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An Annexin 1 N-Terminal Peptide Activates Leukocytes by Triggering Different Members of the Formyl Peptide Receptor Family

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The human N-formyl peptide receptor (FPR) is a key modulator of chemotaxis directing granulocytes toward sites of bacterial infections. FPR is the founding member of a subfamily of G protein-coupled receptors thought to function in inflammatory processes. The other two members, FPR-like (FPRL)1 and FPRL2, have a greatly reduced affinity for bacterial peptides or do not bind them at all, with FPRL2 being considered an orphan receptor so far. In this study we show that a peptide derived from the N-terminal domain of the anti-inflammatory protein annexin 1 (lipocortin 1) can activate all three FPR family members at similar concentrations. The annexin 1 peptide initiates chemotactic responses in human monocytes that express all three FPR family members and also desensitizes the cells toward subsequent stimulation with bacterial peptide agonists. Experiments using HEK 293 cells stably expressing a single FPR family member reveal that all three receptors can be activated and desensitized by the N-terminal annexin 1 peptide. These observations identify the annexin 1 peptide as the first endogenous ligand of FPRL2 and indicate that annexin 1 participates in regulating leukocyte emigration into inflamed tissue by activating and desensitizing different receptors of the FPR family. The Journal of Immunology, 2004, 172: 7669–7676.
receptor and does not bind fMLP. The only FPRL2 ligand identified so far is the unnatural synthetic peptide WKYMVM (24).

Because annexin 1 might also signal through a low affinity fMLP receptor on granulocytes or other leukocytes, we investigated whether it can act through FPRL1 or FPRL2. We show that the mimetic N-terminal annexin 1 peptide Ac1-25 is able to activate and desensitize not only FPR but also FPRL1 and FPRL2. Human embryonic kidney (HEK) 293 cells solely expressing FPRL1 or FPRL2 respond to the annexin 1 peptide with Ca\(^{2+}\) fluxes and chemotaxis revealing that the peptide is an agonist for all members of the FPR subfamily and the first endogenous ligand for FPRL2.

Materials and Methods

Peptides and Abs

The synthetic chemotactic peptide fMLP and its analog NfNleLFNleYK (which has a slightly higher affinity for FPR) were obtained from Sigma-Aldrich (St. Louis, MO). The annexin 1 peptide (Ac1-25, N-acetyl-AM-VSEFLKQAFWIEENEEQYVQTVK), which was acetylated during synthesis to mimic the physiological situation (25) and the unnatural synthetic peptide WKYMVM or the D-methionine containing WKYMVM peptides were purchased from Advanced Biotechnology Centre (London, U.K.). All experiments were reproduced with Ac1-25 from different preparations and preparations to exclude possible effects of potential impurities.

Isolation of human PBls

Peripheral blood granulocytes and monocytes were isolated from buffy coats using Ficoll-Paque gradient centrifugation (26). Erythrocytes were removed from sedimented granulocytes through hypotonic lysis.

Stable expression of FPR, FPRL1, and FPRL2 in HEK 293 cells

Cloning and stable expression of the FPR has been previously described (14). The cDNAs encoding the human receptors FPRL1 and FPRL2 were obtained by PCR using a human leukocyte cDNA library as template (Clontech Laboratories, Palo Alto, CA). To create FPRL fusion proteins C-terminal-tagged with a 10-aa myc epitope (FPRL1\(^\text{myc}\), FPRL2\(^\text{myc}\)), a PCR was performed using oligonucleotide primers introducing a XhoI restriction site immediately 5′ of the initial ATG and a myc epitope sequence followed by a translational Stop signal and an EcoRI site 3′ to the last codon of the receptor DNA. For FPRL1, oligonucleotides used were 5′-gatccgacctgagcaagagtgaacactctctcact-3′ and 5′-gatcgaattcttacaagtcctcttcagatatcagctgttgctcggccattctctcact-3′; FPRL2 was amplified with oligonucleotides 5′-gatccgacctgagcaagagtgaacactctctcact-3′ and 5′-gatcgaattcttacaagtcctcttcagatatcagctgttgctcggccattctctcact-3′. The PCR products were inserted into the pcDNA3.1 vector (Invitrogen, Karlsruhe, Germany). All constructs were confirmed by sequencing (SEQLAB, Göttingen, Germany). Plasmids encoding FPRL1 or FPRL2 were transfected into HEK 293 cells by electroporation (Trenton, NJ) performing the transfection. The remaining cells were then incubated in the presence or absence of fMLP or the annexin 1 peptide. After washing, cells were fixed for 4 min in −20°C methanol and stained for filamentous actin (F-actin) using rhodamine-phalloidin. Subsequently, the cells were washed extensively in PBS and mounted using Mowiol containing 4% propylgalact. Images were acquired using a cooled CCD camera (Micromax; Princeton Instruments, Trenton, NJ) installed on a DM RXA fluorescence microscope (Leica, Wetzlar, Germany).

