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A Homolog of Formyl Peptide Receptor-Like 1 (FPRL1) Inhibitor from Staphylococcus aureus (FPRL1 Inhibitory Protein) That Inhibits FPRL1 and FPR

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The members of the formyl peptide receptor (FPR) family are involved in the sensing of chemoattractant substances, including bacteria-derived N-formylated peptides and host-derived peptides and proteins. We have recently described two chemoattractant receptor inhibitors from Staphylococcus aureus. Chemotaxis inhibitory protein of S. aureus (CHIPS) blocks the formyl peptide receptor (FPR) and the receptor for complement C5a (C5aR), while FPR-like 1 (FPRL1) inhibitory protein (FLIPr) blocks the FPRL1. Here, we describe another staphylococcal chemoattractant-inhibiting protein with 73% overall homology to FLIPr and identical first 25 aa, which we termed FLIPr-like. This protein inhibits neutrophil calcium mobilization and chemotaxis induced by the FPRL1-ligand MMK-1 and FPR-ligand FMLP. While its FPRL1-inhibitory activity lies in the comparable nanomolar range of FLIPr, its antagonism of the FPR is ~100-fold more potent than that of FLIPr and comparable to that of CHIPS. The second N-terminal phenylalanine was required for its inhibition of the FPR, but it was dispensable for the FPRL1. Furthermore, the deletion of the first seven amino acids reduced its antagonism of the FPRL1, and the exchange of the first six amino acids with that of CHIPS-confferred receptor specificity. Finally, studies with cells transfected with several chemoattractant receptors confirmed that FLIPr-like specifically binds to the FPR and FPRL1. In conclusion, the newly described excreted protein from S. aureus, FLIPr-like, is a potent inhibitor of the FPR- and FPRL1-mediated neutrophil responses and may be used to selectively modulate these chemoattractant receptors. The Journal of Immunology, 2009, 183: 6569–6578.

Neutrophils are crucial in the initial host defense against microorganisms, but they also contribute to the pathogenesis of inflammatory diseases. To migrate from the blood stream toward the site of infection, they are guided by a gradient of chemotactic factors. These chemoattractants are derived from pathogens, the complement system, host cells and tissues, or even phagocytes themselves and signal via a family of seven-transmembrane, G protein-coupled receptors (GPCRs).1

The formyl peptide receptor (FPR) gene cluster contains the FPR (formylated peptide receptor; FPR1), FPRL1 (formyl peptide receptor-like 1; FPR2/ALX), and FPRL2 (formyl peptide receptor-like 2; FPR3) (1–3). The FPR is the high-affinity receptor for formylated peptides leaking from growing bacteria and therefore serves as an important innate immune recognition receptor (4). Formylated peptides, such as fMLP, are some of the most potent chemoattractants for human leukocytes, and they mediate directed motility, phagocytosis, exocytosis, and superoxide anion generation (5, 6). In addition to formylated peptides, the FPR can be activated by peptides derived from several viral proteins (7, 8). The FPRL1, on other hand, recognizes a variety of ligands of different origin and structure. These include the endogenous anti-inflammatory lipid mediator lipoxin A4 and amyloid-related peptides, bacterial and viral peptides, and peptides derived from synthetic libraries (1–3, 7). Initially the FPR expression has been described in monocytes, neutrophils, and in microglial and dendritic cells but it is now also found in nonhematopoietic cells and tissues. The FPRL1 is also expressed in a large variety of cells and organs including monocytes, neutrophils, macrophages, and microglial cells. Neutrophils express FPR and FPRL1 but not FPRL2, while monocytes express all three members (1, 7). FPRL1 does not only play a role during different stages of the innate immune response, but increasing evidence shows its implication in the pathogenesis of amyloidogenic diseases (9). Both the FPR and FPRL1 trigger many neutrophil functions, including chemotaxis, exocytosis, and superoxide generation. A transient rise in intracellular calcium is required, albeit not sufficient, for triggering optimal chemoattractant-induced responses (5).

Staphylococcus aureus is a commensal of the human skin but can cause a wide spectrum of infections spanning from trivial skin infections to severe septic diseases. This pathogen can produce infections in essentially every human organ or tissue. It exports a variety of virulence factors to the cell surface and extracellular milieu that contribute to the pathogenesis of infection. These proteins cause direct cellular damage and/or interact with host defense factors (10, 11). To cause deep infections and survive in the host, the bacteria must evade the immune system by producing molecules that target critical processes in nearly every stage of the

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3 Abbreviations used in this paper: GPCR, G protein-coupled receptor; Aβ, amyloid β; CHIPS, chemotaxis inhibitory protein of S. aureus; FLIPr, FPRL1 inhibitory protein; FPR, formyl peptide receptor; FPRL; FPR-like receptor; HSA, human serum albumin; LTB4, leukotriene B4; PAF, platelet-activating factor; PIP2, prion protein.

