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Structural Characterization and Inhibitory Profile of Formyl Peptide Receptor 2 Selective Peptides Descending from a PIP2-Binding Domain of Gelsolin

Huamei Forsman,* Emil Andréasson,* Jennie Karlsson,* Francois Boulay,*†‡§¶ and Claes Dahlgren*

The neutrophil formyl peptide receptors, FPR1 and FPR2, play critical roles for inflammatory reactions, and receptor-specific antagonists/inhibitors can possibly be used to facilitate the resolution of pathological inflammatory reactions. A 10-aa-long rhodamine-linked and membrane-permeable peptide inhibitor (PBP10) has such a potential. This FPR2 selective inhibitor adopts a phosphatidylinositol 4,5-bisphosphate–binding sequence in the cytoskeletal protein gelsolin. A core peptide, RhB-QRLFQV, is identified that displays inhibitory effects as potent as the full-length molecule. The phosphatidylinositol 4,5-bisphosphate–binding capacity of PBP10 was not in its own sufficient for inhibition. A receptor in which the presumed cytoplasmic signaling C-terminal tail of FPR2 was replaced with that of FPR1 retained the PBP10 sensitivity, suggesting that the tail of FPR2 was not on its own critical for inhibition. This gains support from the fact that the effect of cell-penetrating lipopeptide (a pepducin), suggested to act primarily through the third intracellular loop of FPR2, was significantly inhibited by PBP10. The third intracellular loops of FPR1 and FPR2 differ in only two amino acids, but an FPR2 mutant in which these two amino acids were replaced by those present in FPR1 retained the PBP10 sensitivity. In summary, we conclude that the inhibitory activity on neutrophil function of PBP10 is preserved in the core sequence RhB-QRLFQV and that neither the third intracellular loop of FPR2 nor the cytosplasmic tail of the receptor alone is responsible for the specific inhibition.

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Abbreviations used in this article: [Ca2+]i, intracellular Ca2+ concentration; CL, chemoiluminescence; FPR, formyl peptide receptor; β-gal, β-galactosidase; GPCR, G protein-coupled receptor; HA, hemagglutinin; MFI, mean fluorescence intensity; PAF, platelet-activating factor; PIP2, phosphatidylinositol 4,5-bisphosphate; PSM, phenol-soluble modulin; ROS, reactive oxygen species.

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the inhibitory effect of PBP10 is, in contrast to the conventional antagonists, dependent on its ability to pass membranes and block FPR2- but not FPR1-mediated cellular responses. Nevertheless, the precise mechanism underlying PBP10-mediated inhibition on FPR2 signaling has not yet been defined.

In the present study, we sought to characterize the structural requirements of both PBP10 and FPR2 in mediating the PBP10-sensitive pathway. We could show that a chimeric FPR2/FPR1 receptor, in which the entire cytoplasmic tail of FPR2 (a signaling domain that differs substantially between FPR1 and FPR2) was replaced for its FPR1 counterpart, retained its sensitivity to PBP10, and the PBP10 insensitivity was not transferred to a "mutated" FPR2 in which the two amino acids differing between FPR1 and FPR2 were exchanged. Taken together, these data suggest that neither the cytoplasmic tail of FPR2 nor the third intracellular loop do alone determine the PBP10 sensitivity. The neutrophil activity induced by a cell-permeable peptide (a pepducin) derived from the third intracellular loop of FPR2 was dose-dependently inhibited by PBP10, suggesting similarities between the PBP10-sensitive pathway and that triggered by pepducin. Furthermore, our data show that the PIP2-binding capacity alone was not enough for inhibition of FPR2, and a hexapeptide with a C-terminal truncation, PBP1<sub>1.6</sub> (RhB-QRLFQV), was as potent as the original PBP10 in inhibiting the FPR2-mediated response, but the receptor specificity was partly lost, as this shorter peptide inhibits also the FPR1-mediated cellular response.

Materials and Methods

Peptides and reagents

Rhodamine B-conjugated PBP10 peptide (RhB-QRLFQVKKGR) and PBP2-related peptides as well as the FPR2/FPR1-derived pepducins F2pal-16F1-pal-16 were synthesized by Caslo Laboratory (Lyngby, Denmark). The formulated peptide IMIFL and the hexapeptide WKYMVM were synthesized and purified by AltaBioscience (University of Birmingham, Birmingham, U.K.), and the phenol-soluble modulin (PSM#o2 peptides (PSM#o2, MCIIAGIKPKIKLIEKRKTG: PSMα3, MEFVAKLFKFLLLQGLG-NN) were synthesized in their formulated form by American Peptide Company (Sunnyvale, CA). The formulated peptide 3MLF and 3Sa were purchased from Sigma-Aldrich (St. Louis, MO). IL-8 was from R&D Systems (Abingdon, U.K.) and platelet-activating factor was from Avanti Polar Lipids (Alabaster, AL). The FITC-INLPNTL and the Cy5-WKYMVM peptides were from Phoenix Pharmaceutical ( Burlingame, CA), and compound 43 was a gift from Agen (Thousand Oaks, CA). All peptides were dissolved in dimethyl sulfoxide to a concentration of 10<sup>−2</sup> M and stored at −70°C until use. Further dilutions were made in Krebs-Ringer phosphate buffer that was supplemented with glucose (10 mM), Ca<sup>2+</sup> (1 mM), and Mg<sup>2+</sup> (1.5 mM) (KRG; pH 7.3).

