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Utilization of Two Seven-Transmembrane, G Protein-Coupled Receptors, Formyl Peptide Receptor-Like 1 and Formyl Peptide Receptor, by the Synthetic Hexapeptide WKYMVm for Human Phagocyte Activation[1]

Yingying Le,* Wanghua Gong,† Baoqun Li,† Nancy M. Dunlop,* Weiping Shen,* Shao Bo Su,* Richard D. Ye,‡ and Ji Ming Wang[2]*

Trp-Lys-Tyr-Val-D-Met (WKYMVm) is a synthetic leukocyte-activating peptide postulated to use seven-transmembrane, G protein-coupled receptor(s). In the study to characterize the receptor(s) for WKYMVm, we found that this peptide induced marked chemotaxis and calcium flux in human phagocytes. The signaling induced by WKYMVm in phagocytes was attenuated by high concentrations of the bacterial chemotactic peptide fMLP, suggesting that WKYMVm might use receptor(s) for fMLP. This hypothesis was tested by using cells over expressing genes encoding two seven-transmembrane receptors, formyl peptide receptor (FPR) and formyl peptide receptor-like 1 (FPRL1), which are with high and low affinity for fMLP, respectively. Both FPR- and FPRL1-expressing cells mobilized calcium in response to picomolar concentrations of WKYMVm. While FPRL1-expressing cells migrated to picomolar concentrations of WKYMVm, nanomolar concentrations of the peptide were required to induce migration of FPR-expressing cells. In contrast, fMLP elicited both calcium flux and chemotaxis only in FPR-expressing cells with an efficacy comparable with WKYMVm. Thus, WKYMVm uses both FPR and FPRL1 to stimulate phagocytes with a markedly higher efficacy for FPRL1. Our study suggests that FPR and FPRL1 in phagocytes react to a broad spectrum of agonists and WKYMVm as a remarkably potent agonist provides a valuable tool for studying leukocyte signaling via these receptors. ***The Journal of Immunology*****, 1999, 163: 6777–6784.**

Leukocyte infiltration is an important feature of host response to invading microbial pathogens. Numerous exogenously and endogenously produced leukocyte chemoattractants that initiate leukocyte migration and activation have been identified. They include several classical chemotactic factors such as the bacterial derived fMLP, activated complement component 5 (C_{5a}), and leukotriene B_4, as well as a superfamily of chemokines that selectively induce infiltration, trafficking, and homing of leukocyte subpopulations (1–5). Both classical chemoattractants and chemokines bind and activate G protein-coupled, seven-transmembrane (STM)[3] cell receptors (3–5). The activation of these STM receptors by chemotactic agonists results in a G protein-mediated signaling cascade that promotes cellular calcium (Ca^{2+}) mobilization, phosphoinositide hydrolysis, chemotaxis, and activation of mitogen-activated protein kinase (1–5). The cells thus activated are essential constituents of host defense.

In addition to bacterium- and tissue-derived chemoattractants, many synthetic peptides that exhibit chemotactic activity for leukocytes have been discovered. In fact, one of the most potent phagocyte chemotactic factors, fMLP, was synthesized even before its isolation from Gram-negative bacterial supernatants (1). These synthetic chemotactic peptides have become very useful probes for the study of leukocyte receptor expression and activation. Recently, WKYMVm, a hexapeptide isolated and modified from a peptide library, has been reported to be a very potent stimulant of several human leukocytic cell lines as well as peripheral blood neutrophils (6–9). This peptide stimulated a typical STM receptor-mediated signaling pathway, including the activation of phospholipase C, phosphoinositide hydrolysis, and Ca^{2+} mobilization in target cells. Since the signaling of WKYMVm in human cells was not cross-desensitized by a number of known chemoattractants, including fMLP, C_{5a}, platelet-activating factor, and the chemokine IL-8, it was postulated that this peptide may use an undefined leukocyte STM receptor (7, 8). Due to the high potency of WKYMVm in leukocyte activation, we considered it desirable to identify the leukocyte receptor(s) that might be utilized by this peptide and to explore its potential as a potent inducer of cell signaling and desensitization. In this study, we report that MKYMVm uses two STM receptors expressed by phagocytic leukocytes for its chemotactic and calcium (Ca^{2+})-mobilizing activity: the high affinity fMLP receptor FPR and the low affinity fMLP receptor FPRL1, which has been identified as an efficacious receptor mediating the chemotactic and Ca^{2+}-mobilizing activity of serum amyloid A (SAA) (10–12) and a synthetic peptide domain of HIV-1 envelope gp41 (13). Since the peptide WKYMVm

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[3] Abbreviations used in this paper: STM, seven-transmembrane; CI, chemotaxis index; FPR, formyl peptide receptor; FPRL1, formyl peptide receptor-like 1; LXA_4, lipoxin A_4; SAA, serum amyloid A.

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exhibits an extraordinarily high efficacy on FPRL1, it could be used as a very useful agent in the study of FPRL1 signaling.

Materials and Methods

Chemicals

The WKYMVm (Trp-Lys-Tyr-Met-Val-D -Met, designated W peptide) was synthesized and purified by the Department of Biochemistry, Colorado State University (Fort Collins, CO), according to the published sequence (7). The purity was greater than 90%, and the amino acid composition was verified by mass spectrometer. The endotoxin levels in the dissolved peptide were undetectable. The synthetic formyl peptide fMLP was purchased from Sigma (St. Louis, MO). Tritiated [^{3}H]fMLP was purchased from Dupont NEN (Boston, MA).

Cells

The human PBMC were isolated from leukopacks through the courtesy of Transfusion Medicine Department, National Institute of Health Clinical Center (Bethesda, MD). Monocytes were further purified by elutriation to yield >90% pure preparations. Human neutrophils were purified from the same leukopacks by 3% dextran sedimentation with a purity of >98%. Rat basophilic leukemia cells stably transfected with epitope-tagged high affinity fMLP receptor FPR (designated ETFR cells) were a kind gift of Drs. H. Ali and R. Snyderman, Duke University (Durham, NC). Human embryonic kidney 293 cells stably expressing FPRL1 (designated FPRL1/293 cells) were a kind gift of Drs. P. M. Murphy and J.-L. Gao (National Institute of Allergy and Infectious Diseases, Bethesda, MD). All of the transfected cells were maintained in DMEM, 10% FCS, and 0.8 mg/ml geneticin (G418; Life Technologies, Rockville, MD).