Fluorescence microscopy of stained actin coats using Ficoll-Paque gradient centrifugation (26). Erythrocytes were removed from sedimented granulocytes through hypotonic lysis.

Annexin 1 peptide-induced activation of human leukocytes

Previous studies revealing the anti-inflammatory effect of annexin 1 in different models of inflammation have demonstrated that the activities displayed by full-length annexin 1 are faithfully and completely retained within the N-terminal peptide Ac1-25 (for example, see Refs. 27, 9, 23). Moreover, proteolytic cleavage at position 26 of the N-terminal domain was previously shown to occur in different cells and N-terminal proteolysis was markedly induced upon neutrophil extravasation with only 25% of neutrophil annexin 1 remaining intact (28, 29). Because this strongly suggested that the N-terminal peptide Ac1-25 is the physiological and pathophysiological active compound, Ac1-25 was used throughout the study. With this N-terminal peptide as well as full-length annexin 1 we could show previously that its inhibitory effect on the transendothelial migration of granulocytes is due to an interaction with and desensitization of FPR (14). To further characterize the ability of annexin 1 to activate leukocytes, we analyzed whether the N-terminal peptide Ac1-25 can also act as a chemoattractant. As shown in Fig. 1A, the annexin 1 peptide induced the directional migration of human peripheral blood granulocytes and monocytes in a dose-dependent manner. When equal concentrations of Ac1-25 were present in both the lower and the upper wells of the migration chamber, no increase in cell migration was observed (data not shown). Thus, the observed migration induced by Ac1-25 was based on chemotaxis rather than chemokinesis.

Granulocytes stimulated by chemoattractants respond by rapidly adopting a polarized morphology, with distinctive leading and trailing edges, and localized actin polymerization. Comparison of the granulocyte morphology and F-actin content upon stimulation with the prototypical bacterial chemoattractant fMLP or with the annexin 1 peptide Ac1-25, respectively, revealed that both FPR agonists caused rearrangements of F-actin and a substantial alteration in cell shape, leading to cell polarization (Fig. 1B). Thus, in addition to inhibiting granulocyte migration by desensitizing FPR (14), annexin 1 can also elicit chemotaxis most likely by activating FPR or a related family member.
To determine the specificity in the activation mediated by the annexin 1 peptide and to elucidate the range of leukocyte responses elicited, we performed desensitization experiments using human monocytes which are known to express all three members of the FPR subfamily (24). Desensitization induced by one agonist toward another generally indicates activation of the same receptor. To measure such homologous desensitization in the case of FPR/FPRLs on human monocytes, we recorded intracellular Ca$^{2+}$ mobilization in response to different peptide ligands, the fMLP analog NfNleLFNleYK and fMLP used at low or high concentrations to trigger FPR or FPRL1, respectively, the artificial WKYMVM peptide known to activate FPRL2 and FPRL1 (but not FPR) (24) and the annexin 1 peptide Ac1-25. As shown in Fig. 2, stimulation with all peptides elicited a dose-dependent sustained increase in intracellular Ca$^{2+}$. When monocytes were first stimulated with the known FPR family agonists, the fMLP analog NfNleLFNleYK, fMLP or WKYMVM given at saturating concentrations (Fig. 2), no significant second Ca$^{2+}$ mobilization was observed in response to a second stimulation with the same agonist (data not shown). In contrast, in each case a response was obtained with the annexin 1 peptide as second challenge (Fig. 3, A–C). These results suggest that the annexin 1 peptide might either use a completely different receptor or act agonistically on all three members of the FPR family, thereby activating the remaining two susceptible receptors when one family member is desensitized upon binding of the specific peptide ligand. The fact that when cells were restimulated with NfNleLFNleYK, fMLP, or WKYMVM after a first challenge with the annexin 1 peptide at saturating concentrations (100 nM), no significant second rise in intracellular Ca$^{2+}$ could be observed (Fig. 3, D–F) indicates that Ac1-25 was able to activate all FPR family members. Likewise, cells stimulated with annexin 1 peptide were unable to generate a second rise in intracellular Ca$^{2+}$ upon a second challenge with this peptide (data not shown).