Human neutrophils and cell lines overexpressing FPRs

Neutrophil granulocytes were isolated from buffy coats obtained from healthy adults by dextran sedimentation at 1 × g, hypotonic lysis of the remaining erythrocytes, and centrifugation in a Ficoll-Paque gradient (18). The neutrophils were washed twice and resuspended in KRG and stored on melting ice until use.

The stable expression of FPR1 and FPR2 in undifferentiated HL-60 cells has been described elsewhere (19, 20). Control experiments with the specific agonists (IMLF for FPR1 and WKYMVM for FPR2) were performed at each experimental event. The hemagglutinin (HA)-epitope–tagged FPR2/FPR1 chimeric receptor was constructed as follows: a PvuII-XhoI fragment that contains the C-terminal cytoplasmic region of FPR1 (aa 296–350) was amplified by PCR using the CDMS/FPR1 plasmid as a template. The purified fragment was substituted for the corresponding fragment in 3HA-tagged FPR2 in the pCI expression vector (Clontech Laboratories). The nucleotide sequence encoding the 3HA-tagged FPR2/FPR1 chimeric receptor was further excised by XbaI and Nhel from pCI and inserted into the pEFneo plasmid cleaved by XbaI (21). The wild-type form of 3HA-tagged FPR1/ALX was purchased from the cDNA Resource Center at the University of Missouri–Rolla. A mutant 3HA-tagged FPR (3HA-FPR2oopR1) in which the sequence of the third intracellular loop of FPR2 (KIHKKG-MIKSSRPLRV) has been mutated to the sequence of the third intracellular loop of FPR1 (KIHKKQGLKKSSRPLRV) was created by using the PCR strategy described by Yon and Fried (22). Briefly, using 3HA-tagged FPR2/ALX in pCDNA3.1 as a template, two intermediary PCR fragments, PCR1 and PCR2, were generated with two couples of primers. PCR1 was produced with a sense primer that is located upstream the start codon. The 5’ end of the primer contained an extension with an appropriate restriction site for further ligation in an expression vector, whereas the reverse primer carried the desired mutations. Likewise, PCR2 was created with a sense primer, which is complementary to the reverse primer used for the synthesis of PCR1, and a reverse primer, located downstream from the stop codon, with an appropriate restriction site. After purification, the two PCR fragments were mixed, denatured, and hybridized. The mixture was used to generate PCR3, which contains the mutated sequence. Ten cycles of amplification were first performed in the absence of primers to increase the number of copies of full-length cDNA carrying the mutations on both strands. Then, 35 cycles of amplification were carried out in the presence of the sense and reverse primers used to generate PCR1 and PCR2. PCR3 was cleaved with the appropriate restriction enzymes for ligation in pCDNA3.1 (Invitrogen, Cergy Pontoise, France). The entire sequence of the 3HA-tagged mutant was cloned in pEF-neo (21) for stable expression in HL-60 cells. The open reading frame was entirely sequenced.

Chinese hamster ovary cells expressing FPR1 and FPR2 fused to a nonfunctional β-galactosidase (β-gal) fragment were obtained from DiscoveRx (Fremont, CA).

**Determination of changes in cytosolic calcium and translocation of β-arrestin**

HL-60 cells were labeled with Fura 2-AM (Molecular Probes, Eugene, OR), and the change in intracellular Ca<sup>2+</sup> was followed as described (19, 20).

The translocation of β-arrestin was measured using the PathHunter system with a bioluminescence enzyme fragment complementation assay developed by DiscoveRx and based on the manufacturer’s instructions. Briefly, cells stably expressing FPR1 or FPR2 (containing a nonfunctional β-gal fragment) were cultured and stimulated with respective ligands, and the activation of the receptor upon ligand binding induces the recruitment of cytosolic β-arrestin tagged with a complementary fragment of β-gal.

**NADPH oxidase activity measurements**

The release of superoxide anion was measured using the isoluminol-amplified chemiluminescence (CL) technique (23, 24). Neutrophils were incubated with or without inhibitors PBP10 or PBP10 derivatives at 37°C for 5 min, after which the stimulus (0.1 ml) was added. The peptide inhibitors were used at 1 μM final concentration unless otherwise stated.

**Determinations by FACS analysis of PBP10 binding and effect of agonist binding**

To determine the role of receptor exposure for PBP10 binding, the peptide (10 μM final concentration) was added to undifferentiated HL-60 cells stably transfected to express FPR1 or FPR2. The cells were incubated for 10 min at 37°C and the amount of peptide associated to the cells was determined by FACS analysis and given as mean fluorescence intensity (MFI) values.

To determine the effect of PBP10 on ligand-binding to FPR1 and FPR2, a FITC-conjugated formylated hexapeptide (FITC-INLPNTL; 10<sup>−7</sup> M final concentration) or a Cy5-conjugated FPR2-specific hexapeptide (Cy5-WKYMVM; 10<sup>−7</sup> M final concentration) was added to neutrophils incubated on ice with or without PBP10. The labeled peptides were added to neutrophils in the absence or presence of nonlabeled 3MLF (10<sup>−7</sup> M) or WKYMVM (10<sup>−7</sup> M). The cells were then incubated for 30 min, and no washing was performed after labeling. Specific binding was calculated by subtracting the MFI for unspecific binding (with unlabeled probe) from the MFI for total binding (without unlabeled probe) for each sample using an Accuri C6 flow cytometer and given in fluorescence units.