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innate host response to infection (12–14). We have previously reported the molecular properties of several secreted proteins from *S. aureus* that contribute to the evasion of the human defense system. These newly described proteins interfere with several arms of the innate immune response, such as initial rolling of neutrophils (15), complement activation (16), and chemotactic-mediated activation and migration of phagocytes (17). The FPR1-inhibitory protein or FLIPr (18) is a secreted staphylococcal antiinflammatory protein that potently inhibits FPR1-specific ligands, such as the synthetic peptides MMK-1 (19) and WKYMVM (20), and the endogenous proteins amyloid β 1–42 (Aβ42) (21, 21) and prion protein fragment PrP106–126 (22). FLIPr binds directly to the GPCR FPR1, thereby acting as an antagonist. A BLAST search through sequenced *S. aureus* genomes revealed that *S. aureus* encodes for a protein with 73% homology to FLIPr. This protein, which we named FLIPr-like, consists of 104 aa and is preceded by a classical signal peptide with an AXA motif. In this study we show that FLIPr-like inhibits FPR1-mediated neutrophil activation as determined by intracellular calcium mobilization and chemotaxis. Moreover, FLIPr-like also efficiently inhibits FMLP-induced responses comparable to CHIPS. This newly described excreted protein of *S. aureus* is an antagonist of both the FPR and FPR1. Deletion and substitution mutants indicate that the dual activity of FLIP-like is allocated to different sites within the protein.

Materials and Methods

Reagents

MMK-1 (H-LESIFRSSLFRVM-OH) was synthesized by Sigma-Genosys. The hexapeptide WKYMVM-NH2 was from Bachem AG (Switzerland), its D-conformer WKMVMw was synthesized by J. A. Krujitzer (Department of Medicinal Chemistry, Utrecht Institute for Pharmaceutical Sciences, Utrecht, The Netherlands), and WRWWWWW-NH2 (WRW6) and carboxyfluorescin (FAM)-labeled WKYMVM were from Phoenix Pharmaceuticals. IMLP, recombinant C5a, propidium iodide, and anti-FLAG mAb were from Sigma-Aldrich. The fluorescein conjugate of formyl-Nle-Met-Leu-Phe-Nle-Tyr-Lys (abbreviated as FITC-MLLP) and Fluo-3-AM (acetoxyxymethyl ester) were obtained from Molecular Probes/Invitrogen. IL-8 and neutrophil-activating protein 2 were from PeproTech. Platelet-activating factor (PAF)-16 and purified human C3a were from Calbiochem and neutrophil-activating protein 2 were from PeproTech. fMLP, recombinant C5a, propidium iodide, and anti-FLAG mAb were from Sigma-Aldrich. The fluorescein conjugate of formyl-Nle-Met-Leu-Phe-Nle-Tyr-Lys (abbreviated as FITC-MLLP) and Fluo-3-AM (acetoxyxymethyl ester) were obtained from Molecular Probes/Invitrogen. IL-8 and neutrophil-activating protein 2 were from PeproTech. Platelet-activating factor (PAF)-16 and purified human C3a were from Calbiochem and neutrophil-activating protein 2 were from PeproTech. The fluorescein conjugate of formyl-Nle-Met-Leu-Phe-Nle-Tyr-Lys (abbreviated as FITC-MLLP) and Fluo-3-AM (acetoxyxymethyl ester) were obtained from Molecular Probes/Invitrogen. IL-8 and neutrophil-activating protein 2 were from PeproTech. Platelet-activating factor (PAF)-16 and purified human C3a were from Calbiochem and neutrophil-activating protein 2 were from PeproTech.

Cloning, expression, and purification of FLIPr-like

Primers were designed according to the published sequence of the gene MW1038 (from *S. aureus* subsp. aureus MW2) for the cloning of FLIPr-like into pRSETB vector (Invitrogen) and were manufactured by Invitrogen. Recombinant protein was generated by PCR and cloned into the EcoRI and XbaI site of the pRSETB vector by overlap extension PCR and propagated in TOP10F' Escherichia coli (Novagen) as described before (18). After verification of the correct sequence, the protein was expressed in Rosetta (DE3) lysachar E. coli (Novagen) by induction with 1 mM isopropyl-thiogalactopyranoside (Roche). HIS-tagged protein was isolated under denaturing conditions using a 5-mL HiTrap chelating HP column (GE Healthcare Europe) following the manufacturer’s protocol and cleaved afterward with enterokinase (Invitrogen) to obtain the native protein. To remove the HIS-tag, the cleaved protein mixture was passed over a nickel column for a second time and selectively eluted with phosphate buffers of pH 6 and pH 5.3. The separate fractions were analyzed on a 15% SDS-PAGE gel and showed two different bands of purified protein corresponding to 12 and 11 kDa, respectively. The corresponding fractions were pooled and dialyzed separately toward PBS and stored at −20°C. The native protein FLIPr-like was mixed with 0.1 mg/ml FITC (Sigma-Aldrich) in 0.1 M carbonate buffer (pH 9.5) and subsequently separated from free FITC by a desalting column, as described earlier for FLIPr and CHIPS (17, 18).

Construction of mutants and chimeras

Site-directed mutagenesis was performed on the N-terminus of both FLIPr and FLIPr-like by deletion of the first or the first two amino acids, both phenylalanines, and cloning in pRSETB vector by overlap extension PCR as described above. Three chimeras were also constructed. For CHIPS1–4-FLIPr-like106–104 and CHIPS1–2-FLIPr-like105, the first six N-terminal amino acids of FLIPr-like and FLIPr were substituted for the first six amino acids of CHIPS, and for the reverse chimera FLIPS1–2-CHIPS1–121, the amino acids 1–6 of CHIPS were exchanged for those of FLIPr. The competent cells BL21(DE3) E. coli (Novagen) were used to express the mutants and chimeras. After verification of the correct sequence, all HIS-tagged proteins were expressed and purified using the Pro-Bond resin (Invitrogen) or HiTrap chelating HP column following the manufacturer’s instructions and cleaved afterward with enterokinase as described above.

