Structural changes of the ligand and of the receptor alters the receptor preference for neutrophil activating peptides starting with a formylmethionyl group

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A B S T R A C T

Pathogenic Staphylococcus aureus strains produce N-formylmethionyl containing peptides, of which the tetrapeptide fMIFL is a potent activator of the neutrophil formyl peptide receptor 1 (FPR1) and the PSMα2 peptide is a potent activator of the closely related FPR2. Variants derived from these two peptide activators were used to disclose the structural determinants for receptor interaction. Removal of five amino acids from the C-terminus of PSMα2 gave rise to a peptide that had lost the receptor-independent neutrophil permeabilizing effect, whereas neutrophil activation capacity as well as its preference for FPR2 was retained. Shorter peptides, PSMα2_1–10 and PSMα2_1–5, activate neutrophils, but the receptor preference for these peptides was switched to FPR1. The fMIFL–PSMα2_1–10 Peptide, in which the N-terminus of PSMα2_1–10 was replaced by the sequence fMIFL, was a dual agonist for FPR1/FPR2, whereas fMIFL–PSMα2_1–10 preferred FPR1 to FPR2. Further, an Ile residue was identified as a key determinant for interaction with FPR2. A chimeric receptor in which the cytoplasmic tail of FPR1 was replaced by the corresponding part of FPR2 lost the ability to recognize FPR1 agonists, but gained function in relation to FPR2 agonists.

Taken together, our data demonstrate that the C-terminus of the PSMα2 peptide plays a critical role for its cytotoxicity, but is not essential for the receptor-mediated pro-inflammatory activity. More importantly, we show that the amino acids present in the C-terminus, which are not supposed to occupy the agonist-binding pocket in the FPRs, are of importance for the choice of receptor.

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1. Introduction

Peptides starting with an N-formylated methionyl group constitute a unique hallmark of bacterial as well as mitochondrial metabolism [1,2], and professional phagocytes of our innate immune system recognize this microbial/mitochondrial pattern as a danger signal that guides innate immune cells, such as neutrophil granulocytes, to infected/inflamed tissues [3,4]. The molecular basis for this recognition in neutrophils is the expression of pattern recognition receptors, e.g., FPRs, for which the formylated methionyl group at the N-terminus of the peptide is a critical determinant. These cell surface receptors belong to the group of G-protein coupled receptors, a group that also comprises other receptors expressed in neutrophils such as the receptors for C5a (C5aR), LTB4 (BLT1), and PAF (PAFR) [5,6]. Human neutrophils express two structurally very similar formyl peptide receptors, FPR1 and FPR2 [6]. The precise biological roles of FPRs are incompletely understood, but the identification of both exogenous and endogenous ligands involved in inflammation strongly indicates a pivotal role of these receptors in regulating defense reactions and the resolution of inflammation [5,7,8].

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The two neutrophil members of the FPR family (FPR1 and FPR2) share a high degree of amino acid identity and signaling properties but they bind different agonists that either activate or inhibit cellular functions depending on the triggering agonist [5]. FPR1 is one of the best-characterized chemoattractant receptors for which the Staphylococcus aureus-derived tetrapeptide fMLF is a very potent agonist and the Escherichia coli-produced attractant FMLF is a slightly less potent agonist. The latter is a commonly used prototypic chemoattractant hardly recognized by FPR2 [5,6,9]. The ability of this prototypic peptide and variants thereof to activate/bind the wild type FPR1 and chimeric receptors, as well as receptor mutants [10-12], has been the basis for defining the structural demands for ligand recognition by FPR1. The high-affinity binding site in FPR1 for formylmethylion peptides most likely involves multiple non-contiguous residues that must be positioned by the proper folding of all extracellular domains as well as the transmembrane parts in the receptor [13,14]. The size of the proposed binding pocket that is available for receptor agonists has been estimated to be limited and comprises four or at most five amino acids [15]. Virtually no studies of structural demands for ligand recognition have been performed with FPR2.

The short S. aureus-derived peptide fMLF displays high-affinity binding to FPR1, but community-associated methicillin resistant S. aureus strains (CA-MRSA) also secrete a group of somewhat longer formylated peptides that interact primarily with FPR2 [16]. These peptides belong to the family of phenol soluble modulins (PSM), α-helical molecules with a high degree of amphipathicity, and they are usually secreted from the bacteria without deformylation (removal of the formyl group at the N-terminal methionine). All PSMα peptides investigated have the same basic functions and promote virulence through effects on distinct neutrophil functions (i.e. chemotaxis) and by being cytotoxic at higher concentrations [16-18]. We have recently shown that PSMα2 and PSMα3 bind to FPR2 and trigger superoxide release in neutrophils at low nanomolar concentrations. In addition, at high nanomolar concentrations they display cytotoxicity selectively on apoptotic neutrophil membranes and this occurs in an FPR2 independent manner [18].