To further test this hypothesis, we aimed at activating and desensitizing all FPR/FPRL receptors expressed in leukocytes. We therefore used the synthetic D-methionine containing peptide WKYMVm, which had been shown to activate all receptors of the FPR family (24, 30, 31). When monocytes or granulocytes were exposed to WKYMVm, the cells were unable to respond to a second stimulation with either the same agonist (Fig. 4, A and E), NfNleLFNleYK (Fig. 4, B and F) or fMLP (Fig. 4, C and G), toward another generally indicates activation of the same receptor.
respectively. This indicates that after the first stimulation with WKYMVm, the cells were subsequently unable to mobilize intracellular Ca²⁺ through the FPR family receptors due to homologous desensitization. Activation by WKYMVm also blocked annexin 1 peptide-induced Ca²⁺ mobilization (Fig. 4, D and H), indicating that both peptides act on the same receptors. Thus, the

FIGURE 3. Densensitization of Ca²⁺ mobilization in monocytes triggered with FPR/FPRL agonists. Cells were loaded with fura 2-AM and the fluorescence ratios were recorded to analyze the agonist-induced rise in intracellular Ca²⁺. Stimulation with the fMLP analog NfNleLFNleYK (A), fMLP (B), or WKYMVM (C) at the concentrations indicated did not desensitize the cells toward a second challenge with Ac1-25. No significant second peak indicative of a second stimulation was observed in cells first activated with Ac1-25 and then restimulated with the fMLP analog NfNleLFNleYK (D), fMLP (E), or WKYMVM (F) at the concentrations indicated (homologous desensitization). Curves show representative experiments. Arrows indicate the time point of agonist addition.

FIGURE 4. Simultaneous desensitization of all FPR family members inhibits Ac1-25 induced Ca²⁺ mobilization. Fura 2-AM loaded monocytes (A–D) or granulocytes (E–H) were first stimulated with WKYMVm, an agonist for all known FPRs. WKYMVm was used at 4 μM, a concentration fully desensitizing the cells toward a second WKYMVm challenge (data not shown). Cells were then challenged with an agonist specific for a given receptor. Arrows indicate agonist addition. Tracings of representative experiments are shown.
annexin 1 peptide Ac1-25 appears to stimulate monocytes and granulocytes through all FPR family receptors. The annexin 1 peptide is an agonistic ligand of all members of the human FPR family.

To elucidate whether the annexin 1 peptide can indeed activate all three FPR family members, we next measured intracellular Ca\(^{2+}\) mobilization upon Ac1-25 stimulation of HEK 293 cells stably expressing only FPR, FPRL1, or FPRL2. We did not observe Ca\(^{2+}\) mobilization in the parental HEK 293 cells with any of the peptides tested, i.e., Ac1-25 or the known agonists N\(\text{N}\)l\(\text{N}\)eL\(\text{N}\)leYK, fMLP, and WKYMVM, respectively (data not shown). As expected, low nanomolar concentrations of the fMLP analog N\(\text{N}\)l\(\text{N}\)eL\(\text{N}\)leYK or micromolar concentrations of the annexin 1 peptide induced Ca\(^{2+}\) transients in FPR expressing HEK 293 cells (Fig. 5). This reflects the ~10,000-fold higher affinity of FPR for fMLP as compared with the annexin peptide (14).

