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Identification of an *N*-Formyl Peptide Receptor Ligand Binding Domain by a Gain-of-Function Approach

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Replacement of N-formyl peptide receptor (FPR) domains with those from a homologous receptor, FPR2, resulted in chimeric receptors displaying low binding affinity to fMet-Leu-Phe (fMLF). To characterize fMLF binding domain, we adopted a "gain-of-function" approach by selective replacement of non-conserved residues in the FPR2 portion of the chimeric receptors with those from the FPR. This led to the identification of 3 clusters of residues required for high-affinity fMLF binding. Introduction of 2 positively charged amino acids, Arg^{84} and Lys^{85}, dramatically improved binding affinity of one chimeric receptor (K_d from 105 nM to 1.6 nM). Similarly, restoration of either Gly^{89}/His^{90} or Phe^{102}/Thr^{103} improved the binding affinity of another chimeric receptor from a K_d of 275 nM to a 2.3 K_d and 3.3 nM, respectively. Increased ligand binding affinity was accompanied by a gain in calcium mobilization capability, suggesting functional coupling to G proteins. These results demonstrate the presence of structural determinants in the first extracellular loop and its adjacent transmembrane domains that are essential for high affinity fMLF binding.

Leukocyte chemoattractant receptors form a subgroup within the G protein-coupled receptor superfamily (1,2). A large number of chemoattractant receptors interact with short peptide ligands which vary in size from 3 to 80 amino acid residues. While the binding sites for biogenic amine receptors and the rhodopsin have been well characterized, much less is known about the interaction of peptide ligands with G protein-coupled chemoattractant receptors. It is not clear, for example, whether these peptide chemoattractants have binding pockets that extend into the transmembrane domains. Recent studies suggest that initial contact sites on the extracellular domains of chemoattractant receptors are important for subsequent high-affinity binding, which requires the transmembrane domains (3-5). A better understanding of the ligand interaction with this class of receptors may facilitate future design of specific antagonists.

In this study, we attempted to identify the structural basis for agonist interaction with the N-formyl peptide receptor (FPR), a relatively abundant chemoattractant receptor expressed primarily in phagocytes. Binding of the prototype formyl peptide N-formyl-Met-Leu-Phe (fMLF), a chemotactic product primarily secreted by bacteria (6) but also found in mitochondria of mammalian cells (7), induces a full range of neutrophil responses including chemotaxis, degranulation and generation of superoxide anions (reviewed in (8)). Due to its small size, fMLF may interact with extracellular as well as transmembrane domains of its receptor. This notion has not been thoroughly tested, although published data suggest that the binding pocket of the FPR is of limited depth capable of accommodating up to 5 amino acids (9). Molecular cloning of the FPR and structurally related low-affinity homologues has permitted more detailed studies by construction and analysis of chimeric and point mutant receptors (10,11). We report here the use of a "gain-of-function" approach and the identification of 3 non-contiguous clusters of amino acids in the first extracellular loop and its adjacent transmembrane domains that appear to be necessary for high-affinity binding of fMLF.

MATERIALS AND METHODS

Construction of mutant receptors. The cDNA sequences of the FPR and FPR2 have been described previously (12,13). The chimeric receptors CH40-86 and CH87-105 were constructed as reported before (11). In short, chimeric FPR/FPR2 gene encoding the CH40-86

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was generated by exchange of FPR DNA restriction fragments between the *Ava*I and *Nco*I sites with those of the corresponding FPR2 fragments. The resulting chimeric receptor CH40-86 contains the FPR2 amino acid sequence between the residues Gly^{40} and Ala^{86} in the FPR framework (Fig.1). CH87-105, generated by a similar approach, contains the FPR2 amino acid sequence introduced between the residues Met^{87} and Val^{105} (Fig. 1). Point mutations were introduced by PCR, using primers containing appropriate restriction sites in addition to the mutated bases to facilitate subcloning of the amplified DNA fragments. For the mutations on CH40-86, DNA fragments were amplified between the *Ava*I site encoding Gly^{40} and the *Nco*I site encoding Ala^{86}. For the mutations on CH87-105, DNA fragment between the *Nco*I recognition site and the *Sal*I site (Val^{106}) was amplified by PCR, digested with these two endonucleases and subcloned into the FPR framework. In some cases site-directed mutants were prepared by the method of Kunkel (14) using M13mp18 and the dut^- ung^- strain of *Escherichia* coli CJ236. The nucleotide sequence of all mutants was confirmed by dideoxy DNA sequencing. Chimeric and mutant receptors were stably expressed in mouse L cell fibroblasts as described previously (11).