In this study, we investigated the structural-functional relationship for peptide activation of the FPRs in neutrophils. We found that both the N- and C-terminus of PSMα2 are essential for cytotoxicity, whereas the C-terminus could be removed without any major loss in the ability to induce superoxide release. However, the C-terminus plays a critical role in determining receptor preference for FPR2 upon neutrophil activation.

2. Materials and methods

2.1. Reagents

Isoluminol was purchased from Sigma-Aldrich and horseradish peroxidase (HRP) from Roche Diagnostics (Bromma, Sweden). The PSMα2–peptide and its variants were synthesized by an American Peptide Company (Sunnyvale, CA). All peptide stocks were made in DMSO and further dilutions were made in Krebs-Ringer phosphate buffer containing glucose (10 mM), CaCl2 (1 mM) and MgCl2 (1.2 mM) (KRG, pH 7.3). WR247777 (WRW4) was from GenScript Corporation (Scotch Plains, NJ, USA) and Cyclosporin H (CysH) was kindly provided by Novartis Pharma (Basel, Switzerland). Ficoll-Paque was obtained from Amersham Biosciences. The FPR2 specific gelsolin-derived inhibitory peptide PBP10 (gelsolin residues 160-169; [19]) was synthesized by CASLO Laboratory (Lyngby, Denmark). RPMI 1640, fetal calf serum (FCS), PEST and G418 were from PAA Laboratories GmbH, Austria. Annexin V-FLUOS was from Roche Diagnostics (Mannheim, Germany) and ToPro3 from Invitrogen, Molecular Probes (Eugene, OR). The fluorescent peptides WKYVM (Cy5 labeled) and FNeLFnEiYK (FITC labeled) were from Phoenix Pharmaceuticals (Berlingame, CA, USA) and Invitrogen, Molecular Probes (Eugene, OR), respectively.

2.2. Overexpression of wild type FPRs and FPR1/FPR2tail chimeric receptor in HL-60 cells

The procedures used to obtain stable expression of FPR1 and FPR2 in undifferentiated HL-60 cells have been previously described [20]. To prevent possible auto-differentiation due to the accumulation of differentiation factors in the culture medium, cells were passed twice a week after a density of 2 × 10⁶ cells/ml. At each passage, an aliquot of the cell culture was centrifuged, the supernatant was discarded and the cell pellet was resuspended in fresh medium RPMI 1640 containing FCS (10%), PEST (1%), and G418 (1 mg/ml). A 3xHA-tagged FPR1/FPR2tail chimeric receptor was constructed as follows: 3xHA-FPR1 and 3xHA-FPR2 in pCDNA3.1 (+) were purchased from the cDNA Resource Center at the University of Missouri-Rolla and the KpnI–XhoI fragments that contain the entire 3xHA tagged receptors were subcloned in the pCI expression vector (Clontech). The KpnI–PvuII DNA fragment of pCI-3xHA-FPR1 containing the C-terminal cytoplasmatic region of FPR1 was excised and replaced by the corresponding fragment (amino acid 296–351) retrieved from 3xHA-FPR2 in the pCI vector after digestion with KpnI and PvuII. The nucleotide sequence encoding the 3xHA-tagged FPR1/FPR2tail chimeric receptor was further excised from pCI by XbaI and Nhel and inserted in the pEFneo expression vector cleaved by XbaI.

2.3. Isolation of human neutrophils from peripheral blood

Blood neutrophils were isolated, as described by Böyum [21], from buffy coats from healthy volunteers. After dextran sedimentation at 1 g, hypotonic lysis of the remaining erythrocytes, the neutrophils obtained by centrifugation in a Ficoll–Paque gradient were washed twice in KRG. The cells were resuspended in KRG (1 × 10⁶/ml) and stored on ice until use.

