The Journal of Immunology

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J Immunol 2001; 166:4099-4105; ;
doi: 10.4049/jimmunol.166.6.4099
http://www.jimmunol.org/content/166/6/4099

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Differential Roles of the NPXXY Motif in Formyl Peptide Receptor Signaling[1]

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The NPXXY motif (X represents any amino acid) in the seventh transmembrane domain of the chemotactic formyl peptide receptor (FPR) is highly conserved among G protein-coupled receptors. Recent work suggested that this motif contributes to G protein-coupled receptor internalization and signal transduction; however, its role in FPR signaling remains unclear. In this study we replaced Asn^{297} and Tyr^{301} in the NPXXY motif of the human FPR with Ala (N297A) and Ala/Phe (Y301A/Y301F), respectively, and determined the effects of the substitutions on FPR functions in transfected rat basophilic leukemia cells. Whereas all the mutant receptors were expressed on the cell surface, the N297A receptor exhibited reduced binding affinity and was unable to mediate activation of phospholipase C-β and the p42/44 mitogen-activated protein kinase (MAP kinase). The Y301F receptor displayed significantly decreased ligand-stimulated internalization and MAP kinase activation, suggesting that the hydrogen bonding at Tyr^{301} is critical for these functions. The Y301F receptor showed a chemotactic response similar to that of wild-type FPR, indicating that cell chemotaxis does not require receptor internalization and hydrogen bonding at the Tyr^{301} position. In contrast, the Y301A receptor displayed a left-shifted, but overall reduced, chemotaxis response that peaked at 0.1–1 nM. Finally, using a specific MAP kinase kinase inhibitor, we found that activation of MAP kinase is required for efficient FPR internalization, but is not essential for chemotaxis. These findings demonstrate that residues within the NPXXY motif differentially regulate the functions of FPR. *The Journal of Immunology,* **2001, 166: 4099–4105.**

The human *N*-formyl peptide receptor (FPR)[3] belongs to the seven-transmembrane domain G protein-coupled receptor (GPCR) superfamily and is expressed predominately in myeloid cells (1, 2). The FPR, which binds bacterial and mitochondrial *N*-formyl peptides such as *N*-formyl-Met-Leu-Phe (fMLF), couples to pertussis toxin-sensitive G proteins and activates a complex signaling cascade leading to integrin activation, chemotaxis, superoxide production, and degranulation (3). It has been established that FPR plays an important role in host defense against bacterial infection (4).

Hundreds of GPCRs from different species have been cloned and sequenced in the last decade. Although these receptors all possess the basic seven-transmembrane structure, they share only a limited number of conserved amino acid residues. One such conserved sequence is the characteristic NPXXY motif found in the seventh transmembrane domain of most GPCRs (5). The positions of Asn, Pro, and Tyr residues are almost invariable within the motif. The inner two residues are generally hydrophobic in nature, while the residues that flank Tyr and Asn may vary (5). In the human FPR, this NPXXY sequence is NPMLY, or Asn^{297}-Pro^{298}-Met^{299}-Leu^{300}-Tyr^{301} (Fig. 1).

The NPXXY sequence is similar to the NPXY sequence that is found in the cytoplasmic portion of single-transmembrane receptors such as receptors for the low density lipoprotein, insulin, and epidermal growth factor (6–8). The NPXY motif in the cytoplasmic segment of low density lipoprotein receptor was first described as an internalization signal important for both constitutive and agonist-induced receptor endocytosis (9). The NPXY sequence is believed to be directly involved in the association of the receptors with the HA-2 adaptor protein of the membrane clathrin lattice (6), a process that leads to efficient endocytosis via clathrin-coated pits (10).

For GPCRs, the NPXXY motif in the seventh transmembrane domain has been assumed to be an internalization sequence (11, 12). However, the mechanism by which NPXXY regulates agonist-induced internalization is unknown. It has been reported that the Tyr residue in the NPXXY motif is essential for the sequestration of the β_2-adrenergic receptor (13) and neurokinin 1 receptor (14). However, the NPXXY motif is not essential for internalization of the angiotensin II receptor (15) and gastrin-releasing peptide receptor (16). Thus, regulation of receptor internalization by the NPXXY motif may vary among GPCRs.

It has not been determined whether the NPXXY motif is a receptor internalization sequence in the FPR that undergoes rapid internalization upon agonist stimulation (17). Although phosphorylation of the C-terminal tail of FPR by GPCR kinase 2 is required for receptor internalization (18), a recent study has shown that internalization of the FPR occurs in an arrestin-independent manner and may not involve clathrin-coated pits (19). These findings as well as the importance of receptor internalization in down-regulation of GPCR signaling prompted us to examine the role of NPXXY in FPR internalization and transmembrane signaling. Our results, based on analysis of mutant receptors, indicate that the NPXXY motif differentially regulates several functions of the FPR.