Ligand binding assays. Membranes were prepared from transfected cells by N_2 cavitation and centrifugation (11). For equilibrium binding studies, [^{3}H]fMLF at different concentrations was added to the membranes (30 μg membrane proteins) in a total volume of 0.2 ml of bind buffer as described elsewhere (11). The binding data were analyzed with the computer program LIGAND (15) and the dissociation constant (K_d), number of sites (B_{max}) and nonspecific binding were determined. After incubating with [^{3}H]fMLF at different concentrations in binding buffer at 4°C, the unbound ligand was removed by filtration, the cells were rinsed 3 times with the binding buffer, lysed with 0.2 M NaOH and the retained radioactivity was measured by scintillation counting.

Calcium mobilization assay. Functional expression of the mutant receptors and their ability to transduce signals were determined by the release of Ca^{2+} from intracellular stores. The cells were resuspended at 5×10^6 cells/ml in RPMI 1640 medium containing 10% fetal bovine serum and loaded with indo-1 AM (5 μM). The change of intracellular calcium concentration was estimated by the ratio of the fluorescence intensity at 400 and 490 nM as described before (16).

RESULTS

Construction and expression of mutant FPR receptors. We previously constructed chimeric receptors by sequential replacement of the FPR domains with similar segments from a homologous receptor, FPR2 (11). This receptor has been recently shown to mediate the biological functions of lipoxin A_4 (17). FPR2 is 69% identical in sequence to the of FPR (13), yet it binds fMLF with nearly 500-fold lower affinity (11). This discrepancy provides an experimental window for the localization of the FPR ligand binding domains by an approach using chimeric receptors. Of the 10 chimeras analyzed, 2 displayed low affinity binding properties of the FPR2, which has a K_d of 430 nM for fMLF (11). The first chimera, CH40-86, contains a segment of 47 residues (Gly^{40} to Ala^{86}) from the FPR2 and binds fMLF with a K_d of 105 nM. For the second chimeric receptor, a 19-residue replacement of the FPR with an FPR2 fragment resulted in an even lower binding affinity for fMLF (K_d = 275 nM). Both chimeric receptors were

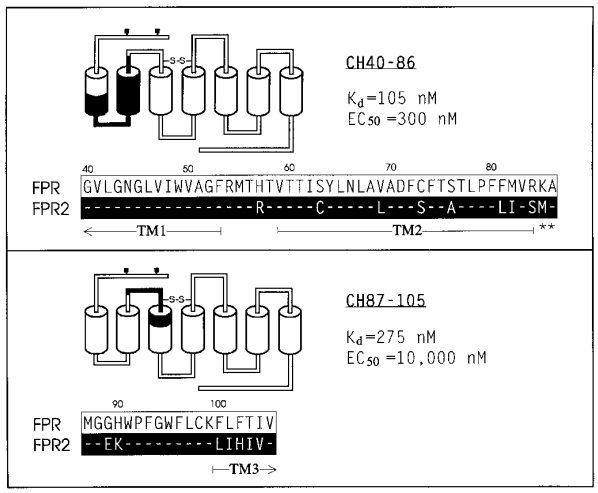

FIG. 1. Mutants based on the FPR/FPR2 chimeric receptors. The chimeric receptors were constructed between the human FPR (shown as open cylinders and lines) and FPR2 (shown as closed cylinders and solid lines). The name, dissociation constant, and EC_{50} for calcium mobilization are displayed on the right side of each chimera. The sequence of the replaced segments in the chimeras are aligned below each construct, and only the different FPR2 residues are shown. Putative transmembrane domains are marked underneath the aligned sequence. The two asterisks indicate the residues that are not present in the chimeric CH40-86, as described in the text.

expressed on the cell surface as they were able to mediate agonist-specific calcium mobilization (11).