Synthetic peptides

The peptides of the N-terminal amino acids 1–6 of FLIPr as well as FLIPr-like (H-FFSYEW-NH2) were synthesized by Dr. R. van der Zee (Institute of Infectious Diseases and Immunology, Utrecht University) and of CHIPS (H-FTFEPF-NH2) by Dr. John A.W. Krujitzer as described before (23).

Leukocyte isolation

Venous blood was collected from healthy volunteers into tubes containing sodium heparin. Blood was diluted with an equal volume of PBS and layered onto a gradient of Ficoll (Amersham Biosciences) and Histopaque (Sigma-Aldrich). After centrifugation for 20 min at 397 × g and 21°C, mononuclear cells and polymorphonuclear neutrophils were collected separately from Ficoll and Histopaque interfaces, respectively. Cells were washed with cold RPMI 1640 containing 25 mM HEPES, t-glutamine (BioWhittaker), and 0.05% human serum albumin (HSA; Sanquin) (RPMI-HSA). For elimination of erythrocytes, the neutrophil pellet was subjected to a hypotonic shock was distilled H2O for 30 s and centrifuged PBS to restore isotonicity. After washing, cells were resuspended in RPMI-HSA.

Calcium mobilization

To determine activation of neutrophils by chemoattractants the transient increase in free intracellular calcium concentration was measured by flow cytometry (18, 24). For this purpose, neutrophils (5 × 106 cells/ml) were loaded with 2 μM Fluo-3-AM for 20 min at room temperature, protected from light with gentle agitation. The cells were washed, resuspended in RPMI-HSA to 5 × 106 cells/ml and incubated with buffer or protein for at least 1 min. Before stimulation, cells were diluted to 1 × 106 cells/ml in a volume of 180 μl while maintaining the inhibitory protein concentration. The basal fluorescence level for Fluo-3 was monitored at 530 nm for −8 s after which 12.5 μl of 10X concentrated stimulus was added. The sample tube was rapidly placed back to the sample holder and the fluorescence measurement continued up to 52 s. The fluorescence was gated based on scatter parameters to exclude cell debris, and the mean fluorescence value at basal level was subtracted from the value at peak value (at 30 s). The different stimulus concentrations were expressed relative to the maximal response for each individual stimulus.

Chemotaxis

Neutrophil migration was measured in a 96-multifwell transmembrane system (ChemoTX; Neuro Probe) using an 8-μm pore size polycarbonate membrane (18). Cells were labeled with 2 μM calcine-AM for 20 min, washed, and resuspended to a concentration of 2.5 × 106 cells/ml in HBSS supplemented with 1% HSA, Wells were filled with 29 μl of chemottractant, and the membrane holder was assembled. Cells were incubated with different concentrations of FLIPr or FLIPr-like and 25 μl was placed as a droplet on the membrane. After incubation for 30 min at 37°C in a humidified 5% CO2 atmosphere, the membrane was washed extensively with PBS, and the fluorescence was measured in a FlexStation (Molecular Devices). Percent migration was calculated relative to wells containing 25 μl of cells.

Binding to leukocytes

To determine the binding of FLIPr-like to different cell types, 50 μl of blood leukocytes at 5 × 106 cells/ml was incubated for 30 min on ice with buffer or a concentration range FITC-labeled protein. Cells were washed and resuspended in RPMI-HSA. The fluorescence of 17,500 cells was measured by flow cytometry, and the different leukocyte populations were identified based on forward and sideward scatter parameters. For binding in whole blood, 50 μl of EDTA anticoagulated blood was used, and the samples were treated with FACS lysing solution (BD Biosciences) before analysis. To discriminate between different leukocyte subpopulations, leukocytes were labeled with mAbs and selected by specific gating on scatter and fluorescence. Specific labels used were anti-CD3-RPE/Cy5 for T cells, anti-CD4-PE/Cy5 for B cells, anti-CD16-PE/Cy5 for monocytes, and anti-CD11a-PE/Cy5 for NK cells.
anti-CD19-PE for B cells, anti-CD16/CD56-PE (plus negative for anti-CD3-PE/Cy5) for NK cells, and anti-CD14-PE for monocytes.

Binding to HEK293 and HL60 transfecteds

Human embryonic kidney cells (HEK293; obtained from the American Type Culture Collection) were transfected with plasmids containing a FLAG-tagged FPR, FPRL1, or C5AR or HA-tagged FPRL2, as described before (18, 25). After 2 days, transfected cells were harvested with EDTA and incubated with 10 μg/ml mouse anti-FLAG or anti-HA mAb for 45 min on ice. Cells were then washed and subsequently incubated with saturation concentration of allophycocyanin-labeled goat anti-mouse IgG together with different concentrations of FITC-labeled protein, FLIPr-like, FLIPr, or CHIPS. Cells were incubated for 45 min on ice, washed, and resuspended in 200 μl of RPMI-HSA containing 5 μg/ml propidium iodide. FITC (FITC), FL2 (propidium iodide) and FL4 (allophycocyanin) were measured in a FACScalibur flow cytometer (BD Biosciences). As a control, HEK293 cells transfected with an empty pcDNA3.1 vector were used. The binding of FITC-labeled proteins was determined by selecting live cells (propidium iodide-negative) expressing the receptor as indicated by positive staining with anti-FLAG or anti-HA mAb (18). Binding to nontransfected cells within the same sample was used to establish background values. Human promyelocytic leukemia HL60 cells stable transfected with the FPR, FPRL1, and FPRL2 were provided by F. Boulay (Laboratoire Biochimie et Biophysique des Systemes Integres, Grenoble, France) (26, 27). Cells were cultured in RPMI 1640 medium containing 7% fetal bovine serum (FBS). Cells were maintained below 2 × 10^6 cells/ml. Cells were centrifuged at each passage. Fresh cells (5 × 10^6 cells/ml in RPMI-HSA) were incubated with fluorescent-labeled ligands for 45 min on ice under gentle agitation, washed once, and fixed with 1% paraformaldehyde. For competition experiments, cells were first incubated for 15 min with unlabeled protein or peptide. Curve fitting and calculation of inhibitory concentration value (IC_{50}) was performed by nonlinear regression analysis of the dose-response curves generated using Prism 5 (GraphPad Software).

