A single amino acid substitution (N297A) in the conserved NPXXY sequence of the human *N*-formyl peptide receptor results in inhibition of desensitization and endocytosis, and a dose-dependent shift in p42/44 mitogen-activated protein kinase activation and chemotaxis

Jeannie M. GRIPENTROG, Algirdas J. JESAITIS and Heini M. MIETTINEN¹

Department of Microbiology, Montana State University, 109 Lewis Hall, Bozeman, MT 59717-3520, U.S.A.

The formyl peptide receptor (FPR) is a G-protein-coupled receptor (GPCR) that mediates chemotaxis and stimulates the mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase pathway. We have examined the functional effects of substitutions of a conserved aspartic acid residue in the second transmembrane domain (D71A) and of residues in the conserved NPXXY motif in the seventh transmembrane domain (N297A and Y301A). These mutated receptors, expressed in Chinese hamster ovary (CHO) cells, bind ligand with affinities similar to wild-type FPR, but the D71A mutant is uncoupled from G-protein [Miettinen, Mills, Gripenroeg, Dratz, Granger and Jesaitis (1997) *J. Immunol.* **159**, 4045–4054]. In the present study, we show that both the D71A and N297A mutations resulted in defective endocytosis. The N297A substitution also prevented desensitization, as determined by intracellular calcium mobilization by sequential stimulation with ligand. In chemotaxis

assays, the N297A mutation resulted in cell migration towards gradients of up to 100 nM *N*-formyl-methionyl-leucyl-phenylalanine (fMLF), whereas cells expressing the wild-type FPR and the Y301A mutant were no longer chemotactically responsive at 10–100 nM fMLF. Maximal activation of p42/44 MAPK occurred in CHO cells expressing wild-type FPR at 10 nM–100 nM fMLF, whereas cells expressing the N297A mutant showed a dose-dependent increase in the amount of phosphorylated p42/44 MAPK up to 1–10 μ M fMLF. Since the MAPK kinase inhibitor PD98059 blocked fMLF-induced chemotaxis, our results suggest that the dose-dependent increase in p42/44 MAPK activation may correlate with the increased chemotactic migration of N297A transfectants at 10 nM–100 nM fMLF.

Key words: G-protein-coupled receptor, MAPK, migration, signal transduction.

INTRODUCTION

The human formyl peptide receptor (FPR) is expressed in neutrophils and monocytes, and mediates various host defence functions, such as chemotaxis towards the source of the chemo-attractant and an oxidative burst in the close proximity of the bacterium [1,2]. The FPR binds *N*-formyl peptides, such as *N*-formyl-methionyl-leucyl-phenylalanine (fMLF), which is the prototype ligand isolated from *Escherichia coli* [3]. Signal transduction by the FPR and other G-protein-coupled receptors (GPCRs) is attenuated within seconds of exposure to ligand, a process termed homologous desensitization. The termination of the signal is initiated by phosphorylation of the ligand-bound GPCR by a GPCR kinase [4]. The phosphorylation is thought to promote the direct or indirect interaction of various cytosolic molecules that are required for receptor endocytosis. Phosphorylation-deficient cytoplasmic deletion mutants or site-specific mutants of various GPCRs do not become desensitized and remain on the cell surface. It was previously thought that endocytosis of GPCRs is required for activation of the mitogen-activated protein kinase (MAPK) cascade [5]. However, recent studies using endocytosis-defective GPCR mutants or dominant-

negative mutants of β -arrestin or dynamin (which are involved in clathrin-mediated endocytosis) showed that activation of the p42/44 MAPK pathway can occur without receptor internalization [6–8].

Until recently, it was also thought that receptor desensitization, internalization and recycling are essential for cell migration in the presence of increasing concentrations of chemoattractant. However, new evidence suggests that this may not be the case. For example, a CCR2B mutant with all cytoplasmic serine and threonine residues substituted resulted in impaired receptor internalization, but intracellular calcium mobilization and chemotaxis were unaffected [9]. Similarly, replacement of all serine and threonine residues by alanine and glycine in the cytoplasmic tail of the FPR resulted in a mutant (Δ ST) that was neither phosphorylated nor internalized, but showed no change in the concentration-dependence for migration towards fMLF [10].

We analysed the effects of three FPR mutations (D71A, N297A and Y301A) on receptor desensitization and internalization, and on fMLF-induced chemotaxis and activation of the p42/44 MAPK pathway. Asp-71 is located in the putative second transmembrane domain and is conserved among GPCRs.

Abbreviations used: FPR, *N*-formyl peptide receptor; GPCR, G-protein-coupled receptor; MAPK, mitogen-activated protein kinase; fMLF, *N*-formyl-methionyl-leucyl-phenylalanine; CHO, Chinese hamster ovary; FBS, fetal bovine serum; f-NleLFNle YK-fluorescein, *N*-formyl-norleucine-leucine-phenylalanine-norleucine-tyrosine-lysine-fluorescein derivative; FACScan, fluorescence-activated cell scanner; fura 2/AM, fura 2 acetoxymethyl ester; BODIPY, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene; f-NleLFNleYK-BODIPY-FL, *N*-formyl-norleucine-leucine-phenylalanine-norleucine-tyrosine-lysine-BODIPY-fluorescein derivative; DMEM, Dulbecco's minimal essential medium; ARF, ADP-ribosylation factor.

¹ To whom correspondence should be addressed (e-mail heini@montana.edu).

Figure 1 Flow cytometric analysis of cell-surface expression of wild-type (wt) FPR and of the D71A, N297A and Y301A mutants in transfected CHO cells

Cells were incubated on ice with 20 nM f-NleLFNleYK-fluorescein. The results are from a single experiment that was representative of at least three separate experiments.