To identify the structural determinants responsible for fMLF binding, we compared sequences of the two receptors in the switched domains. The FPR2 sequence is highly homologous to that of the FPR in the two segments, with only a few non-conserved amino acids clustered in 3 groups separated by several amino acids (Fig. 1). These amino acids are located at positions 84/85, 89/90 and 100-104. We reasoned that if these residues are essential for fMLF binding, replacement of the FPR2 sequence by the FPR sequence may restore the binding affinity. This hypothesis was tested by point mutagenesis of the chimeric receptor cDNA and functional expression and characterization of the resultant receptor in stably transfected cells.

The role of Arg^{84}/Lys^{85} in fMLF binding. The chimeric receptor CH40-86 lacks 2 amino acids present in the wild-type FPR2 (Met^{85} and Ala^{86}) (11). We first examined whether restoration of these two residues could revive high affinity ligand binding. Addition of Met^{85} and Ala^{86} (M1.0) did not result in high-affinity fMLF binding, suggesting that residues other than Met^{85} and Ala^{86} contribute more to ligand binding (Table I). A sequence analysis identified 9 different amino acids in an FPR2 segment of 47 residues within the chimeric receptor CH40-86 (Fig. 1). Of the 9 different residues $Arg^{84} \rightarrow Ser^{84}$ and $Lys^{85} \rightarrow Met^{85}$ represent major structural changes of the amino acid side chains. The positively charged Arg^{84} and Lys^{85} are predicted

TABLE I

The Effects of Point Mutations on Ligand Binding

Receptors	Residue changes	K_d (nM)	B_{max} (fmol/mg)
CH40-86	None (template)	105.0 ± 4.6	435 ± 18
M1.0	Restore M^{85}, A^{86}	63.0 ± 2.5	894 ± 33
M1.1	$S^{84}\rightarrow R^{84}$, $M^{85}\rightarrow K^{85}$	1.6 ± 0.1	1692 ± 27
M1.2	$M^{85}\rightarrow K^{85}$	34.7 ± 1.2	1083 ± 49
M1.3	$S^{84}\rightarrow R^{84}$	4.6 ± 0.4	1307 ± 38
CH87-105	None (template)	275.0 ± 14.5	605 ± 45
M2.1	$E^{89}\rightarrow G^{89}$, $K^{90}\rightarrow H^{90}$, $H^{102}\rightarrow F^{102}$, $I^{103}\rightarrow T^{103}$	3.3 ± 0.3	906 ± 28
M2.2	$E^{89}\rightarrow G^{89}$, $H^{102}\rightarrow F^{102}$, $I^{103}\rightarrow T^{103}$	1.8 ± 0.1	523 ± 8
M2.3	$H^{102}\rightarrow F^{102}$, $I^{103}\rightarrow T^{103}$	3.3 ± 0.2	1163 ± 17
M2.4	$H^{102}\rightarrow F^{102}$	NB	NB
M2.5	$I^{103}\rightarrow T^{103}$	4.5 ± 0.3	2381 ± 83
M2.6	$E^{89}\rightarrow G^{89}$, $K^{90}\rightarrow H^{90}$	2.3 ± 0.1	745 ± 6
M2.7	$E^{89}\rightarrow G^{89}$	81.5 ± 11.3	907 ± 37
M2.8	$K^{90}\rightarrow H^{90}$	63.3 ± 5.6	1183 ± 48
FPR	None (template)	0.95 ± 0.06	1125 ± 12