Results

Identification of FLIPr-like

The tblastn algorithm search of the sequenced S. aureus genomes with FLIPr identified a protein with 73% homology in two of the nine genomes: hypothetical protein MW1038 (S. aureus subsp. aureus MW2) and hypothetical protein SAS1089 (S. aureus subsp. aureus MSSA476). The genes encode for an extracellular protein of 104 aa preceded by a classical signal peptide with an AXA motif that is cleaved at the bacterial membrane (Fig. 1A). The homology of this newly identified protein to CHIPS is 27% for the processed mature protein with a high degree of homology in the leader peptides. This homolog of FLIPr, which we named FLIPr-like, was cloned, expressed in E. coli as a HIS-tagged protein without the leader sequence, and purified using nickel affinity chromatography. To remove the HIS-tag, the protein was treated with enterokinase and the cleaved protein mixture was passed over a nickel column again. Selective elution with phosphate buffers of different pH showed two different bands of purified protein corresponding to 12 and 11 kDa, respectively, on a 15% SDS-PAGE gel (Fig. 1B).

N-terminal sequencing of the two proteins identified the 12-kDa band as the native FLIPr-like (first five N-terminal amino acids: FFSYE) and the 11-kDa band as a cleavage product lacking the first seven amino acids, FLIPr-like_{1-104} (first five N-terminal amino acids: GLEIA; Fig. 1A, underlined).

FLIPr-like inhibits neutrophil activation by MMK-1 and fMLP

As FLIPr antagonizes the FPR1, we examined whether FLIPr-like also blocks the activation of this receptor. For this purpose, neutrophils were preincubated with 3 μg/ml FLIPr-like, FLIPr, or CHIPS and compared with control cells for mobilization of intracellular calcium in response to a concentration range of the FPR1-specific ligand MMK-1. Fig. 2A shows a representative change in intracellular calcium concentration of control and FLIPr-like–treated neutrophils stimulated with MMK-1 as measured by an increase in fluorescence. Increasing concentrations of MMK-1 gradually increased the mobilization of intracellular calcium in control cells. Both FLIPr and FLIPr-like completely inhibited the cell response to MMK-1, while CHIPS did not (Fig. 2B). As FLIPr is also a moderate inhibitor of the FPR, the effect of FLIPr-like on fMLP-induced neutrophil activation was examined. Incubation of neutrophils with FLIPr-like shifted the fMLP-induced calcium mobilization curve toward higher concentrations (Fig. 2, C and D). In comparison to FLIPr, its homolog FLIPr-like was a more potent inhibitor of fMLP-induced neutrophil activation and was almost as active as CHIPS (Fig. 2D). Because FLIPr-like mimicked the activity of CHIPS on fMLP, the ability of FLIPr-like to block the C5a-induced calcium mobilization was tested as well. FLIPr-like and its homolog FLIPr did not affect the C5a-mediated calcium mobilization, while CHIPS effectively inhibited this response (Fig. 2, E and F). Additionally, FLIPr-like and FLIPr did not inhibit the calcium mobilization in neutrophils stimulated with optimal concentrations of C3a (C3aR), IL-8 (CXCR1 and CXCR2), neutrophil-activating protein 2 (CXCR2), PAF (PAFR), or LTB_{4} (BLTR) (data not shown). Finally, to examine the effects of these proteins on the FPR2, we performed experiments with F2L, the acetylated peptide derived from cleaved heme-binding protein with high affinity for FPR2. Although neutrophils do not express FPR2, a concentration of 4 μM F2L induced a significant calcium mobilization that was inhibited by 3 μg/ml FLIPr-like and FLIPr but not by CHIPS. This response was probably mediated via the FPR1 and could therefore be inhibited by FLIPr-like and FLIPr. While monocytes are described to express FPR2, we were unable to show stimulation by nanomolar concentrations F2L. FLIPr-like itself at concentrations up to 30 μg/ml did not induce a calcium mobilization in human neutrophils.

FLIPr-like inhibits neutrophil chemotaxis to MMK-1 and fMLP

Both MMK-1 and fMLP induce chemotactic migration of neutrophils. We investigated the effects of FLIPr-like, FLIPr, and CHIPS on neutrophil chemotaxis in a 96-well transmembrane system. Both FLIPr-like and FLIPr showed a comparable concentration-dependent inhibition of chemotaxis to MMK-1, while even 10^{-6} M CHIPS had no effect (Fig. 3A). FLIPr-like and CHIPS effectively inhibited neutrophil chemotaxis toward fMLP (Fig. 3B), and FLIPr only showed an effect at a high concentration (Fig. 3B).