FIGURE 6. Cross-desensitization of HEK 293 cells stably expressing FPR, FPRL1, or FPRL2, respectively. HEK 293 cells stably expressing the receptors indicated; FPR (A and B), FPRL1 (C and D), FPRL2 (E and F) were loaded with fura 2-AM and subjected to Ca\(^{2+}\) mobilization recordings following different peptide challenges. To identify cross-desensitization, the cells were first triggered with the annexin 1 peptide Ac1-25 followed by the receptor-specific agonistic peptide (A, C, and E) or subjected to peptide stimulation in the opposite order, i.e., first the specific agonist and then Ac1-25 (B, D, and F). Fluorescence ratios of representative recordings are given. Note the desensitization of each FPR by Ac1-25. Some residual Ca\(^{2+}\) mobilization is seen when high fMLP concentrations are used as a second challenge (C), possibly due to a minimal receptor reactivation during the time between the first and second challenge.
been reported to activate both FPRL1 and FPRL2 (24), induced mobilization of Ca^{2+} in the FPRL2 expressing cells (Fig. 5C). Interestingly, the annexin 1 peptide not only triggered Ca^{2+} release in the FPR-293 cells but also induced Ca^{2+} fluxes in both, FPRL1–293 and FPRL2-293 cells, in a dose-dependent manner (Fig. 5, B and C). This shows that Ac1-25 is an agonist of both FPR receptors and also identifies the peptide as the first endogenous ligand of FPRL2, so far considered an orphan receptor. In each case the annexin 1 peptide concentrations required to obtain a marked Ca^{2+} flux were in the low micromolar range, corresponding to the levels shown to elicit chemotaxis in granulocytes and monocytes (as previously mentioned). Desensitization experiments revealed that annexin 1 peptide-mediated activation of each of the receptors desensitized the respective cells toward a subsequent stimulation by the known specific agonist and vice versa (Fig. 6), suggesting desensitization due to two agonists sharing the same receptor.

**Discussion**

In this study, we have demonstrated that a peptide derived from the unique N-terminal domain of annexin 1 elicits chemotaxis in human peripheral blood granulocytes and monocytes. Moreover, we could show that this peptide is capable of activating all three members of the human FPR family, FPR, FPRL1, and FPRL2. In each case analyzed, this activation induced a chemotactic response indicating that the annexin 1 derived peptide can function as an endogenous and activating ligand of all three FPRs known in humans. Although fMLP is a high affinity agonist for FPR, it binds to FPRL1 with low affinity and transduces signals via this receptor only when applied at high concentrations (18, 19). FPRL1 is a rather promiscuous receptor that is activated by a broad spectrum of unrelated agonists, including the SAA protein (21) and even a lipid metabolite, lipoxin A₄ (22). It was shown recently to participate in mediating the anti-inflammatory activities of aspirin and glucocorticoids by responding to aspirin-triggered lipoxin A₄ and dexamethasone-induced annexin 1 (Ac1-25) with a resulting down-regulation of neutrophil diapedesis (23). The second FPR homologue, termed FPRL2, is expressed in monocytes, but not in granulocytes. Although it shares 83% amino acid identity with FPRL1, FPRL2 is not activated by fMLP (24, 19) and the only reported agonist has been the synthetic hexapeptide WKYMVM. Our observations identify the annexin 1-derived peptide Ac1-25 as the first endogenous ligand of FPRL2.