Note. Construction and binding analysis of the mutant receptors have been described in the *Experimental Procedures.* The numbers presented in this table represent the mean ± SE of three determinations. NB, no binding (this mutant, M2.4, was not expressed on the cell surface). Fluorescence staining with an anti-FPR carboxyl terminal peptide antibody (24) revealed a pattern of perinuclear staining (data not shown), suggesting improper folding and transport of the M2.4 receptor. Single-letter codes for amino acids are used.

to reside near the end of the second transmembrane domain (Fig. 1). To examine the function of these two residues, we prepared mutant receptor M1.1 by restoration of the two charged residues. The resultant receptor displayed a nearly wild-type binding affinity for fMLF with a K_d of 1.6 nM (Table I). These residues were then mutated individually. Conversion of Ser^{84} to Arg resulted in a great increase in binding affinity for fMLF (M1.3, K_d = 4.6 nM), whereas conversion of Met^{85} to Lys alone improved ligand binding affinity to a lesser extent (M1.2, K_d = 34.7 nM).

Localization of two other clusters of amino acids that affect fMLF binding. An earlier analysis of the CH87-105 chimeric receptor suggested that replacement of a 19-residue segment of the FPR with that of the FPR2 significantly reduced binding affinity for fMLF, regardless of the presence of Arg^{84} and Lys^{85} (Fig. 1 and Table I). An alignment of the sequences of this segment revealed seven different residues between the two receptors, of which three non-conservative substitutions were found at positions 89, 102 and 103 (Fig. 1). In addition, His^{90} of the FPR became a similarly charged Lys but with a quite different side chain in the FPR2-derived segment of the chimera. These residues form two non-contiguous clusters located in the first extracellular loop and the proximal portion of the adjacent TM3 (Fig. 1). To evaluate the functional role of these residues in fMLF binding, we mutated all 4 residues to the FPR sequence. The mutant receptor, M2.1, displayed near wild-type binding affinity (K_d = 3.3 nM, Table I). A similar improvement in binding affinity was observed when three of the four residues were converted to the FPR sequence ($Glu^{89} \rightarrow Gly^{89}$, $His^{102} \rightarrow Phe^{102}$, $Ile^{103} \rightarrow Thr^{103}$; M2.2). To examine whether both clusters contribute to high-affinity binding, amino acids at positions 89/90 and 102/103 were separately converted to the FPR sequence. Binding data from these experiments indicate that either cluster by itself was sufficient to convey high affinity fMLF binding (M2.3 and M2.6). Within each cluster, however, the functions of individual residues vary. While a switch from Ile to Thr at position 103 dramatically increased ligand binding affinity (M2.5, K_d = 4.5 nM), replacement of His^{102} by a Phe resulted in an apparently aberrant receptor unable to express on the cell surface, as confirmed by direct fMLF binding and fluorescent Ab staining (not shown). Replacement of individual residue at positions 89 and 90 was less effective in improving ligand binding affinity (M2.7, M2.8; Table II). This latter finding suggests a requirement for both Gly^{89} and His^{90} as a cluster in the first extracellular loop for high-affinity interaction with fMLF.

Selective reduction in binding affinity for fMLF but not for tBOC-FLFLF. Replacement of the amino terminal CHO group with tBOC results in a ligand with antagonistic properties (18). To confirm that the lower binding affinities displayed by some of the chimeric and mutant receptors are direct consequences from changes in the primary structure, we conducted competition binding assays with four receptors showing significantly reduced affinity for fMLF. Our results indicate that tBOC-FLFLF was able to compete effectively for binding of fMLF to the mutant receptors with essentially no reduction in binding affinity. In contrast, the binding affinity of these mutant receptors for the agonist fMLF was reduced by 63-105-fold (Table II). We

TABLE II

Binding Affinities of Selected Mutant Receptors for fMLF and tBOC-FLFLF

Receptors	Residue changes	K_d for fMLF (nM)	K_i for tBOC-FLFLF (μM)
FPR (WT)	None	1.00 ± 0.12	3.25 ± 0.76
M1.0	M^{85}/A^{86}	63.00 ± 2.50	3.40 ± 1.90
M2.7	$E^{89}\rightarrow G^{89}$	81.50 ± 11.30	4.70 ± 2.80
M2.8	$K^{90}\rightarrow H^{90}$	63.30 ± 5.60	0.50 ± 0.23