Chemotaxis

Migration of leukocytes, ETFR, and FPRL1/293 cells was assessed using a 48-well microchemotaxis chamber technique, as previously described (14–16). Different concentrations of stimulants were placed in wells of the lower compartment of the chamber (Neuro Probe, Cabin John, MA); the cell suspension was seeded into wells of the upper compartment, which was separated from the lower compartment by a polycarbonate filter (Osmonics, Livermore, CA; 5 μm pore size for leukocytes, 10 μm pore size for ETFR and FPRL1/293 cells). The filters for ETFR and FPRL1/293 cell migration were precoated with 50 μg/ml collagen type I (Collaborative Biomedical Products, Bedford, MA) to favor cell attachment. After incubation at 37°C (90 min for monocytes, 60 min for neutrophils, and 300 min for ETFR or FPRL1/293 cells), the filters were removed and stained, and the number of cells migrating across the filter were counted by light microscopy after coding the samples. The experiments were performed at least five times with each cell type and the results are presented as the chemotaxis indexes (CI) representing the fold increase in the number of migrating cells in response to stimuli, over the spontaneous cell migration (in response to control medium). The CI of 1.8 or greater are statistically significant compared with cell migration in the absence of chemoattractant, as assessed by Student's *t* test.

Calcium mobilization

Calcium mobilization was assayed by incubating 10^7/ml of monocytes, neutrophils, FPRL1, or FPR transfectants in loading buffer containing 138 mM NaCl, 6 mM KCl, 1 mM $CaCl_2$, 10 mM HEPES (pH 7.4), 5 mM glucose, 0.1% BSA, with 5 μM fura 2 (Sigma) at 37°C for 30 min. The dye-loaded cells were washed and resuspended in fresh loading buffer. The cells were then transferred into quartz cuvettes (10^6 cells in 2 ml), which were placed in a luminescence spectrometer LS50 B (Perkin-Elmer Limited, Beaconsfield, U.K.). Stimulants at different concentrations were added in a volume of 20 μl to the cuvettes at indicated time points. The ratio of fluorescence at 340 and 380 nm wavelength was calculated using the FL WinLab program (Perkin-Elmer).

Binding assays

A single concentration of [^{3}H]fMLP was added simultaneously with different concentrations of unlabeled fMLP or W peptide to a cell suspension (FPRL1/293 or ETFR, 2×10^6 cells/200 μl in RPMI 1640, 1% BSA, and 0.05% NaN_3) in duplicate samples. The samples were incubated under constant rotation for 30 min at 37°C. After incubation, the samples were filtered onto Whatman GF/C discs (Whatman International, Kent, U.K.) on a 12-well manifold, followed by extensive washing with ice-cold PBS. The discs were air dried at 65°C, submerged in liquid scintillation mixture, and counted for β emission.

Statistical analysis

Unless specified, all experiments were performed three to five times and the results presented are from representative experiments. The significance of the difference between test and control groups was analyzed with a Student's *t* test.

Results

W peptide has been reported to induce a series of signaling events in human leukocytes that were probably mediated by G protein-coupled STM receptors (6–9). However, there have been no reports showing this peptide could induce leukocyte migration, a

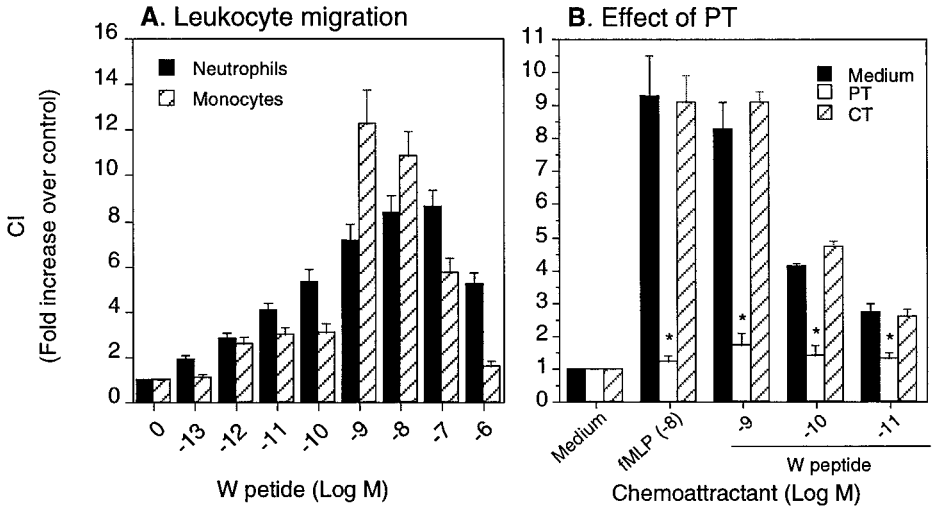

FIGURE 1. Induction of phagocyte migration by W peptide. Different concentrations of W peptide were placed in the lower wells of the chemotaxis chamber; cell suspension was placed in the upper wells. The upper and lower wells were separated by polycarbonate filters. After incubation, the cells migrated across the filters were stained and counted. *A*, Fold increase of monocyte and neutrophil migration in response to W peptide over control medium. Chemotaxis index greater than 1.8 is statistically significant compared with spontaneous migration in the absence of chemoattractant. *B*, Inhibition of monocyte migration in response to W peptide by pretreatment of the cells with 100 ng/ml pertussis toxin (PT) or cholera toxin (CT) at 37°C for 30 min. fMLP was used as a control. *, $p < 0.01$ compared with migration of cells incubated with medium alone.

Table I. *Checkerboard analysis of monocyte migration in response to W peptide*[a]

W Peptide in Lower Wells (M)	Number of Migrated Cells in 1 HPF (mean ± SE)			
	W peptide in upper wells (M)			
	Medium	10^{-10}	10^{-9}	10^{-8}
Medium	26 ± 3	31 ± 3	28 ± 3	29 ± 2
10^{-10}	84 ± 5*	52 ± 6	47 ± 3	31 ± 3
10^{-9}	183 ± 11*	160 ± 13*	120 ± 15*	49 ± 4
10^{-8}	326 ± 21*	297 ± 32*	236 ± 5*	105 ± 6*

[a] Different concentrations of W peptide were placed in the upper and/or lower wells of the chemotaxis chamber; monocytes at 2×10^6/ml were placed in the upper wells. The upper and lower wells were separated by a polycarbonate filter. After incubation, the nonmigrating cells were removed, the filter was fixed and stained, and the cells migrated across the filter were counted in three high-powered fields (HPF, ×400). The results are expressed as the mean number (±SE) of the cells in 1 HPF.

*, $p < 0.01$ compared with migration in the presence of medium alone in both upper and lower wells as determined by Student's *t* test.

feature commonly associated with STM receptor activation and a crucial step for cell accumulation at sites of inflammation or injury. We therefore first tested the ability of W peptide to induce migration of human phagocytes. Human peripheral blood monocytes and neutrophils migrated in a dose-dependent manner in response to a concentration gradient of W peptide (Fig. 1*A*). The chemotactic activity of W peptide was very potent and already evident at picomolar concentrations for both monocytes and neutrophils. The dose-response curves are bell shaped with maximal cell migration at low nanomolar peptide concentrations. We next examined whether phagocyte migration induced by W peptide was based on chemotaxis or chemokinesis. Checkerboard analyses showed that monocytes migrated well only when higher concentrations of W peptide were present in the lower wells of the chemotaxis chamber (Table I). There was no increased cell migration when higher concentrations of W peptide were present in the upper wells. Equal concentrations of W peptide in both upper and lower wells induced a slight but significant increase in cell migration. These results suggest that the cell migration induced by W peptide was mainly due to a chemotactic effect with a minor contribution of chemokinesis.

