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Chemotaxis of Chinese hamster ovary cells expressing the human neutrophil formyl peptide receptor: role of signal transduction molecules and $\alpha_5\beta_1$ integrin

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SUMMARY

Activation of the *N*-formyl peptide receptor (FPR) of human neutrophils by ligands such as *N*-formyl-methionine-leucine-phenylalanine (*f*MLP) induces mobilization of intracellular calcium, cell adhesion, chemotaxis, superoxide production and degranulation. Chinese hamster ovary (CHO) cells are normally devoid of FPR and unresponsive to *f*MLP, but when stably transfected with a human FPR cDNA, exhibited some of these same responses. Specifically, stimulation with *f*MLP resulted in release of intracellular calcium and chemotactic migration toward a gradient of *f*MLP. As in neutrophils, both processes were inhibited through receptor desensitization by prior exposure to a higher or equal concentration of ligand or by treatment with pertussis toxin. Soluble and membrane-bound fibronectin greatly increased *f*MLP-induced chemotaxis of CHO cells expressing FPR, but not of wild-type CHO cells, suggesting a role for FPR in activation of integrin function. Evidence for this hypothesis was obtained by demonstrating that CHO cells expressing FPR rapidly increased their adhesion to a fibronectin-coated surface after stimulation with *f*MLP. Both chemotaxis and adhesion were largely inhibited by RGDS peptide and a function-blocking antibody against α_5 integrin. FPR-mediated chemotaxis of the CHO transfectants was partly inhibited by a tyrosine kinase inhibitor, herbimycin A, and blocked by a phosphoinositide 3-kinase inhibitor, wortmannin. These data suggest that stimulation of CHO FPR transfectants with a gradient of *f*MLP results in phosphoinositide 3-kinase-dependent chemotactic migration, which is enhanced by binding of activated $\alpha_5\beta_1$ to fibronectin. This non-myeloid, non-lymphoid fibroblastic cell line will thus serve as a useful model to investigate additional requirements of signal transduction molecules, adhesion molecules and cytoskeletal elements in FPR-mediated chemotaxis.

Key words: Migration, Chemoattractant receptor, Cell adhesion, Phosphoinositide 3-kinase

INTRODUCTION

Directed cell migration induced by chemoattractants and chemokines is an important process in a variety of biological functions, such as inflammation, embryogenesis and wound healing. In bacterially induced inflammation, the most potent chemoattractants are formylated peptides, such as *N*-formyl-methionyl-leucyl-phenylalanine (*f*MLP), which are released from living and dead bacteria (Schiffmann et al., 1975). Binding of *f*MLP to its receptor on neutrophils, the formyl peptide receptor (FPR), transmits signals through pertussis toxin-sensitive G proteins, triggering an intracellular signal cascade that induces chemotaxis, calcium flux, superoxide production and release of degradative compounds. Chemotaxis involves a complex interplay between different chemotactic receptors, signal transduction molecules, cell adhesion molecules, and the force-generating cytoskeleton. One of the initial steps in the migration of neutrophils from the circulation to sources of formylated peptides is the firm attachment of the neutrophils to endothelial cells. This is accomplished through the binding of neutrophil surface integrins and endothelial cell adhesion molecules. Stimulation of neutrophils with *f*MLP leads to a three- to fivefold increase in the levels of surface β_2 integrin (Derian et al., 1995; Kishimoto et al., 1989) and an activation of preexisting cell surface β_2 integrins, possibly through a conformational change (Buyon et al., 1988; Ginsberg et al., 1992; O'Toole et al., 1994). This increased activity enhances the neutrophil adherence to the endothelium. In concert with the activation of β_2 integrin, a rapid and transient increase in F-actin can be observed upon stimulation with *f*MLP (Wallace et al., 1984). Interestingly, a more sustained actin polymerization can be detected when the β_2 integrins are crosslinked with antibodies, suggesting that the engagement of β_2 integrins results in their association with the cytoskeleton (Löfgren et al., 1993). A similar observation was made in fibroblasts, where β_1 integrins, which were labeled without

crosslinking, diffused freely on the cell surface, whereas crosslinking with fibronectin resulted in a directed movement of the β_1 integrins, apparently along the actin filaments (Felsenfeld et al., 1996).

The sequence of events and the various molecules controlling chemoattractant-mediated cell adhesion and chemotaxis is still largely unknown. Due to the complex nature of neutrophil chemotaxis, where several different chemoattractant receptors affect each other's activity, we decided to generate a simplified cell model where FPR serves as the only chemoattractant receptor. We transfected Chinese hamster ovary (CHO) cells with an expression vector containing the human FPR cDNA and found that the cells migrated in a pertussis toxin-sensitive fashion toward *f*MLP. Addition of fibronectin to the cells greatly enhanced chemotaxis toward *f*MLP, whereas addition of RGDS peptide or a function-blocking antibody against α_5 integrin greatly reduced the chemotaxis, suggesting that the engagement of $\alpha_5\beta_1$ integrin is important for the cell migration. Cell surface expression levels of $\alpha_5\beta_1$ integrin were not altered upon *f*MLP treatment, but the increased number of cells that adhered to fibronectin suggested that the preexisting integrins were activated. Wortmannin inhibited cell adhesion and abolished chemotaxis, suggesting a central role for phosphoinositide 3-kinase (PI3K) in the regulation of directed cell migration.