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Received for publication August 23, 2000. Accepted for publication January 5, 2000.

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[1] This work was supported by grants from the National Institutes of Health (AI33503), the Arthritis Foundation, and the American Heart Association. D.D.B. is the recipient of a fellowship from the Arthritis Foundation. R.D.Y. is an established investigator with the American Heart Association.

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[3] Abbreviations used in this paper: FPR, formyl peptide receptor; G proteins, guanine nucleotide-binding regulatory proteins; GPCR, G protein-coupled receptor; fMLF, *N*-formyl-Met-Leu-Phe; PLC, phospholipase C; IP, inositol phosphates; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase.

0022-1767/01/$02.00

Materials and Methods

Chemicals

The *N*-formyl peptide fMLF was purchased from Sigma (St. Louis, MO). Pertussis toxin was obtained from List Laboratories (Campbell, CA). [^{3}H]fMLF and myo-[^{3}H]inositol were obtained from DuPont-NEN (Boston, MA). Indo-1/AM was purchased from Molecular Probes (Eugene, OR). The MEK inhibitor U0126 was purchased from Biomol (Plymouth Meeting, PA).

Mutagenesis of human FPR

Mutants were generated by PCR using human FPR cDNA (Clone 26) (20) as a template. The overlapping PCR method (21) was used to introduce a mutation either at bases 889 and 890 of the FPR open reading frame (to replace Asn^{297} with Ala) or at bases 901 and 902 (to change Tyr^{301} to Ala or Phe; Fig. 1*B*). The mutations were confirmed by DNA sequencing. cDNA inserts coding for wild-type FPR (FPRwt) or mutant FPRs were subcloned into the *Eco*RI site of the SFFV.neo expression vector (22). This vector contains a neomycin resistance cassette, allowing selection of stable transfectants by G418 (Life Technologies, Gaithersburg, MD).

Cell culture and transfection

RBL-2H3 cells (provided by John Apgar, The Scripps Research Institute, La Jolla, CA) were maintained in DMEM containing 20% heat-inactivated FBS, 2 mM L-glutamine, 100 IU/ml penicillin, and 50 μg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cell transfection and selection of stable transfectants were conducted essentially as described in a recent publication (23). The transfected cells were sorted by flow cytometry (Becton Dickinson, San Jose, CA) using *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys-fluorescein (Molecular Probes). The positive cells were pooled and further cultured before analysis.

Flow cytometry

RBL cells, transfected with FPRwt or mutant FPRs, were harvested by trypsin-free dissociation buffer (Life Technologies), washed with PBS containing 0.5% BSA, and resuspended in the same buffer to 1×10^6 cells/ml. Receptor expression was determined using FPR-specific Ab and flow cytometry. Cells were prelabeled with the anti-FPR mAb 5F1 (2 μg/ml) (24), washed with PBS containing 0.5% BSA, and then incubated with FITC-conjugated goat anti-mouse IgG (5 μg/ml). All incubations were performed on ice for 60 min; the cells were washed three times with PBS containing 0.5% BSA between each incubation and before flow cytometric analysis. Binding was confirmed with the high affinity FPR ligand *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys-fluorescein derivative (100 nM) using flow cytometry (data not shown).

Calcium mobilization, chemotaxis, and mitogen-activated protein (MAP) kinase activation assays

These assays were conducted using procedures described in our recent publication (23), except that for chemotaxis assays the filter was scanned using a CanoScan FB620U flat-bed scanner (Canon Computer Systems, Salt Lake City, UT). The density was taken as directly proportional to the number of cells that had migrated across the filter onto the lower side of the filter and were trapped. Quantitative measurement of density was conducted using ImageQuant software (Molecular Dynamics, Mountain View, CA). Data were presented as the chemotaxis index, which is the ratio of the density of the area where cells are migrating toward fMLF over the density of the area where cells are migrating toward medium. Each experiment was conducted in quadruplicate, and the values were averaged. In some experiments the MAP kinase kinase inhibitor U0126 (5 μM) was included in the assay buffer.