The migration of phagocytes to W peptide was completely inhibited by pretreatment of the cells with pertussis toxin, but not by cholera toxin (Fig. 1*B*) or herbimycin A (data not shown), suggesting that a G_i protein-coupled receptor was involved (2–5, 17–19). This was supported by the potent induction of a dose-dependent, and pertussis toxin-sensitive, Ca^{2+} mobilization in monocytes and neutrophils by W peptide (Fig. 2, *A* and *E*, and data

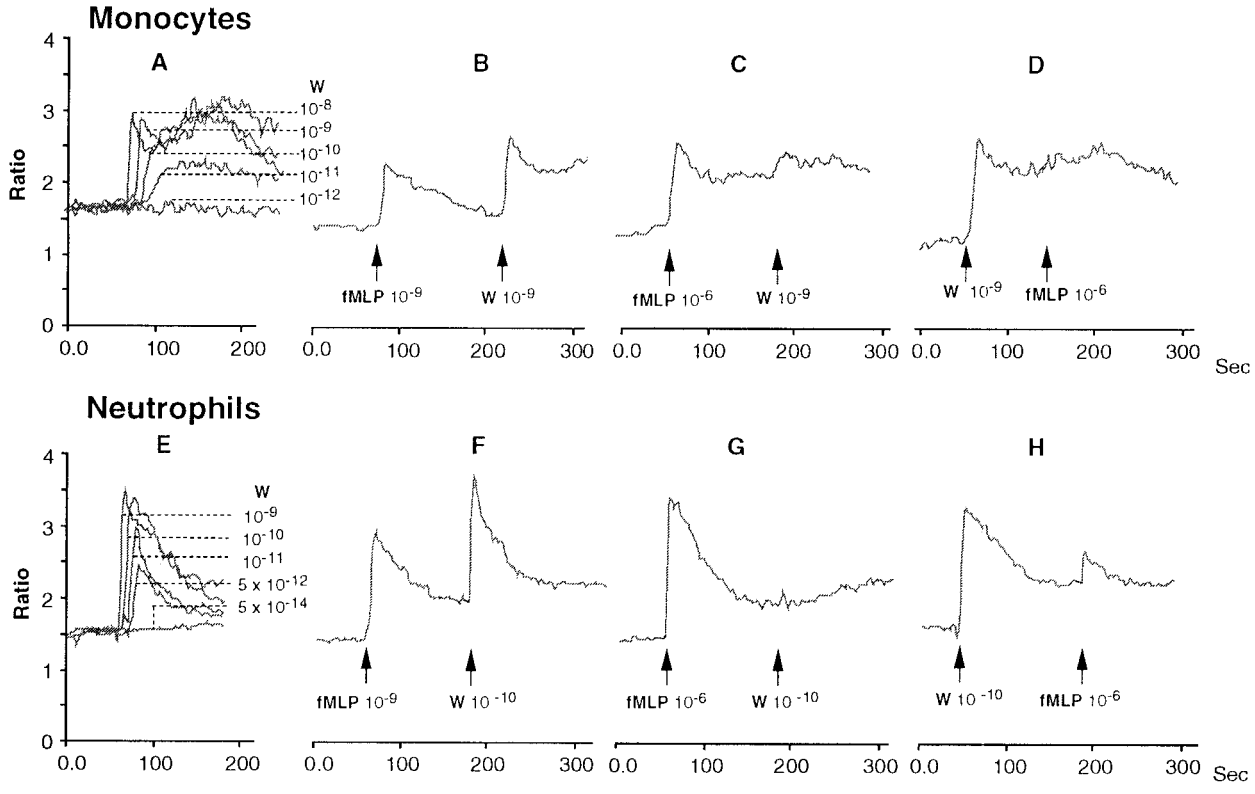

FIGURE 2. Ca^{2+} mobilization in phagocytes induced by W peptide. Human monocytes or neutrophils were loaded with fura 2 and then were stimulated with various concentrations of W peptide (*A* and *E*). The ratio of fluorescence at 340 and 380 nm wavelength was calculated using the FLWinLab program. Desensitization of W peptide-induced Ca^{2+} flux by fMLP in monocytes (*B* and *C*) or neutrophils (*F* and *G*) was measured by sequentially stimulating the cells with both agonists and vice versa (*D* and *H*).