MATERIALS AND METHODS

Materials, cells and cell culture

Antibody against hamster α_5 integrin subunit (Brown and Juliano, 1985) was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, IA. Laminin, a gift from Dr Jean Starkey and Deb Berglund, Montana State University, Bozeman, MT, was purified according to a published procedure (Timpl et al., 1979). Fibronectin, vitronectin, collagen, RGDS-peptide, pertussis toxin, wortmannin, bis indolyl-maleimide and herbimycin A were from Sigma (St Louis, MO).

Human neutrophils were isolated as described previously (Quinn et al., 1989). CHO cells stably expressing wild-type FPR were maintained as described previously (Miettinen et al., 1997). Fourteen to eighteen hours before each experiment, expression was increased by adding 6 mM sodium butyrate to the medium of the CHO transfectants (Gorman et al., 1983).

Calcium assay

Transfected CHO cells were detached from culture plates with a cell scraper after incubation with 1 mM Na-EDTA, pH 8.0, in Ca^{2+}/Mg^{2+}-free PBS. Cells were harvested by centrifugation, resuspended in PBS + Ca^{2+}/Mg^{2+} (PBS^{2+}) containing 5% FBS, and incubated with 2.5 μM Fura-2AM (Molecular Probes, Eugene, OR) for 45 minutes at 37°C. Cells were centrifuged and resuspended in the above buffer at room temperature, where they were left until the assay. Each measurement contained ~5×10^6 CHO cells in a volume of 1 ml. Continuous fluorescent measurements of calcium-bound and free Fura-2 were made at 37°C with a double excitation monochromator fluorescence spectrofluorometer (Photon Technologies International, Mammoth Junction, NJ) with excitation at 340 nm and 380 nm and emission at 510 nm.

Chemotaxis assay

CHO cells were removed from tissue culture plates by trypsinization and allowed to equilibrate in complete medium for 1 hour at 37°C. Cells were centrifuged and resuspended in serum-free medium. 3×10^5 cells in 300 μl were added to 6.5 mm diameter Transwell inserts of 8 μm pore size (Corning Costar, Cambridge, MA). 500 μl serum-free medium with or without *f*MLP and/or fibronectin (at concentrations as indicated in the table and figure legends) were placed in the wells of a 24-well culture plate. The Transwell inserts containing the cells were moved into the wells, and chemotaxis was allowed to proceed for 4 hours at 37°C. The optimal time for chemotaxis was previously determined by allowing the cells to migrate 0.5-5 hours toward 1 nM *f*MLP (data not shown). Cells on the upper face of the insert were removed with cotton swabs; the cells that adhered to the underside of the filter were fixed with 2.5% paraformaldehyde (PFA) in PBS, stained with Hematoxylin Gill's stain, and quantified. The cells could not be reliably counted by eye using a light microscope due to cell clumping, so they were quantified by analysis of the surface area of the migrated cells using an image analyzer (M4 True Color Image Analysis System, Imaging Research, St Catherines, Ontario). The CHO cells did not drop from the filters; no CHO cells could be detected in the lower well, other than those on the filter, even after culturing the 24-well plates several days.

Some filters were coated overnight at 4°C with 10 μg of fibronectin, laminin or collagen, or 5 μg of vitronectin prior to the chemotaxis experiment. Excess liquid was removed and the filters were allowed to dry in the laminar flow hood before use.

Neutrophils in Hanks' balanced salt solution (HBBS) (10^6 cells in 200 μl) were added to 6.5 mm diameter Transwell inserts of 5 μm pore size and allowed to migrate for 2 hours at 37°C. The neutrophils that had migrated through the holes and detached were quantified using a myeloperoxidase assay, as previously described (Parkos et al., 1991).

When the effect of various inhibitors of signal transduction was examined, the CHO cells or neutrophils were preincubated for 10 minutes at 37°C with the desired inhibitor prior to the chemotaxis assay. Effector and inhibitor molecules were used at the following concentrations: 20 μg/ml fibronectin, 2.5 μg/ml anti-α_5 integrin antibody, 500 μg/ml Arg-Gly-Asp-Ser (RGDS), 500 ng/ml pertussis toxin, 100 nM wortmannin, 50 μM 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7), 5 μM bis indolyl-maleimide, and 10 μg/ml herbimycin A.

Cell adhesion assay

24-well tissue culture plates were coated overnight at 4°C with 250 μl fibronectin at 20 μg/ml in PBS. The liquid was removed, the wells were allowed to dry, and the culture plate was placed in a 37°C waterbath. The CHO cells expressing FPR (5×10^5 cells in 500 μl of serum-free medium) were incubated for 10 minutes at 37°C in the absence of inhibitor or in the presence of either 100 nM wortmannin, 250 μg/ml RGDS-peptide, or 2.5 μg/ml function-blocking antibody against α_5 integrin (a concentration that has previously been shown to inhibit CHO cell adhesion to fibronectin; Brown and Juliano, 1985), followed by a 2 minute incubation with or without 10 nM *f*MLP. Cells were plated on fibronectin-coated wells and allowed to adhere for 8 minutes. The incubation time on fibronectin-coated wells had been previously determined based on the maximal % difference in cell adherence ± *f*MLP over a time period of 2-20 minutes. These studies showed the largest difference in adherence ± *f*MLP at about 4-8 minutes, whereas the difference was minor at later time points 15-20 minutes (data not shown). Cells were fixed and stained with 10% ethanol containing 0.2% Crystal Violet. The wells were washed extensively with PBS and the stain was extracted from the cells with 300 μl 0.05 M Na_2HPO_4 in 50% ethanol. 100 μl of the stain was quantified by reading the absorbance at 540 nm.

