The N-terminal part of the calcium-regulated and phospholipid-binding protein annexin AI contains peptide sequences with pro- and anti-inflammatory activities. We have earlier shown that a proinflammatory signal triggered by one of these peptides, Gln9–Lys25, is mediated by FPR1, a member of the formyl peptide receptor family expressed in human neutrophils. To determine the core structure in Gln9–Lys25, smaller peptides were generated, and their capacity to activate neutrophils was determined. A peptide spanning from amino acid Glu14 to Lys25 was inactive, whereas the activity was retained in the Gln9–Tyr20 peptide. Removal of amino acids from the C and N terminus of Gln9–Tyr20 revealed that the first amino acid (Gln9) was of the utmost importance for activity. The core structure that activated the neutrophil NADPH oxidase to release superoxide anions was Gln9–Ala10–Trp11–Phe12. This peptide also inhibited the activity induced by N-formyl-Met-Leu-Phe and WKYMVM. A structural model of the peptide agonist-FPR1 complex suggests that the transmembrane part of the binding pocket of the receptor binds optimally to a tetrapeptide. According to the model and the results presented, the N-terminal amino acid glutamine in Gln9–Phe12 is located close to the bottom of the binding cleft, leaving for steric reasons insufficient space to extend the peptide at the N terminus. The addition of amino acids at the C terminus will not affect binding. The model presented may be helpful in developing specific FPR1 ligands.

The binding of inflammatory mediators to G-protein-coupled receptors (GPCRs) expressed in phagocytic leukocytes regulates cell function during infection and inflammation. The most studied human neutrophil GPCR is FPR1 (formyl peptide receptor 1), a chemoattractant receptor that binds several peptide agonists that are either endogenous or derived from microbial pathogens. The biological effects induced by these agonists can be mimicked by synthetic peptides (1, 2). N-Formylated peptides generated during prokaryotic protein synthesis were the first identified high affinity agonists for FPR1, and formyl-Met-Leu-Phe (fMLF) is a commonly used prototype peptide, which induces chemotaxis, granule mobilization, and production of reactive oxygen metabolites in receptor-expressing neutrophils (3).

FPR1 belongs to the group of “seven-transmembrane receptors,” and the agonist binding structure is expressed on the extracellular spanning regions of the receptor (1). It has been suggested that the binding pocket in the receptor accommodates at the most five amino acids (4) and that agonist binding involves the second, third, and fourth extracellular domains of the receptor with adjacent transmembrane domains (5). One of the important differences between FPR1 and a closely related receptor, FPR2, belonging to the same receptor family, is that the latter is a low affinity receptor for fMLF, and this affinity difference has been exploited to identify which amino acid residues are involved in binding of formylated peptides (6). FPR1 is a promiscuous receptor, and non-formylated proteins and peptides that are structurally distinct from fMLF have been shown to trigger phagocyte function via FPR1 (1, 2). Several peptides derived from the N-terminal domain of human annexin I are ligands for FPR1 and for FPR2 and FPR3, the two other members of the FPR family in myeloid cells (7–11).

Annexin I belongs to a family of calcium-dependent phospholipid binding proteins. The C-terminal domain of the annexins is highly conserved and mediates the calcium-regulated binding of the protein to membranes, whereas the unique N-terminal part defines the precise function of the protein. In annexin I, the N terminus is sensitive to cleavage by endogenous proteases (12, 13), and peptides derived from this domain possess immunoregulatory effects (14). An N-terminally acetylated annexin I peptide corresponding to the sequence Gln9–Lys25 has been shown to mediate both activating and inhibitory effects on neutrophil function (7, 10).

This study aimed at identifying the core structure within the Gln9–Lys25 annexin I peptide that activates FPR1. Our results...
suggest that neutrophil activation is strictly mediated by the acetylated Gln<sup>-9</sup>-Ala<sup>-10</sup>-Trp<sup>-11</sup>-Phe<sup>-12</sup> (Gln<sup>-9</sup>-Phe<sup>-12</sup>) sequence. In addition, we have generated a computational homology model of this peptide in complex with FPR1, based on the rhodopsin crystal structure, to interpret the biological outcome and to suggest new peptide variants for testing.