The D71A mutation in the FPR results in uncoupling from G-protein [11,12]. Asn-297 and Tyr-301 are located within the conserved NPXXY (Asn-Pro-Xaa-Xaa-Tyr) sequence in the putative seventh transmembrane domain. This motif has been found to play a role in desensitization and/or resensitization of the β_2 -adrenergic receptor and the platelet-activating factor receptor [13–16], but not in that of the gastrin-releasing hormone receptor or the angiotensin II receptor [17–20]. Here we show that the N297A mutant FPR is endocytosis-defective and does not become desensitized. However, the N297A mutant does support maximal chemotactic migration and activation of p42/44 MAPK at 10–100-fold higher fMLF concentrations than for wild-type FPR.

MATERIALS AND METHODS

Cell culture, immunofluorescence microscopy and flow cytometry

FPR mutagenesis and transfection of Chinese hamster ovary (CHO) cells were carried out as described previously [12,21]. Transfected CHO cells were maintained in selection medium containing 0.5 mg/ml G418 in α -modified Eagle's medium containing 5% (v/v) fetal bovine serum (FBS), 50 units/ml penicillin and 50 μ g/ml streptomycin. Increased expression was induced by adding 6 mM sodium butyrate to the medium 14–16 h before each experiment [22]. To examine receptor expression levels and localization in the cell, cells on coverslips were fixed and stained as described previously [21]. To examine cell surface expression of the FPR, CHO transfectants and wild-type CHO cells were incubated with 20 nM *N*-formyl-norleucine-leucine-phenylalanine-norleucine-tyrosine-lysine-fluorescein derivative (f-NleLFNleYK-fluorescein) (Molecular Probes, Eugene, OR, U.S.A.) in the presence or absence of a 1000-fold excess of fMLF and analysed using a FACScan (fluorescence-activated cell scanner) flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.), as described previously [21].

Chemotaxis assay

The chemotaxis assay was carried out essentially as described previously [23]. Briefly, CHO transfectants at 3×10^5 cells/300 μ l

were added to 6.5 mm-diam. Transwell inserts of 8 μ m pore size (Corning Costar, Cambridge, MA, U.S.A.). Then 500 μ l of serum-free medium with or without fMLF (at concentrations as indicated in the Figure legends) was 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 h at 37 °C. 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 in PBS, stained with Hematoxylin Gill's stain, and quantified using an image analyser (M4 True Color Image Analysis System; Imaging Research, St. Catharines, ON, Canada).

Calcium assay

Transfected CHO cells were detached from culture plates with a cell scraper after incubation with 1 mM NaEDTA (pH 8.0) in Ca^{2+} / Mg^{2+} -free PBS. Cells were harvested by centrifugation (200 *g*, 5 min), resuspended in PBS containing Ca^{2+} and Mg^{2+} /5% (v/v) FBS, and incubated with 2.5 μ M fura 2 acetoxymethyl ester (fura 2/AM) (Molecular Probes) for 45 min at 37 °C. Cells were centrifuged (200 *g*, 5 min) and resuspended in the above buffer at room temperature, where they were left until the assay. Each measurement contained $\sim 5 \times 10^6$ CHO cells in a volume of 1 ml. Continuous fluorescence measurements of calcium-bound and free fura 2 were made at 37 °C with a double-excitation monochromator fluorescence spectrofluorimeter (Photon Technology Int., Monmouth Junction, NJ, U.S.A.) with excitation at 340 nm and 380 nm and emission at 510 nm. Cells were exposed to various concentrations of fMLF, as indicated in Figure 4. ATP was added as a heterologous ligand to provide a standard stimulus for calcium mobilization. In one set of experiments (see Figure 4C), the loading of fura 2/AM was carried out in the presence of 10 nM fMLF for 90 min and the cells were resuspended in PBS containing Ca^{2+} and Mg^{2+} /5% FBS/10 nM fMLF. The cells were exposed to 10 nM fMLF for a minimum of 2 h prior to the calcium assay.

Endocytosis assays

CHO transfectants were removed from tissue culture plates as described for the calcium assay. Cells were resuspended in medium and incubated either on ice (to prevent endocytosis) or at 37 °C for 2 h or 4 h with 20 nM *N*-formyl-norleucine-leucine-phenylalanine-norleucine-tyrosine-lysine-BODIPY-fluorescein derivative (f-NleLFNleYK-BODIPY-FL, where BODIPY is 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) (Molecular Probes). Cells were then moved to ice, centrifuged (200 *g*, 5 min) and washed two times with PBS containing Ca^{2+} and Mg^{2+} /5% FBS to remove ligand from the cell surface. Samples were divided in half and incubated with 20 nM f-NleLFNleYK-BODIPY-FL in the presence or absence of a 1000-fold excess of fMLF (to measure non-specific binding). The internalized fluorescent ligand was analysed by measuring the fluorescence of 10000 cells using a FACScan flow cytometer. The results showed that approx. 85% of the surface-bound f-NleLFNleYK-BODIPY-FL from each cell line could be removed by washing with PBS. The percentage of binding sites remaining on the cell surface after 2 h and 4 h of endocytosis was calculated based on the fluorescence compared with that for cells that were incubated on ice (= 100%).