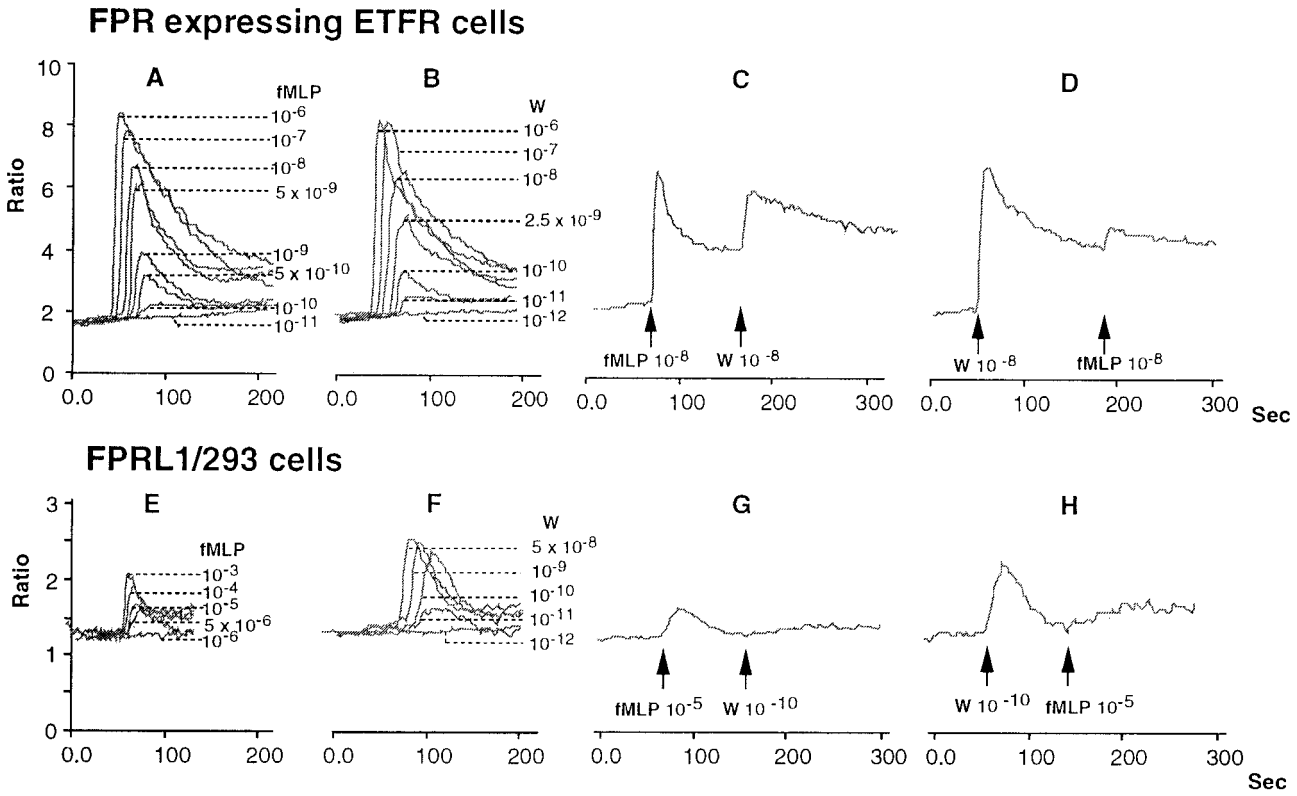

FIGURE 3. Calcium mobilization in ETFR and FPRL1/293 cells induced by W peptide. ETFR cells (*upper panels*) and FPRL1/293 cells (*lower panels*) were used to evaluate Ca^{2+} flux induced by fMLP (*A* and *E*) or W peptide (*B* and *F*). *C*, *D*, *G*, and *H* show cross-desensitization of cell signaling between W peptide and fMLP.

not shown). In agreement with the chemotactic activity, the Ca^{2+}-mobilizing activity of W peptide was also significant at picomolar concentrations. These results confirm the original reports that W peptide is a potent activator of phagocytic cells (6–9) and extended the observation of function of this peptide as a chemoattractant of monocytes and neutrophils.

To characterize the molecular nature of the receptor(s) possibly used by W peptide on phagocytic cells, a series of experiments examining cross-desensitization of Ca^{2+} mobilization were performed using a variety of chemoattractants. The Ca^{2+} flux induced by W peptide in monocytes or neutrophils was not desensitized by chemokines such as monocyte chemoattractant protein-1, RANTES, monocyte chemoattractant protein-3, macrophage-inflammatory protein-1α, IL-8, and stromal cell-derived factor 1α, or vice versa (data not shown), suggesting W peptide does not share a receptor with any of these chemokines. Low concentrations (in nM range) of fMLP also had a limited effect on W peptide-induced Ca^{2+} flux (Fig. 2, *B* and *F*). This is in agreement with the previous reports that fMLP in such concentration range did not desensitize the cell response to W peptide (7, 8). However, when the concentrations of fMLP were increased to the micromolar range, the cell response to W peptide in monocyte (Fig. 2*C*) or neutrophils (Fig. 2*G*) was significantly attenuated. Moreover, W peptide was able to desensitize the Ca^{2+} flux response to 10^3–10^4-fold higher concentrations of fMLP in both monocytes (Fig. 2*D*) and neutrophils (Fig. 2*H*). These results strongly suggest that W peptide shares receptor(s) with fMLP on human phagocytic cells and activates the receptor(s) with much higher efficacy than fMLP.

Since fMLP was known to induce Ca^{2+} flux through at least two STM, G protein-coupled receptors, the high affinity FPR and the low affinity FPRL1 (4, 17, 20), we tested the effect of W peptide on these two receptors transfected and overexpressed in human cells that originally were not responsive to fMLP stimulation. fMLP over a wide range of concentrations induced Ca^{2+} mobilization in an FPR-transfected rat basophilic leukemia cell line (ETFR cells), with a minimal effective dose at 10^{-10} M (Fig. 3*A*). In contrast, the minimal effective concentration for fMLP to induce Ca^{2+} mobilization in FPRL1-transfected cells (FPRL1/293 cells) was in the μM range (Fig. 3*E*). These results confirmed the previous conclusion that FPR is a high affinity receptor for fMLP, whereas FPRL1 has much lower affinity for fMLP (4, 17, 20). The W peptide also induced Ca^{2+} mobilization in cells transfected with either of these receptors (Fig. 3, *B* and *F*). However, the minimal effective doses for W peptide to activate both FPR and FPRL1 were at 10^{-11} M, suggesting that W peptide activate these receptors with higher efficacy. This was further supported by cross-desensitization of Ca^{2+} flux between W peptide and fMLP in both receptor transfectants. As shown in Fig. 3, *G* and *H*, although sequential stimulation of the cells expressing FPRL1 with W peptide and fMLP resulted in bidirectional desensitization, a 10^5-fold excess of fMLP was required to desensitize the effect of W peptide in FPRL1/293 cells. Likewise, in ETFR cells, with equal concentrations, W peptide also more potently desensitized the effect of fMLP (Fig. 3, *C* and *D*). In control experiments, W peptide and fMLP did not induce any Ca^{2+} mobilization in parental or mock-transfected rat basophilic cell line and human embryonic kidney 293 cells (data not shown). Our results indicate that W peptide activates both FPR and FPRL1 with high potency, whereas fMLP is a highly efficacious agonist only for FPR.

The migration of the cells transfected with FPR or FPRL1 was tested to establish the contribution of each of these receptors to the cell chemotaxis in response to W peptide. FPRL1/293 cells showed a marked migratory response to W peptide with an EC_{50} of ~1 pM (Fig. 4, *A* and *B*), but these cells failed to migrate in