2.4. Measurement of superoxide anion production

The production of superoxide anion by the neutrophil NADPH-oxidase was measured by isoluminol-amplified chemiluminescence (CL) in a six-channel Biolumat LB 9505 (Berthold Co, Wildbad, Germany) as described earlier [22,23]. In short, 2 × 10⁵/ml neutrophils were mixed (in a total volume of 900 μl) with HRP (4 U) and isoluminol (6 × 10⁻⁵ M) in KRG, pre-incubated at 37 °C for 5 min, after which the stimulus (100 μl) was added. The light emission was recorded continuously. When required, the specific receptor inhibitors were included in the CL mixture for 5 min at 37 °C before stimulation. By direct comparison of the SOD inhibitable reduction of cytochrome C and SOD inhibitable CL, 7.2 × 10⁷ counts were found to correspond to a production of 1 nmol of superoxide (a millimolar extinction coefficient for cytochrome C of 21.1 was used).

2.5. Assessment of PSMα2 peptides-induced cytotoxicity on apoptotic neutrophils

Freshly isolated neutrophils were resuspended in RPMI 1640 medium supplemented with 10% FCS and 1% PEST to a density of 5 × 10⁶ cells/ml. Cells (200 μl) were incubated at 37 °C in 5% CO₂ for 20 h to allow a part of the neutrophil population to spontaneously go into apoptosis [24,25]. The mixed neutrophil population was washed and resuspended in 100 μl Annexin V binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4) with addition of Annexin V-FLUOS (2 μl). Samples were stained with Annexin V-FLUOS in the dark for 5 min at ambient temperature, after which ToPro3 was added and the staining was continued for another 5 min. Interaction
of cells with PSMα peptides was carried out on ice for 30 min before examination with an Accuri C6 flow cytometer (Accuri, UK).

2.6. Determination of changes in cytosolic calcium

Cells were resuspended at a density of 5 × 10^7 cells/ml in KRG containing 0.1% BSA and loaded with 2 μM Fura 2-AM (Molecular Probes, Eugene, OR) for 30 min at RT. The cells were then washed and resuspended in KRG at a density of 2 × 10^7 cells/ml. The amount of cells used in the assay was 2 × 10^6 cells/measuring cuvette. Calcium measurements were carried out with a PerkinElmer fluorescence spectrophotometer (LS50) with excitation wavelengths of 340 and 380 nm, and an emission wavelength of 509 nm. The transient rise in intracellular calcium is presented as a ratio of fluorescence changes (340:380 nm) [26].

2.7. Binding of FPR ligands

Ligand binding to the FPR1/FPR2 chimeric receptor was determined using a Cy5-conjugated hexapeptide (Cy5-WKYMVm) and a FITC-labeled formylated peptide (FITC-fNleFnileYK) with preferences for FPR2 and FPR1, respectively. HL-60 cells overexpressing the chimeric receptor (2 × 10^6 cells/ml) were incubated with the fluorescent peptides in the absence or presence of unlabeled FPR ligands. The cells were incubated with the labeled peptides on ice for 45 min and the amount of cell bound fluorescence was determined and expressed as the mean fluorescence intensity (MFI) using an Accuri C6 flow cytometer (Becton Dickinson Sparks, MD, USA).

3. Results

3.1. A C-terminally truncated PSMα2 peptide loses its cytotoxic activity but retains pro-inflammatory activity

Nanomolar concentrations of the peptide PSMα2 have been shown to trigger pro-inflammatory neutrophil responses, such as activation of the superoxide generating NADPH-oxidase, and this activation is mediated through FPR2 [18]. In addition, the full length PSMα2 peptide (hereafter referred to as PSMα2) at high nanomolar concentrations was cytolytic, with effects primarily on apoptotic neutrophils (Fig. 1A top panel and B). Based on the observation that the specific FPR2 inhibitor PB810 had no effect on the ability of the peptide to permeabilize the membranes of apoptotic neutrophils, we concluded that the cytotoxic effect occurs independent of FPR2 [18].

To gain further insight into the mechanism underlying the pro-inflammatory and cytotoxic activity of PSMα2, we designed a series of peptide variants (Table 1). PSMα2, a truncated peptide in which the five most distant (the C-terminus) amino acids in PSMα2 were omitted, lacked cytotoxic effects on apoptotic neutrophils (Fig. 1A lower panel and B). The ability to activate neutrophils was retained, however, although with reduced potency (Table 1). The C-terminal part of PSMα2, required for the cytotoxic effect, was not alone sufficient to mediate the cytoytic activity, as illustrated by the fact that the N-terminally truncated peptides PSMα2 or PSMα2 did not have cytotoxic effects on apoptotic neutrophils (Fig. 1B). The two peptides lacking the N-terminal amino acids were also unable to activate neutrophils to produce superoxide anions (Table 1). Taken together, these data show that the full length PSMα2 is required for cytotoxicity and that the peptide PSMα2, lacking the C-terminus, retains the pro-inflammatory activity.