**Statistical analysis**

The two tailed Student t test and one-way ANOVA with a Dunnett posttest were used for statistical evaluation. The p values were as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

**Results**

The gelsolin-derived peptide PBP10 inhibits FPR2-mediated NADPH oxidase activity in neutrophils

Activation of human neutrophils with FPR1- or FPR2-specific agonists results in an assembly of the NADPH oxidase and a cell-
lular production and release of reactive oxygen species (ROS) (16). Accordingly, the high-affinity FPR2 agonist WKYMVM (Fig. 1A) and the prototype FPR1 agonist fMLF (Fig. 1B) give rise to a rapid release of ROS from neutrophils. The fMLF response (FPR1-mediated) was almost identical in magnitude, as well as with respect to the peak/duration, to the activity induced by WKYMVM (FPR2-mediated), suggesting that the two receptors share very similar signaling transduction pathways. In agreement with our earlier findings in neutrophils as well as in monocytes (16, 25), FPR2-mediated ROS production was almost completely inhibited by the cell-permeable gelsolin-derived peptide PBP10 (RhB-QRLFQVKGR), whereas no such effect was found on the response induced by the FPR1-specific agonist (Fig. 1). The inhibitory effect was most likely linked to the receptor rather than to the specific agonist examined as illustrated by the fact that PBP10 completely abolished the ROS production induced also by the recently described formylated FPR2 agonists PSMa2 and PSMa3, peptides derived from pathogenic strains of Staphylococcus aureus (Fig. 1A). Two other FPR1 agonists, the S. aureus-derived peptide fMIFL and compound 43, a synthetic nonpeptide agonist, were found to be insensitive to PBP10 (Fig. 1B).

There was no increased binding/association of the PBP10 peptide related to receptor exposure. The amount of peptide bound (as measured by FACS analysis) was represented by a MFI value of ~200 when interacting with the stable transfectant expressing FPR1 (210 ± 80 [mean ± SD], n = 9) compared with ~150 when interacting with cells expressing FPR2 (160 ± 60 [mean ± SD], n = 9).

Based on the fact that an unconjugated peptide (QRLFQVKGR) lacks effects on cells, we as well as others have suggested that membrane permeability is of importance for the cellular effects (15, 16, 26, 27). Note that the rhodamin group of PBP10 could not be replaced by a fatty acid (palmitoylated). Such a peptide had no effect on the neutrophil response to the FPR2 agonist WKYMVM or to the FPR1 agonist fMLF (data not shown). To determine whether the PBP10 peptide affects agonist binding, we used a conventional ligand-binding competition assay. To achieve full functional inhibition by the PBP10 peptide, an inhibitor/agonist molar ratio of 5:10 is needed, but to inhibit binding a higher molar ratio was required; in the presence of such concentrations, PBP10 displaced some of the binding to neutrophils of a specific FPR2 agonist peptide (Cy5-WKYVMV), but binding of a specific FPR1 agonist (FITC-fNLPLNTL) was also affected. At a molar ratio of 100 (100 nM PBP10 and 1 nM agonist peptide), binding of the FPR2-specific agonist was reduced by ~30% (31 ± 18% [mean ± SD], n = 5), and binding of the FPR1-specific agonist was also reduced by ~15% (17 ± 14% [mean ± SD], n = 4).

The ability to bind PIP2 alone is not sufficient for inhibition

The PIP2-binding capacity has been suggested to be of importance for the basic functions of the PBP10 sequence when present in gelsolin, the protein from which the peptide sequence is derived. In an attempt to determine the role of the PIP2-binding capacity for the FPR2-specific inhibitory effect of PBP10, we investigated the effect of Rhb-HVKGKK, a peptide derived from villin (a cytoskeletal protein containing multiple gelsolin-like domains) that possesses the same PIP2-binding activity as PBP10 but with no sequence similarity to PBP10 (15). However, no inhibitory effect was observed by the peptide Rhb-HVKGKK (Fig. 2A), suggesting that the PIP2-binding capacity alone is not sufficient to mediate the inhibitory effect. PBP10 is a peptide conjugated with rhodamine at its N terminus, a conjugation that makes the peptide membrane permeable. To rule out the possibility that the lack of inhibition by Rhb-HVKGKK on FPR2 signaling is not simply due to a reduced cell association and membrane permeability, we measured the amount of cell-associated peptide by flow cytometry. Addition of Rhb-HVKGKK and PBP10 to human neutrophils displayed similar amounts of cell associated fluorescence, indicating that the two peptides associate and pass the neutrophil membrane to the same extent (Fig. 2B).