Ligand binding assay

Direct binding assays were conducted as described previously (25). Briefly, cell membranes were prepared by nitrogen cavitation in a buffer containing 100 mM KCl, 2 mM NaCl, 1 mM ATP, 3.5 mM $MgCl_2$, and 10 mM PIPES, pH 7.3. Membranes collected after centrifugation at $150{,}000 \times g$ for 45 min were resuspended in a buffer containing 25 mM HEPES (pH 7.0) and 0.2 M sucrose and stored at −80°C until use. Direct binding with [^{3}H]fMLF (NEN; sp. act., 80 Ci/mmol) was performed at 23°C for 60 min with 30 μg of the membrane proteins and in a buffer containing 5 mM KCl, 140 mM NaCl, 1.9 mM KH_2PO_4, 1.1 mM Na_2HPO_4, 5.5 mM glucose, 0.15 mM $CaCl_2$, 0.3 mM $MgSO_4$, and 1 mM $MgCl_2$. The radiolabeled fMLF was used at eight different concentrations ranging from 0.1–100 nM, in the presence or the absence of a 1000-fold molar excess of unlabeled fMLF. Unbound ligand was removed by filtration through Whatman GF/C filters (Clifton, NJ). The amount of bound ligand was estimated by scintillation counting, and data were collected in triplicate. Specific binding and Scatchard analyses were conducted using Prism software (version 3.0, GraphPad, San Diego, CA).

Assay for phosphoinositide hydrolysis

Cells were seeded in 24-well tissue culture plates (0.2×10^6 cells/well) and cultured overnight with 1 μCi/ml of myo-[^{3}H]inositol in inositol-free medium supplemented with 10% dialyzed FBS (Life Technologies). Cells were washed with HBSS supplemented with 100 mM $LiCl_2$ and 0.1% BSA and preincubated with 200 μl of the same buffer for 10 min at 37°C. After incubation, cells were stimulated with different concentrations of fMLF for the indicated times. The reaction was terminated by addition of 750 μl of chloroform/methanol/4 M HCl (100/200/2). Then, 250 μl of 0.1 M HCl and 250 μl of chloroform were added to the sample. After vortex and centrifugation, the total [^{3}H]inositol phosphates in the aqueous phase were separated on columns of Dowex AG1-X8 and eluted with a buffer containing 1 M ammonium formate and 0.1 M formic acid. The radioactivity of [^{3}H]inositol phosphates was measured in a Beckman LS 3801 scintillation counter (Palo Alto, CA).

Assay for receptor internalization

Cells grown in six-well plates (1×10^6 cells/well) were washed twice with serum-free DMEM containing 1% BSA and preincubated in the same medium for 1 h. Cells were then stimulated for 90 min with 100 nM [^{3}H]fMLF (600 mCi/mmol) at 4°C (for cell surface receptor binding) and 37°C (for receptor binding plus internalization or total cell-associate radioactivity). The reaction was stopped by adding 1 ml of ice-cold PBS supplemented with 0.5% BSA. The unbound [^{3}H]fMLF was removed by washing cells three times, each with 3 ml of ice-cold PBS supplemented with 0.5% BSA. After this washing step, 200 μl of the lysis buffer (0.5% Nonidet P-40 and 0.5% Triton X-100 in PBS) was added. The contents were incubated on ice for 30 min, and the associated radioactivity was determined in a Beckman LS 3801 scintillation counter. Receptor internalization was determined by subtracting cell surface binding from total cell-associated radioactivity. The internalization index was calculated as the fold increase over basal, which was measured with the untransfected RBL cells under the same experimental conditions.

Results

Expression and binding properties of the mutant FPRs

RBL-2H3, a rat basophilic leukemia cell line devoid of endogenous response to fMLF (26), was stably transfected with cDNA encoding human FPRwt or the mutant FPRs N297A, Y301A, and Y301F (Fig. 1). Flow cytometric analysis was conducted to determine the expression of the receptors on cell surface. Fig. 2 shows the histograms of representative flow cytometric experiments. In all cases, the receptors were expressed in >80% of the transfected cells. Direct binding assays were performed to determine whether mutations of the NPXXY motif affected agonist binding. Data summarized in Table I indicate that the Y301A and Y301F mutant receptors displayed slightly decreased binding affinity for [^{3}H]fMLF, whereas the N297A mutation reduced binding affinity significantly. The N297A and Y301A mutant receptors were expressed at slightly higher levels compared with the FPRwt.

Effect of the NPXXY mutations on FPR signaling

To examine the effects of the three mutations on transmembrane signaling of the FPR, we first determined whether ligand binding to the mutant receptors induced intracellular calcium release. It has been demonstrated that agonist stimulation of FPR results in an increase in the cytosolic calcium concentration in the cells via G_i protein-mediated phospholipase C-β (PLC-β) activation and generation of inositol 1,4,5-trisphosphate (27). Calcium mobilization assays were conducted in indo-1/AM-labeled cells. As shown in Fig. 3, receptor-mediated calcium mobilization under the agonist-saturating condition was not significantly altered in cells expressing Y301A or Y301F compared with the FPRwt cell. However, the N297A cells displayed significantly decreased receptor-mediated