3.2. The residues 5–16 of the PSMα2 peptide are critical determinants for receptor preference

We have earlier shown that the activation of the NADPH-oxidase in neutrophils triggered with the full length PSMα2 peptide is inhibited by FPR2 selective antagonists/inhibitors. This shows a receptor preference for FPR2 and accordingly, the response induced is virtually insensitive to the FPR1 specific antagonist CysH [18]. Similar to the full length PSMα2, the PSMα2 triggered neutrophil activation was also inhibited by the FPR2 inhibitor PB810 (Fig. 2A). The effect of the FPR1 antagonist CysH was fairly small (Fig. 2A), suggesting that FPR2 is the preferred receptor also for PSMα2. This suggestion is supported by the fact that the response was also inhibited by WRW4, another FPR2 selective antagonist (data not shown).

To gain insight into the structure–function relationship of PSMα2 in activating neutrophils, peptides lacking larger segments of the peptide to permeabilize the membranes of apoptotic neutrophils, Annexin V+ToPro3−; lower right in the two control fields without added peptide). In this cell population around 15% were necrotic (Annexin V+ToPro3−; upper right in each field) as assessed by Annexin V-FUOIS and ToPro3 staining by flow cytometry. Incubation of this mixed cell population with the full length PSMα2 (800 nM; upper panel) but not PSMα2 (800 nM; lower panel) for 30 min resulted in a necrotic shift of almost all apoptotic cells. Representative plots out of at least five independent experiments are shown. B) The cytotoxic effect of different PSMα2 derivatives (800 nM final concentration) was examined by incubation of peptides with the mixed cell population on ice for 30 min after which the cells were subjected for flow cytometry analysis. Control cells received buffer only. Data are presented as % necrotic of cell, mean + SEM (n = 3). *p < 0.05 analyzed by one-way ANOVA with Dunnett's comparisons to control.
Fig. 2. Changed receptor preference for the PSMα2−21 derivatives. A) Primary human neutrophils were left untreated or incubated with the FPR1 specific inhibitor CysH and the FPR2 specific inhibitor PBP10 (Fig. 2B). When the concentration of PSMα2−12 was lowered, the FPR1 selective inhibitor was actually slightly more potent than the FPR2 specific inhibitor (data not shown). Further, when the two inhibitors were combined, the inhibition was total (Fig. 2B), suggesting that PSMα2−12 is a dual agonist that is recognized by both FPR1 and FPR2. The PSMα2−10 and PSMα2−5 peptides, with additional deletions of amino acids from the C-terminus, retained the capacity to activate neutrophils to produce superoxide. EC50 values, however, reflected lower potency (Table 1). More importantly, the responses induced by these peptides (PSMα2−10 and PSMα2−5) were totally inhibited by CysH but not by PBP10 (Fig. 2C, D), suggesting that FPR1 was the preferred receptor.

The receptor preference and relative potency of the PSMα2 variants were also determined with FPR1 and FPR2 overexpressing cells and the results obtained with primary neutrophils were confirmed. In agreement with the receptor preference observed in neutrophils expressing both receptors, PSMα2−16 at 1 nM concentration triggered a robust calcium transient in FPR2 overexpressing cells, whereas no such activity was induced in cells overexpressing FPR1 (Fig. 3). In addition, the shorter peptides PSMα2−10 and PSMα2−5 evoked a transient rise in calcium solely in FPR1 overexpressing cells (Fig. 3). With respect to potency, a much higher concentration of PSMα2−5 was needed to trigger a calcium response in FPR1 overexpressing cells, compared to the concentration needed for PSMα2−10 to induce a response in FPR2 overexpressing cells (Fig. 3). In agreement with the dual agonism of PSMα2−12 in neutrophils (Fig. 2B), this peptide triggered a calcium response in both FPR1- and in FPR2 overexpressing cells (Fig. 3). Taken together, these data show that the presence of the C-terminal amino

Table 1
Forsman H et al. Description of the PSMα2 and fMIFL peptides.