**FIGURE 1.** PBP10 selectively inhibits FPR2-induced signaling in neutrophils. Neutrophils (2 × 10⁶ cells) were incubated at 37°C for 5 min with (dashed lines) or without (solid lines) PBP10 (1 µM), after which the cells were stimulated by addition of agonists specific for either of the two neutrophil FPRs. The activity induced by the FPR2-specific agonists WKYMVM (100 nM), PSMa2 (50 nM), or PSMa3 (50 nM) are shown in (A), whereas activity induced by the FPR1-specific agonists fMLF (100 nM), fMIFL (10 nM), or compound 43 (1 µM) are shown in (B). Representative CL responses (n > 6) are shown. The arrow indicates the addition of agonists. Abscissa indicates time of study (min); ordinate indicates superoxide production expressed in cpm × 10⁶ (Mcpm).

**PBP10 inhibits the FPR2-induced rise in intracellular calcium and membrane translocation of β-arrestin**

A rapid rise in intracellular calcium is one of the earliest signals induced upon FPR1/FPR2 stimulation (28). The presence of PBP10 completely abolished the response mediated by FPR2 when triggered by WKYMVM (Fig. 3A). Although stimulation of FPR1 and FPR2 with their respective ligands induced very similar Ca²⁺ responses, the fMLF-induced Ca²⁺ response was not affected by the presence of PBP10 (data not shown). A unique signaling pathway, suggested to be triggered without any involvement of the generation of classic second messengers such as Ca²⁺, can be monitored as a coupling of the cytoplasmic domains of an activated G protein–coupled receptor (GPCR) to cytosolic β-arrestin (29). To further determine the effects of PBP10 on FPR signaling, we used an FPR1/2-β-arrestin–coupled system in which the cells express β-arrestin tagged with a nonfunctional moiety of the enzyme β-gal, together with a receptor tagged with the complementary fragment of the enzyme. In the presence of a receptor-specific ligand, β-arrestin is translocated to the activated FRPs and this brings the two parts of β-gal together, and the formation of a functional holoenzyme is picked up as enzyme activity (30). We showed that when WKYMVM was added to cells overexpressing FPR2-β-gal, a dose-dependent translocation of β-arrestin was induced as measured by the β-gal activity, and the EC₅₀ value was 30 nM (Fig. 3B). We also showed that the FPR2-triggered (WKYMVM-induced) translocation of β-arrestin was inhibited by PBP10 in a dose-dependent manner (Fig. 3B, inset). In contrast, no inhibitory effect was induced in an fMLF/FPR1β-gal

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system by 1 μM PBP10 (data not shown), a concentration that induces an almost complete inhibition in all FPR2-mediated cellular responses examined (Fig. 3B, inset). These data thus clearly demonstrate that PBP10 inhibits multiple signaling pathways downstream of FPR2 but not of FPR1. Given the facts that PBP10 enters into the cells and inhibits multiple signaling pathways downstream of FPR2 independent of the nature of receptor-specific agonist used, we hypothesize that most likely PBP10 is targeting a specific intracellular signaling domain of FPR2 rather than a specific signaling pathway downstream of the receptor.

Inhibition of FPR2 signaling by PBP10 does not solely rely on the C-terminal cytoplasmic tail or the third intracellular loop of the receptor

To explore the role of the cytoplasmic tail of FPR2 in mediating the PBP10-sensitive signaling, we designed chimeric FPR2/FPR1 receptors in which the entire cytoplasmic tail of FPR2 was replaced by the corresponding asas 296–350 (shown in black circles for the FPR2/FPR1 chimera in Fig. 4A) present in FPR1. This design was chosen based on the facts that this domain presents the largest sequence difference at the amino acid level among the intracellular regions between the two receptors and that this part has been implicated in FPR1/G protein coupling (9). The FPR2-specific ligand WKYMVM triggered an increase in intracellular Ca²⁺ concentration ([Ca²⁺]) in cells expressing the chimeric FPR2/FPR1 receptor. The addition of PBP10 revealed an inhibition of the transient rise in [Ca²⁺], also in cells expressing the chimeric receptors, and the IC₅₀ value of PBP10 inhibition was comparable to that of the wild-type FPR2 (Fig. 4B). An FPR1 mutant in which the cytoplasmic tail was replaced by the corresponding amino acids present in FPR2 was not responsive to any of our FPR1 agonists, suggesting an importance of cytosolic structures for ligand binding on the opposite side of the membrane (data not shown). In summary, these data clearly show that although the cytoplasmic tail of FPR2 is the domain that differs the most from that of FPR1, this is not the signaling domain that alone confers the receptor selectivity of PBP10 between the two receptors.

A membrane-permeable lipopeptide (F2pal-16) containing a peptide sequence derived from the third intracellular loop of FPR2 (aas 227–242; shown in gray circles in Fig. 4A) present in FPR1. This activation was restricted to FPR2-expressing cells and shown to be insensitive to a receptor antagonist that blocks binding of classical agonists (31). Using the F2pal-16 peptide we showed that this peptide activates also human neutrophils to produce superoxide anions (Fig. 4C), whereas no such effect was obtained with the corresponding F1-pal-16 peptide derived from FPR1 (data not shown). Of particular interest is that PBP10 dose dependently inhibited the ROS production induced by F2pal-16, suggesting an interaction/competition between PBP10 and F2pal-16 (Fig. 4D). A significant inhibition of PBP10 on the F2pal-16–induced activity was observed already at a molar ratio (PBP10/F2pal-16) of 0.1.