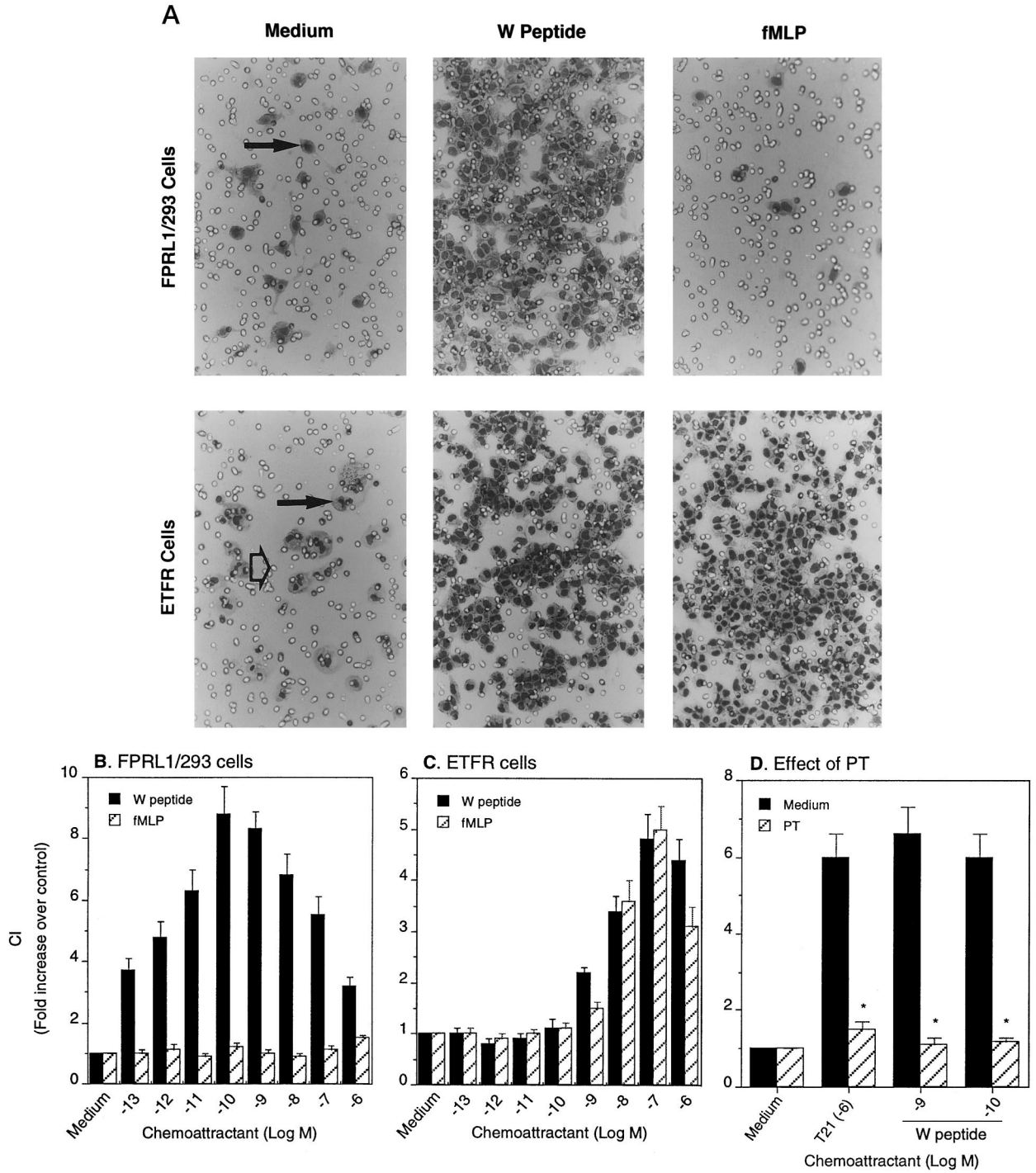

FIGURE 4. Migration of FPRL1/293 and ETFR cells in response to W peptide. *A*, Visualization (magnification: ×200) of FPRL1/293 (*upper panels*) and ETFR (*lower panels*) cell migration in response to control medium (Medium), W peptide and fMLP (10^{-8} M for ETFR cells, 10^{-9} M for FPRL1/293 cells). Black arrows in the figure denote cells migrated across the filters; the open arrow in the *left panel* indicates one of the micropores in the filter. *B* and *C*, Fold increase (chemotaxis index) of FRPL1/293 cell or ETFR cell migration in response to W peptide or fMLP over control medium. A CI greater than 1.8 was statistically significant compared with spontaneous cell migration in the absence of chemoattractant. *D*, Inhibition of FPRL1/293 cell migration in response to W peptide by pretreatment of the cells with 100 ng/ml pertussis toxin (PT) at 37°C for 30 min. T21, a FPRL1-stimulating peptide domain of HIV-1 envelope gp 41, was used as a control. ∗, $p < 0.01$ compared with migration of cells incubated with medium alone.

response to a wide concentration range of fMLP (Fig. 4, *A* and *B*). On the other hand, both fMLP and W peptide induced the migration of the ETFR cells with comparable dose-response curves (Fig. 4, *A* and *C*). These chemotaxis experiments indicate that fMLP is only a partial and low affinity agonist for FPRL1 since it does not induce migration of FPRL1-expressing cells. W peptide, on the other hand, appears to be a very efficient agonist for both FPR and FPRL1, with even higher efficacy for FPRL1 than FPR, as low picomolar concentrations of W peptide are sufficient to induce both Ca^{2+} flux and chemotaxis in these cells. As observed with phagocytic cells, the migration of FPRL1/293 and ETFR cells induced by W peptide was also inhibited by pretreatment of the cells with

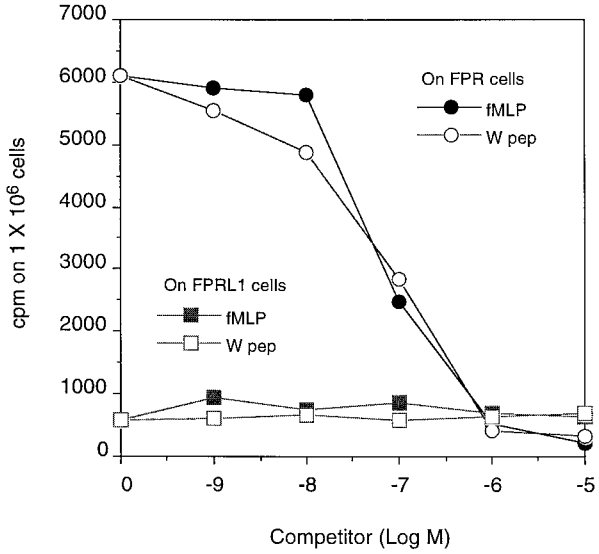

FIGURE 5. Displacement of [^{3}H]fMLP binding to ETFR or FPRL1/293 cells by W peptide. Aliquots of cells (2×10^6/200 μl) were incubated with constant concentrations of [^{3}H]fMLP (0.2 nM) in the presence of different concentrations of unlabeled fMLP or W peptide at 37°C for 30 min. The cells were then washed with ice-cold PBS and filtered onto Whatman discs. The radioactivity associated with cells was measured with a beta counter. Three experiments were performed yielding similar results.

pertussis toxin (Fig. 4D and data not shown). To further confirm that W peptide shares receptors with fMLP, we used ligand-binding competition experiments with ^{3}H-labeled fMLP. In agreement with the chemotactic and Ca^{2+} mobilization activity for the ETFR cells, W peptide effectively competed with [^{3}H]fMLP for binding to ETFR cells (Fig. 5). [^{3}H]fMLP did not significantly bind to FPRL1/293 cells, presumably due to its low affinity for FPRL1 (Fig. 5).

