also by its D₁₀₀ fragment and by peptides duplicating its γ191–202 and γ373–395 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 α₃β₂-dependent cell migration. This difference in activity is consistent with the greater apparent affinity of P2 for α₃β₂ (21).

When P2 was added to the α₃β₂ transfectants, it enhanced cell migration. This increase appears to reflect an activation of α₃β₂ by the peptide. This interpretation is supported by the observation that the epitope for c7E3, which is expressed by activated α₃β₂ (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 α₃β₂ (59). Particularly relevant to the present observation, an α₃β₂ ligand peptide derived from ICAM-2 activates α₃β₂-mediated cell migration (60). The fact that P2, but not P1, induced such activation implies that, even though both peptides bind to the α₃ I domain, they must interact in a fundamentally different way to initiate a differential response in the receptor. Activation of α₃β₂ 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 α₃ I domain (61), or outside of α₃β₂ altogether (PMA stimulation). β-Glucan is a ligand for the lectin-binding domain of α₃β₂ (61), and its activation of the receptor has been studied extensively (62). β-Glucan activation of α₃β₂ also is known to result in expression of the α₃ I domain activation neotope (61) recognized by mAb CBRM1/5, and this mAb eliminates α₃β₂ adhesion to Fg (63). Nevertheless, mAbs CBRM1/5 and c7E3 do not compete with each other for binding to activated α₃β₂ (54). Our data support a model in which β-glucan and P2 stimulation results in an activated α₃β₂ conformation, which expresses the c7E3 epitope. The greater inhibition by c7E3 of β-glucan activation of α₃β₂ also is known to result in expression of the α₃ I domain activation neotope (61) recognized by mAb CBRM1/5, and this mAb eliminates α₃β₂ adhesion to Fg (63). Nevertheless, mAbs CBRM1/5 and c7E3 do not compete with each other for binding to activated α₃β₂ (54). Our data support a model in which β-glucan and P2 stimulation results in an activated α₃β₂ 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 α₃β₂ activation may be different. Also, P2 was shown to enhance neutrophil migration to Fg, emphasizing that modulation of α₃β₂ 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 α₃β₂ 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 α₃ I domain and is influenced by other domains of α₃β₂. While our earlier studies identified a negative role for the β₂ subunit in modulating adhesion (37), this study identifies a positive role for the β₂ subunit in influencing cell migration. Thus, the structural requirements for α₃β₂-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 αMβ2 and stimulate cell migration to Fg.

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