In vivo phosphorylation and immunoprecipitation assay

Cells on 5 cm tissue culture dishes were rinsed with phosphate-free Dulbecco's minimal essential medium (DMEM). Cells were

Figure 2 Maximal chemotaxis of the N297A mutant FPR occurs at higher fMLF concentrations than for wild-type (wt) FPR

Cells were allowed to migrate towards 0, 1 or 100 nM fMLF for 4 h. Cells that had migrated through 8 μ m pores and attached to the other side of the semi-permeable membrane were fixed, stained and photographed. The small round circles on the filters are the 8 μ m pores, and the black structures are the cells that have migrated through the pores or are in the process of migrating through the pores.

incubated for 3 h at 37 °C in phosphate-free DMEM containing 0.3–0.5 mCi of [32 P]P_i in 1 ml, and subsequently for 10 min with or without 100 nM fMLF. Cells were moved on to ice, washed with phosphate-free DMEM and extracted with 800 μ l of RIPA buffer (10 mM Tris, 0.1 % SDS, 1 % deoxycholate, 1 % Nonidet P40 and 150 mM NaCl, pH 7.4) containing 5 μ g/ml leupeptin, 20 μ g/ml pepstatin, 1 μ g/ml aprotinin, 1 mM PMSF, 0.1 mM bromotetramisole, 1 μ M cantharidin, 100 μ M sodium orthovanadate and 10 mM NaF. Insoluble material was pelleted by centrifugation (20 800 g, 5 min) and the supernatant was pre-cleared using an irrelevant antibody and 50 μ l of 10 % (w/v) washed *Staphylococcus aureus* bacteria. The immunoprecipitation was carried out using a rabbit polyclonal antibody against a peptide corresponding to the C-terminal 14 amino acids of the FPR, followed by 50 μ l of 10 % (w/v) washed *S. aureus* bacteria. The bacteria were washed three times with RIPA buffer con-

taining protease and phosphatase inhibitors. Then 2 $\times$ Laemmli sample buffer was added to the final bacterial pellet and the samples were heated at 37 °C for 20 min prior to loading on to an SDS/10 % -polyacrylamide gel. After electrophoresis, the gel was dried and exposed in a fluorimeter cassette and/or on X-ray film.

MAPK assay

CHO transfectants were incubated for 5 min with 0–10 μ M fMLF and lysed with boiling Laemmli sample buffer. The incubation time was based on results from previous experiments that showed maximal p42/44 MAPK activation at 5 min for FPR- and N297A-transfected cells. Extract corresponding to $\sim 1 \times 10^5$ cells was run on an SDS/10 % -polyacrylamide gel. The proteins were transferred on to nitrocellulose and the MAPKs,

p44 and p42, were detected using a mouse monoclonal antibody against human p42/44 MAPK phosphorylated on Thr-202 and Tyr-204, or a rabbit polyclonal antibody against total human p42/44 MAPK (New England Biolabs, Beverly, MA, U.S.A.). The proteins were detected using horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence (Amersham, Arlington Heights, IL, U.S.A.). The intensity of the bands on the X-ray films was quantified using the Image Quant program after scanning with a Hewlett Packard Scanjet IIcx scanner.

RESULTS

D71A, N297A and Y301A mutant FPRs are expressed on the cell surface and bind ligand with similar affinities as wild-type FPR

To examine the role of the conserved aspartic acid residue, D71 (second transmembrane domain), and of the NPXXY motif (seventh transmembrane domain) in FPR-mediated signalling and chemotaxis, each amino acid was replaced with an alanine residue. The effects of these mutations on ligand binding and G-protein coupling have been published previously [12]. In short, the D71A, N297A and Y301A mutations did not affect ligand binding, but the D71A mutant protein appeared to be uncoupled from G-protein, based on a lack of fMLF-induced binding of guanosine 5'-[γ - 35 S]thio]triphosphate to membranes. However, higher concentrations of fMLF (100 nM–1 μ M) resulted in intracellular Ca^{2+} release and activation of p42/44 MAPK, suggesting that the uncoupling is not complete or that a G-protein-independent pathway is utilized [12].

The cell-surface expression levels of these mutant proteins were confirmed by flow cytometric analysis of the surface-bound ligand f-NleLFNleYK-fluorescein (Figure 1). The N297A mutant showed similar surface expression levels as wild-type FPR (1.1×10^6 and 1.2×10^6 receptors/cell respectively), whereas the expression levels of the D71A and Y301A mutants were somewhat lower (5.3×10^5 and 4.9×10^5 receptors/cell respectively). The P298A substitution led to a mutant that was not transported to the cell surface, presumably due to misfolding, since the protein could be detected in endoplasmic-reticulum-like structures by immunofluorescence (results not shown).

The N297A mutant FPR induces increased chemotaxis at concentrations that inhibit chemotaxis of cells expressing the wild-type receptor

We have shown previously that CHO cells expressing wild-type FPR migrate towards a concentration gradient of fMLF in a similar fashion to human neutrophils [23]. To examine the effects of the mutations on chemotaxis, we allowed the cells to migrate through 8 μ m pores into a lower well containing various concentrations of fMLF. Figure 2 shows examples of the filters after migration of CHO transfectants towards 0, 1 or 100 nM fMLF. Cells expressing wild-type FPR or the Y301A mutant migrated best towards 1 nM fMLF, whereas cells expressing the D71A mutant did not migrate at all, and N297A mutant-containing cells migrated more efficiently towards 100 nM than towards 1 nM fMLF (Figure 2). The cell surface area of the migrated CHO FPR wild-type and N297A cells was quantified using an image analyser (Figure 3). Maximal migration of CHO FPR wild-type cells was observed at 1 nM fMLF, whereas higher concentrations were shown to cause a decrease in migration. In contrast, the CHO N297A cells showed increased migration with higher concentrations of fMLF, with maximal chemotaxis at 10–100 nM (Figure 3). This result is unlikely to have been caused

Figure 3 Chemotaxis induced by wild-type FPR decreases at fMLF concentrations above 1–10 nM, whereas the N297A mutant FPR induces increased chemotaxis up to approx. 100 nM fMLF

Cells were allowed to migrate for 4 h towards the indicated concentrations of fMLF. The cells that had migrated through the pores were quantified by measuring the cell surface area by image analysis of six randomly chosen fields. Values are means $\pm$ S.E.M. from three separate experiments.

by differences in ligand binding affinity, since the N297A mutant FPR did not exhibit a decreased affinity for the ligand [21].