RESULTS

Chinese hamster ovary cells express a functional formyl peptide receptor

We previously generated a CHO cell line stably expressing ~10^6 FPR/cell with a dissociation constant (K_d) of ~6 nM (Miettinen et al., 1997). Furthermore, we have shown that binding of ligand

to FPR approximately doubled the binding of [^{35}S]GTPγS to CHO membranes, suggesting that the receptor couples to G protein (Miettinen et al., 1997). To examine whether ligand binding to FPR induces signal transduction in CHO cells, we examined the effect of *f*MLP addition on the release of intracellular calcium. Cells were loaded with the fluorescent calcium chelator, Fura 2-AM, and the calcium release was measured after addition of various concentrations of *f*MLP. As a reference value, calcium release was also examined after addition of 10 μM ATP. ATP binds the purinergic receptor and induces release of intracellular calcium in a G protein-independent fashion. It thus serves as a control for adequate loading of Fura 2-AM and for the responsiveness of the cells. The amount of calcium released by *f*MLP was calculated as the proportion of that released subsequently by ATP. As seen in Fig. 1, maximal calcium release was obtained with 10-100 nM *f*MLP; a small response could be detected at concentrations as low as 0.1 nM *f*MLP (Fig. 1A). Addition of 1 nM *f*MLP did not desensitize the cells to a subsequent exposure of the cells to 10 nM *f*MLP (data not shown), whereas 10 nM *f*MLP completely desensitized the cells to subsequent exposure of the cells to 10 nM *f*MLP (Fig. 1E) and resulted in only a minor response to subsequent exposure to 100 nM *f*MLP (Fig. 1F). These results are comparable to those seen with human neutrophils, suggesting that FPR in transfected CHO cells may be functionally similar to endogenous FPR in neutrophils.

CHO FPR cells migrate toward a gradient of formylated peptide

We next determined whether FPR was capable of mediating chemotactic migration of the CHO transfectants toward formylated peptide. Cells were seeded onto polycarbonate filter inserts and allowed to migrate in the presence of 0-10 nM *f*MLP through 8 μm holes into a lower well containing 0-10 nM *f*MLP in serum-free medium. As shown in Table 1, the migration of CHO cells expressing FPR is directional and maximal at 1 nM. This value is in good agreement with FPR-mediated chemotaxis of neutrophils, which are reported to exhibit maximal chemotaxis through semi-permeable filters at about 1 nM (for review see Snyderman and Pike, 1984). Limited chemokinesis was also observed when cells were incubated with higher concentrations of *f*MLP (5 and 10 nM) in the upper well (Table 1).

FPR-mediated chemotaxis of CHO cells is enhanced in the presence of fibronectin

Addition of *f*MLP to neutrophils has been shown to result in upregulation and activation of β_2 integrins, resulting in enhanced adhesion and chemotaxis (Derian et al., 1995; Kishimoto et al., 1989; Buyon et al., 1988). Incubation of neutrophils with soluble fibronectin has also been shown to enhance the chemotaxis toward *f*MLP, suggesting a possible role for the $\alpha_5\beta_1$ integrin (Everitt et al., 1996). We examined