FIGURE 1. Sequence and purification of FLIPr-like. A. Sequence alignment of FLIPr and FLIPr-like using ClustalW. The putatively excreted protein is depicted in bold with black boxes marking mismatched residues and gray boxes indicating the signal-peptidase cleavage site. B. Coomassie blue-stained SDS-PAGE gel (15%) showing purified FLIPr-like protein (lane 1; 12 kDa), mixture after enterokinase cleavage (lane 2), FLIPr-like_{1-104} (lane 3; 11 kDa), and HIS-tagged protein (lane 4; 15.5 kDa). M indicates marker.
The first seven amino acids of FLIPr-like are crucial for fMLP but not MMK-1 inhibition

Cleavage of the HIS-tag from the recombinant FLIPr-like protein with enterokinase generated the native protein as well as an additional cleavage product lacking the first seven amino acids (FLIPr-like8–104). This deletion mutant was also isolated and used to investigate the importance of the N terminus in FLIPr-like activity. Cells were incubated with increasing concentrations FLIPr-like8–104, native FLIPr-like, FLIPr, or CHIPS and stimulated with an optimal concentration MMK-1 or fMLP. Loss of the first seven amino acids in FLIPr-like (FLIPr-like8–104) resulted in diminished inhibitory potency of the MMK-1-induced neutrophil activation as compared with native FLIPr-like (Fig. 4A) that showed maximal inhibition up to $10^{-6}$ M MMK-1 ($B$), $10^{-6}$ M fMLP ($D$), or $10^{-8}$ M C5a ($F$). For reasons of clarity, the gray line was set slightly offset. Cells were stimulated with increasing concentrations of agonist, and the difference in fluorescence values after and before stimulation was expressed relative to the maximal response induced in control cells stimulated with $10^{-6}$ M MMK-1 ($B$), $10^{-6}$ M fMLP ($D$), or $10^{-8}$ M C5a ($F$). Data are means ± SEM of three independent experiments.

Function of FLIPr and FLIPr-like N-terminal mutants and chimeras

To further investigate which residues are important in the activity of FLIPr-like, calcium mobilization assays were performed with several mutants, chimeras, and peptides. Deletion of the first N-terminal amino acid, a phenylalanine, in FLIPr-like (FLIPr-likeF1) did not affect the inhibition of MMK-1- and fMLP-stimulated neutrophils (Fig. 5, $A$ and $B$). However, the mutant lacking the first two N-terminal phenylalanines (FLIPr-likeF2F3) lost the inhibition for the fMLP-induced activation (Fig. 5B) but retained full inhibition for the MMK-1-induced response (Fig. 5A). It was remarkable that both mutants

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**FIGURE 2.** FLIPr-like inhibits MMK-1- and fMLP-induced calcium mobilization. Fluo-3-loaded neutrophils were incubated with buffer (●), 3 µg/ml FLIPr-like (■), FLIPr (○), or CHIPS (▲) for 20 min at room temperature. To monitor calcium mobilization by flow cytometry, each sample was first measured for ~8 s to determine the basal fluorescence. Subsequently, MMK-1 ($A$ and $B$), fMLP ($C$ and $D$), or C5a ($E$ and $F$) was added, and the sample was immediately placed back in the sample holder to continue the measurement. Examples are shown of the change in fluorescence for control (dark line) and FLIPr-like- (gray line) treated cells stimulated with $3 \times 10^{-7}$ M MMK-1 ($A$), $3 \times 10^{-7}$ M fMLP ($C$), and $3 \times 10^{-10}$ M C5a ($E$). For reasons of clarity, the gray line was set slightly offset. Cells were stimulated with increasing concentrations of agonist, and the difference in fluorescence values after and before stimulation was expressed relative to the maximal response induced in control cells stimulated with $10^{-6}$ M MMK-1 ($B$), $10^{-6}$ M fMLP ($D$), or $10^{-8}$ M C5a ($F$). Data are means ± SEM of three independent experiments.