Since the migration of cells containing the mutant N297A FPR was significantly reduced at 1 μ M fMLF, we decided to examine the rate of diffusion of 1 μ M f-NleLFNleYK-fluorescein from the lower well through the pores into the upper well. At 1 h, the calculated concentration of this fluoresceinated hexapeptide in the upper well was approx. 50 nM, and at 4 h it was approx. 300 nM. Since the maximal migration of CHO N297A cells was seen at a starting concentration of 10–100 nM in the lower well, the almost undetectable migration at a concentration of 1 μ M formulated peptide may be due to desensitization.

To examine whether the shift in fMLF concentration-dependence for migration was the result of a decrease in G-protein coupling to the N297A mutant FPR, we measured the binding of guanosine 5'-[γ - 35 S]thio]triphosphate to membranes after stimulation with various concentrations of fMLF. The EC_{50} s for wild-type and N297A FPRs were 15 nM and 10 nM respectively, suggesting that the mutation did not result in a significant change in G-protein coupling.

Finally, we determined whether the migration of the CHO N297A cells was chemotactic or chemokinetic. As shown in Table 1, an increasing concentration of chemoattractant in the upper well did not significantly increase migration and prevented migration to the lower well. This suggests that the increased migration at 10–100 nM fMLF was directional chemotaxis, rather than random chemokinesis.

The N297A mutant FPR does not become desensitized at low concentrations of fMLF

To examine further the shift in concentration-dependence of chemotaxis of cells containing the N297A mutant FPR, we determined whether the mutant receptor is desensitized to a second exposure of fMLF. We have shown previously that CHO cells expressing wild-type FPR mobilize intracellular calcium in response to the addition of 10 nM–1 μ M fMLF. Since additional calcium release cannot be detected upon subsequent addition of a 10 times higher concentration of fMLF, the receptor is operationally desensitized [23]. Interestingly, in CHO cells ex-

Table 1 Checkerboard analysis of fMLF-stimulated migration of CHO cells expressing the N297A mutant FPR

Concentrations of fMLF added to the upper wells are shown as column headings, and those added to the lower wells are shown in the first column. Relative cell surface areas of the migrating cells from a single experiment are shown as means $\pm$ S.E.M. of six randomly chosen fields.

fMLF concn. in lower well (nM)	fMLF concn. in upper well (nM)...	Relative cell surface area of migrating cells				
		0	1	10	100	1000
0		9 $\pm$ 2	8 $\pm$ 2	6 $\pm$ 2	5 $\pm$ 2	5 $\pm$ 1
1		126 $\pm$ 16	6 $\pm$ 2	7 $\pm$ 3	5 $\pm$ 2	7 $\pm$ 2
10		237 $\pm$ 38	141 $\pm$ 22	11 $\pm$ 4	4 $\pm$ 2	6 $\pm$ 1
100		307 $\pm$ 26	180 $\pm$ 16	100 $\pm$ 23	14 $\pm$ 3	12 $\pm$ 2
1000		32 $\pm$ 8	30 $\pm$ 7	15 $\pm$ 5	10 $\pm$ 2	9 $\pm$ 3

Figure 4 CHO cells expressing the N297A mutant FPR do not become desensitized to fMLF

(A) Fura 2/AM-loaded CHO cells expressing wild-type or N297A mutant FPR were induced to release intracellular calcium by the addition of 10 nM fMLF. After 50 s the cells were exposed to a 10-fold higher concentration of fMLF, resulting in a total fMLF concentration of 110 nM. ATP (1 μ M) was added at 150 s to confirm that the cells were in good condition. (B) Cells expressing the N297A mutant FPR were exposed at 50 s to 100 nM (left panel) or 1 μ M (right panel) fMLF, followed by the addition of 1 μ M fMLF at 100 s and of 1 μ M ATP at 150 s. (C) Cells expressing wild-type or N297A mutant FPR were exposed for 2 h to 10 nM fMLF prior to the calcium assay; 1 μ M fMLF was added at 50 s and 10 μ M ATP was added at 100 s. The graphs are representative of a minimum of three experiments with similar results.

Figure 5 The D71A and N297A mutants are endocytosed with lower efficiency than wild-type FPR

Cells were incubated for 4 h on ice (background control), or for 2 and 4 h at 37 °C with a formylated hexapeptide, f-NleLFNleYK, conjugated to a pH-insensitive fluorescent dye, BODIPY-FL. Cells were washed as described in the Materials and methods section, then the samples were divided in two and incubated with f-NleLFNleYK-BODIPY-FL as above in the presence or absence of a 1000-fold excess of fMLF, to distinguish between internalized and total cell-associated FPR. The fluorescence of 10000 cells was analysed using a FACScan flow cytometer. The internalized FPR was calculated by subtracting the wash-resistant background fluorescence (approx. 10–15% of total) from the fluorescence obtained after 2 h and 4 h of internalization of ligand. The results are means $\pm$ S.E.M. from four separate experiments. Significance of differences compared with wild type: *** $P < 0.0004$; ** $P < 0.009$; * $P < 0.03$.

pressing the N297A mutant FPR, the amount of calcium released from intracellular stores was quite significant on addition of 100 nM fMLF after an initial exposure to 10 nM fMLF (Figure 4A). These results suggest that the N297A mutant does not become desensitized during the 50 s interval between additions of ligand. However, if the cells were first exposed to 100 nM or 1 μ M fMLF, they no longer responded to an additional exposure to fMLF (Figure 4B). This effect may be due to saturation of the receptor sites or to desensitization.