To explore the role of the third intracellular loop of FPR2 in mediating the PBP10-sensitive signaling, a mutated FPR2 was expressed in HL-60 cells. The two amino acids K231 and M233 specific for FPR2 were exchanged for the FPR1-specific ones (Q231 and L233). The amino acid sequence of the third intracellular loop of this mutated FPR2 is thus identical to that of FPR1. The FPR2-specific ligand WKYMVM triggered an increase in [Ca²⁺], in cells expressing the mutated FPR2, and also this receptor retained the PBP10 sensitivity with an IC₅₀ value of PBP10 inhibition comparable to that of the wild-type FPR2 (Fig. 4B).

Shortening of the PBP10 peptide with one amino acid from the N terminus or five amino acids from the C terminus generates peptides with a reduced inhibitory activity

Next, we examined the role of different structural domains of the PBP10 peptide for its inhibitory effect on FPR2 signaling. The inhibitory potencies of a series of PBP10 peptide derivatives were examined and compared with those of the full-length PBP10 molecule (hereby named PBP1–10 starting with the first amino acid bound to rhodamine). The inhibitory effect of PBP2–10, a peptide with the first glutamine deleted, was significantly reduced com-
pared with that of PBP1–10 (Fig. 5A), showing the importance of glutamine in mediating the PBP1–10 effect. Peptides with additional amino acid sequentially removed from the N terminus (tested up to PBP5–10) inhibited the WKYMVM-induced neutrophil response to the same degree as PBP2–10 (data not shown), further demonstrating the importance of the first amino acid glutamine in mediating the PBP10 inhibition.

We then determined the inhibitory potencies of a series of progressive truncations from the C terminus of PBP1–10. The PBP1–8, PBP1–7, and PBP1–6 peptides with two, three, and four amino acids deleted, respectively, from the C terminus were found to be as potent as the full-length peptide PBP1–10 in inhibiting the FPR2–mediated (WKYMVM-induced) ROS production (Fig. 5A, shown for PBP1–6). The hexapeptide PBP1–6 actually displayed an even greater inhibitory effect on ROS production than PBP1–10 (Fig. 5A).

### FIGURE 4

**A** Predicated transmembrane disposition of human FPR/FPR1 based on hydrophobicity of the amino acid sequence and on similarities with other GPCRs. The amino acids (aa) in the part of seventh transmembrane region and the entire cytoplasmic tail of FPR1 replaced by the corresponding residues in FPR1 are indicated by filled black circles. The third intracellular loop of FPR2 with an amino acid sequence identical to that in the pepducin F2Pal-16 is highlighted in gray. **B** Fura 2-AM–labeled HL cells expressing the chimeric FPR2/FPR1 receptor (filled circles) or FPR2 mutant with the third intracellular loop identical to that in FPR1 (filled squares) were stimulated with WKYMVM (100 nM) and the inhibitory effect of PBP10 was determined relative to control, which received no PBP10. Abscisca indicates concentration of PBP10; ordinate indicates inhibition of the [Ca^{2+}] signal (percentage of control). **C** PBP10-mediated inhibition of pepducin (F2Pal-16)-induced superoxide production in human neutrophils. Neutrophils were incubated at 37°C for 5 min with PBP10, and the degree of inhibition was determined relative to control, which received no PBP10. Abscisca indicates concentration of PBP10; ordinate indicates inhibition of the [Ca^{2+}] signal (percentage of control). **D** Dose-dependent inhibition of PBP10 on F2Pal-16–induced superoxide production. Abscisca indicates concentration of PBP10; ordinate indicates inhibition of superoxide production in percentage of control (mean ± SEM; n = 4). ***p < 0.001.

### FIGURE 5

Inhibitory effect of truncated peptides originating from the PBP1–10 sequence. **A** Neutrophils were incubated with 1 μM either of the truncated peptides PBP1–10, PBP2–10, PBP1–5, or PBP1–6 at 37°C for 5 min prior to addition with WKYMVM (100 nM; added at arrow). The inhibitory potency of the truncated peptides on the WKYMVM-induced superoxide release is shown. Results are expressed as percentage of control without any inhibitor (mean ± SEM; n = 3). **B** Neutrophils were incubated without (control) or with the truncated peptide PBP1–5 (1 μM) or PBP10–6 (1 μM) derived from PBP10 at 37°C for 5 min prior to addition with WKYMVM (100 nM; added at arrow). The release of superoxide was recorded continuously. A representative CL response (n > 3) is shown. Abscisca indicates time of study (min); ordinate indicates superoxide production expressed in cpm × 10^6 (Mcpm). ***p < 0.001.
lower IC_{50} value (15 nM) than did the full length PBP_{1-10} peptide (33 nM). When the valine was further removed from PBP_{1-6}, the resulting peptide PBP_{1-5} displayed significantly reduced inhibition compared with that of PBP_{1-6} (Fig. 5). These data thus reveal the hexapeptide PBP_{1-6} as the shortest derivative exerting the full inhibitory potency of PBP_{1-10}. A similar pattern of inhibition was obtained when these truncated PBP10 peptides were examined in a calcium mobilization assay with FPR2 overexpressing HL-60 cells (data not shown). Furthermore, these truncated PBP10 peptides were found to enter neutrophils to the same extent as the full-length PBP10 as determined by flow cytometry (data not shown), suggesting that the difference in inhibition among the peptides cannot be simply explained by a difference in their membrane permeability.