Discussion

W peptide was derived from a biologically active peptide WKYMVM-NH2 (7) isolated from a peptide library. WKYMVM-NH2 stimulates phosphoinositide hydrolysis in a human B cell line, and based on its inhibition by pertussis toxin, the usage of a G_i protein-coupled, STM receptor was postulated (6). A modification of the methionine at the NH2 end with D-type amino acid yielded WKYMVm (W peptide), which exhibited more than 100-fold increase in its biological effect than its prototype WKYMVM-NH2 and stimulated a variety of STM, G protein receptor-mediated signaling events in human monocytes (9) and neutrophils (7) in addition to B lymphocytes. The possible homology of W peptide with bacterium- and tissue-derived molecules has not been identified as yet. Also, the D-amino acid modification will not occur under physiological conditions, yet may render the peptide resistant to proteolytic cleavage. W peptide increases the Ca^{2+} mobilization, phospholipase D activation, superoxide generation, bactericidal activity of human leukocytes, as well as phagocyte migration as shown in this study, features typical of cell activation through STM chemoattractant receptors. It has been proposed that W peptide use a receptor different from those of fMLP, platelet-activating factor, or chemokines (7). This conclusion was derived from the failure of a saturable concentration of fMLP (up to 10^{-6} M) as well as other chemoattractants to attenuate the cell response to relatively high concentrations of W peptide (at high nanomolar range). In fact, W peptide is chemotactic and also a highly efficacious inducer of Ca^{2+} flux in both human monocytes and neutrophils and is active over a picomolar to low nanomolar concentration range. A more careful evaluation of cross-desensitization of Ca^{2+} flux in our study revealed that fMLP at micromolar concentrations was able to attenuate the cell response to a 10^3-10^4-fold lower concentration of W peptide. These results led to our identification of two fMLP receptors used by W peptide.

Leukocyte infiltration at the sites of inflammation in vivo is believed to be a consequence of cell migration toward a gradient of chemoattractant(s), either derived from microorganisms or the local tissues. The discovery of synthetic *N*-formyl oligopeptide chemoattractants for phagocytes greatly facilitated the study of leukocyte locomotion (1). Several natural *N*-formyl peptide chemoattractants, including the prototype fMLP, have subsequently been purified from bacterial supernatants, providing evidence in support of them being biologically relevant ligands for formyl peptide receptors on phagocytic cells. Mitochondrial proteins are also *N* formylated and are chemotactic for neutrophils (21), and thus may constitute an endogenous source of chemotactic peptides released by damaged tissue. Although the *N*-formyl group was considered essential for optimal agonist potency (22), nonformylated peptides may also attract and activate phagocytes (4, 17). The prototype receptor for formyl peptides designated FPR is expressed by neutrophils and monocytes and was cloned several years ago (4, 17). FPR was subsequently shown to have a much broader spectrum of agonists than initially expected. In fact, the synthetic pentapeptide Met-Nle-Leu-Phe-Phe-OH, either *N* formylated or *N* acetylated, is more potent than the parental prototype fMLP in the induction of Ca^{2+} flux in human neutrophils (19). Amino-terminal urea-substituted and carbamate-modified peptides are also potent agonists for FPR (23, 24). In our study, W peptide does not contain any modification groups at the N terminus, yet exhibits comparable Ca^{2+}-mobilizing and chemotactic activity as fMLP on FPR. These observations support the hypothesis derived from structural analysis of FPR that the binding pocket of this receptor is able to accommodate larger amino-terminal groups (23, 24), accounting for the capacity of this receptor to interact with a great variety of endogenously derived as well as exogenous peptide ligands.

FPRL1 was cloned from human phagocytic cells (20, 25–27) and a genomic library (28). FPRL1 possesses 69% identity at the amino acid level to FPR (18, 19), and both receptors are expressed by monocytes and neutrophils and are clustered on human chromosome 19q13 (28, 29). While fMLP is a high affinity agonist for FPR, it interacts with and induces Ca^{2+} flux in FPRL1 only at high concentrations (Fig. 3*E* and Refs. 20, 26, 29) and did not induce significant migration of FPRL1/293 cells at a wide range of concentrations tested (Fig. 4*B* and Ref. 10). In addition, [^{3}H]fMLP bound only to FPR, but not to FPRL1-transfected cells, as shown in this study. Thus, fMLP is not an efficacious agonist for FPRL1. In contrast, W peptide, although also activating both FPR and FPRL1, showed a much higher efficacy than fMLP on FPRL1 and induced migration and Ca^{2+} flux in FPRL1/293 cells at low picomolar concentrations, suggesting W peptide has even greater efficacy on FPRL1 than on FPR. This may also explain the failure for fMLP to desensitize Ca^{2+}-mobilizing activity in phagocytes when W peptide was used at high nanomolar concentrations (7). FPRL1 is expressed by a variety of cell types, including monocytes, neutrophils, and cells other than the hemopoietic origin, such as hepatocytes (17). The expression of this receptor was also highly inducible in epithelial cells by selected cytokines such as IL-13 and IFN-γ (30). Since W peptide was also reported to promote phosphoinositol hydrolysis in B lymphoma cells (7), we tested the chemotactic activity of this peptide on peripheral B cells and found a

significant migration of B cells in response to low nanomolar concentrations of W peptide (data not shown). Preliminary experiments suggest that human B lymphocytes express FPRL1, but not FPR transcripts (Le et al., data not shown). Due to the expression of FPRL1 in a great variety of cell types, it may play an important role in inflammatory and immunological responses. In support of this hypothesis, we recently identified FPRL1 to be a functional receptor for a normal serum protein, SAA (10), which increases its concentration by up to several hundred-fold during acute phase responses, and is a potent phagocyte chemoattractant and activator (11, 12). We also identified several synthetic peptide domains derived from HIV-1 envelope proteins as activators of FPRL1 (13, 31). Moreover, several peptides derived from a plasmid-based random library have been shown also to be highly efficacious agonists for FPRL1 (32). However, W peptide does not bear any significant sequence homology to either fMLP, SAA, or HIV-1 envelope protein domains. Therefore, FPRL1, like its prototype FPR, has the capacity to interact with a broad spectrum of agonists.

In addition to peptide and protein agonists, a lipid metabolite lipoxin A_4 (LXA_4) has been reported to be a high affinity ligand and potent agonist for FPRL1 (also thus termed LXA_4R) (33). LXA_4 is an eicosanoid generated during a number of host reactions such as inflammation, thrombosis, and atherosclerosis, and was initially discovered as an inhibitor of immune responses (reviewed in Ref. 34). LXA_4 was subsequently reported to inhibit neutrophil chemotaxis (35) and transepithelial migration induced by chemotactic agents (36). LXA_4 bound to CHO cells transfected with FPRL1(LXA_4R) with high affinity and increased GTPase activity and the release of esterified arachidonate (33). Thus, LXA_4 has been proposed to be an endogenously produced ligand for FPRL1 (33, 37). Although LXA_4 has not been documented to induce Ca^{2+} mobilization in neutrophils or FPRL1-transfected cells (33), it was reported to induce Ca^{2+} flux and chemotaxis in monocytes presumably through FPRL1 (38, 39). Based on these observations, differential activation of second messengers in monocytes vs neutrophils by LXA_4 was postulated. Further study is needed to elucidate the structural basis for the interaction of FPRL1 with unrelated protein/peptide ligands such as W peptide, SAA, HIV-1 envelope protein domains, and fMLP, vs the lipid ligand LXA_4.