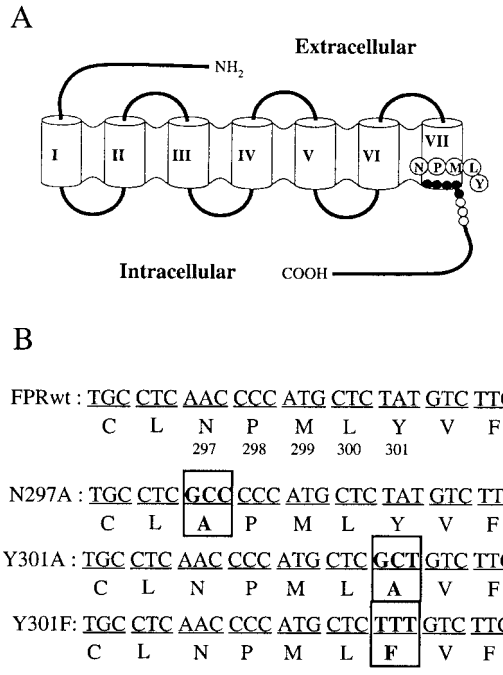

FIGURE 1. A topographical model of the FPR and its NPXXY motif. *A*, A model of the FPR shown with its seven-transmembrane domains numbered. The NPMLY sequences are circled. *B*, The nucleotide and protein sequences of the NPMLY motif, where mutations were introduced at Asn^{297} and Ty^{301} (boxed). The name of each mutant receptor is given on the *left*.

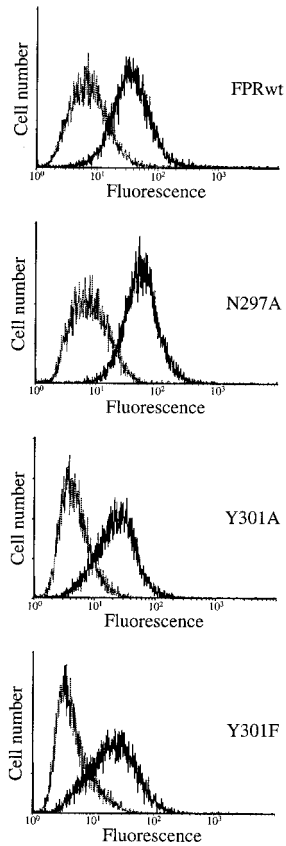

FIGURE 2. Cell surface expression of wild-type and mutant forms of the FPR. RBL cells were transfected with the wild-type FPR (FPRwt), N297A, Y301A, or Y301F mutant constructs. Cell surface expression of FPRwt and the three mutant receptors was detected by flow cytometry with the anti-FPR Ab 5F1 (solid line) or control (dashed line). FITC-conjugated second Ab was used for detection.

Table I. *Binding properties of the wild-type and mutant FPRs*[a]

Receptors	K_d (nM) (mean ± SE)	B_{max} (pmol/mg protein) (mean ± SE)
FPRwt	4.20 ± 0.76	0.37 ± 0.02
N297A	30.22 ± 7.49	0.47 ± 0.05
Y301A	8.20 ± 1.62	0.55 ± 0.03
Y301F	7.88 ± 2.17	0.38 ± 0.03

[a] Direct binding assays were conducted using [^{3}H]fMLF and membranes prepared from transfected RBL-2H3 cells. Data in triplicate were subject to specific binding and Scatchard analyses using Prism software (ver. 3.0).

calcium mobilization. We then investigated the effects of these mutations on activation of PLC-β by measurement of the accumulation of total inositol phosphates (IP) following fMLF stimulation. As illustrated in Fig. 4, fMLF induced a 3.5-fold increase in total IP production in cells expressing the wild-type FPR. Under similar experimental conditions, there were 2.5- and 2.8-fold increases in IP accumulation detected in Y301A and Y301F cells, respectively. Little IP production was seen in the N297A cells following fMLF stimulation. This result when combined with the result shown in Fig. 3, indicates that Asn^{297} is essential for activation of the PLC-β pathway.

To further analyze the different effects of the mutations on FPR signaling, we investigated whether fMLF binding to the mutant receptors could activate the MAP kinases. It has been shown that

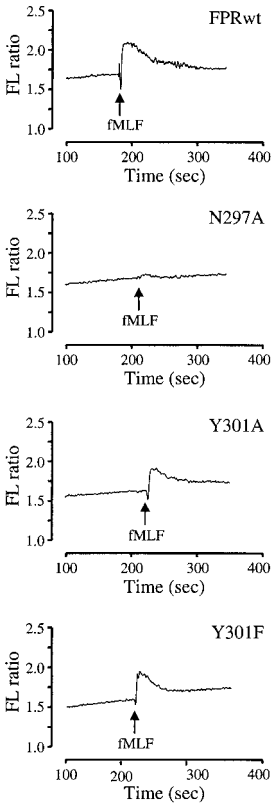

FIGURE 3. Agonist-induced intracellular calcium mobilization in cells expressing FPRwt and the three mutant receptors. The cells were labeled with indo-1/AM and subjected to stimulation with fMLF (100 nM) as marked by arrows. Changes in the intracellular free calcium level were measured every 0.5 s and expressed as a ratio of fluorescence (FL) detected at 405 and 485 nm with an excitation wavelength of 340 nm. Data are from one representative experiment. A total of three independent measurements were made, and similar results were obtained.