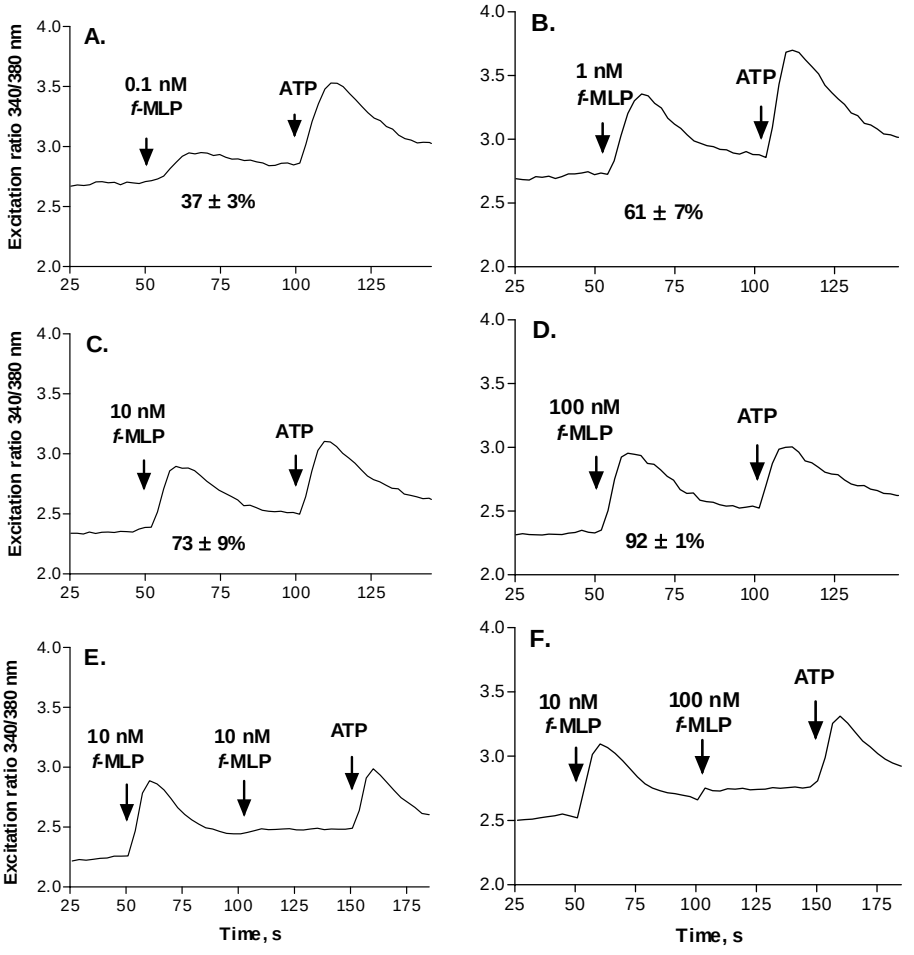

Fig. 1. CHO cells expressing FPR mobilize intracellular calcium in response to addition of *f*MLP. CHO transfectants were loaded with Fura 2-AM and the calcium release was measured in a dual wavelength excitation spectrofluorometer after addition of *f*MLP. ATP, which induces release of intracellular calcium in a G protein-independent manner, was added as a positive control. The numbers below the *f*MLP-induced calcium peak indicate the percentage of *f*MLP-mobilized calcium compared to ATP. Numbers show the results from at least three separate experiments (± s.e.m.); the curves represent single experiments.

Table 1. Checkerboard analysis of *f*MLP-stimulated migration of CHO cells expressing FPR

*f*MLP (nM)	0	0.5	1.0	5.0	10.0
0	**100±42**	105±45	127±64	186±72	246±82
0.5	1415±210	**494±149**	281±95	273±112	237±85
1.0	1906±87	614±86	**289±134**	278±94	257±97
5.0	1149±280	939±188	673±137	**145±37**	235±141
10.0	342±90	221±102	388±104	125±42	**222±45**

Concentrations of *f*MLP (nM) added to the upper wells (horizontal line) or lower wells (vertical line) are shown. The relative cell surface area of the migrated cells is shown as mean of six randomly chosen fields ± s.e.m.

the effect of fibronectin in chemotaxis of the CHO transfectants. As seen in Fig. 2, fibronectin alone at 20 μg/ml resulted in significant migration as shown before with fibroblastic and melanoma cells (Aznavoorian et al., 1990; McCarthy and Furcht, 1996). When both fibronectin and *f*MLP were present, the migration of the CHO transfectants was greatly enhanced whether the fibronectin was present in the upper chamber with the cells or in the lower chamber with *f*MLP. To examine whether adherent fibronectin would also enhance chemotaxis, the polycarbonate filters were coated either on the upper side or the lower side with fibronectin. As seen in Fig. 3A, the fibronectin-coated filters, together with *f*MLP, enhanced chemotaxis to similar extent as when fibronectin was added to the medium, suggesting that over the time course of the 4 hour chemotaxis some of the fibronectin is likely to coat the filters.

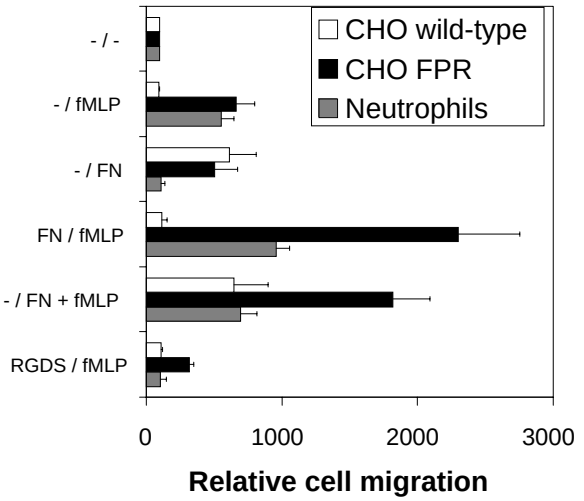

Fig. 2. The chemotaxis of CHO FPR cells is enhanced in the presence of fibronectin and inhibited in the presence of RGDS peptide. Wild-type CHO cells, CHO FPR cells and human neutrophils were allowed to migrate without chemotactic stimulus or with either 1 nM *f*MLP, 20 μg/ml fibronectin (FN) or both in the lower chamber. Some cells were pretreated for 10 minutes at 37°C with 20 μg/ml fibronectin or 500 μg/ml RGDS peptide prior to the chemotaxis assay. CHO cells were quantified from the underside of the polycarbonate filter after fixing and staining; the neutrophils were quantified from the medium in the lower well using a myeloperoxidase assay. Numbers are relative, such that migration in the absence of chemoattractant = 100. The bars indicate the mean from five experiments (CHO wild-type and CHO FPR) or four experiments (neutrophils) ± s.e.m.