<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>Number of amino acids</th>
<th>EC50 (nM)</th>
<th>95% (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>f-MIFL</td>
<td>4</td>
<td>0.12</td>
<td>0.09–0.17</td>
</tr>
<tr>
<td>f-MIFL-PSM5−10</td>
<td>10</td>
<td>0.40</td>
<td>0.23–0.67</td>
</tr>
<tr>
<td>f-MIFL-PSM5−12</td>
<td>12</td>
<td>0.60</td>
<td>0.38–0.95</td>
</tr>
<tr>
<td>f-MIFL-PSM5−16</td>
<td>16</td>
<td>0.73</td>
<td>0.51–1.05</td>
</tr>
</tbody>
</table>

Peptide sequences and the number of lengths (number of amino acids) are shown. The activating potencies of the peptides for neutrophil FPRs were examined in the superoxide release assay. Human neutrophils were stimulated with different concentrations of peptides and dose-dependent superoxide release was recorded. The EC50 values and 95% confidence intervals (CI) were calculated from the normalized peak superoxide release from three independent experiments. Data are shown as the mean EC50 in (nM)."--": poor or inactive in the assay.

C-terminus were generated (Table 1). The PSMα2−12 peptide (four additional amino acids deleted from the C-terminus of PSMα2−16) activated neutrophils to release superoxide with an EC50 value only slightly higher than that of PSMα2−16 (Table 1), suggesting that these four amino acids do not significantly contribute to the activation potency. They do, however, contribute to the choice of receptor, as shown by the fact that the neutrophil response induced by PSMα2−12 was inhibited to the same degree by the FPR1 specific inhibitor CysH and the FPR2 specific inhibitor PBP10 (Fig. 2B). When the concentration of PSMα2−12 was lowered, the FPR1 selective inhibitor was actually slightly more potent than the FPR2 specific inhibitor (data not shown). Further, when the two inhibitors were combined, the inhibition was total (Fig. 2B), suggesting that PSMα2−12 is a dual agonist that is recognized by both FPR1 and FPR2. The PSMα2−10 and PSMα2−5 peptides, with additional deletions of amino acids from the C-terminus, retained the capacity to activate neutrophils to produce superoxide. EC50 values, however, reflected lower potency (Table 1). More importantly, the responses induced by these peptides (PSMα2−10 and PSMα2−5) were totally inhibited by CysH but not by PBP10 (Fig. 2C, D), suggesting that FPR1 was the preferred receptor.

The receptor preference and relative potency of the PSMα2 variants were also determined with FPR1 and FPR2 overexpressing cells and the results obtained with primary neutrophils were confirmed. In agreement with the receptor preference observed in neutrophils expressing both receptors, PSMα2−16 at 1 nM concentration triggered a robust calcium transient in FPR2 overexpressing cells, whereas no such activity was induced in cells overexpressing FPR1 (Fig. 3). In addition, the shorter peptides PSMα2−10 and PSMα2−5 evoked a transient rise in calcium solely in FPR1 overexpressing cells (Fig. 3). With respect to potency, a much higher concentration of PSMα2−5 was needed to trigger a calcium response in FPR1 overexpressing cells, compared to the concentration needed for PSMα2−10 to induce a response in FPR2 overexpressing cells (Fig. 3). In agreement with the dual agonism of PSMα2−12 in neutrophils (Fig. 2B), this peptide triggered a calcium response in both FPR1- and in FPR2 overexpressing cells (Fig. 3). Taken together, these data show that the presence of the C-terminal amino

![Image](image-url)
Shorter chimeric fMIFL-PSM peptides (i.e., fMIFL-PSM$_{12}$ and fMIFL-PSM$_{4}$) also activated neutrophils to release superoxide, with EC$_{50}$ values very similar to those of the fMIFL-PSM$_{21}$ peptide (Table 1). The fMIFL-PSM$_{12}$ peptide was also a dual agonist, as illustrated by the fact that neither CysH nor PBP10 alone inhibited the response (Fig. 4D). In contrast, the activity induced by fMIFL-PSM$_{4}$ was fully inhibited by CysH, whereas PBP10 was without effect (Fig. 4D), suggesting that FPR1 was the preferred receptor.

In conclusion, there was a clear difference in receptor preference between fMIFL-PSM$_{4}$ and fMIFL-PSM$_{12}$, in that fMIFL-PSM$_{4}$ activated neutrophils primarily through FPR1, whereas fMIFL-PSM$_{12}$ was a dual agonist as the response was inhibited by the combined action of CysH and PBP10 (Figs. 4D, 5A). To investigate the role of the amino acids Ile at position 11 and Lys at position 12 present in the fMIFL-PSM$_{12}$, but absent in fMIFL-PSM$_{4}$, we designed peptide variants and determined the receptor preference of these variants through the inhibitory effects of CysH and PBP10. The inhibition profile for fMIFL-PSM$_{4}$ was not changed when Lys in position 12 was exchanged for a Gln (Fig. 5B). The dual agonism was, however, lost when Ile in position 11 was omitted or exchanged for an Ala, as the response induced by these peptides was markedly inhibited by CysH, suggesting a preference for FPR1 over FPR2 (shown for the peptide with Ile omitted in Fig. 5B). These variants were as potent as the fMIFL-PSM$_{12}$ peptide in triggering superoxide release in neutrophils (data not shown). Taken together, the data strongly suggests that size matters when it comes to FPR preference, and the C-terminal part of the peptides constitutes a key determinant for FPR2 interaction.