In summary, we show that the first glutamine at the N terminus plays a critical role and the C-terminal–truncated peptide RhB-QRLFQV (PBP_{1-6}) adopts a core sequence of PBP10 to mediate a potent inhibitory effect on FPR2 signaling.

**Inhibitory activities of peptide RhB-QRLFQV derivatives on FPR2 signaling**

To further investigate the importance of individual amino acids in the core peptide RhB-QRLFQV (PBP_{1-6}) for the inhibitory effect on FPR2 signaling, several peptide mutants were synthesized and their effect on the FPR2-mediated response was evaluated. The PBP_{1-6Q1→G} or PBP_{1-6Q1→H}, in which the first amino acid glutamine was substituted with either glycine or histidine, displayed a very similar inhibitory potency to that induced by the core peptide PBP_{1-6} (Fig. 6, shown for PBP_{1-6Q1→G}), suggesting that glutamine does not play a specific role, although an amino acid has to be included in the first position in order for the PBP peptide to mediate inhibition (Fig. 5A). When the arginine at the second position was replaced by an alanine, the inhibitory effect on FPR2-mediated ROS production of the resulting peptide PBP_{1-6R2→A} was significantly reduced (Fig. 6). A significant reduction was obtained also when the last valine was exchanged for either histidine (PBP_{1-6V6→H}) or threonine (PBP_{1-6V6→T}) (Fig. 6), suggesting a critical role of the arginine and valine in mediating FPR2 inhibition. A replacement of the third amino acid leucine for glycine generated the peptide PBP_{1-6L3→G} with similar but slightly lower inhibitory capacity compared with the core peptide PBP_{1-6} (Fig. 6). The PBP_{1-6} mutants were found to enter neutrophils to the same extent as the core peptide, as determined by the amount of cell-associated fluorescence measured by flow cytometry, indicating that the difference in inhibition is not due to a difference in their membrane permeability to human neutrophils (data not shown).

Taken together, these data clearly show that the glutamine and leucine at positions 1 and 3, respectively, of the PBP_{1-6} peptide are of minor importance, whereas the arginine and valine located at positions 2 and 6 play a key role in the inhibition of FPR2 signaling.

The core peptide PBP_{1-6} (RhB-QRLFQV) also inhibits ROS production mediated through the homologous receptor FPR1

The results presented above show that the peptide RhB-QRLFQV adopts a core sequence for inhibition of FPR2 signaling, but whether the receptor specificity of this peptide to FPR2 is retained is not known. We addressed this by examining the effect of this peptide on ROS production mediated through several other neutrophil GPCRs. The PBP_{1-6} peptide did not inhibit neutrophil activation mediated through CXCR1 (receptor for IL-8), C5aR (receptor for C5a), or platelet-activating factor receptor (Table I). In contrast, the neutrophil response induced by the prototype FPR1 agonist fMLF was inhibited up to 50% in the presence of high concentration (2 μM) of PBP_{1-6} (Fig. 7A), suggesting that PBP_{1-6} displays receptor specificity for both FPRs in neutrophils, although the inhibition potency was much lower for FPR1 (IC_{50} value of 1.5 μM) than for FPR2 (IC_{50} of 15 nM). The extended receptor specificity of the core PBP_{1-6} peptide also for FPR1 was evident from the data showing that PBP_{1-6} inhibited ROS production induced also when fMLF was replaced for other FPR1 agonists, that is, the formyl peptide fMIFL and compound 43 (Table I).

A gain of receptor specificity also for FPR1 was found to be restricted to the hexapeptide PBP_{1-6} as neither PBP_{1-5} nor PBP_{1-7} displayed any inhibitory effect on FPR1 (Fig. 7B). Moreover, the inhibition potency of two peptide mutants in which the terminal valine of PBP_{1-6} was exchanged for either histidine (PBP_{1-6V6→H}) or threonine (PBP_{1-6V6→T}) was significantly reduced compared with PBP_{1-6} (Fig. 7B), suggesting that the valine at the C terminus plays a critical role for interaction with both FPRs. In summary, our data show that the core sequence of PBP10 (PBP_{1-6}; RhB-QRLFQV) displays receptor specificity not only for FPR2 but also gained specificity for the homologous receptor FPR1, and the last amino acid valine plays a critical role.

**Discussion**

The pattern recognition receptors FPR1 and FPR2 play important roles in infection and inflammation by recognition of various in-
flammmatory mediators (9, 12, 29, 32, 33). Although the two receptors share large sequence similarities and induce almost identical cellular responses, there is one fundamental difference in their downstream signaling; that is, the FPR2 signaling is PBP10 sensitive, whereas the FPR1 signaling is PBP10 insensitive (16, 17). We have now characterized the PBP10-sensitive pathway in more detail and we show that a peptide possessing the same PIP2-binding capacity as PBP10 lacked the inhibitory effects on FPR2 signaling, suggesting that the PIP2-binding capacity of the molecule alone is not sufficient for the inhibition. There is only one amino acid difference between the first cytoplasmic loops of FPR1 and FPR2 and two amino acids that differ in the second and third loops. A substantial number of amino acids (13 to be precise) differ in the tails of the two receptors, making this an attractive domain for the selective inhibitor. Our finding that chimeric receptors, in which the entire cytoplasmic tail is exchanged between the two receptors, retained the PBP10 sensitivity suggest, however, that the cytoplasmic tail is not the only part that confers the PBP10 sensitivity.