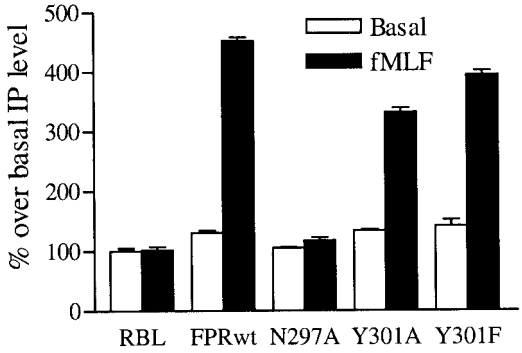

FIGURE 4. Agonist-induced generation of inositol phosphates. The cells expressing FPRwt or the three mutant receptors were labeled overnight with [^{3}H]inositol before a 10-min incubation with (or without) fMLF (100 nM) at 37°C. The accumulated IP were measured in quadruplicate and expressed as a percentage of change over the basal level. Mock-transfected RBL cells were used as controls. A total of three independent experiments were conducted, and results from a representative experiments are shown.

fMLF stimulation of human neutrophils (28, 29) and FPR-transfected cells (30) results in phosphorylation of the p42/44 MAP kinases, extracellular signal-regulated kinases (ERK). To examine ERK activation, we incubated the cells in the presence or the absence of fMLF for 5 min and analyzed the cell lysates by Western blotting using an Ab against the phosphorylated ERKs. As shown in Fig. 5, fMLF stimulated ERK activation in the FPRwt-transfected cells and, to a lesser extent, in cells expressing the Y301F mutant. However, cells that express N297A and Y301A responded poorly to fMLF in the ERK activation assay. It is notable that fMLF at the same concentration (100 nM) induced nearly normal calcium mobilization (Fig. 3) and only slightly reduced IP generation (Fig. 4) in the Y301A-transfected cells. Therefore, the FPR mutations in the NPXXY motif impacted differently on MAP kinase activation and calcium mobilization, although pertussis toxin treatment (200 ng/ml, 16 h) blocked both responses (Fig. 5*B* and data not shown).

Effect of the NPXXY mutations on FPR internalization

To examine whether the NPXXY motif is essential for FPR internalization, we measured the amounts of [^{3}H]fMLF accumulated in the cell following a period of exposure to fMLF. As shown in Fig. 6*A*, after a 90-min incubation with fMLF, internalization of the FPRwt was observed. The amount of [^{3}H]fMLF accumulated in FPRwt cells was approximately 9-fold greater than that in untransfected cells. In comparison, all three mutant FPRs exhibited markedly impaired internalization. Both the N297A and Y301A receptors lost their function of internalization, although some internalization was observed with the Y301F receptor. The significant (~80%) loss of receptor internalization with the Y301F mutation indicates the importance of hydrogen bonding at Tyr^{301} for this receptor-mediated function.

Since the loss of receptor internalization paralleled impairment of MAP kinase activation with all three mutant receptors, we speculated that there might be a correlation between these two events. To examine this possibility, we tested whether a specific inhibitor of MAP kinase kinase, U0126 (31), has an effect on FPR internalization. It was found that treatment of the cells with U0126 (5 μM) inhibited internalization of FPRwt while having less effect on the already reduced internalization of the mutant receptors (Fig. 6*B*). Inhibition of the p42/44 MAP kinase activation by U0126 was confirmed by Western blotting using an anti-phospho-ERK Ab