In most cases, chemotactic responses are elicited through high affinity ligand-receptor interactions whereas binding of the annexin 1 peptide most likely represents a low affinity ligand-receptor interaction. Previously identified ligand-G protein–coupled receptor interactions of relatively low affinity include those of different chemokines and their receptors, e.g., macrophage inflammatory protein-1β or RANTES and their interactions with CCR3 or CCR4, respectively (32, 33). Low affinity FPR (or FPRL) ligands might be essential for leukocyte recruitment toward the focus of inflammation where high concentrations of the chemoattractant are met and in which high affinity ligand-receptor interactions would result in receptor saturation, desensitization and/or sequestration. This scenario would require high (micromolar) extracellular concentrations of the annexin 1 peptide in close proximity to the focus of inflammation. Such concentrations are higher than annexin 1 levels reported in normal serum. However, annexin 1 expression is induced by glucocorticoids and IL-6 (34–36) and systemic annexin 1 concentrations have been shown to increase at least 10-fold in a number of inflammatory and infectious diseases (37–39). Furthermore, leukocytes have been reported to release annexin 1 under certain conditions (40) and thus may produce relatively high concentrations of the protein in their microenvironment. Finally, annexin 1

**FPRL1- and FPRL2-mediated chemotaxis is elicited by the annexin 1 peptide**

Because the annexin 1 peptide initiated a migratory response in monocytes, we next examined the ability of the Ac1-25 peptide to elicit chemotaxis in the HEK transfectants. Control cells, i.e., non-transfected HEK 293 cells, did not migrate in response to any of the peptides tested (data not shown). The fMLP analog NiNleLFNiYK induced migration of FPR-293 cells at nanomolar concentrations (Fig. 7A), whereas FPRL1-293 cells only responded to high (micromolar) levels of fMLP in a chemotactic manner (Fig. 7B). FPRL2-expressing 293 cells failed to migrate in response to fMLP (data not shown) but migrated upon exposure to WKYMVM. When challenged with the annexin 1 peptide, all three ectopically expressed receptors mediated a potent migratory response, indicating that Ac1-25 can induce chemotaxis through FPR, FPRL1, and FPRL2, respectively (Fig. 7).
could be released by tissue or cell damage locally generating high extracellular concentrations. In such cases the released annexin 1 is most likely proteolytically cleaved and the active N-terminal peptides are generated. In fact, annexin 1 added to activated human granulocytes is quickly cleaved within its N-terminal domain, most likely by a liberated granulocyte protease (U. Rescher and A. Wibers, unpublished observation). A substantial degree of N-terminal annexin 1 proteolysis also occurs within cells, e.g., in neutrophils upon extravasation into the subendothelium (29). Collectively, these data indicate that the N-terminal annexin 1 peptide is the physiologically active entity released from cells or generated extracellularly at sites of inflammation. The importance of the free N-terminal peptide is also underscored by recent crystal structure data of full-length annexin 1. In this structure a substantial part of the N-terminal domain is buried in the protein core being inaccessible for protein (e.g., FPR/L) interaction and it requires high Ca²⁺ concentrations to release this part (41, 42). Thus, the proteolytically generated N-terminal peptide is most likely the annexin 1 derivative regulating leukocyte activities through FPR family member interactions and its concentration at local inflammatory sites is likely to be within the range necessary to attract and activate leukocytes. Interestingly, other known ligands of FPRL1, namely SAA and lipoxin A₄, also show markedly increased levels during inflammation (43, 44).

Apart from inducing chemotaxis, the annexin 1 N-terminal peptide can also desensitize FPR family members thereby rendering leukocytes unresponsive to additional stimuli and thus limiting the degree of inflammation. Such anti-inflammatory and anti-migratory activity is observed when the annexin 1 peptide is applied exogenously in mouse models of inflammation (9, 23) or when it is present at the luminal side of the endothelium in in vitro models of human granulocyte diapedesis (14). The balance between the anti-migratory and chemotactic activities of annexin 1 could depend on the receptors being used (FPR, FPRL1, or FPRL2) and the type of inflammation occurring. Although annexin 1 released during granulocyte extravasation (40) could trigger through its N-terminal peptide receptor desensitization and thereby reduce the extent of inflammation occurring in response to bacterial infections, annexin 1 peptides liberated during noninfectious tissue damage, i.e., in the absence of bacterial peptides, could chemotact leukocytes.

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