It is clear that it is hard to conclusively state that activators and inhibitors such as the pepducins (see below) and the PBP10 peptide really trigger the sensitive/specie receptor from the inside of the plasma membrane, even though earlier reports provide convincing evidence that these peptides permeate the plasma membrane of a variety of cell types and that this is required for their activities. Note that very high concentrations of PBP10 are required to determine an association with neutrophils, much higher concentrations than those required to inhibit cell function, and the amount of PBP10 associated is not dependent on the receptor expressed. The peptide’s biochemical activities and their abilities to affect cell functions are not by any means obvious and likely to be complex. The basic physicochemical properties, due in part to the linked fluorophore/fatty acid, allow the peptides to enter cells passively and to affect many cellular functions (15, 26, 34, 35), and support for their intracellular actions is the clear differences between the clean/naked peptides and the ones made membrane permeable. Furthermore, the observation that a selective non-permeant receptor antagonist (16, 31) has no effect on the inhibitory properties of PBP10-derived peptides or the activating properties of the F2Pal-16 pepducin is also supportive for an intracellular effect. The fact that PBP10 inhibits the activity induced by the F2pal-16 peptide (see below) also provides support to the suggestion that the cellular effects are mediated through a similar type of interaction with FPR2. Remember, however, that even if the some basic requirements are fulfilled, that does not automatically mean that the cellular effects must be mediated through an interaction with the cytosolic part of the receptor.

The results obtained using different peptide derivatives from the 10-aa-long PBP10 peptide clearly show that the N terminus of PBP10 is of vital importance for the inhibitory effects, whereas the C terminus can be substantially shortened without any change in the inhibitory activity. A shorter peptide (PBP1-6), lacking the last four amino acids, retained the inhibitory effects as potent as the full-length PBP10 on FPR2 signaling, but this peptide also partly inhibited the FPR1-triggered response. Our data thus provide strong evidence that the signals downstream of FPR1 and FPR2 link in different ways to the receptors. It is a great challenge to disclose this difference, knowing that the two receptors share large sequence similarities in their signaling transduction domains and that they transduce very similar cellular responses (9, 17, 36). It has been shown/suggested that certain FPR2 agonists trigger a unique downstream signaling cascade in which no classic second messengers, such as the transient rise in [Ca2+], are generated. A β-arrestin/β-gal reporter system was recently introduced, which allowed us to follow this unique signaling pathway (29). We showed that PBP10 inhibits also this signaling pathway downstream of FPR2, whereas the same signaling pathway downstream of FPR1 is unaffected. From the results presented it is clear that PBP10 inhibits calcium signaling as well as translocation of β-arrestin, the two earliest steps in the signaling cascade generated by this receptor. Consequently, all cellular responses downstream of the signals we have studied will be inhibited by PBP10, including the final functional output, granule secretion, and superoxide anion production. It would be interesting to determine also the pathophysiological implications for this inhibitor in, for example, a murine disease model. However, this is easy to suggest but hard to realize, and the background to this is that the biology of murine FPRs is so much more complex than that of the human receptors that a direct translation from human FPR knowledge to the murine system is impossible. A potent and specific ligand for murine FPR2 is currently lacking, and in our hands the effects of PBP10 on murine neutrophils is very limited. For a more complete description of the human/murine problem with respect to the FPRs, we refer to a recent review about the family of FPRs (9).

A variety of approaches have been taken to define the contact sites in FPR1 that transduce the signals from the activated receptor to the G protein (for a review see Ref. 9). The results from the investigations with FPR1 identify the C-terminal tail together with the second intracellular loop as the candidate sites for the contact between the receptor and the signaling G protein. Despite the fact that the C terminus of FPR1 differs the most from the tail of FPR2, we could exclude this region as the domain of importance for the PBP10 sensitivity of FPR2 (see the results with the chimeric receptors). In adrenergic and muscarinic receptors the third intra-
cellular loop has been suggested to have an important role for the downstream signaling (37), but mutagenesis studies performed to identify FPR1 domains of importance for signaling suggest that the third intracellular loop of the receptor does not play an important role in mediating G protein coupling (38). With respect to FPR2, no such studies have been performed, but results with a novel cell-penetrating pepducin, presented in a recent publication, suggest some type of role for the third intracellular loop in FPR2 signaling (31). Pepducins are lipidated peptides designed to target signaling loops of a particular receptor, and they can act as intracellular inhibitors or activators of signal transduction for the targeted receptor, but note that the exact receptor domains or the precise mechanism for how these peptides activate cells is still unclear (34, 39). It is of importance to have this in mind when discussing the recent data showing that a lipidated peptide fragment derived from the third intracellular receptor loop of FPR2 specifically activates cells expressing this receptor (31). Our data confirm the published pepducin results and imply that the third intracellular loop of FPR2 is involved in G protein signaling either alone or together with other portions of the receptor. No neutralophil activation was obtained using an FPR1 pepducin, that is, a lipopeptid containing the amino acids present in the third intracellular loop in FPR1, but more interestingly we found that PBP10 inhibits the pepducin F2pal-16–induced neutrophil activation, suggesting that the two peptides (PBP10 and the F2pal-16 pepducin) in one way or another target the same signaling structure. Whether PBP10 inhibits FPR2 signaling by competing with the pepducin-like region for G protein coupling or the interaction with other portions of the receptor is not known. The amino acid sequence differences between FPR1 and FPR2 in the third intracellular loop are limited to two residues, that, K231 in FPR2 is replaced by a glutamine in FPR1 and M233 is replaced by a leucine. This subtle difference between the two receptors, however, is not the basis for the sensitivity to PBP10, since a mutated FPR2 in which the K231 and the M233 were exchanged for a glutamine and a leucine, respectively, was as sensitive to PBP10 as was the wild-type receptor. The F2pal-16 pepducin activated also the mutated FPR2 (data not shown), supporting the suggestion that although the sequence of a pepducin corresponds to a distinct receptor region, different parts of a receptor may confer the pepducin reactivity (35).