4.1. A chimeric receptor in which the cytoplasmic tail of FPR1 was replaced by that in FPR2 recognizes FPR2 agonists

The calculations of the proposed agonist-binding pocket for fMet peptides present in FPR1 suggest that there is room for four or maybe five amino acids [15], but the results presented in this study show that the receptor preference is determined also by parts of the agonists that most probably are located outside of the proposed binding pocket. Changed binding properties could possibly also be induced by structural changes of the receptors, and these changes might involve domains of the receptors not directly engaged in agonist binding. This suggestion gains support from earlier published results with an FPR1 chimeric receptor having a cytoplasmic tail identical to that of FPR2 (amino acids 296–351; Fig. 6A). This chimeric receptor was no longer able to respond to fMIFL, an FPR1 specific agonist ((19) and Fig. 6B). This finding supports the suggestion that the ligand-binding characteristics may be influenced by parts of the receptor not directly involved in agonist binding. We have now extended these findings when showing that the loss of function of the FPR1/FPR2tail chimeric receptor is also accompanied by a gain of function, as the fMIFL-PSM$_{12}$ hybrid peptide, shown to be a dual agonist for the two FPRs, triggered an increase in [Ca$^{2+}$]$_i$ in cells expressing the chimeric FPR1/FPR2tail receptor (Fig. 6C). Moreover, the response induced by fMIFL-PSM$_{16}$ in cells expressing the FPR1/FPR2tail receptor was completely abolished by PBP10 (an FPR2 specific inhibitor) but not by CysH (an FPR1 specific inhibitor; Fig. 6C). Taken together this suggests that the chimeric receptor (FPR1/FPR2tail) has a preference for FPR2 ligands, which gained further support from the activation profile obtained when using several other well-characterized agonists that have been shown to be either dual agonists or specific receptor agonists for FPR1 and FPR2, respectively [5,6]. The receptor preferences of all ligands used were confirmed using FPR1- and FPR2 overexpressing cells (Fig. 6D), and all dual and FPR2 specific agonists were found to induce a rise in [Ca$^{2+}$]$_i$ in cells expressing the chimeric FPR1/FPR2tail receptor, whereas no such response was induced by FPR1 specific agonists (Fig. 6D).

Binding experiments performed with fluorescent-labeled FPR agonists support the suggestion about the changed receptor preference. Accordingly, we could not show any binding of the fluorescent FPR1

**Fig. 3.** Transient changes in cytosolic calcium induced by the PSM$_{21}$ derivatives in HL-60 cells overexpressing FPR1 or FPR2. Fura-2 labeled HL-60 cells overexpressing FPR1 (left panel) or FPR2 (right panel) were stimulated with PSM$_{21}$ derivatives at different concentrations as indicated. The time points for peptide addition are further illustrated by arrows and an increase in cytosolic free Ca$^{2+}$ was monitored by the change in Fura-2 fluorescence. Representative experiments out of at least three are shown. Abscissa, time of study (s); ordinate, fluorescence (arbitrary units).
specific ligand fNleLFNleYK [27] to cells expressing the chimeric receptor (data not shown) whereas the dual agonist (Cy5-WKYMVm) binds. This binding was specific as illustrated by the fact that a non-labeled peptide inhibited binding and in addition this binding was also inhibited by the FPR2 specific ligand WKYMVM whereas the FPR1 specific ligand fMIFL was without any effect (Fig. 7).