**EXPERIMENTAL PROCEDURES**

**Peptides and Reagents**—Dextran was from Amersham Biosciences, and Ficoll-Paque was from GE Healthcare. The annexin AI-derived peptides were synthesized and HPLC-purified by Ross-Petersen ApS (Holte, Denmark), dissolved in alkaline phosphate-buffered saline, and stored at −70 °C. The hexapeptide Trp-Lys-Tyr-Met-Met-NH<sub>2</sub> (WKYMVM) was synthesized and HPLC-purified by Alta Bioscience (University of Birmingham, Birmingham, UK). The formylated peptide N-formyl-Met-Leu-Phe (fMLF), phorbol myristate acetate, and N-terminal hexapeptide Trp-Lys-Tyr-Met-Met-NH<sub>2</sub> were from Sigma. The FPR2 antagonist WR247775 was purchased from Genscript Corp. (Scotch Plains, NJ). These agonists/antagonists were dissolved in DMSO to 10<sup>-6</sup> M and stored at −70 °C until used. Cyclosporin H was kindly provided by Novartis Pharma (Basel, Switzerland) and diluted in DMSO to 10<sup>-3</sup> M and stored at −70 °C. Horseradish peroxidase was from Roche Applied Science. Luminol was purchased from Sigma.

**Computer Model**—Structure models of FPR1 were created using the crystal structure of the bovine rhodopsin receptor (Protein Data Bank code 1u19) (16). A multiple sequence alignment containing structure-determined GPCR and selected amine receptors was manually adjusted, and pairwise sequence alignments for the modeling target and structure templates were extracted (supplemental Fig. 1). A homology model was built using the standard tool of the program ICM (17) (Molsoft LLC, La Jolla, CA), which includes loop modeling by a library search, followed by geometry optimization of the model. The side chains of non-conserved residues were optimized, and the extracellular loop between helices 4 and 5 was removed. residues Arg<sup>163</sup>-Ile<sup>191</sup> to avoid steric clashes with the peptide while maintaining the majority of the proposed binding site residues. A number of complex structures were generated by means of torsion space biased probability Monte Carlo structure optimization (17) of the peptide, with the all-atom receptor model replaced by potential energy maps representing van der Waals, electrostatic, hydrogen bonding, and hydrophobic interactions (ICM version 3.5 manual). This method is aimed at broad sampling of conformational space, meaning that many different binding modes are explored. Therefore, we biased the optimizations by the introduction of a weak harmonic constraint between the N-terminal α-carbons of the simulated and manually docked ligands to increase sampling efficiency. Several alternative low energy solutions were inspected, and those that best matched available binding data were selected for further analysis.

**Isolation of Human Neutrophils**—Neutrophil granulocytes were isolated from buffy coats obtained from healthy adults by dextran sedimentation at 1 × g, centrifugation in a Ficoll-Paque gradient, and hypotonic lysis of remaining erythrocytes (18). The neutrophils were washed twice and resuspended (1 × 10<sup>7</sup> cells/ml) in Krebs-Ringer phosphate buffer containing glucose (10 mM), Ca<sup>2+</sup> (1 mM), and Mg<sup>2+</sup> (1.5 mM) (KRG; pH 7.3) and stored on melting ice until used.

**Stable Expression of Formyl Peptide Receptors in Undifferentiated HL-60 Cells**—The stable expression of FPR1 and FPR2 in undifferentiated HL-60 cells has been described elsewhere (19, 20). Cells were cultured in RPMI 1640 medium/glutaMax I, and the maximal density was maintained below 2 × 10<sup>6</sup> cells/ml. The control experiments with the specific agonists (fMLF for FPR1 and WKYMVM for FPR2) were performed at each experimental event and turned out positive.