**FIGURE 3.** FLIPr-like inhibits neutrophil chemotaxis toward MMK-1 and fMLP. Calcein-labeled neutrophils were incubated with different concentrations FLIPr-like (■), FLIPr (○), or CHIPS (▲) and allowed to migrate across a 8-µm pore membrane toward $3.3 \times 10^{-8}$ M MMK-1 ($A$) or $3.3 \times 10^{-8}$ M fMLP ($B$). Results are expressed as percentage inhibition of the migration of buffer-treated cells, and all samples were run in triplicate for two experiments. Percentage migration of control cells toward fMLP was 44.4 ± 4.6 (mean ± SD) and toward MMK-1 was 51.8 ± 3.6, and spontaneous migration was 3.4 ± 1.3.
showed a slightly more potent activity toward MMK-1 as compared with the parent FLIPr-like protein. The same N-terminal deletion mutants were constructed for FLIPr. Both mutants, FLIPr\_H9004\_F1 and FLIPr\_H9004\_F1F2, retained all inhibitory activity toward MMK-1 (Fig. 5C). As shown for FLIPr-like, deletion of both phenylalanines (FLIPr\_H9004\_F1F2) eliminates the inhibitory activity for fMLP (Fig. 5D). To further elucidate the importance of the N terminus, chimeras were constructed in which the first six amino acids were exchanged with the remaining sequence of FLIPr-like, FLIPr, or CHIPS. The chimera CHIPS\_1-6-FLIPr\_7-105 showed no inhibition of MMK-1 (Fig. 5E) or fMLP-induced (Fig. 5F) neutrophil activation. Surprisingly, the chimera CHIPS\_1-6-FLIPr-like\_7-104 showed only limited inhibition of the MMK-1 response (Fig. 5E) and no inhibition of the fMLP response (Fig. 5F). The reverse chimera FLIPr\_1-6-CHIPS\_7-121 retained inhibition of the fMLP response (Fig. 5F) and, like CHIPS, did not inhibit the MMK-1 response (Fig. 5E). These results indicate the N terminus as the active site of both FLIPr-like, FLIPr, or CHIPS. The activities of different N-terminal-deletion mutants (ΔF1, ΔF1F2, and FLIPr-like\_8-104) and chimeras of the first six amino acids of CHIPS with FLIPs (CHIPS-FLIPr-like), CHIPS with FLIPr-like (CHIPS-FLIPr-like), and FLIPs (equal to FLIPr-like) with CHIPS (FLIPr-CHIPS) were tested in a calcium mobilization assay with neutrophils stimulated with MMK-1 (3 × 10^{-7} M; A, C, and E) or fMLP (3 × 10^{-9} M; B, D, F). Cells were preincubated with increasing concentrations of antagonists. Data are the means of three or more experiments.
and FLIPr-like for the FPR as was shown before for CHIPS (16). Additionally, part of the N terminus seems to be important for the activity toward the FPRL1. The N-terminal HIS-tagged forms of the proteins were also tested for their inhibitory capacity. Both HIS-tagged FLIPr and FLIPr-like retained their activity on MMK-1-induced responses but lost their effect on fMLP-induced responses (data not shown). We have previously described the importance of the first phenylalanine in CHIPS for the FPR-blocking activity and showed that a synthetic peptide compromising the first six N-terminal amino acids blocks fMLP-induced activation (23). Because the first six amino acids of FLIPr closely resemble the allowed substitutions within this six-amino acid CHIPS peptide (23), synthetic peptides of CHIPS and FLIPr were compared. The FLIPr1–6 peptide, representing the first six amino acids of FLIPr as well as FLIPr-like (see Fig. 1A), effectively inhibited the fMLP-induced response, even more potently when compared with the CHIPS1–6 peptide. Complete FLIPr-like protein was ~100 times more potent than the peptides (Fig. 6). Neither FLIPr1–6 nor CHIPS1–6 peptide inhibited the MMK-1-induced response (data not shown).

FLIPr-like binds to cells expressing FPR or FPRL1

To verify the direct association of FLIPr-like with leukocytes expressing the FPRL1, the binding of FLIPr-likeFITC to neutrophils, monocytes, and lymphocytes was measured by flow cytometry. Fig. 7A shows a concentration-dependent binding of FLIPr-likeFITC to neutrophils, monocytes, and a proportion of lymphocytes. Also, in whole blood, FLIPr-likeFITC bound to neutrophils and monocytes (data not shown). Further analysis of lymphocyte subpopulations revealed a strong binding to B and NK cells and not to T cells, as determined by double labeling with specific mAbs (Fig. 7B). These results are similar to those previously observed for FLIPrFITC (18). Because B and NK cells are not known to definitively express members of the FPR family, cells were preincubated with unlabeled FLIPr-like and FPR agonists to displace FLIPr-likeFITC binding (Fig. 7C). FLIPr-like displaced the binding of FILR-likeFITC to all leukocyte subtypes. For B and NK cells none of the FPR agonists inhibited the binding of FILR-likeFITC. For monocytes and neutrophils that express all FPRs, only WKYMVm showed displacement of FLIPr-likeFITC binding while the high-affinity FPR ligand iMLP and FPRL1-specific ligand MMK-1 were ineffective (Fig. 7C).

The FITC-labeled protein was also used in binding experiments with HEK293 cells transiently transfected with FLAG- or HA-tagged versions of FPR, FPRL1, FPRL2, or C5aR. HEK293 cells expressing the different receptors were analyzed for binding of FLIPr-likeFITC, while the nontransfected cells within the same sample were used to determine background binding. FLIPr-likeFITC bound clearly to HEK293 cells transfected with FPRL1, with some binding to cells transfected with FPR and FPRL2 and not with C5aR (Fig. 8A). As a control, CHIPSFITC...
bound only to FPR- and C5aR-transfected cells as previously described (18) (data not shown). Additionally, undifferentiated HL60 cells stably transfected with the FPR, FPRL1, or C5aR or HA-tagged FPRL2. Binding of 3 μg/ml FLIPr-likeFITC to transfecants was determined by selecting cells stained positive for anti-FLAG or anti-HA mAb and allophycocyanin-labeled goat antimouse IgG Ab (open histogram). The binding to nontransfected cells was determined in the same sample (gray histogram). Data are representative of three experiments. B, Concentration-dependent binding of FLIPr-likeFITC to HL60 cells stably expressing the FPR, FPRL1, or FPRL2 as well as control parent cells. Results are presented as mean fluorescence after subtraction of autofluorescence from a representative experiment. C, Mean fluorescence values ± SEM (n = 3) for binding of 3 μg/ml (250 nM) FLIPr-likeFITC, (D) 100 nM fMLPFITC, and (E) 300 nM WKYMVMFAM to the different HL60 transfectants. ND indicates not done.