We also examined the migration of human neutrophils under identical conditions (Fig. 2). The results indicated that neutrophils do not exhibit increased migration toward fibronectin; however, fibronectin added to the upper chamber containing the neutrophils significantly increased the migration toward *f*MLP, as previously shown (Everitt et al., 1996). Although neutrophils have been reported to express β_1 integrins, they are not thought to play a significant role in neutrophil migration relative to β_2 integrins. However, recent evidence suggests that β_1 integrins may be important in neutrophil migration after extravasation (Gao and Issekutz, 1997; Shang and Issekutz, 1997; see also Discussion). In our in vitro system, fibronectin enhanced *f*MLP-induced neutrophil chemotaxis, suggesting a role for a fibronectin-binding integrin, possibly $\alpha_5\beta_1$ integrin (Fig. 2). The wild-type CHO cells did not exhibit any chemotaxis toward *f*MLP, but, as expected, migrated toward fibronectin in a similar manner as the transfected cells (Fig. 2).

To further establish that $\alpha_5\beta_1$ integrin rather than some other fibronectin-binding integrin is critical for chemotaxis of the CHO transfectants, we examined the migration in the presence of a function-blocking antibody against hamster α_5 integrin. This antibody reduced the migration by about 50%, suggesting that $\alpha_5\beta_1$ integrin is the major cell adhesion molecule promoting migration of CHO FPR transfectants toward *f*MLP in the absence and presence of fibronectin (Fig. 3B). Incubation with higher concentrations of the anti-α_5 integrin antibody did not further decrease the migration, suggesting that the antibody concentration was saturating and that other cell adhesion molecules may be responsible for some of the migration (Fig. 6A).

Laminin, vitronectin and collagen do not significantly enhance FPR-mediated chemotaxis of CHO cells

To examine the possible role of other integrins and extracellular matrix molecules in chemotaxis of the CHO FPR transfectants, we measured the cell migration toward *f*MLP after coating the polycarbonate filters with laminin, vitronectin or collagen. As seen in Fig. 4, only vitronectin appeared to have a minor enhancing effect on chemotaxis. It is thus possible that $\alpha_V\beta_3$ which can serve as receptor for both fibronectin and vitronectin, plays a minor role in chemotaxis of the CHO transfectants. Without access to function-blocking antibodies to these integrin subunits, we were unable to further confirm this possibility.

*f*MLP-induced chemotaxis is abolished by the PI3K inhibitor wortmannin and reduced by pertussis toxin, herbimycin A and bis indolyl-maleimide

We tested the effect of various inhibitors of signal transduction molecules on the chemotaxis of CHO transfectants. The first step in the neutrophil signaling cascade after exposure to *f*MLP is the activation of G protein. The G protein that interacts with FPR in neutrophils is most likely G_i (Okajima et al., 1985), but G_o may also interact (Goetzl et al., 1994). These G proteins are pertussis toxin sensitive; i.e. they are inactivated upon ADP-ribosylation. In our chemotaxis assay, we found that incubation of the CHO transfectants with pertussis toxin for 10 minutes

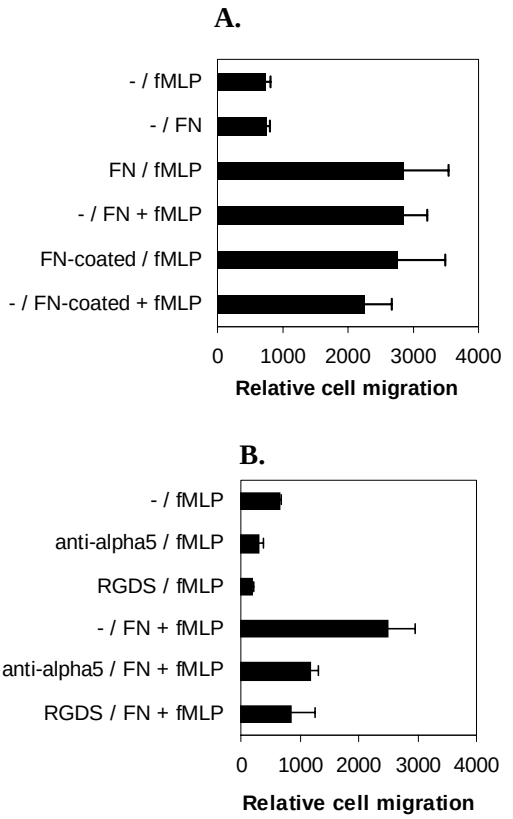

Fig. 3. (A) Fibronectin-coated polycarbonate filters enhance chemotaxis of CHO cells expressing FPR. Polycarbonate filters were coated with 10 μg fibronectin or left uncoated. Chemotaxis of CHO transfectants in the absence or presence of fibronectin toward 1 nM *f*MLP and/or 20 μg/ml of fibronectin was quantified as above. (B) A function-blocking antibody against α_5 integrin inhibits chemotaxis toward *f*MLP. Cells were preincubated for 10 minutes with no additions (-), 2.5 μg/ml of a function-blocking antibody against α_5 integrin (anti-alpha5), or with 500 μg/ml RGDS peptide and allowed to migrate for 4 hours toward 1 nM *f*MLP or toward 1 nM *f*MLP + 20 μg/ml fibronectin (FN). Filters were quantified as in Fig. 2. Results are from three separate experiments ± s.e.m.