**Neutrophil NADPH Oxidase Activity**—Neutrophil production and release of superoxide anions was measured by a luminol-enhanced chemiluminescence assay (21). The chemiluminescence activity was measured in a six-channel Biolumat LB 9505 apparatus (Berthold Co., Wildbad, Germany), using disposable 4-ml polypropylene tubes with a 500-µl reaction mixture containing neutrophils (5 × 10<sup>6</sup> cells), horseradish peroxidase (4 units), and luminol (20 µM). A volume of 50 µl of cyclosporin H (10 µM) or WR247775 (5 µM) was added as indicated under “Results.” The measuring tubes were equilibrated for 5 min at 37 °C, and the cells were activated by the addition of one or two agonists at different time points. The concentration of fMLF and WKYMVM was 1 × 10<sup>-7</sup> M. In some experiments the concentration for fMLF and WKYMVM was titrated to give the same peak response as 50 µM Gln<sup>-9</sup>-Tyr<sup>20</sup> (Ac-QAWFIENEEQY). The light emission was recorded continuously.

**Determination of Changes in Cytosolic Calcium**—Cells were loaded with 2 µM Fura-2/AM (Molecular Probes, Inc., Eugene, OR) according to the instructions given by the manufacturer. Calcium measurements were carried out as described earlier (20) using a LC50 fluorescence spectrophotometer (Perkin Elmer Life Sciences model LS50B). Intracellular calcium concentrations were calculated using the formula,

\[
[F] = K_d(F - F_{min})/(F_{max} - F) + F_{min}
\]

where \(F\) is the fluorescence in the presence of 0.04% Triton X-100 and \(F_{min}\) is the fluorescence after the addition of 5 mM EGTA and 30 mM Tris-HCl, pH 7.4.

**Statistical Analysis**—A two-tailed paired Student’s t test was performed to determine statistical significance. A p value of <0.05 was regarded as significant.

**RESULTS**

**The N-terminal Part of the Gln<sup>-9</sup>-Lys<sup>-25</sup> Annexin I Peptide Is Crucial for Receptor Interaction**—The peptide Gln<sup>-9</sup>-Lys<sup>-25</sup> (Ac-QAWFIENEEQYVQTVK), corresponding to amino acids 9–25 in the N terminus of annexin I, activates the neutrophil NADPH oxidase primarily by interaction with FPR1 (7). Two truncated peptides were synthesized from this sequence and assessed for neutrophil-activating properties. A peptide spanning amino acids Glu<sup>-13</sup>-Lys<sup>-25</sup> (Ac-ENEEQYVQTVK) showed no activating or inhibitory effect on neutrophil superoxide...
anion production (data not shown). A peptide from amino acid Gln9 to Tyr20 (Ac-QAWFIENEEQEY) induced a rapid and pronounced release of superoxide with peak activation at 1 min, after which the response rapidly declined (Fig. 1A); the kinetics of this response thus was similar to that induced by the annexin I peptide Gln9–Lys25 and other chemotactic peptides, such as fMLF (7). The activity of Gln9–Tyr20 was concentration-dependent with maximal activity at around 50 μM.

The receptor involved was identified using transfected HL60 cells selectively expressing FPR1 or FPR2. The Gln9–Tyr20 peptide induced a distinct increase in intracellular calcium in FPR1-expressing cells (Fig. 1B), whereas only a minimal response was induced in FPR2-expressing cells (Fig. 1C), thus implying that the Gln9–Tyr20 peptide activates neutrophils preferentially via FPR1.

This was confirmed using receptor-specific antagonists; the FPR1 antagonist Boc-MLF inhibited the Gln9–Tyr20-induced response (Fig. 2A, first arrow, dotted and gray lines), whereas the FPR2-specific antagonist WRWWWW (W5) was ineffective (Fig. 2B, first arrow, dotted and gray lines). The receptor specificity was also determined in desensitization experiments using the FPR1 agonist fMLF and the FPR2 agonist WKYMVM together with receptor-specific antagonists. The neutrophil response to fMLF (Fig. 2B, second arrow, black and dotted lines) and C) and WKYMVM (Fig. 2A).
(second arrow, black and dotted lines) and C) was strongly reduced when the cells had first been challenged with Gln9–Tyr20. The FPR2-specific antagonist WRWWWW (W5) could not reverse the inhibitory effects of Gln9–Tyr20 on the fMLF-induced response (Fig. 2, B (gray and dotted lines) and D), suggesting that the receptor is down-regulated by homologous desensitization. In contrast, the state of desensitization to WKYMVM was reversed by the FPR1 antagonist Boc-MLF (Fig. 2, A and D), indicating that the binding of Gln9–Tyr20 to FPR1 also induces a heterologous desensitization of FPR2 that can be blocked by an FPR1 antagonist.