Discussion

The activation and migration of phagocytes to the site of inflammation is a key event in host defense against invading microorganisms and in the pathogenesis of several inflammatory diseases (28). We have previously described a secreted protein from S. aureus, FLIPr, which is a potent antagonist of the FPRL1 (18). Here, we identify a highly homologous protein from S. aureus, named FLIPr-like, which inhibits both FPR and FPRL1. FLIPr-like has an overall homology of 73% with FLIPr, and their first 25 aa are identical (Fig. 1). The homology with CHIPS, which inhibits the FPR and C5aR, is only 27% for the native excreted and processed protein. The genes for both FLIPr and FLIPr-like encode an identical signal peptide with an AXA cleavage motive and shows high homology with the signal peptide encoding CHIPS. CHIPS
can be identified in the supernatant of growing *S. aureus* by Western blot analysis (17). Also, FLIPr (SA1001) can be identified in the extracellular proteome of *S. aureus*, as was determined by 2D-PAGE and MALDI-TOF mass spectrometry (11). The presence of a leader peptide in the gene encoding FLIPr-like that is identical to that of FLIPr and is highly homologous to the leader peptide of CHIPS makes it likely that the processed protein can be found in the secretome of *S. aureus*. Additionally, a specific rabbit antiserum identified FLIPr-like in the secretome of *S. aureus* strain MW2 by Western blot (data not shown). The genes for FLIPr (*flr*) or FLIPr-like (*fll*) cluster with other known and potential immune evasion molecules on the genomes of all sequenced *S. aureus* strains. These include SCIN (staphylococcal complement inhibitor) homologs, Efb (extracellular fibrinogen-binding protein), and its homolog Ecb (29). This region represents a novel immune evasion cluster (IEC-2) in *S. aureus*, whereas CHIPS is located on a bacteriophage-localized cluster IEC-1 (30).

Phagocytes, but also many other cell types including cells of the nervous system, express the formyl peptide receptors FPR (FPR1), FPR1 (FPR2/ALX), and FPR2 (FPR3) (3). Expression of FPR2 is restricted to myeloid cells, including monocytes and dendritic cells, and human neutrophils only express FPR and FPR1 (2, 3, 7). This receptor family plays a crucial role in the recognition of microorganisms and inflammatory responses, but only a few antagonists have been reported. Replacement of the formyl group of fMLP with butyloxylcarbonyl resulted in an antagonistic peptide (31), and other modifications of such peptides can also influence their antagonistic power (32, 33). Cyclosporine H has been developed as a potent and selective FPR antagonist and has been reported to inhibit calcium mobilization, chemotaxis, and superoxide generation (34, 35). The antiinflammatory drug piroxicam also inhibits neutrophil activation by FPR but not by FPR1 agonists due to the competition with the natural ligand (36, 37). Furthermore, through screening of hexapeptide libraries, WRW4 was identified as a novel, potent, FPR1 antagonist (37). This peptide inhibits the increase in intracellular calcium induced by several FPR1 agonists. WRW4 also inhibits the activation of FPR2 by the specific ligand F2L, a heme-binding protein fragment peptide (38). Recently, modifications of a substituted quinazoline compound (Quin-C1) convert this selective nonpeptide FPR1 ligand into an antagonists (39). We have previously described a potent FPR1 antagonist that is secreted by *S. aureus*. FLIPr inhibits the chemotaxis and intracellular calcium mobilization induced by the synthetic FPR1 agonists WKYMVM, WKYMVm, and MMK-1 as well as the endogenous peptides A/H9252 and prion protein fragment PrP106–126 (18). Additionally, FLIPr modestly inhibits the FMLP-induced activation of neutrophils. Here, we show that the newly identified *S. aureus* protein FLIPr-like inhibits both FPR and FPR1 agonist-induced calcium mobilization and chemotaxis in human neutrophils at nanomolar concentrations. The hexapeptide WRW4 inhibits the WKYMVM-induced calcium mobilization in FPR1-expressing RBL-2H3 cells at micromolar concentrations (37). Ligands acting on other neutrophil GPCRs are not affected by FLIPr-like. The FPR2 selective agonist F2L (40) induced a calcium flux in neutrophils at micromolar range and could be inhibited by both FLIPr and FLIPr-like. F2L is described to activate human FPR1 as well and to
target the mouse low-affinity receptor Fpr2 (41). Because neutrophils do not express FPRL1, we think that the response was mediated by FPRL1 and could therefore be inhibited by FLIPr-like and FLIPr. Others have indeed shown that F2L is a chemotactic stimulus for neutrophils that binds to the FPRL1 and FPR. In contrast to our data, they observed that F2L fails to stimulate the intracellular calcium increase and superoxide generation (42). FLIPr-like is an antagonist that directly binds to the FPRL1 and FPR, thereby preventing peptide ligands from binding and inducing subsequent signaling. Specific binding was demonstrated using HEK293 transfectants expressing appropriate GPCRs. HL60 cells stably expressing each of the FPRs were used to investigate the receptor specificity of FLIPr-like. Undifferentiated HL60 cells, a cell line of myeloid origin that does not express these receptors (26), showed some minor association of FLIPr-like. This observation can be attributed to the low endogenous expression of FPRL1 or another unknown receptor. However, the specific FPRL1 ligand MMK-1 only induced a calcium response in HL60/FPRL1 cells (data not shown). Strong binding with FPRL1-expressing HL60 cells confirms the data obtained with transiently expression of the receptor in HEK293 cells. FLIPr-like also binds to the FPR- and FPRL2-expressing HL60 cells. Furthermore, competitive binding assays with FPR and FPRL1 demonstrated that all of the FPRL1 specific peptides, WKYMVM, WKYVMVM, MMK-1 and WRW4, displace FLIPr-likeFITC as well as WKYVMVMFAM bound to HL60/FPRL1 cells. A different type of interaction may exist for the FPR where only a partial competition by high-affinity peptide ligands for FLIPr-likeFITC was observed. In line with these observations unlabeled FLIPr-like efficiently competes with WKYVMVMFAM binding to the FPRL1 and partly with the FPR while the high-affinity ligand fMLPPTTTC was only displaced by CHIPS. In our hands, the fluorescent WKYVMVMFAM effectively bound to HL60/FPRL1 cells indicative for a high-affinity binding (as shown for CHO cells expressing the different FPRs) (40), whereas published data indicate specificity for the FPRL1 (20, 26, 27, 43). Apparently both FPR and FPRL1 contain structures necessary and sufficient for binding of FLIPr-like, resulting in prevention of calcium mobilization by fMLP and MMK-1, respectively. The discrepancy between displacement of fluorescent ligands and ability to block functional responses could indicate that nonoverlapping binding sites are involved. FLIPr-like binds to neutrophils and monocytes, which express the FPR as well as FPRL1 (1, 7). Interestingly, FLIPr-like also binds to subpopulations of lymphocytes. Both CD19-positive B cells and CD16/CD56-positive NK cells show strong binding of FLIPr-like that is comparable to its binding to CD14-positive monocytes and neutrophils. Binding is specific and effectively displaced by unlabeled FLIPr-like. However, only the peptide WKYVMVM, with high affinity for FPRL1 as well as FPR, inhibited FLIPr-likeFITC binding to monocytes and neutrophils but not with B and NK cells. Because peripheral blood lymphocytes are not known to definitively express one or more functional FPRs when unstimulated (7, 44), it is likely that FLIPr-like recognizes yet another unknown receptor (or receptors) on these cells that is restricted to certain leukocyte subpopulations. Also on monocytes and neutrophils, FLIPr-like binds probably to another unknown receptor (or receptors) next to members of the FPR family. The homologous protein FLIPr also mimics the profile for this unknown receptors (or receptors) as previously described (18).