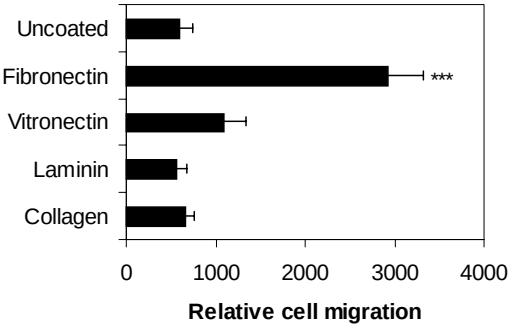

Fig. 4. Vitronectin, laminin, and collagen do not produce statistically significant increases in chemotaxis of CHO FPR-transfectants toward *f*MLP. Cells were allowed to migrate toward 1 nM *f*MLP through semipermeable polycarbonate filters that were either uncoated or coated with fibronectin, vitronectin, laminin or collagen. Filters were quantified as above. Data are from two experiments with triplicate filters ± s.e.m. ***P<0.001.

at 37°C prior to exposure to *f*MLP reduced the rate of migration by about 64% (Fig. 5). A small inhibition was also seen in random migration of untransfected CHO cells, suggesting that other G protein-regulated molecules may play a role in random migration. Similar effects were obtained with the tyrosine kinase inhibitor, herbimycin A, and the protein kinase C inhibitor, bis-indolyl maleimide, whereas the protein kinase C inhibitor, H7, appeared to increase migration (Fig. 5). Only wortmannin, a fungal metabolite that inhibits PI3K, completely blocked cell migration (Fig. 5), exhibiting an IC_{50} of about 4 nM (Fig. 6C).

Stimulation of CHO cells expressing formyl peptide receptor with fMLP increases the cell adhesion to fibronectin

To further examine the basis for the fibronectin-enhanced chemotaxis, we determined whether stimulation of CHO transfectants with *f*MLP upregulates and/or activates the fibronectin-binding integrins. Cells were incubated with or without 10 nM *f*MLP for 2 minutes at 37°C and then allowed to adhere to fibronectin-coated wells at 37°C for 8 minutes before fixing and staining. As seen in Fig. 7, cell adhesion was about twice as efficient after stimulation with *f*MLP compared to cells that were not stimulated. By measuring the average diameter of the adhered cells by image analysis it was also determined that *f*MLP-treatment lead to more extensive cell spreading on fibronectin. However, due to high variability among individual cells, the difference in average cell diameter ± *f*MLP was not statistically significant (data not shown). To confirm that the increased adhesion of the CHO transfectants was integrin-dependent, we preincubated the cells with 250 μg/ml RGDS-peptide or 2.5 μg/ml of the α_5-integrin function blocking antibody (Brown and Juliano, 1985) before plating onto fibronectin-coated wells. These pretreatments resulted in reduced adhesion both of *f*MLP-stimulated and unstimulated cells (Fig. 7).

Increased adhesion after *f*MLP treatment could be due to upregulation of β_1 integrin and/or activation of the receptor. To distinguish between these two possibilities, we determined by flow cytometry whether the α_5 and β_1 integrin subunits were upregulated on the cell surface after *f*MLP addition. Unlike neutrophils, where β_2-integrin has been found to be upregulated upon stimulation with *f*MLP, the surface expression of α_5 and β_1-integrins was not found to change in CHO FPR-transfectants after addition of *f*MLP (data not shown). It thus appears that the increased binding of *f*MLP-stimulated cells is due to enhanced adhesion of the integrins to fibronectin.

Finally we also examined the requirement of PI3K in FPR-induced cell adhesion. Pretreatment of CHO transfectants with wortmannin prior to activation with *f*MLP reduced adhesion to fibronectin, emphasizing the close connection between regulated cell adhesion and migration (Fig. 7).

DISCUSSION

In this study, we have shown that expression of FPR in CHO cells, a non-leukocytic cell line, allows the cells to migrate in a chemotactic fashion toward a gradient of *f*MLP (Fig. 2, Table

1). Chemotaxis is increased in the presence of fibronectin and decreased in the presence of RGDS peptide or a function-blocking antibody against α_5 integrin, suggesting that $\alpha_5\beta_1$ integrin enhances the cell migration (Figs 2 and 3). In neutrophils, the β_1 integrin is thought to be of secondary importance to β_2 integrin in migration. However, recent evidence suggests that neutrophil β_1 integrins enhance transendothelial migration (Roussel and Gingras, 1997), migration through connective tissue fibroblast barriers (Gao and Issekutz, 1997) and migration across fibronectin-coated microporous filters (Everitt et al., 1996). Furthermore, a function-blocking antibody against the α_5 subunit of human integrin was shown to reduce the migration of neutrophils across synovial and lung fibroblast barriers, suggesting that $\alpha_5\beta_1$ integrin, along with other β_1 integrins, play a role in neutrophil migration in the tissue (Shang and Issekutz, 1997; Gao et al., 1995). Similarly, our results showed that fibronectin increased the number of neutrophils that migrated toward *f*MLP. This effect may be due to fibronectin cross-linking of integrin leading to a regulated actin polymerization, as noted in the Introduction