4. Discussion

The FPRs play important roles in the defense reaction against microbial infections and in regulation of inflammation. Their basic function is to recognize danger signals in the form of N-formylated peptides and various other inflammatory mediators [5,16,28].
two neutrophil FPRs (FPR1 and FPR2) interact with formylated peptides, but they possess somewhat different recognition profiles. It has been known for long that FPR1 recognizes N-formylated methionyl (N-fMet) peptides generated by bacteria or mitochondria, forming the molecular basis for professional phagocytes (neutrophils and monocytes/macrophages) to sense gradients of chemoattractants and to migrate directionally to the sites of infection and inflammation [7,8]. Following the deorphanization of FPR2 [20,29,30], a receptor closely related to FPR1, a number of different non-formylated peptides and small molecules were found to be recognized by this receptor, but despite the large similarities with FPR1, none of the high affinity ligands initially described for FPR2 belonged to the group of peptides starting with an N-fMet group [5,6]. The recent characterization of peptides from mitochondria [1] and a group of phenol-soluble modulins (PSMs), a family of peptides that are secreted by a dedicated transport system in pathogenic strains of S. aureus, changed this situation as these peptides start with an N-fMet and have proinflammatory properties that are mediated primarily via interaction with FPR2 [16,18]. It is thus clear that both FPR1 and FPR2 have the ability to recognize N-fMet peptides but with different binding profiles depending on the amino acids linked to the initial fMet moiety.

In this study, we investigated the structural–functional relationship of PSMox2 peptides in interaction with FPR1 and FPR2, using a rapid and sensitive functional assay (an NADPH-oxidase activity assay). This assay allows us to fairly rapidly measure and quantify receptor specific activities using many different agonist concentrations in the presence and absence of defined antagonists, and by that the technique is well suited to determine receptor preference for the peptide agonists. The signaling molecules downstream of the FPRs are many and the pathways are complicated [5,6], but since our work is focused on the receptor preference for the PSM-peptides, cells overexpressing FPR1 or FPR2 and a read out system (the PLC/Ca²⁺ route) that picks up a signal as close as possible to the receptors were used to confirm the receptor selectivity. Our results clearly show that the C-terminus of PSMox2 is of prime importance for activation of FPR2 by this peptide, as deletion of the C-terminal region generates peptide variants of
PSMox2 with a changed receptor preference, going from FPR2 selective over a dual FPR1/FPR2 agonism to a preference for FPR1 for the shortest peptides. The importance of the C-terminus of PSMox2 in FPR2 interaction is further supported by the fact that an attachment of a twelve-amino-acid long sequence (amino acids 5–16) in PSMox2 to the potent FPR1 agonist FMFL give rise to a dual agonist that activates both FPR1 and FPR2. A peptide that is devoid of the N-terminal part has no FPR-activating effects, and the sequence starting with an N-formylated methionyl group is required for the interaction with both FPR1 and FPR2. The amino acids of the N-terminus in PSMox21–16 are identical to those in PSMox21–5 and PSMox21–10, respectively, yet the peptides activate different receptors. It is known from earlier studies that the binding pocket in FPR1 has room for no more than five amino acids [15], and when the charge of an FPR1 selective pentapeptide is reduced, such peptides will interact also with FPR2 [31], even if FPR1 is still the preferred receptor [32]. This means that receptor preference is not determined solely by the amino acids that have access to the presumed agonist binding pocket of the receptor, but in part also by amino acids in the peptide that do not have direct access to the binding pocket.

In addition to the FPR2-mediated pro-inflammatory activity, PSMox peptides also exert cytotoxicity on leukocytes [16,18]. This is a result of the PSMox peptide adopting an amphiphilic α-helical structure, which may contribute to membrane disruption and cytotoxicity. Our results obtained using PSMox2 variants suggest that the full-length peptide is required for cytolytic activity on apoptotic neutrophils, but since the N-terminus is the part that is essential for neutrophil activation there is no direct link between the cytolytic and the pro-inflammatory activities. The lack of cytolytic activity of the C-terminal truncated peptides may be related to a reduced capacity to form an α-helix, although it has been suggested that the α-helicity is not correlated with peptide functions, including cytolytic activity [31]. In line with our findings, a recent study using an alanine substitution screen of PSMox3, a closely related S. aureus modulin, demonstrates that the physicochemical property of the amino acids present in C-terminus of PSMox3 (i.e., Lys6, Lys12, Lys17, Asn21 or Asn22) is crucial for the FPR2 binding capacity [31]. It should be noticed, however, that no change in the receptor preference was described, meaning that all the PSMox3 peptide variants tested preferred FPR2 over FPR1. This suggests that the C-terminus of PSMox3 is important for the potency, but not for receptor preference [31]. With respect to potency, our results support the role of the C-terminus, as illustrated by the fact that the truncated peptide PSMox21–15 retains FPR2 selectivity, but with a substantially reduced potency compared to PSMox21–21. Whether this is due to a direct interaction of the C-terminus of the peptide with the receptor, or more indirect effects due to an interaction with the membrane, remains to be determined. Although PSMox2 and PSMox3 share a common three-dimensional structure, their primary peptide sequences are very different, and it is possible that the C-termini of PSMox2 and PSMox3 are differently involved in FPR interaction. More studies attempting to understand the structure-function relationship of individual PSM peptides should help elucidate whether a common mechanism is applicable for all PSM peptides.