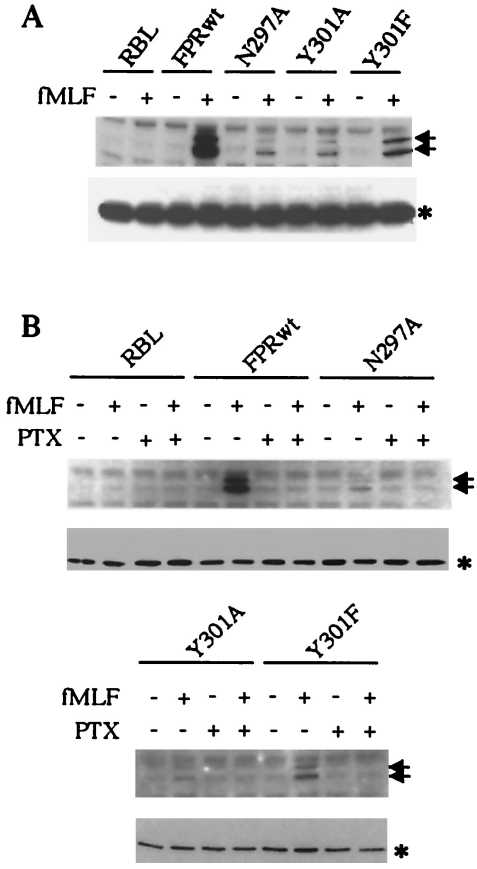

FIGURE 5. Pertussis toxin (PTX)-sensitive activation of the p42/44 MAP kinases. The FPRwt and mutant receptor-expressing cells were treated with (or without) 100 nM fMLF for 5 min at 37°C. Cell lysates were denatured, and soluble proteins were subjected to SDS-PAGE followed by Western blot analysis. The phosphorylated (arrows) and total (*) p42/p44 MAP kinases were detected by anti-phospho-ERK and anti-ERK Abs, respectively. *A*, fMLF stimulated MAP kinase activation in the FPRwt and Y301F cells, but only weakly in the N297A and Y301A cells. *B*, Cells were incubated overnight (16 h) in the absence or the presence of 200 ng/ml pertussis toxin and then stimulated with fMLF for 5 min. Phosphorylation of the p42/44 MAP kinase was inhibited by pertussis toxin in cells expressing either FPRwt or mutant receptors.

(Fig. 6*C*). This finding suggests a possible link between ERK activation and FPR internalization.

Role of the NPXXY motif in chemotaxis

The FPR mediates neutrophil chemotaxis in vivo, and this function can be reconstituted in transfected cells that express the receptor (32–34). We examined whether chemotaxis of FPR-transfected RBL cell is affected by mutations of the NPXXY motif. As shown in Fig. 7, the fMLF-induced chemotactic responses in both FPRwt and Y301F cells followed a normal distribution (bell-shaped) dose curve. The optimal concentration for fMLF to induce chemotaxis in the Y301F cells was 1 order of magnitude higher than that for the FPRwt, but the degree of chemotaxis to fMLF was similar. In contrast, the ability of the N297A cell to undergo chemotaxis was greatly impaired. Of interest is that the Y301A cells responded better to a lower dose of fMLF in chemotaxis assays, although the overall chemotactic response was decreased. Peak chemotaxis for the Y301A-transfected cells was between 0.1 to 1 nM fMLF and was at least 1 order of magnitude lower than the FPRwt and 2 orders of magnitude lower than the Y301F peak values. The discordance between the Y310A and Y301F in mediating chemotaxis

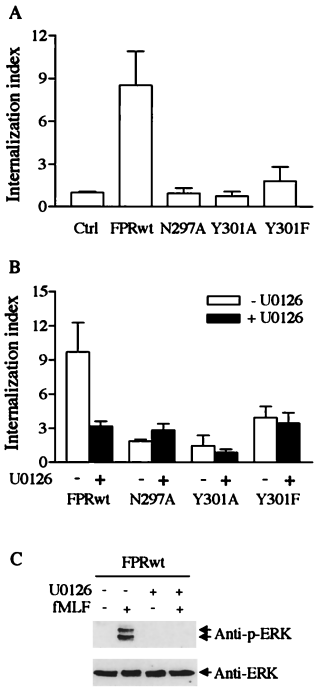

FIGURE 6. Agonist-induced FPR internalization. Receptor internalization was measured as cell-associated radioactivity (with [^{3}H]fMLF, 100 nM, 600 mCi/mmol) minus cell surface binding and expressed as the internalization index (fold increase over basal, as described in *Materials and Methods*). *A*, All three mutant receptors exhibited significantly reduced endocytosis compared with the wild-type FPR. Ctrl, control with untransfected RBL. *B*, Internalization was measured in the absence or the presence of a specific MAP kinase kinase inhibitor, U0126 (5 μM). A total of three experiments were conducted, and data from a representative experiment are shown. With each experiment, triplicate measurements were taken. *C*, Western blot data showing that U0126 at 5 μM blocked phosphorylation of the p42/44 MAP kinase in fMLF-stimulated cells.