The FPR1 agonist activity of the Gln9–Tyr20 peptide, in contrast to the Glu14–Lys25 peptide, suggests that the N terminus is critical for receptor interaction. This was verified using shorter peptides generated by a stepwise removal of amino acids. The 4-amino acid peptide Gln9–Phe12 (Ac-QAWF) was the shortest peptide that retained both the capacity to trigger a neutrophil NADPH oxidase response and to inhibit the activity induced by another FPR1 agonist (i.e. fMLF) or an FPR2 agonist (i.e. WKYMVM) (Fig. 3, A and B). Variants of this peptide were used for further characterization of the ligand-receptor interaction.

Molecular Modeling of FPR1 and Binding of the Annexin I-derived Peptide Gln9–Phe12—Five GPCRs have been crystallized to date: bovine (22) and squid (23) rhodopsin, turkey β1-adrenergic receptor (24), human β2-adrenergic receptor (25), and human A2A adenosine receptor (26). Their ligand binding sites for retinal and catecholamines are located near the extracellular side of the transmembrane (TM) region and engage mainly residues in helices 3, 5, and 6. The same region has been proposed to be critical for peptide binding, and additional binding regions are plausible, considering that tetrameric peptides are substantially larger. Miettinen et al. (6) have suggested 10 amino acids of FPR1 to be important for high affinity binding of fMLF. These authors also presented a model in which these residues were located in positions below the second extracellular loop (ECL2), which coincides with the bottom of the binding pocket for retinal rhodopsin (22) as well as for several ligands that bind amine receptors (27). These data suggest that the region below the ECL2 is of importance for the contact between peptide ligands and the receptor.

We generated several models of FPR1 with docked peptides and used these to interpret our experimental data. Homology models of FPR1 were based on the bovine rhodopsin crystal structure (Protein Data Bank code 1u19) (16), which has a sequence identity of 20% for 348 aligned residues corresponding to the full transmembrane domain. The ECL2 blocks to a large extent the binding pocket of the receptor. This loop and the N-terminal region preceding helix TM1 were excluded in the model in order to allow docking of the peptide into the binding pocket. The fact that there are large sequence variations and a structural variability in ECL2 among the different GPCRs suggests that it is difficult to predict the conformation of this region. This is also illustrated by the fact that the ECL2 region in bovine rhodopsin forms two β-strands that are part of a β-sheet and that the corresponding segment in amine receptors forms a single helix located on top of the helix bundle. Due to these difficulties, the ECL2 region was not modeled.

FIGURE 3. Activation and inhibition of NADPH oxidase by annexin I-derived peptides. Neutrophils were stimulated with or without (black line, 0 µM; gray line, 50 µM) Ac-QAWF (i.e. Gln9–Phe12) (A and B), Ac-AAWF (C and D), Ac-WF (E and F), and Ac-QAWFIE (G and H) before restimulation with 3·10−8 M fMLF (left) or 5·10−8 M WKYMVM (right). The release of superoxide anions was measured by a luminol-enhanced chemiluminescence assay. The curves are from representative experiments. The arrows indicate the addition of annexin I peptide and fMLF or WKYMVM.