With respect to receptor preference it is obvious that the Ile at position 11 in the PSMox2 peptide is of prime importance, as illustrated by the preference change from FPR2 to FPR1 when this non-polar residue was omitted or exchanged for an Ala, but it is hard to fit this into the binding model for the FPRs. Receptor mutagenesis studies with FPR1 suggest that amino acids in both extracellular and transmembrane domains are of importance for agonist binding [10,12,14,33], but only a limited number of these (i.e., the ones in positions 84, 85, 89, 90, 102, and 103) differ between FPR1 and FPR2. Recent computer modeling and site directed mutagenesis experiments have suggested that the amino acid difference in position 281 (an Asp in FPR2 and a Gly in FPR1) is of importance for the selectivity in agonist binding [32], and an exchange of the Asp in FPR2 for a Gly may change the activation potency of certain fMet containing peptides [32]. The chimeric receptor approach has also been used to define the molecular determinants involved in agonist recognition [5,6], and it is evident that an exchange of receptor parts that are not directly involved in agonist binding may also affect the selectivity. In line with this, it has been shown that a chimeric receptor in which the cytoplasmic tail in FPR1 was replaced by that present in FPR2 possesses reduced the potency of the FPR1 agonist FMFL [34], and a chimeric receptor in which the second intracellular loop or the cytoplasmic tail of FPR1 was exchanged for the corresponding parts of FPR2 actually results in an enhanced calcium response to FPR2 agonists [35]. We also recently described that a chimeric FPR1/FPR2tail receptor in which the cytoplasmic tail in FPR1 was replaced with that from FPR2 (amino acids 296–351), was unable to recognize FPR1 agonists [19]. Interestingly this chimeric receptor also gained function, a conclusion drawn from our results showing that all FPR2 agonists investigated activated this receptor. It is not very likely that the cytoplasmic tail is directly involved in ligand binding, but rather that it affects distant parts of the receptor involved in agonist recognition. Taken together, these observations suggest an importance of the composition/structure of cytosolic receptor domains for ligand binding. This is supported by the fact that a chimeric FPR1/FPR2tail receptor, containing all the structural elements supposed to be of importance for recognition of FPR1 agonists, is activated by agonists that have FPR2 as their prime receptor. It is reasonable to assume that the formation of a high-affinity agonist-binding site is a highly dynamic process that must be apposed by coordinate folding of multiple non-contiguous domains, including the sites that are directly involved in binding. This indicates that very similar binding structures on the molecular level may be present in FPR1 and FPR2, and that binding is affected by more general conformational differences between the two receptors. The fact that FPR2-selective agonists, but not FPR1-selective agonists, activate cells expressing the chimeric receptor in which the cytoplasmic tail of FPR1 has been exchanged for that of FPR2, support this suggestion.

Although the two FPRs share large sequence similarities and induce almost identical neutrophil responses, we have earlier described some fundamental differences that distinguish these receptors from each other. One such characteristic is that the two receptors differ in their sensitivity to allosteric modulation from the cytosolic side of the plasma membrane. We have earlier suggested this as the mechanism at hand for the FPR2 selective inhibition mediated by the gelsolin derived peptide PB10 [19], as well as for the FPR2 selective activation induced by the pepducin F2Pal10 (with a peptide sequence derived from the third intracellular loop of FPR2) [36]. The fact that these FPR2 selective allosteric modulators also inhibit and activate, respectively, the cells that express the chimeric FPR1/FPR2tail, in which the cytoplasmic tail of FPR1 has been exchanged for that in FPR2, suggests that very similar binding structures on the molecular level may be present in FPR1 and FPR2, and that interaction with allosteric modulators is also affected by more general conformational differences between the two receptors.

In conclusion, our data show that the length of a peptide agonist and the structure of the FPRs together determine the activation potency and receptor preference of a given agonist. The PSMox2 derivatives identified, with distinct receptor preference, together with the unique allosteric modulators for FPRs, should serve as valuable molecular tools to approach the ligand-binding pocket and other regulatory domains present in FPR1 vs. FPR2, as more structural information, including crystal structures for FPRs, becomes available.

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Authorship

Contribution: HF and CD designed and wrote the manuscript together. MG, MW, and HF performed the experiments and analyzed the data together with CD and SLS. FB and M-JR provided suggestions on the project, constructed the chimeric receptor and, revised the manuscript.

References