We further examined whether cells stimulated with *f*MLP show a difference in their adhesion properties by analyzing the adhesion of CHO transfectants to fibronectin-coated plates before and after stimulation with *f*MLP. The incubation with chemoattractant prior to plating resulted in increased cell adhesion, which could be detected as early as 4 minutes after plating (not shown). Similar although more pronounced results were obtained by Honda et al. (1994), who showed that mouse pre-B cells stably expressing FPR exhibited, after stimulation with *f*MLP, increased adhesion to mouse VCAM-1 through the $\alpha_4\beta_1$ integrin. In agreement with our results, stimulation with *f*MLP did not change the surface expression of α_4, suggesting that increased adhesion was due to augmented binding affinity to fibronectin. This type of integrin activation has been referred to as 'inside-out signal transduction' and shown to be mediated by the cytoplasmic domains of integrin (O'Toole et al., 1994). Further analysis showed that the adhesion of CHO transfectants to fibronectin was inhibited 50-80% by pertussis toxin, wortmannin and RGDS, irrespective of *f*MLP treatment, suggesting that adhesion is modulated by G proteins, PI3K and integrins (Fig. 7).

Several studies have previously examined the effect of various inhibitors of signal transduction molecules on chemotaxis of various cell lines. Wortmannin inhibited chemotaxis of platelet-derived growth factor receptor-expressing endothelial cells with an IC_{50} of about 15-20 nM (Hooshmand-Rad et al., 1997), whereas interleukin-8-induced

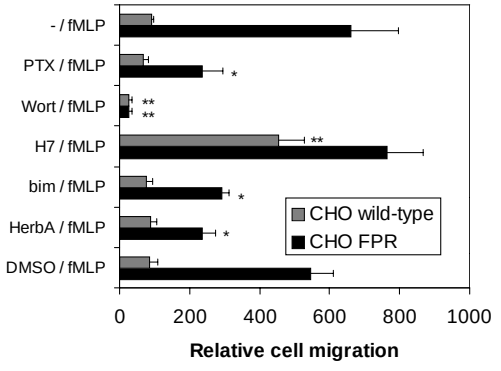

Fig. 5. Wortmannin and herbimycin A inhibit migration of wild-type CHO cells and CHO cells expressing FPR. Cells were preincubated for 10 minutes at 37°C with medium alone, 500 ng/ml pertussis toxin (PTX), 100 nM wortmannin (Wort), 50 μM H7, 5 μM bis indolyl-maleimide (bim), 10 μg/ml herbimycinA (HerbA), or 1% DMSO (carrier for herbimycinA). Cells were allowed to migrate toward 1 nM *f*MLP in the lower chamber for 4 hours prior to quantification. The bars represent the mean from five different experiments ± s.e.m. **P*<0.05; ***P*<0.01.