The signal transduction pathways utilized by FPRL1 have not been extensively studied. However, the high level of homology to FPR, sensitivity to inhibition by pertussis toxin, and potent induction of phagocyte migration and activation by their agonists suggest that FPRL1 and FPR may share many signal transduction steps following activation. The binding of FPR by agonists results in a G protein-mediated signaling cascade, which results in cell adhesion, chemotaxis, release of oxygen intermediates, enhanced phagocytosis, and bacterial killing, as well as mitogen-activated protein kinase activation and increased gene transcription (4, 17). Activation by fMLP can lead to heterologous desensitization of the subsequent cell response to other G protein receptor ligands (40, 41), including chemokines. In our previous study, incubation of human monocytes with a synthetic FPRL1-specific peptide domain of HIV-1 gp120 resulted in down-regulation of chemokine receptors CCR5 and CXCR4 (31), and a reduced cell response to a number of chemoattractants (12). It is therefore reasonable to propose that activation of FPRL1 by W peptide may also activate signaling events that culminate in heterologous desensitization of other G protein-coupled chemotactic receptors. Whether W peptide will be potent desensitizer of CCR5 and CXCR4 thus inhibits M-tropic or T-tropic HIV fusion/entry is being investigated.

After studies of considerable number of peptide variants, W peptide has been identified as one of the most potent activators of phagocyte chemotaxis and Ca^{2+} flux, in addition to reported activation of phospholipase D, phosphoinositide hydrolysis, and bacterial killing of phagocytes (6–9). In receptor-transfected cells, W peptide showed similar potency as fMLP in activating FPR, but it is far more potent than fMLP in activating FPRL1. In fact, among the chemotactic and Ca^{2+}-mobilizing agonists identified to date for FPRL1 in this laboratory, W peptide is the most efficacious, acting at picomolar concentrations. Therefore, this peptide will provide a very useful probe for the study of leukocyte receptor expression, activation, and desensitization. Furthermore, immunomodulatory agents can be designed based on the structure-function property of W peptide.

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References

1. Schiffmann, E., B. A. Corcoran, and S. M. Wahl. 1975. *N*-formylmethionyl peptides as chemoattractants for leukocytes. *Proc. Natl. Acad. Sci. USA 72:1059.*
2. Oppenheim, J. J., C. O. Zachariae, N. Mukaida, and K. Matsushima. 1991. Properties of the novel proinflammatory supergene "intercrine" cytokine family. *Annu. Rev. Immunol. 9:617.*
3. Murphy, P. M. 1994. The molecular biology of leukocyte chemoattractant receptors. *Annu. Rev. Immunol. l2:593.*
4. Murphy, P. M. 1996. The *N*-formyl peptide chemotactic receptors. In *Chemoattractant Ligands and Their Receptors.* CRC Press, Boca Raton, p. 269.
5. Balkwill, F. 1998. The molecular and cellular biology of the chemokines. *J. Viral Hepatitis. 5:1.*
6. Baek, S. H., J. K. Seo, C. B. Chae, P. G. Suh, and S. H. Ryu. 1996. Identification of the peptides that stimulate the phosphoinositide hydrolysis in lymphocyte cell lines from peptide libraries. *J. Biol. Chem. 271:8170.*
7. Seo, J. K., S. Y. Chio, Y. Kim, S. H. Baek, K. T. Kim, C. B. Chae, J. D. Lambeth, P. G. Suh, and S. H. Ryu. 1997. A peptide with unique receptor specificity: stimulation of phosphoinositide hydrolysis and induction of superoxide generation in human neutrophils. *J. Immunol. 158:1895.*
8. Seo, J. K., Y. S. Bae, H. Song, S. H. Baek, B. S. Kim, W. S. Choi, P. G. Suh, and S. H. Ryu. 1998. Distribution of the receptor for a novel peptide stimulating phosphoinositide hydrolysis in human leukocytes. *Clin. Biochem. 31:137.*
9. Bae, Y. S., S. A. Ju, J. Y. Kim, J. K. Seo, S. H. Baek, J. Y. Kwak, B. S. Kim, P. G. Suh, and S. H. Ryu. 1999. Trp-Lys-Tyr-Met-Val-D-Met stimulates superoxide generation and killing of *Staphylococcus aureus* via phospholipase D activation in human monocytes. *J. Leukocyte Biol. 65:241.*
10. Su, S. B., W. Gong, J.-L. Gao, W. Shen, P. M. Murphy, J. J. Oppenheim, and J. M. Wang. 1999. A seven-transmembrane, G-protein-coupled receptor, FPRL1, mediates the chemotactic activity of serum amyloid A for human phagocytic cells. *J. Exp. Med. 189:395.*
11. Badolato, R., J. M. Wang, W. J. Murphy, A. R. Lloyd, D. F. Michiel, L. L. Bausserman, D. J. Kelvin, and J. J. Oppenheim. 1994. Serum amyloid A is a chemoattractant: induction of migration, adhesion, and tissue infiltration of monocytes and polymorphonuclear leukocytes. *J. Exp. Med. 180:203.*
12. Badolato, R., J. A. Johnston, J. M. Wang, D. McVicar, L. L. Xu, J. J. Oppenheim, and D. J. Kelvin. 1995. Serum amyloid A induces calcium mobilization and chemotaxis of human monocytes by activating a pertussis toxin-sensitive signaling pathway. *J. Immunol. 155:4004.*
13. Su, S. B., J. Gao, W. Gong, N. M. Dunlop, P. M. Murphy, J. J. Oppenheim, and J. M. Wang. 1999. T21/DP107, a synthetic leucine zipper-like domain of the HIV-1 envelope gp41, attracts and activates human phagocytes by using G-protein-coupled formyl peptide receptors. *J. Immunol. 162:5924.*
14. Gong, W., O. M. Howard, J. A. Turpin, M. C. Grimm, H. Ueda, P. W. Gray, C. J. Raport, J. J. Oppenheim, and J. M. Wang. 1998. Monocyte chemotactic protein-2 activates CCR5 and blocks CD4/CCR5-mediated HIV-1 entry/replication. *J. Biol. Chem. 273:4289.*
15. Gong, X., W. Gong, D. B. Kuhns, A. Ben-Baruch, O. M. Howard, and J. M. Wang. 1997. Monocyte chemotactic protein-2 (MCP-2) uses CCR1 and CCR2B as its functional receptors. *J. Biol. Chem. 272:11682.*
16. Ben-Baruch, A., L. Xu, P. R. Young, K. Bengali, J. J. Oppenheim, and J. M. Wang. 1995. Monocyte chemotactic protein-3 (MCP3) interacts with multiple leukocyte receptors: C-C CKR1, a receptor for macrophage inflammatory protein-1α/Rantes, is also a functional receptor for MCP3. *J. Biol. Chem. 270: 22123.*
17. Prossnitz, E. R., and R. D. Ye. 1997. The *N*-formyl peptide receptor: a model for the study of chemoattractant receptor structure and function. *Pharmacol. Ther. 74:73.*
18. Murphy, P. M., and D. McDermott. 1991. Functional expression of the human formyl peptide receptor in *Xenopus* oocytes requires a complementary human factor. *J. Biol. Chem. 266:12560.*