Note. Competition binding assays were performed with membranes containing mutant receptors as described in Table I. Unlabeled tBOC-FLFLF of various concentrations (0.1-500 μM) were added to the binding buffer in order to compete with [^{3}H]-fMLF for receptor binding. Binding was conducted at room temperature for 60 min and data were collected for calculation of the binding affinity. Binding data presented in this table represent the mean ± SE of 3 determinations. WT, wild-type.

were not able to obtain accurate measurements for the chimeras CH40-86 and CH87-105 due to their very low binding affinities for fMLF. These data suggest that the reduction in binding affinities for fMLF seen in the chimeric receptor is likely a direct effect of the amino acid substitutions that appeared to selectively affect agonist but not antagonist binding.

Correlation between reduced ligand binding affinities and calcium mobilization capabilities. Effective transmembrane signaling requires the interaction of G proteins with multiple intracellular domains of the receptor (19). Slight changes in receptor conformation can lead to dissociation of the receptor from G protein coupling, preventing transmembrane signal transduction. We examined several mutant receptors with reduced binding affinity for their capability of mobilizing calcium in response to fMLF stimulation, an event downstream from receptor-G protein coupling. Dose-response curves shown in Fig. 2A suggest that all these mutant receptors were capable of mobilizing Ca^{2+} from intracellular stores with the exceptions of M2.4, which was not expressed on cell surface. Although the extent of Ca^{2+} mobilization for each mutant was different, there was a good correlation between the EC_{50} and K_d values (R value = 0.93) (Fig. 2B). These data suggest that the majority of the mutant receptors retained a structure capable of G protein coupling, although higher agonist concentrations were necessary for maximal calcium mobilization due to reduced binding affinities for fMLF.

DISCUSSION

Point mutagenesis has been widely used as a method for identification of amino acid residues responsible for receptor-ligand and receptor-G protein interactions. In this study, we employed a different approach to positively identify amino acids important for ligand binding by a "gain of function" approach. Two chimeric receptors with low binding affinities were chosen as templates and potentially important non-conservative residues of the FPR2 were converted to the wild-type FPR sequence. Analysis of the binding affinity and signaling capability of these mutant receptors led to the identification of three clusters of residues that contribute to high affinity ago-