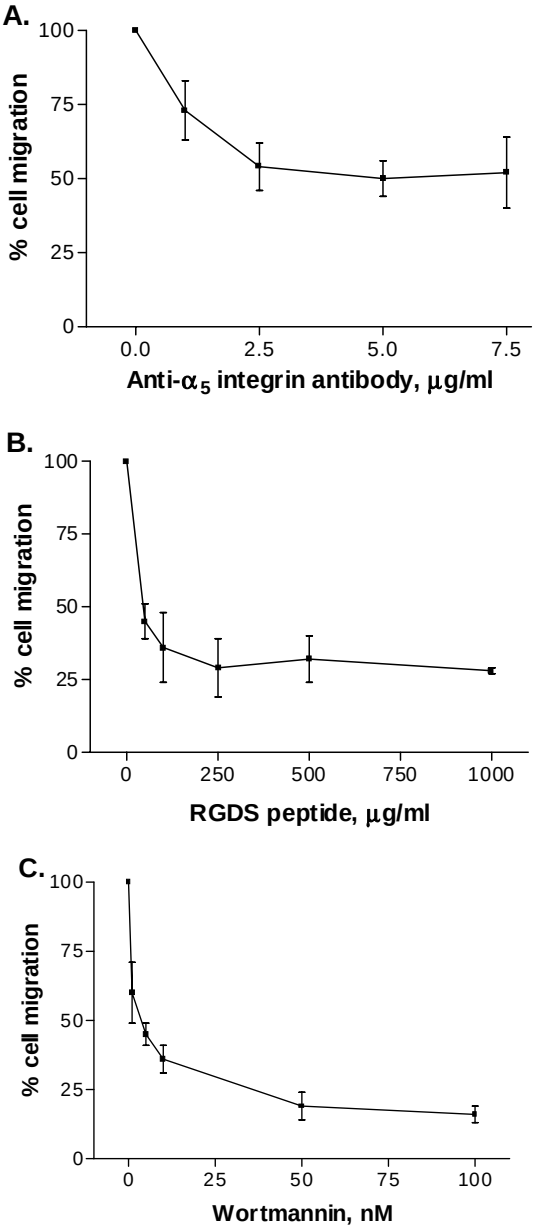

Fig. 6. Function-blocking antibody against α_5 integrin, RGDS peptide and wortmannin inhibit chemotaxis of CHO cells expressing FPR toward 1 nM *f*MLP. Cells were incubated for 10 minutes at 37°C with (A) 0-7.5 μg/ml anti-α_5 integrin antibody, (B) 0-1 mg/ml RGDS peptide or (C) 0-100 nM wortmannin prior to chemotaxis toward *f*MLP. The data represent the mean ± s.e.m. from a single experiment with triplicate filters (A) or of three separate experiments with single filters (B and C).

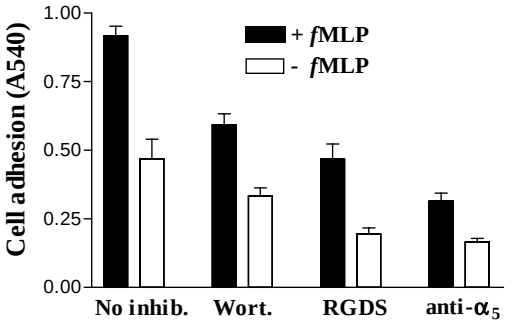

Fig. 7. Adhesion of CHO cells expressing FPR to a fibronectin-coated surface is enhanced by activation with *f*MLP and inhibited by wortmannin, RGDS peptide and the function-blocking antibody against α_5 integrin. Cells were incubated 10 minutes with 100 nM wortmannin (Wort.), 250 μg/ml RGDS peptide or 2.5 μg/ml function-blocking antibody against α_5 integrin (anti-α_5). After a 2 minute incubation with or without *f*MLP, the cells were allowed to adhere to fibronectin-coated wells for 8 minutes, after which they were quantified as described in Materials and Methods. The bars represent the means from three different experiments ± s.e.m.

neutrophil chemotaxis was shown to be inhibited with an IC_{50} of 26 nM (Knall et al., 1997). In one study, 100 nM wortmannin was not found to inhibit *f*MLP-induced chemotaxis of neutrophils (Thelen et al., 1995), whereas two other studies showed a 30% and 100% inhibition, respectively (Harakawa et al., 1997; Niggli and Keller, 1997). Wortmannin inhibits PI3K activity in fibroblasts with an IC_{50} of about 5 nM (Wymann and Arcaro, 1994) and myosin light chain kinase activity with an IC_{50} of about 160 nM in vitro (Nakanishi et al., 1992). We found that wortmannin inhibited *f*MLP-induced chemotaxis of transfected CHO cells with an IC_{50} of about 4 nM (Fig. 6C), which is in good agreement with the previously reported half-maximal inhibitory concentration for PI3K in fibroblasts (Wymann and Arcaro, 1994).

The tyrosine kinase inhibitor, herbimycin A, was previously shown to inhibit chemotaxis of neutrophils, whereas the protein kinase C inhibitor, H7, did not (Yasui et al., 1994; Harakawa et al., 1997). Similar results were obtained in our studies; herbimycin A inhibited *f*MLP-mediated chemotaxis of CHO transfectants whereas H7 had no effect. Another PKC inhibitor, bis indolyl-maleimide, decreased chemotaxis by about 56% at 5 μM, suggesting a difference in specificity between these two inhibitors. Harakawa et al. (1997) tested the effect of three different PKC inhibitors, H7, calphostin and hypericin, and found that none of them inhibited *f*MLP-induced random migration or chemotaxis of neutrophils. Together, these results suggest that protein kinase C may not be required for chemotaxis induced by *f*MLP.

FPR has been previously expressed for adhesion studies in mouse pre-B cells (L1-2) (Honda et al., 1994; Laudanna et al., 1996; see above), actin polymerization studies in RBL-2H3 cells (Hall et al., 1997), and for chemotaxis studies in undifferentiated U937 cells (Kew et al., 1997; Hsu et al., 1997). U937 cells transfected with FPR showed pertussis toxin-sensitive migration toward *f*MLP that did not require phosphorylation of FPR, but was inhibited by prior exposure to a high concentration of ligand (Kew et al., 1997; Hsu et al., 1997).

In summary, fibroblastic CHO cells expressing human myeloid FPR exhibited directed migration toward *f*MLP in a fashion similar to that of neutrophils and FPR-transfected leukocytic lines. Cell adhesion to fibronectin was increased after activation with *f*MLP, and chemotaxis was enhanced by fibronectin, suggesting an important link between FPR, actin and integrins in the regulation of cell adhesion and migration toward a gradient of chemoattractant. The CHO transfectants thus serve as a useful model for examining the various adhesion and signal transduction molecules that are involved in *f*MLP-induced chemotaxis. Since CHO cells lack known chemoattractant receptors, the activities of FPR can be studied in isolation, without potential interference (such as desensitization) by other molecules of this class. In addition, the cross-talk between two chemoattractant receptors may be investigated by simultaneous expression.

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