The peptide model for the acetylated core annexin I peptide, Gln9–Phe12, was docked to potential maps that represented van der Waals, electrostatic, hydrogen bonding, and hydrophobic properties of FPR1 (lacking the ECL2). We assumed that the acetylated N terminus of the annexin peptide was bound in the
most buried part of the binding pocket near Asp$^{106}$ in the receptor, based on the observation that modifications of the peptide N terminus had obvious effects on peptide binding in our functional studies. Glutamine Gln$^9$ was restrained to this region by means of a harmonic restraint. Only limited structure optimization was employed because the model was expected to be of lower resolution due to low sequence identity with the template, the clear difference in ligand properties, and the lack of detailed structural information for peptide receptors. In the ensemble of energetically most favored solutions, Gln$^9$ interacts with a cavity near Asp$^{106}$ in TM3 and forms an extended structure that points toward Arg$^{201}$ in TM5 and the extracellular ends of helices TM3 and TM4 (Fig. 4, C and B). This places the peptide C terminus near the extracellular side of the transmembrane region. Because cross-linking data indicated the proximity of the peptide to Lys$^{85}$ in TM2 (28, 29), we reviewed the Monte Carlo geometry optimization data and found solutions of slightly higher energy that did include interactions between Lys$^{85}$ and the C-terminal carboxylic acid (Fig. 4A). The direction of the extended peptide was more tilted toward the membrane plane than the other solution, allowing for the ECL2 to fold down onto regions close to TM3 and TM4 that are occupied by the peptide in the other solution. Several residues that affect ligand binding upon mutation were in contact with the peptide. Although the exact location of the peptide could not be predicted, the model suggests that the tetrapeptide length matches the distance from Asp$^{106}$ to the extracellular termini of transmembrane helices, meaning that this is the optimal length for interaction with the transmembrane domain and that few interactions occur with the extracellular loop regions.

Properties of Variant Gln$^9$–Phe$^{12}$ Peptides—As mentioned, the Gln$^9$–Phe$^{12}$ (Ac-QAWF) peptide was the shortest peptide possessing the capacity both to trigger a neutrophil NADPH oxidase response and to inhibit the activity induced by other FPR agonists (Fig. 3, A and B). According to earlier published data on ligand interaction with FPR1, the annexin I peptide most probably interacts with the amino acids near the extracellular side of the transmembrane domain that have been proposed to form a binding pocket in FPR1 (6). According to the model presented, the N-terminal amino acid glutamine in Gln$^9$–Phe$^{12}$ is located close to the bottom of the binding cleft, leaving no extra space for an additional amino acid (Fig. 4A). If this assumption is correct, it should not be possible to extend the peptide at the N-terminal end for steric reasons. This was supported by results obtained from experiments showing that a peptide containing an additional amino acid, alanine, linked to the Gln$^9$ (Ac-QAWF), has lost most of the capacity to activate the neutrophil NADPH oxidase (see Table 2). The results suggest that the location of Gln$^9$ in the binding cleft of FPR1 is of pivotal importance for activation of the receptor. Interestingly, the alanine-elongated peptide retained some of the inhibitory activity, illustrated by the fact that the fMLF as well as the WKYMVM responses were reduced in cells pretreated with the missing loop ECL2 between helices 4 and 5 is indicated with a white dashed line (upper left corner). The surface shows the shape of the ligand binding pocket and represents the isosurface where the van der Waals interaction energy between a carbon probe and the protein is 0 kcal/mol.
The dipeptide Ac-WF, in which both the Gln9 and the Ala10 had been removed, had lost the capacity to activate neutrophils (Fig. 3, E and F, and Table 1). Similarly, an alanine has replaced the Gln9 had a markedly reduced activating peptide WKYMVM (Fig. 3F). This strongly suggests that receptor activation (FPR1) and inhibition (heterologous desensitization of FPR2) rely on the same type of ligand-receptor interactions.

Some of these peptides inhibited the response induced by the FPR1 ligand fMLF and had in common the C-terminal amino acids Trp11 and Phe12 (all but one peptide). This suggests that Trp11 and Phe12 can bind to the receptor but that, in the absence of Gln9 and Ala10, they are unable to induce FPR1 signaling and thus also desensitization of FPR2. Based on the fact that these peptides had neither any NADPH oxidase activity nor any effect on FPR2, the inhibitory effect on fMLF-induced oxidative burst most likely was caused by blockage of the binding pocket rather than signaling-induced desensitization of FPR1.