19. Gao, J. L., E. L. Becker, R. J. Freer, N. Muthukumaraswamy, and P. M. Murphy. 1994. A high potency nonformylated peptide agonist for the phagocyte *N*-formylpeptide chemotactic receptor. *J. Exp. Med. 180:2191.*
20. Gao, J. L., and P. M. Murphy. 1993. Species and subtype variants of the *N*-formyl peptide chemotactic receptor reveal multiple important functional domains. *J. Biol. Chem. 268:25395.*
21. Carp, H. 1982. Mitochondrial *N*-formylmethionyl proteins as chemoattractants for neutrophils. *J. Exp. Med. 155:264.*
22. Freer, R. J., A. R. Day, N. Muthukumaraswamy, D. Pinon, A. Wu, H. J. Showell, and E. L. Becker. 1982. Formyl peptide chemoattractants: a model of the receptor on rabbit neutrophils. *Biochemistry 21:257.*
23. Higgins, J. D., III, G. J. Bridger, C. K. Derian, M. J. Beblavy, P. E. Hernandez, F. E. Gaul, M. J. Abrams, M. C. Pike, and H. F. Solomon. 1996. N-terminus urea-substituted chemotactic peptides: new potent agonists and antagonists toward the neutrophil fMLF receptor. *J. Med. Chem. 39:1013.*
24. Derian, C. K., H. F. Solomon, J. D. Higgins III, M. J. Beblavy, R. J. Santulli, G. J. Bridger, M. C. Pike, D. J. Kroon, and A. J. Fischman. 1996. Selective inhibition of *N*-formylpeptide-induced neutrophil activation by carbamate-modified peptide analogues. *Biochemistry 35:1265.*
25. Murphy, P. M., T. Ozcelik, R. T. Kenney, H. L. Tiffany, D. McDermott, and U. Francke. 1992. A structural homologue of the *N*-formyl peptide receptor: characterization and chromosome mapping of a peptide chemoattractant receptor family. *J. Biol. Chem. 267:7637.*
26. Ye, R. D., S. L. Cavanagh, O. Quehenberger, E. R. Prossnitz, and C. G. Cochrane. 1992. Isolation of a cDNA that encodes a novel granulocyte *N*-formyl peptide receptor. *Biochem. Biophys. Res. Commun. 184:582.*
27. Nomura, H., B. W. Nielsen, and K. Matsushima.1993. Molecular cloning of cDNAs encoding a LD78 receptor and putative leukocyte chemotactic peptide receptors. *Int. Immunol. 5:1239.*
28. Bao, L., N. P. Gerard, R. L. Eddy Jr., T. B. Shows, and C. Gerard. 1992. Mapping of genes for the human C5a receptor (C5AR), human FMLP receptor (FPR), and two FMLP receptor homologue orphan receptors (FPRH1, FPRH2) to chromosome 19. *Genomics 13:437.*
29. Durstin, M., J. L. Gao, H. L. Tiffany, D. McDermott, and P. M. Murphy. 1994. Differential expression of members of the *N*-formylpeptide receptor gene cluster in human phagocytes. *Biochem. Biophys. Res. Commun. 201:174.*
30. Gronert, K., A. Gewirtz, J. L. Madara, and C. N. Serhan. 1998. Identification of a human enterocyte lipoxin A4 receptor that is regulated by interleukin (IL)-13 and interferon γ and inhibits tumor necrosis factor α-induced IL-8 release. *J. Exp. Med. 187:1285.*
31. Deng, X., H. Ueda, S. B. Su, W. Gong, N. M. Dunlop, J. Gao, P. M. Murphy, and J. M. Wang. 1999. A synthetic peptide derived from HIV-1 gp120 down-regulates the expression and function of chemokine receptors CCR5 and CXCR4 in monocytes by activating the seven-transmembrane G protein-coupled receptor FPRL1/LXA4R. *Blood 94:1165.*
32. Klein, C., J. I. Paul, K. Sauve, M. M. Schmidt, L. Arcangeli, J. Ransom, J. Trueheart, J. P. Manfredi, J. R. Broach, and A. J. Murphy. 1998. Identification of surrogate agonists for the human FPRL-1 receptor by autocrine selection in yeast. *Nat. Biotechnol. 16:1334.*
33. Fiore, S., J. F. Maddox, H. D. Perez, and C. N. Serhan. 1994. Identification of a human cDNA encoding a functional high affinity lipoxin A4 receptor. *J. Exp. Med. 180:253.*
34. Samuelsson, B., S. E. Dahlen, J. A. Lindgren, C. A. Rouzer, and C. N. Serhan. 1987. Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. *Science 237:1171.*
35. Lee, T. H., P. Lympany, A. E. Crea, and B. W. Spur. 1991. Inhibition of leukotriene B_4-induced neutrophil migration by lipoxin A4: structure-function relationships. *Biochem. Biophys. Res. Commun. 180:1416.*
36. Colgan, S. P., C. N. Serhan, C. A. Parkos, C. Delp-Archer, and J. L. Madara 1993. Lipoxin A_4 modulates transmigration of human neutrophils across intestinal epithelial monolayers. *J. Clin. Invest. 92:75.*
37. Takano, T., S. Fiore, J. F. Maddox, H. R. Brady, N. A. Petasis, and C. N. Serhan. 1997. Aspirin-triggered 15-epi-lipoxin A4 (LXA4) and LXA4 stable analogues are potent inhibitors of acute inflammation: evidence for anti-inflammatory receptors. *J. Exp. Med. 185:1693.*
38. Romano, M., J. F. Maddox, and C. N. Serhan. 1996. Activation of human monocytes and the acute monocytic leukemia cell line (THP-1) by lipoxins involves unique signaling pathways for lipoxin A4 versus lipoxin B4: evidence for differential Ca^{2+} mobilization. *J. Immunol. 157:2149.*
39. Maddox, J. F., M. Hachicha, T. Takano, N. A. Petasis, V. V. Fokin, and C. N. Serhan. 1997. Lipoxin A4 stable analogs are potent mimetics that stimulate human monocytes and THP-1 cells via a G-protein-linked lipoxin A4 receptor. *J. Biol. Chem. 272:6972.*
40. Ali, H., R. M. Richardson, E. D. Tomhave, J. R. Didsbury, and R. Snyderman. 1993. Differences in phosphorylation of formylpeptide and C5a chemoattractant receptors correlate with differences in desensitization. *J. Biol. Chem. 268:24247.*
41. Ali, H., E. D. Tomhave, R. M. Richardson, B. Haribabu, and R. Snyderman. 1996. Thrombin primes responsiveness of selective chemoattractant receptors at a site distal to G protein activation. *J. Biol. Chem. 271:3200.*