The selectivity of PBP10 for FPR2 over FPR1 was confirmed using several new agonists for the receptors. Note particularly that the neutralophil response to compound 43, earlier described as a high-affinity agonist for both human FPR2 and its murine ortholog (14, 29), is PBP10 insensitive. This, however, is in agreement with our recently published results showing that compound 43 is primarily an FPR1 agonist (40). High-affinity formyl peptides for FPR1 have been found in both Escherichia coli (OMLF) and S. aureus (OMF1), and neither of these peptides interacts with FPR2 (41, 42); accordingly, we found the neutralophil response to these agonists to be insensitive to PBP10. We also found that PBP10 completely abolished the neutralophil response induced by two newly described formyl peptides derived from S. aureus exerting high binding affinity for FPR2 (12). Apparently, the presence of a formyl group does not play a significant role in mediating receptor specificity between FPR1 and FPR2 (43).

We have not determined the effects of the lipid agonist lipoxin A_{2} suggested to use FPR2 (9), simply because we have not been able to confirm the receptor preference (40, 44, 45).

PBP10 is a 10-aa peptide with rhodamine conjugated at its N terminus, and earlier studies show that the rhodamine molecule is required for the peptides to pass the plasma membrane (15, 16), and the passage of the membrane is required for the inhibitory function of the peptide (16). Our data using various truncated peptides derived from PBP10 demonstrate that a six-amino acid-long peptide (PBP_{1-6}) in which the four last amino acids were removed, constitutes the core structure for inhibition, but note that this peptide partly inhibits also FPR1. The second amino acid, arginine, in the PBP_{1-6} peptide was of utmost importance, in that a certain distance from the rhodamine group was needed and the inhibitory potency was very much reduced when an exchange of the arginine for an alanine was performed. The amino acid side chain of arginine is not very complex (a three-carbon aliphatic chain capped by a guanidinium group), but the positive charge of the guanidinium group is delocalized, allowing the formation of multiple hydrogen bonds that might be of importance binding to the receptor. The first residue in PBP_{1-6} must be there but its identity is of no importance, suggesting that the second arginine has to be located at a certain distance from the positively charged rhodamine molecule to allow a proper interaction with the receptor/G protein. Nevertheless, this peptide displays much higher inhibitory effects on FPR2 than on FPR1. The valine at the C terminus of PBP_{1-6} is important for the inhibitory effects on both FPR2 as illustrated by the reduced inhibitory potency of peptides in which this amino acid was removed, masked, or replaced by a theronine or a histidine. Our data thus disclose a core inhibitory peptide displaying effects on intracellular signaling downstream of both FPR1 and FPR2 but with preference for FPR2. The fact that the core peptide inhibits also FPR1 indicates that the PBP10-sensitive structure is actually present also in FPR1 but that it is not available/reachable for the larger molecule, and this should have implications for how future chimeric/mutated receptors should be designed.

In conclusion, our data clearly demonstrate that the PBP10-mediated inhibitory effect on FPR2 signaling is neither entirely dependent on the PIP2 binding capacity of the peptide nor on the cytoplasmic tail of the receptor. We show that PBP10 inhibits neutrophil activation induced by the pepducin derived from the third intracellular loop of FPR2, but the difference in PBP10 sensitivity between FPR1 and FPR2 could not be explained by the amino acid differences (limited to two amino acids) in this intracellular loop. The fact that a shorter peptide, PBP_{1-6}, displays receptor specificity also for FPR1 suggests that the structural element for PBP10 sensitivity is present also in this receptor. Our finding that the two receptors transduce intracellular signaling through pathways that can be regulated selectively, that is, one being PBP10 sensitive and the other being PBP10 insensitive, is in line with the accumulating evidence demonstrating that the two receptors have different regulatory roles in host defense in various animal inflammation models. Although the precise mechanisms underlying PBP_{1-6}-mediated receptor inhibition for both FPR1 and FPR2 remain to be elucidated, the selectivity and specificity of this peptide as well as the full-length PBP10 on the two members of FPR family should provide a basis for the potential design of anti-inflammatory therapeutics.

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Disclosures
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References