**DISCUSSION**

Human neutrophils express two members of the FPR family, FPR1 and FPR2, which share a high degree of amino acid identity. The signals generated by these receptors activate or inhibit cellular functions, depending on the triggering agonist. Peptides derived from the N-terminal domain of annexin I possess activity compared with the original peptide. The inhibitory effect of Ac-QWF was as potent as Ac-QWA (Fig. 3). The importance of the C-terminal domain was further investigated by adding back the original amino acids present in this domain, generating Ac-QAWF1 and Ac-QAWFIE. The computer model of the FPR1-peptide complex suggested that the addition of these amino acids would affect the binding of the peptide to a lesser extent because they extended away from the binding pocket unless important interactions occur with the loop regions (Fig. 4). This observation was supported by data obtained from NADPH oxidase activity measurements showing that the Ac-QAWFIE was as potent as Ac-QAWF (Fig. 3G and H). The peptide Ac-QAWFIE had an EC50 value of $11 \pm 1 \mu M$ for activation as compared with Ac-QAWF with an EC50 of $16 \pm 2 \mu M$ (the original peptide Glu9–Tyr20 has an EC50 of $10 \pm 5 \mu M$). These elongated peptides not only activated the neutrophil NADPH-oxidase but also desensitized neutrophils in their response to fMLF (Fig. 3G) and WKYMVM (Fig. 3H) in a concentration-dependent manner similar to that of Glu9–Phe12 (data shown for 50 $\mu M$ annexin peptide). These data imply that the length of the C-terminal domain extending from Phe12 does not affect the binding capacity to the receptor.

**Inhibition of FPR2 Signaling Is Dependent on FPR1 Activation—** Peptides unable to trigger a release of reactive oxygen species (see Table 1) were also unable to inhibit the release of radicals in WKYMVM-stimulated cells (via FPR2). This strongly suggests that receptor activation (FPR1) and inhibition (heterologous desensitization of FPR2) rely on the same type of ligand-receptor interactions.

**TABLE 2**

| Peptide          | % inhibition of fMLF | % inhibition of WKYMVM |
|------------------|----------------------|------------------------|
| Ac Q A W F       | 90±2                 | 85±15                  |
| Ac Q A W F      ++  | 90±2                 | 85±15                  |
| Ac Q A W F      +   | 66±5                 | 75±12                  |
| Ac Q A W F      -   | 35±11                | 52±10                  |
| Ac Q A A F      -   | 10±8                 | 8±9                    |
| Ac Q A A F      -   | 4±10                 | 9±14                    |

* ROS, reactive oxygen species. Peptides with and without NADPH oxidase activity are indicated by plus (+) or minus (−), respectively. Representative values for production of reactive oxygen species are presented in Fig. 3, n.s., non-significant.
Interaction between QAWF and FPR1

from the N-terminal domain of this phospholipid-binding protein.

The Gln<sup>9</sup>–Phe<sup>12</sup> peptide was the smallest annexin I-derived peptide that possessed both the ability to activate the NADPH oxidase and inhibit fMLF- and WKYVMV-induced NADPH oxidase activity. These data, together with the data showing that the Gln<sup>9</sup>–Tyr<sup>20</sup> activity is inhibited by the FPR1 antagonist Boc-MLF but not by the FPR2 antagonist WRWWW, suggest that the annexin I peptide Gln<sup>9</sup>–Phe<sup>12</sup> activates the NADPH oxidase via FPR1 and desensitizes both FPR1 and FPR2 via homologous and heterologous desensitization, respectively. Ligand occupation of FPR1 is associated with a rapid phosphorylation and/or cytoskeleton coupling of the occupied receptor. This results in a physical separation or an inhibition of interaction between the receptor and the G-protein with a termination of signaling capacity (34). This desensitization process makes the cells unable to generate a burst of superoxide when challenged with a new ligand using the same receptor or a hierarchically subordinated receptor (e.g. CXCR triggered by IL-8) (35).