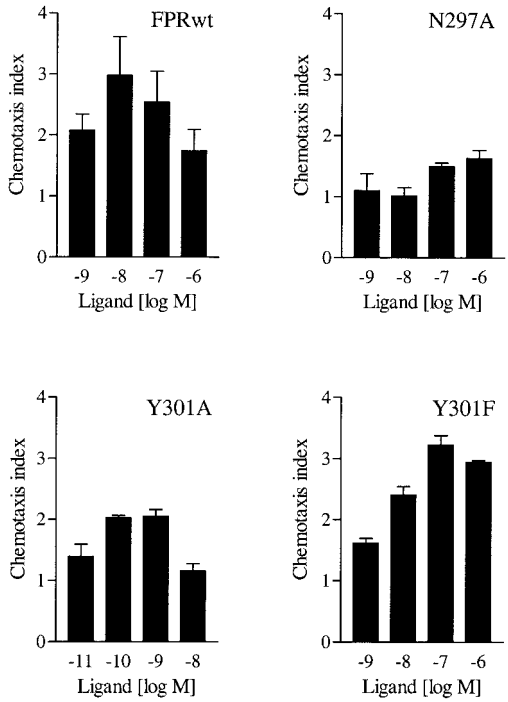

FIGURE 7. Chemotaxis of RBL cells expressing FPRwt and the three mutant receptors. Different concentrations of fMLF were placed in the lower wells of the chemotaxis chamber, and about 35,000 cells were placed in the upper wells. Chemotaxis assay was conducted at 37°C for 4 h, and results are expressed as the chemotaxis index as described in *Materials and Methods*. Data shown are representative of three experiments, each with triplicate measurements. Note that fMLF was present at lower concentrations in chemotaxis assay with the Y301A mutant receptor.

and calcium mobilization suggests that these two functions can be differentially regulated by mutations in the NPXXY motif.

We have shown that inhibition of ERK activation by U0126 blocked FPRwt internalization. It was of interest to determine whether MAP kinase activation is associated with chemotaxis. The FPRwt and cells expressing the three mutant receptors were treated with U0126 before and during chemotaxis assays. Our results indicate that U0126 reduced the degree of chemotaxis of the cells expressing FPRwt by about 15%, but did not change either the optimal concentration of fMLF for chemotaxis or the normal distribution curve of the chemotactic response (data not shown). The chemotactic responses of the cells expressing the three mutant FPRs were essentially unchanged by U0126 treatment (data not shown). We concluded that ERK activation is not essential for FPR-mediated chemotaxis.

Discussion

GPCRs share many biophysical and biochemical properties, probably because of conserved regions or motifs found in their primary structures (5, 35, 36). One such conserved motif is the NPXXY sequence located in the seventh transmembrane domain of these receptors. The NPXXY motif is known primarily as a structure required for receptor internalization (11, 12). In the FPR this motif contains the sequence of NPMLY. The FPR is primarily a GPCR of leukocytes that mediates chemotaxis, degranulation, superoxide production, and transcriptional activation. Thus, it is important to determine whether the NPXXY motif in FPR regulates receptor internalization and transmembrane signal transduction. In the current study selected residues in the NPXXY motif were changed: Asn^{297} to Ala, Tyr^{301} to Ala, and Tyr^{301} to a structurally similar Phe. Cells that express these mutant receptors were analyzed for calcium mobilization, IP generation, MAP kinase activation, internalization, and chemotaxis. Our results indicate that these amino acids in the NPXXY motif play different roles in regulating receptor functions.

Substitution of the Asn residue in the NPXXY motif in different GPCRs has been shown to affect the activation of adenylyl cyclase (37), phospholipase C (38–40), and phospholipase D (41). Asn was also shown to be important for the activation of G_q protein by cholecystokinin B receptor (42). A computational modeling study has shown that the (N/D)P motif introduces a local flexibility in the seventh transmembrane domain of GPCRs that may play a role in receptor activation by serving as a sensitive conformational switch (43). In this study we investigated whether Asn^{297} in the NPXXY motif of the FPR is critical in receptor-mediated signaling. Our results demonstrated that the N297A mutation completely abolished phosphoinositide hydrolysis, MAP kinsae activation, receptor internalization, and chemotaxis. In addition, cells expressing the N297A mutant FPR responded poorly to fMLF stimulation in the calcium mobilization assay. At present it is not clear why the N297A mutant failed to transduce signal, as the receptor is apparently expressed on the cell surface. The lack of responsiveness may be attributed in part to the reduced binding affinity for fMLF. Alternatively, mutation of the Asn residue at position 297 may interrupt G protein coupling, resulting in loss of fMLF-induced cellular functions. This latter possibility may also explain the reduced binding affinity because FPR, like other GPCRs, binds agonist with a higher affinity when it is coupled to G protein. A

recent study, however, suggests that the N297A mutant receptor couples to G protein normally when expressed in Chinese hamster ovary cells (44). It remains to be determined whether the difference comes from the cell lines used in these studies.