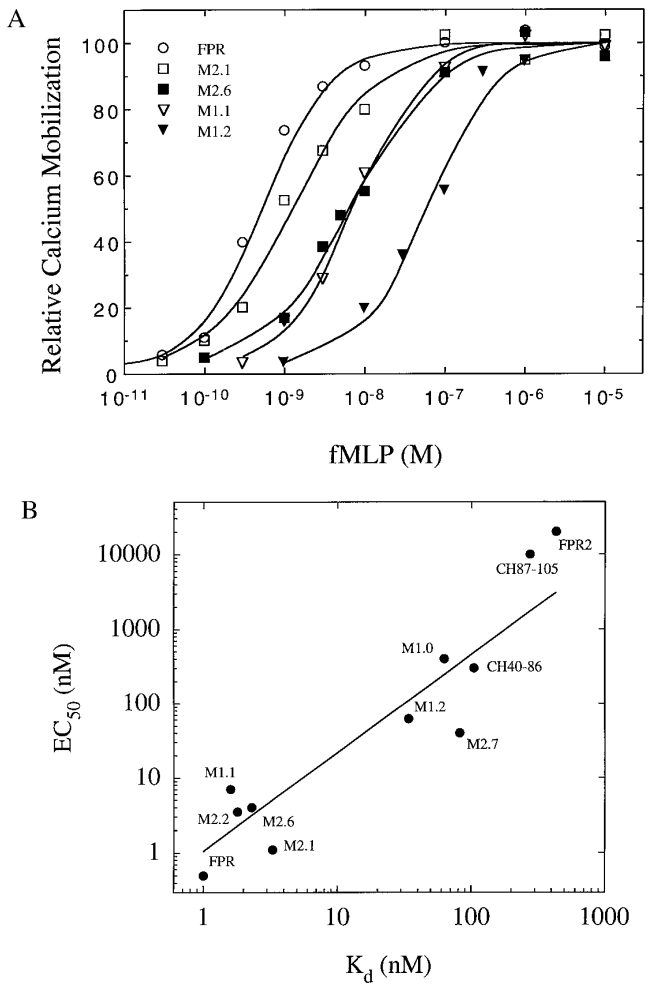

FIG. 2. fMLF-induced calcium mobilization by the wild-type and mutant receptors. (**A**) Ligand concentration-dependent calcium mobilization. Transfected cells expressing the receptors were stimulated with fMLF at various concentrations, and the intracellular free calcium levels were measured as described in **Materials and Methods.** Data from selected mutant receptors, representing a range of different binding affinities, are shown in the figure as a function of fMLF concentration. Maximal calcium mobilization by each receptor was set as 100%. The symbols are: open circle, wild-type FPR; open square, M2.1; closed square, M2.6; open triangle, M1.1; closed triangle, M1.2. (see Table I). (**B**) Correlation of ligand binding affinity with calcium mobilization capability. Shown in the figure is the relationship between fMLF binding, expressed as the K_d values, and the calcium mobilization capability, expressed as the fMLF concentrations that induce half-maximal calcium mobilization (EC_{50}), for the FPR, FPR2, the two chimeric receptors, and the above mutant receptors.

nist binding. These residues are located at the end of the second transmembrane doman, the first extracellular loop, and in the middle of the third transmembrane domain. Together, these residues possibly constitute part of the binding site(s) for fMLF.

Changes in the primary structure of the receptor protein by mutagenesis could conceivably lead to rearrangement of the global receptor structure, thereby affecting ligand binding and G protein coupling. In the absence of a 3-dimensional structure for the FPR, it is difficult to determine whether a particular mutation has caused global structural change. An available although less satisfying method is to examine whether the mutant receptors were able to mediate one of the functions requiring G protein coupling, such as calcium mobilization. Since G protein interaction with the receptor requires multiple, non-contiguous intracellular domains of the receptor (19,20), transmembrane signaling would be as vulnerable as ligand binding to global structural changes of the receptor. Except one mutant (M2.4), all the receptors displayed EC_{50} values for calcium mobilization in proportion to the changes in ligand binding affinities. Furthermore, ligand binding to these receptors was sensitive to GTPγS (not shown), indicating that these receptors were coupled to G proteins. Several mutant receptors with drastically reduced binding affinities for fMLF were able to bind the antagonist tBOC-FLFLF with unchanged affinity. Taken together, our data suggest that the global receptor structure was not significantly affected by the mutation. Changes in the ligand binding affinity, therefore, reflect direct results of replacement of individual amino acid residues.

The conclusion that residues in the first extracellular loop and its adjacent regions contribute to high-affinity fMLF binding is supported by two additional studies. Radel et al. (21,22) found that an 18-residue synthetic peptide spanning the entire first extracellular loop of the FPR could compete with the receptor for fMLF binding. Gao and Murphy (23) characterized the mouse cDNA for fMLF receptor which shares 78% sequence identity with the human FPR. The mouse receptor lacks Arg/Lys at position 84/85 (replaced by Ser/Met, as in FPR2), and displays a low binding affinity with a K_d of approximately 100 nM. These results substantiate the hypothesis that a simple structural determinant exists in the extracellular and/or transmembrane domains that allows low-affinity interaction of the receptor with fMLF. High affinity binding, however, requires additional structural features and G protein coupling. More information on the three-dimensional structure of the receptor will be necessary to unequivocally confirm the identified amino acid residues as constituents of the fMLF binding pocket.

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