In addition to FLIPr-like and FLIPr, S. aureus excretes CHIPS that effectively and specifically inhibits two GPCRs involved in early neutrophil migration, namely the C5aR and FPR (17). For CHIPS, structurally important motifs were identified that participate in the interaction with the C5aR and FPR. The first 30 aa are not essential for inhibition of the C5aR, as a variant lacking those amino acids retains full activity (45). In contrast, the N-terminal region of CHIPS is critical for inhibition of the FPR. In particular, the first amino acid, a phenylalanine, is crucial (23). This study demonstrates that an N-terminal phenylalanine of FLIPr-like and FLIPr is also crucial in the inhibition of the FPR. Both proteins start with two phenylalanines, the first of which is dispensable. It must be noted that the initial six N-terminal amino acids of FLIPr-like and FLIPr resemble the allowed substitutions within the hexapeptide FTFEPF, the minimal N-terminal peptide structure of CHIPS that inhibits FPR activation (23). A peptide resembling the first six amino acids of FLIPr-like and FLIPr indeed inhibited fMLP-induced calcium mobilization, even more potently than did the corresponding CHIPS peptide. To further evaluate this effect, chimeras of FLIPr-like, FLIPr, and CHIPS were constructed in which the 6 N-terminal amino acids were exchanged between the proteins. However, introduction of the N terminus of CHIPS in the FLIPr-like or FLIPr backbone abolished FPR activity, which may have been caused by improperly folded proteins or the need of additional structural elements that were absent or masked. In contrast, the reverse substitution resulting in a CHIPS mutant with N-terminal FLIPr-like and FLIPr led to a protein with slightly diminished activity toward the FPR that was comparable to the activity of wild-type FLIPr-like. As FLIPr-like and FLIPr have identical N-terminal 25 aa, other parts of FLIPr-like contribute to the higher efficacy toward the FPR as compared with FLIPr. In addition to its importance in FPR antagonism, the N terminus of FLIPr-like and FLIPr is also important in the inhibition of the FPRL1. The phenylalanines were dispensable, but the truncation of the first seven amino acids did result in a 100-fold drop in FPRL1-inhibitory activity. Replacing the first six amino acids of FLIPr-like with those of CHIPS resulted in a 10-fold drop in inhibition and may be due to masking of the active site.

The FPRL1 shares 69% amino acid identity with FPR but interacts with a variety of ligands (7). Distinct ligands induce different biological responses and have different modes of receptor activation (5, 46). Interestingly, two homologous S. aureus proteins antagonize these two members of the FPR family with a differential profile. FLIPr-like and FLIPr both inhibit the MMK-1-induced calcium mobilization and chemotaxis within nanomolar range, but they differ in their affinity for the FPR. fMLP-induced calcium mobilization is inhibited by nanomolar concentrations of FLIPr-like and low micromolar concentrations of FLIPr. The activity toward these two related GPCRs probably resides in different domains of FLIPr-like and FLIPr. Structural information on both FLIPr-like and FLIPr will contribute to the elucidation of their inhibitory activity toward the FPR and FPRL1 and may serve as a nonpeptide structural basis for future design of potential therapeutic agents for FPR/FPRL1-related pathophysiology. Alternatively, based on the WKYM peptide structure, the core sequence of a potent agonist was identified that provides important information in designing future peptidomimetic agents for therapeutic use (43). Formyl peptide receptors mediate immune responses to infection, but the identification of novel endogenous agonists in recent years expands the spectrum of biological significance and potential therapeutic approaches.

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