To determine whether the lack of desensitization of the N297A cells at 10 nM fMLF could be correlated with the increased migration at ≥ 10 nM fMLF, we carried out the calcium assay after a 2 h continuous exposure to 10 nM fMLF. As shown in Figure 4(C), addition of 1 μ M fMLF resulted in intracellular calcium release in N297A cells, but not in FPR wild-type cells. Thus a 2 h exposure to fMLF does not result in desensitization and down-regulation of the N297A mutant FPR.

The N297A mutant FPR is endocytosed less efficiently than the wild type

To examine the contribution of altered endocytosis of the N297A mutant FPR to the lack of receptor desensitization and down-regulation, we evaluated the internalization of a fluorescent formylated peptide that is not quenched by the low pH in endosomes and lysosomes. Cells were exposed to 20 nM f-NleLFNleYK-BODIPY-FL either for 4 h on ice or for 2 and 4 h at 37 °C. Cell-surface-bound ligand was washed off, the samples were divided in two, and total FPR compared with internalized FPR was detected using 20 nM f-NleLFNleYK-BODIPY-FL in the absence or presence of a 1000-fold excess of fMLF (20 μ M). Figure 5 shows that the percentage of ligand-binding wild-type FPR on the cell surface was significantly lower at both time points than the percentage of ligand-binding N297A mutant FPR. As a control, we also analysed the endocytosis of the D71A mutant, which has been shown previously to be internalized very inefficiently [24]. The results suggest that the N297A mutant is endocytosed with similar very low efficiency as the D71A mutant.

Figure 6 The N297A mutant FPR is not phosphorylated upon stimulation with ligand

Cells loaded with [32 P] P_i were incubated for 10 min with or without 100 nM fMLF. The cells were extracted, and immunoprecipitated FPR was run on an SDS/polyacrylamide gel. The experiment was carried out twice, with identical results.

The N297A mutant FPR is not phosphorylated in response to ligand binding

It has been shown previously that phosphorylation of FPR is required for receptor internalization. A mutant FPR (Δ ST) which lacked all of the serine and threonine residues in the cytoplasmic tail was not desensitized upon repeated exposure to ligand and remained on the cell surface [10]. Similarly, the D71A mutation resulted in a lack of phosphorylation and endocytosis [24]. We therefore determined whether poor endocytosis of the N297A mutant FPR could also be linked to lack of receptor phosphorylation. Cells loaded with [32 P] P_i were incubated for 10 min with or without fMLF, followed by immunoprecipitation and gel electrophoresis. As shown in Figure 6, activation of wild-type FPR with fMLF resulted in phosphorylation of the receptor. In contrast, the N297A mutant was not phosphorylated, again suggesting that phosphorylation may be a critical event preceding receptor internalization.

The N297A mutation results in an fMLF-concentration-dependent increase in p42/44 MAPK activation

To find out whether p42/44 MAPK plays a role in the fMLF-induced chemotaxis of FPR-expressing CHO cells, we employed a specific inhibitor of p42/44 MAPK kinase, PD98059. Cells preincubated for 1 h with 50 μ M PD98059 before exposure to a chemotactic gradient showed an $87 \pm 9\%$ decrease in migration (mean $\pm$ S.E.M.; $n = 4$).

To determine whether the N297A mutant FPR activates p42/44 MAPK with a different efficiency from that of wild-type FPR, we analysed MAPK activation as a function of fMLF concentration. Maximal phosphorylation of p42/44 MAPK was observed at 1–100 nM fMLF in cells expressing wild-type FPR, and at 100 nM–10 μ M fMLF in cells expressing the N297A mutant (Figure 7). Activation of p42/44 MAPK could also be detected through the D71A mutant, but only at higher concentrations of fMLF, in good agreement with our previous results showing calcium release at 1 μ M fMLF but not at 10 nM fMLF [12]. A Western blot with equal loading, as in Figure 7(A), was

Figure 7 The N297A mutant FPR mediates maximal activation of p42/44 MAPK at 10–100-fold higher concentrations than wild-type FPR

Cells were incubated for 5 min with the indicated concentrations of fMLF, and the quantities of phosphorylated and total p42/44 MAPK were examined by Western analysis. **(A)** Autoradiogram of a Western blot probed with a monoclonal antibody against p42/44 MAPK phosphorylated on Thr-202 and Tyr-204. **(B)** Autoradiogram of a Western blot of the same samples as in **(A)** probed with a polyclonal antibody against total p42/44 MAPK. **(C)** Quantitative analysis of phosphorylated p42 MAPK from Western blots. Autoradiograms were scanned and the bands were quantified using the Image Quant program. Due to film saturation at higher concentrations of fMLF, the quantitative results should not be considered absolute, but indicative of the phosphorylation/dephosphorylation trend. The results represent means $\pm$ S.E.M. from three separate experiments. Significance of differences compared with wild type: * $P < 0.02$.

reacted with an antibody that recognizes total p42/44 MAPK to show that each lane had a similar amount of protein (Figure 7B). Densitometric analysis of phosphorylated p42 MAPK from three separate experiments showed that the differences in phosphorylation mediated by wild-type and N297A FPR were statistically significant at 1 μ M and 10 μ M fMLF (Figure 7C). These results suggest that the lack of desensitization of N297A FPR may result in enhanced p42/44 MAPK activation, resulting in a dose-dependent increase in chemotactic migration.