To our knowledge, neither full-length annexin I nor peptides starting with the amino acid Ala<sup>1</sup> have been shown to induce superoxide anion production in neutrophils. The annexin peptide Gln<sup>9</sup>–Lys<sup>25</sup> (Ac<sub>9</sub>–25; 50–200 μM) is known to trigger neutrophil respiratory burst and calcium mobilization (7, 10). Interestingly, a lower concentration of Gln<sup>9</sup>–Lys<sup>25</sup> (20 μM) is without NADPH oxidase activity but antagonizes neutrophil respiratory burst induced by fMLF (10). This difference is also reflected by the inhibitory effect of a p38 mitogen-activated protein kinase inhibitor on Gln<sup>9</sup>–Lys<sup>25</sup> induced radical production but not on calcium mobilization and cell migration (10). Our results imply that the amino acids Gln<sup>9</sup>–Phe<sup>12</sup> are necessary for NADPH oxidase activation, whereas peptides with amino acids at the N terminus of Gln<sup>9</sup>, such as Met<sup>1</sup>–Lys<sup>25</sup> (Ac<sub>1</sub>–26), are without effect on the oxidase activity but can stimulate (or inhibit) calcium mobilization and migration (8) due to different signaling pathways.

Taken together, these data clearly show that the Gln<sup>9</sup>–Phe<sup>12</sup> sequence is of importance for induction of NADPH oxidase activity. To determine the precise amino acids in the receptor that is of importance for transmission of the activating signal, a mutagenesis approach would be required. Such studies cannot, however, be performed because all cells that express a functional oxidase at the same time express also the wild type FPRs. We thus have to rely on a model of the receptor, based on earlier published binding studies to different receptor constructs (see below).

In previous studies by Jesaitis and co-workers (6), several amino acids in helices II–VII were identified as important for the high affinity binding of formylated peptides to FPR1. Due to the lack of determined structures of peptide GPCRs, Mills et al. (29) proposed a model in which the Met-p-benzoyl-i-phenylalanine-Phe-Tyr-N<sup>ε</sup>-(fluorescein)-Lys-OH (fMBpaFYK-fl) peptide binds to a region that overlaps with the conserved location of binding sites of known structures. The modeled peptide (fMBpaFYK-fl) was positioned such that the benzophenone moiety was close to Lys<sup>85</sup>, as suggested by experimental cross-linking data. In a later study, a model was presented in which the N terminus of an N-formyl peptide is hydrogen-bonded to the ion-paired residues Asp<sup>106</sup> and Arg<sup>201</sup>, the leucine side chain of fMLF is close to Lys<sup>85</sup>, and the COOH-terminal carboxyl group of fMLF is ion-paired with Arg<sup>205</sup> (30). Because the arginines are located in helix TM5, which traverses the membrane going inward, this suggests that the fMLF binds with the N terminus closer to the extracellular side.

Our homology model was built using the bovine rhodopsin crystal structure (16), and the Gln<sup>9</sup>–Phe<sup>12</sup> (Ac–QAWF) peptide was docked to the receptor model based on the assumption that the N terminus binds to the most buried part of the pocket. The bottom of the pocket is formed by residues that have been shown to be important for peptide binding (30), many of which are in contact with the peptide in the complex models. In the model, Asp<sup>106</sup> is positioned to form an ion pair with Arg<sup>205</sup> rather than with Arg<sup>201</sup> as previously suggested (30) (Fig. 4C). The energetically most favored solutions of the geometry optimization of the peptide in the pocket suggested that the peptide orients along TM5 toward the extracellular surface in an extended conformation. Due to the uncertainty of the model, in particular with respect to the lack of the extracellular loop 2, the structure model is of low resolution. It is probable, however, that roughly 4 amino acids will be buried within the transmembrane part. The suggested position and direction of the peptide gains support from our functional assay, where the addition of amino acids to the C-terminal is without effect on the abilities of the extended peptides to induce a neutrophil NADPH oxidase activity. In addition, the location of the N-terminal Gln<sup>9</sup> deep in the pocket suggests that modifications of the N terminus should have clear functional consequences. Indeed, elongation of the peptide N-terminally and also removal/replacement of Gln<sup>9</sup> reduce the NADPH oxidase activity. Interestingly, whereas the Gln<sup>9</sup>–Phe<sup>12</sup> peptide seems to bind with the C terminus facing the extracellular side, the bacterial fMLF peptide has been suggested to bind with the C terminus buried in the receptor ion-paired with Arg<sup>205</sup> (30). Other studies of class A peptide receptors have suggested peptide ligand binding with the C terminus in the receptor interior (FMRF-amide bound to mouse MrgC11 receptor) (31) and those with peptides interacting with extracellular loops only (a-factor bound to yeast STE-2 receptor) (32, 33).