It has been shown that the Tyr residue in the NPXXY motif was required for internalization of the β_2-adrenergic receptor (13), but not the angiotensin II receptor (15). In this study we found that mutation of Tyr^{301} to Ala led to a complete loss of fMLF-induced receptor internalization, and the more conservative Phe substitution only restored the internalization level to 20% of the wild-type FPR. Since tyrosine differs from phenylalanine only in the aromatic hydroxyl group, this suggests that hydrogen bonding at Tyr^{301} is important in maintaining a receptor conformation required for efficient internalization. Alternatively, Tyr^{301} may play a role in coupling the receptor to other proteins that dictate receptor internalization. The requirement for other proteins in FPR internalization is also suggested by the finding that, unlike the β_2-adrenergic receptor, FPR does not use arrestin binding for its internalization (19). At present it is not clear whether Tyr^{301} is phosphorylated and therefore facilitates FPR internalization. Tyrosine phosphorylation plays a role in agonist-induced internalization of non-GPCRs (45), and it may also modulate the signal transduction pathway downstream of GPCRs (46). However, in many GPCRs, including the FPR, Ser/Thr phosphorylation of the carboxyl terminus is important for receptor internalization (33). Therefore, although the NPXXY motif in FPR is apparently necessary for internalization, it may not be sufficient. Whether the mutations in the NPXXY motif affect phosphorylation of the FPR is currently under investigation.

A comparison of the signaling properties between the Y301A and Y301F cells revealed marked differences. Whereas both mutant receptors mediated calcium mobilization and activated PLC-β, the Y301A was much less capable of activating the p42/44 MAP kinases. The results from internalization and chemotaxis assays also revealed deficiencies in Y301A. Thus, substitution of Tyr with Ala at position 301 led to more severe impairment of several FPR functions compared with substitution with a structurally similar Phe. It is notable that although the Y301F mutant was only slightly better than the Y301A mutant in receptor internalization and MAP kinase activation assays, it was nearly normal in mediating chemotaxis. The Y301F mutant receptor also displayed a slightly increased basal activity in the inositol phosphate assay (Fig. 4). The mechanism for this change is unknown, but it is possible that mutation of Tyr at position 301 to Phe results in a low level of receptor autoactivation. Together these findings suggest the possibility that fMLF-induced receptor functions, such as calcium mobilization, receptor internalization, and chemotaxis, are differentially regulated by amino acids in the NPXXY motif. As such, the ability of the Y301A mutant receptor to mediate calcium mobilization in response to fMLF (100 nM) did not translate into MAP kinase activation when the cells expressing this mutant receptor were stimulated by fMLF at the same concentration. On the contrary, the Y301A mutant responded better to lower concentrations of fMLF in chemotaxis assays, although the overall chemotaxis response was reduced. Our data are consistent with recent reports that receptor internalization is not a prerequisite for chemotaxis (33, 47).

We found a correlation between MAP kinase activation and receptor internalization, since the integrity of the NPXXY motif in FPR appears to be essential for both functions. The possibility that efficient MAP kinase activation requires receptor internalization was suggested by a study demonstrating that dynamin-mediated endocytosis is essential for activation of the MAP kinase cascade by β_2-adrenergic receptors (48). It appears that β-arrestin recruits Shc to agonist-coupled β_2-adrenergic receptors, which then move to dynamin-regulated and clathrin-coated pits and are internalized to form a scaffold for downstream signaling leading to MAP kinase activation (49). However, there are also conflicting reports. For example, internalization of the α_2-adrenergic receptors is not required for MAP kinase activation, although this process involves both arrestin and dynamin (50). A recent study has also shown that activation of MAP kinase is an internalization-independent event with the opioid receptors (51). Since internalization of the FPR is an arrestin-independent process (19), we believe that MAP kinase activation by FPR does not depend on receptor internalization. On the contrary, MAP kinase may regulate receptor internalization. Our finding that an inhibitor of the MAP kinase pathway blocks FPR internalization provides the first experimental result suggesting a role for MAP kinase activation in GPCR internalization. A recent study of the insulin receptor, which is not coupled to heterotrimeric G proteins but has an NPXY motif, indicates that treatment of the cells with the MAP kinase kinase inhibitor PD98059 blocked its internalization (52). It will be important to further investigate how FPR-mediated MAP kinase activation modulates the internalization process.

In summary, the current study indicates that the NPXXY motif in the FPR not only serves as an essential structure for receptor internalization, but also plays important roles in other receptor-mediated functions. These functions may be differentially regulated by amino acids within the NPXXY motif. Further investigation into how these receptor functions are regulated should help us to better understand the relationships between calcium mobilization, receptor internalization, MAP kinase activation, and chemotaxis.

Acknowledgments

We thank Ping Xie and Ming Yang for helpful discussions, and Laura Viise and Marisa McShane for technical assistance.

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