DISCUSSION

Migratory cells can sense a gradient of chemoattractant that allows them to move in the direction of the source. The mechanism for chemotaxis is still largely unknown, but involves G_i -linked signalling pathways that lead to changes in the cytoskeleton and cell-surface adhesion molecules. The directional sensing does not appear to depend on the distribution of the chemoattractant receptor; both the C5a receptor in neutrophils and the cAMP receptor in *Dictyostelium* remain uniformly

distributed during chemotaxis [25,26]. However, various proteins containing the pleckstrin homology domain translocate to the leading edge of migrating cells; several of these proteins have been shown to be essential for chemotactic migration through their signal-transduction-regulating functions [25,27,28]. These results have led to the hypothesis that the recruitment of these proteins to the plasmalemma may be a general mechanism by which cells can sense the chemoattractant gradient through GPCRs [25,27].

In vitro studies have shown that ~ 1 –10 nM fMLF is the optimal concentration range for chemotaxis of human neutrophils, as well as CHO cells transfected with the FPR, whereas higher concentrations result in reduced migration [23]. The reduced migration is thought to result from receptor desensitization. In the present study we describe an FPR mutant, N297A, which exhibits maximal chemotactic migration at 10–100 nM fMLF, despite normal ligand-binding affinity and G-protein coupling. Intracellular calcium release assays showed that, unlike wild-type FPR, the N297A mutant was not desensitized during a time course of up to 2 h (Figure 4). Subsequent endocytosis

assays suggested that the chemotaxis of cells containing the N297A mutant at higher concentrations of fMLF may be due in part to inefficient internalization of the N297A mutant receptor (Figure 5). As shown previously with other mutant FPRs, the defect in internalization appears to be linked to a lack of receptor phosphorylation (Figure 6) [10,24]. We do not know why this particular mutation prevents phosphorylation of the FPR. However, both mutagenesis and molecular modelling studies have suggested an important role for the conserved NPXXY sequence in the activation of GPCRs. Extensive computational simulations have shown that the NPXXY motif forms a flexible hinge region that may function as a conformational switch [29]. Based on these studies, we speculate that the N297A mutation inhibits the interaction with the GPCR kinase, and thus prevents phosphorylation of the receptor. Nevertheless, this is, to our knowledge, the first report of a mutant receptor showing that inefficiencies in signalling adaptation may result in a shift to 10–100-fold higher concentrations for the range over which chemotaxis occurs.

Previous studies of FPR mutants have shown that a phosphorylation-deficient FPR mutant, Δ ST, inhibited desensitization [30] and receptor internalization, but did not affect the cells' ability to move towards a gradient of fMLF [10]. Together with our results, these observations suggest that desensitization and efficient internalization of the FPR are not required for chemotaxis. However, the Δ ST mutant also exhibited a similar fMLF-concentration-dependence for chemotaxis as wild-type FPR, suggesting that the lack of phosphorylation itself does not result in increased chemotaxis at higher fMLF concentrations. What, then, is the functional difference between mutants N297A and Δ ST? Since neither N297A nor Δ ST is phosphorylated in response to ligand, it is unlikely that the state of receptor phosphorylation contributes to the enhanced migration at normally desensitizing concentrations. Instead, some other characteristic, such as a change in an interaction with a regulatory molecule, may shift the concentration range for chemotaxis. Possible candidates for such molecules are regulatory proteins that have translocated to the leading edge of the cell [27,31], and actin and actin-binding proteins [32,33]. The N297A mutation may directly or indirectly decrease the interaction with such a molecule, possibly resulting in a requirement for a larger number of receptors to bind ligand in order to trigger chemotaxis.

Two possibilities for translocating proteins are the small G-proteins, ADP-ribosylation factor (ARF) and RhoA. These proteins have been shown to interact with some GPCRs, resulting in activation of phospholipase D [31]. Based on mutagenesis studies of the gonadotropin-releasing hormone receptor and the 5-HT_{2A} receptor, the asparagine residue in the NPXXY motif appears to be required for the formation of functional complexes between the GPCR and RhoA/ARF [31]. This possibility will be investigated further by comparing the translocation of RhoA and ARF in CHO cells expressing wild-type FPR and the N297A mutant.

In the present study, we observed that the activation state of p42/44 MAPK might be critical for FPR-mediated chemotaxis: the p42/44 MAPK kinase inhibitor, PD98059, drastically reduced the fMLF-induced migration of CHO transfectants. This result was somewhat unexpected, since PD98059 was found previously to result in no, or very minor, inhibition of fMLF-induced chemotaxis in neutrophils [34–36], although one opposite result was also reported [37]. However, the role of p42/44 MAPK in chemotaxis may be receptor- and cell type-specific, since PD98059 caused approx. 75% inhibition of eotaxin-induced chemotaxis of eosinophils [38], and antisense oligonucleotides that reduced p42/44 MAPK protein levels by 90% resulted in a 75% decrease in the migration of FG carcinoma

cells [39]. We thus examined the relationship between chemotaxis and p42/44 MAPK activation at various concentrations of fMLF in cells expressing wild-type FPR and the N297A mutant. At 1–100 nM fMLF, only minor differences in MAPK activation could be detected between the two cell lines; however, at 1–10 μ M fMLF, cells expressing the N297A mutant contained a significantly larger amount of phosphorylated p42/44 MAPK than cells expressing wild-type FPR (Figure 7).

Taken together, our data suggest that p42/44 MAPK plays an important role in the FPR-mediated chemotaxis of CHO cells. The sustained chemotactic migration mediated by the N297A mutant FPR may be due in part to a defect in desensitization and/or endocytosis of the receptor, that in turn may affect the phosphorylation state of p42/44 MAPK. These results suggest that the activation level of p42/44 MAPK may correlate with the extent of FPR-mediated chemotaxis, possibly through activation/suppression of downstream signalling or regulatory molecules. In addition, it is possible that the N297A mutation alters the interaction between FPR and some chemotactic regulatory molecule(s).