The reduced capacity to activate neutrophils following amino acid substitutions/deletion is associated with a loss in the inhibitory effect on the WKYVMV-induced activity, whereas the modified peptides retain the ability to affect the fMLF-induced response. We thus show that the dipeptide Ac–WF functions as a selective FPR1 antagonist. The binding of agonists and antagonists to a receptor is usually competitive, but there is obviously a difference in that binding by agonists generates an intracellular signal, whereas the binding of antagonists does not. By adding back the amino acids Gln<sup>9</sup> and Ala<sup>10</sup> to the N-terminal, the peptide is converted into an agonist that has the capacity to induce a signal (i.e. to induce a neutrophil respiratory burst). This is most likely due to the interactions between Gln<sup>9</sup> and Asp<sup>106</sup> or a residue close to it in the binding pocket. Structures of both the calcium-bound, active conformation and the calcium-free, inactive form of annexin I have been reported. The N-terminal 12 residues of the inactive porcine
annexin I form an amphipathic helix buried in the third repeat of the core, with the Gln<sup>9</sup>-Ala<sup>10</sup>-Trp<sup>11</sup>-Phe<sup>12</sup> motif facing the exit from the buried region (36). In contrast, the calcium-binding form of annexin I does not crystallize with the N-terminal residues present, including Gln<sup>9</sup>-Phe<sup>12</sup> (37). Upon calcium binding, the N terminus has been suggested to be ejected from the hydrophobic pocket in the third repeat and becomes solvent-exposed (36). This suggests that the generation of annexin I N-terminal peptides as well as the binding of the full-length protein to FPR is dependent on calcium.

Defining the molecular difference between the receptor-agonist complex and the receptor-antagonist complex is central to the understanding of GPCR activation and may have implications for the design of receptor-specific ligands. In the absence of the high throughput flow cytometry screening technique, a small library of annexin I-derived peptides were assayed for their FPR1 activity. By using this approach, a tetrapeptide with NADPH oxidase activity was identified. A structural model of the peptide agonist-FPR1 complex suggests that the transmembrane part of the binding pocket of the receptor binds optimally to a 4-amino acid peptide. Both virtual and physical data show that C terminus elongation of the peptide has no effect on FPR1 binding/activity, whereas N-terminal modifications result in antagonistic peptides.

In conclusion, data presented in this work show that the capacity of annexin I peptides to desensitize FPR1 and FPR2 is dependent on binding and signaling via FPR1, suggesting an FPR1-mediated cross-talk between these two receptors. Peptides generated without NADPH oxidase activity had no effect on FPR2 signaling but specifically antagonized the binding of ligands to FPR1. In addition, our data imply that NADPH oxidase-activating peptides derived from annexin I bind to the transmembrane domain of FPR1 with the N terminus located in the bottom of the binding pocket and the C terminus directed toward the extracellular region. The smallest peptide generated with NADPH oxidase activity was the tetrapeptide Glu<sup>9</sup>-Phe<sup>12</sup>. The elongation of this peptide in the C-terminal had no effect on NADPH oxidase activity, whereas N-terminal elongation reduced the activity. This suggests that the N-terminal amino acid glutamine is localized close to the bottom of the binding cleft, leaving for steric reasons insufficient space to extend the peptide in this direction.

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