This work was supported by an Arthritis Foundation Investigator Award, a Grant-in-Aid from the American Heart Association (to H. M. M.) and a National Institutes of Health Grant AI22735 (to A. J. J.).

REFERENCES

- Ye, R. D. and Boulay, F. (1997) Structure and function of leukocyte chemoattractant receptors. *Adv. Pharmacol.* **39**, 221–289
- Mills, J. S., Miettinen, H. M., Vlasses, M. J. and Jesaitis, A. J. (1999) The N-formyl peptide receptor: structure, signalling, and disease. In *Molecular and Cellular Basis of Inflammation* (Serhan, C. N. and Ward, P. A., eds.), pp. 215–245, Humana Press, Totowa
- Schiffmann, E., Showell, H. V., Corcoran, B. A., Ward, P. A., Smith, E. and Becker, E. L. (1975) The isolation and partial characterization of neutrophil chemotactic factors from *Escherichia coli*. *J. Immunol.* **114**, 1831–1837
- Pitcher, J. A., Freedman, N. J. and Lefkowitz, R. J. (1998) G protein-coupled receptor kinases. *Annu. Rev. Biochem.* **67**, 653–692
- Daaka, Y., Luttrell, L. M., Ahn, S., Della, R., Ferguson, S. S., Caron, M. G. and Lefkowitz, R. J. (1998) Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. *J. Biol. Chem.* **273**, 685–688
- Budd, D. C., Rae, A. and Tobin, A. B. (1999) Activation of mitogen-activated protein kinase pathway by a G_{q/11}-coupled muscarinic receptor is independent of receptor internalization. *J. Biol. Chem.* **274**, 12355–12360
- DeGraff, J. L., Gagnon, A. W., Benovic, J. L. and Orsini, M. J. (1999) Role of arrestins in endocytosis and signaling of α_2 -adrenergic receptor subtypes. *J. Biol. Chem.* **274**, 11253–11259
- Li, J. G., Luo, L. Y., Krupnick, J. G., Benovic, J. L. and Liu-Chen, L. Y. (1999) U50,488H-induced internalization of the human kappa opioid receptor involves a β -arrestin- and dynamin-dependent mechanism. Kappa receptor internalization is not required for mitogen-activated protein kinase activation. *J. Biol. Chem.* **274**, 12087–12094
- Arai, H., Monteclaro, F. S., Tsou, C. L., Franci, C. and Charo, I. F. (1997) Dissociation of chemotaxis from agonist-induced receptor internalization in a lymphocyte cell line transfected with CCR2B: Evidence that the directed migration does not require rapid modulation of signaling at the receptor level. *J. Biol. Chem.* **272**, 25037–25042
- Hsu, M. H., Chiang, S. C., Ye, R. D. and Prossnitz, E. R. (1997) Phosphorylation of the N-formyl peptide receptor is required for receptor internalization but not chemotaxis. *J. Biol. Chem.* **272**, 29426–29429
- Prossnitz, E. R., Schreiber, R. E., Bokoch, G. M. and Ye, R. D. (1995) Binding of low affinity N-formyl peptide receptors to G protein: Characterization of a novel inactive receptor intermediate. *J. Biol. Chem.* **270**, 10686–10694
- Miettinen, H. M., Gripenroeg, J. M., Mason, M. M. and Jesaitis, A. J. (1999) Identification of putative sites of interaction between the human formyl peptide receptor and G protein. *J. Biol. Chem.* **274**, 27934–27942
- Barak, L. S., Tiberi, M., Freedman, N. J., Kwatra, M. M., Lefkowitz, R. J. and Caron, M. G. (1994) A highly conserved tyrosine residue in G protein-coupled receptors is required for agonist-mediated β_2 -adrenergic receptor sequestration. *J. Biol. Chem.* **269**, 2790–2795

- 14 Barak, L. S., Ménard, L., Ferguson, S. S. G., Colapietro, A.-M. and Caron, M. G. (1995) The conserved seven-transmembrane sequence NP(X)₂Y of the G protein-coupled receptor superfamily regulates multiple properties of the β_2 -adrenergic receptor. *Biochemistry* **34**, 15407–15414
- 15 Ferguson, S. S. G., Ménard, L., Barak, L. S., Koch, W. J., Colapietro, A.-M. and Caron, M. G. (1999) Role of phosphorylation in agonist-promoted β_2 -adrenergic receptor sequestration. *J. Biol. Chem.* **270**, 24782–24789
- 16 Le Gouill, C., Parent, J.-L., Rola-Pleszczynski, M. and Stankova, J. (1997) Structural and functional requirements for agonist-induced internalization of the human platelet-activating factor receptor. *J. Biol. Chem.* **272**, 21289–21295
- 17 Slice, L. W., Wong, H. C., Sternini, C., Grady, E. F., Bunnett, N. W. and Walsh, J. H. (1994) The conserved NPX_nY motif present in the gastrin-releasing peptide receptor is not a general sequestration sequence. *J. Biol. Chem.* **269**, 21755–21762
- 18 Thomas, W. G., Baker, K. M., Motel, T. J. and Thekkumkara, T. J. (1995) Angiotensin II receptor endocytosis involves two distinct regions of the cytoplasmic tail: A role for residues on the hydrophobic face of a putative amphipathic helix. *J. Biol. Chem.* **270**, 22153–22159
- 19 Hunyady, L., Bor, M., Baukal, A. J., Balla, T. and Catt, K. J. (1999) A conserved NPLFY sequence contributes to agonist binding and signal transduction but is not an internalization signal for type 1 angiotensin II receptor. *J. Biol. Chem.* **270**, 16602–16609
- 20 Laporte, S. A., Servant, G., Richard, D. E., Escher, E., Guillemette, G. and Leduc, R. (1996) The tyrosine within the NPX_nY motif of the human angiotensin II type 1 receptor is involved in mediating signal transduction but is not essential for internalization. *Mol. Pharmacol.* **49**, 89–95
- 21 Miettinen, H. M., Mills, J. S., Gripenrot, J. M., Dratz, E. A., Granger, B. L. and Jesaitis, A. J. (1997) The ligand binding site of the formyl peptide receptor maps in the transmembrane region. *J. Immunol.* **159**, 4045–4054
- 22 Gorman, C. M., Howard, B. H. and Reeves, R. (1983) Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate. *Nucleic Acids Res.* **11**, 7631–7648
- 23 Miettinen, H. M., Gripenrot, J. M. and Jesaitis, A. J. (1998) Chemotaxis of Chinese hamster ovary cells expressing the human neutrophil formyl peptide receptor: Role of signal transduction molecules and $\alpha_5\beta_1$ integrin. *J. Cell Sci.* **111**, 1921–1928
- 24 Prossnitz, E. R., Gilbert, T. L., Chiang, S., Campbell, J. J., Qin, S. X., Newman, W., Sklar, L. A. and Ye, R. D. (1999) Multiple activation steps of the *N*-formyl peptide receptor. *Biochemistry* **38**, 2240–2247
- 25 Parent, C. A., Blacklock, B. J., Froehlich, W. M., Murphy, D. B. and Devreotes, P. N. (1998) G protein signaling events are activated at the leading edge of chemotactic cells. *Cell* **95**, 81–91
- 26 Servant, G., Weiner, O. D., Neptune, E. R., Sedat, J. W. and Bourne, H. R. (1999) Dynamics of a chemoattractant receptor in living neutrophils during chemotaxis. *Mol. Biol. Cell* **10**, 1163–1178
- 27 Parent, C. A. and Devreotes, P. N. (1999) A cell's sense of direction. *Science* **284**, 765–770
- 28 Lemmon, M. A., Ferguson, K. M. and Schlessinger, J. (1996) PH domains: Diverse sequences with common fold recruit signaling molecules to the cell surface. *Cell* **85**, 621–624
- 29 Konvicka, K., Guarnieri, F., Ballesteros, J. A. and Weinstein, H. (1998) A proposed structure for transmembrane segment 7 of G protein-coupled receptors incorporating an Asn-Pro/Asp-Pro motif. *Biophys. J.* **75**, 601–611
- 30 Prossnitz, E. R. (1997) Desensitization of *N*-formylpeptide receptor-mediated activation is dependent upon receptor phosphorylation. *J. Biol. Chem.* **272**, 15213–15219
- 31 Mitchell, R., McCulloch, D., Lutz, E., Johnson, M., MacKenzie, C., Fennell, M., Fink, G., Zhou, W. and Sealfon, S. C. (1998) Rhodopsin-family receptors associate with small G proteins to activate phospholipase D. *Nature (London)* **392**, 411–414
- 32 Jesaitis, A. J., Erickson, R. W., Klotz, K.-N., Bommakanti, R. K. and Siemsen, D. W. (1993) Functional molecular complexes of human *N*-formyl chemoattractant receptors and actin. *J. Immunol.* **151**, 5653–5665
- 33 Klotz, K.-N., Krotec, K. L., Gripenrot, J. and Jesaitis, A. J. (1994) Regulatory interaction of *N*-formyl peptide chemoattractant receptors with the membrane skeleton in human neutrophils. *J. Immunol.* **152**, 801–810
- 34 Downey, G. P., Butler, J. R., Tapper, H., Fialkow, L., Saltiel, A. R., Rubin, B. B. and Grinstein, S. (1998) Importance of MEK in neutrophil microbicidal responsiveness. *J. Immunol.* **160**, 434–443
- 35 Hii, C. S. T., Stacey, K., Moghaddami, N., Murray, A. W. and Ferrante, A. (1999) Role of the extracellular signal-regulated protein kinase cascade in human neutrophil killing of *Staphylococcus aureus* and *Candida albicans* and in migration. *Infect. Immun.* **67**, 1297–1302
- 36 Heuertz, R. M., Tricoli, S. M., Ezekiel, U. R. and Webster, R. O. (1999) C-reactive protein inhibits chemotactic peptide-induced p38 mitogen-activated protein kinase activity and human neutrophil movement. *J. Biol. Chem.* **274**, 17968–17974
- 37 Kuroki, M. and O'Flaherty, J. T. (1997) Differential effects of mitogen-activated protein kinase kinase inhibitor on human neutrophil responses to chemotactic factors. *Biochem. Biophys. Res. Commun.* **232**, 474–477
- 38 Kampen, G. T., Stafford, S., Adachi, T., Jinquan, T., Quan, S., Grant, J. A., Skov, P. S., Poulsen, L. K. and Alam, R. (2000) Eotaxin induces degranulation and chemotaxis of eosinophils through the activation of ERK2 and p38 mitogen-activated protein kinases. *Blood* **95**, 1911–1917
- 39 Klemke, R. L., Cai, S., Giannini, A. E., Gallagher, P. J., de Lanerolle, P. and Cheresih, D. A. (1997) Regulation of cell motility by mitogen-activated protein kinase. *J. Cell Biol.* **137**, 481–492

Received 14 March 2000/17 July 2000; accepted 25 September 2000