A Seven-transmembrane, G Protein–coupled Receptor, FPRL1, Mediates the Chemotactic Activity of Serum Amyloid A for Human Phagocytic Cells

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From the *Laboratory of Molecular Immunoregulation, Division of Basic Sciences; the ‡Intramural Research Support Program, SAIC Frederick, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201; and the §Laboratory of Host Defenses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892

Summary

We have previously reported (Badolato, R., J.M. Wang, W.J. Murphy, A.R. Lloyd, D.F. Michiel, L.L. Bausserman, D.J. Kelvin, and J.J. Oppenheim. 1994. J. Exp. Med. 180:203; Xu, L., R. Badolato, W.J. Murphy, D.L. Longo, M. Anver, S. Hale, J.J. Oppenheim, and J.M. Wang. 1995. J. Immunol. 155:1184.) that the acute phase protein serum amyloid A (SAA) is a potent chemotactic for human leukocytes in vitro and mouse phagocytes in vivo. To identify the signaling mechanisms, we evaluated patterns of cross-desensitization between SAA and other leukocyte chemoattractants. We found that the chemotactic bacterial peptide, N-formyl-methionyl-leucyl-phenylalanine (fMLP), was able to specifically attenuate Ca^2+ mobilization in human phagocytes induced by SAA, but only at very high concentrations, suggesting that SAA uses a low affinity fMLP receptor. Here we demonstrate that SAA selectively induced Ca^2+ mobilization and migration of HEK cells expressing FPRL1, a human seven-transmembrane domain phagocyte receptor with low affinity for fMLP, and high affinity for lipoxin A4. Furthermore, radiolabeled SAA specifically bound to human phagocytes and FPRL1-transfected 293 cells. In contrast, SAA was not a ligand or agonist for FPR, the high affinity fMLP receptor. Thus, SAA is the first chemotactic ligand identified for FPRL1. Our results suggest that FPRL1 mediates phagocyte migration in response to SAA.

Key words: serum amyloid A • FPRL1 • chemotaxis • calcium flux • receptor

Serum amyloid A (SAA), an acute phase protein, is normally present in serum at 0.1–μM levels, but increases by 1,000-fold in systemic inflammatory conditions (1–4). It has been proposed that SAA is mainly involved in lipid transportation and metabolism (1–3). Chronic inflammatory conditions with elevated serum SAA may culminate in amyloidosis, characterized by deposition of “amyloid” fibrils in tissues and associated with progressive destruction of organ function (2–4). Although a number of acute phase proteins are known to modulate host immune responses, we recently reported that recombinant human (rh)SAA exhibited considerable chemotactic activity for human monocytes, neutrophils, and T lymphocytes in vitro (5, 6). rhSAA also induced infiltration of phagocytic cells and T lymphocytes into injection sites in mice (5, 6), suggesting that SAA, when present locally, may play a proinflammatory role by recruiting immune cells.

Since SAA induced significant Ca^2+ mobilization in phagocytes (7), and both its chemotactic and Ca^2+ mobilizing effects were inhibitable by pretreatment of the leukocytes with pertussis toxin, we proposed that SAA may use seven-transmembrane, G protein–coupled receptor(s) (6, 7). In an effort to identify the receptor(s) for SAA, we carefully evaluated cross-desensitization of Ca^2+ mobilization in monocytes and neutrophils induced by SAA and other chemoattractants. Among a number of chemoattractants tested, only the bacterial chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP), when used at relatively high concentrations (10 μM and more), was able to attenuate a subsequent cell response to SAA, suggesting that SAA may use a receptor on leukocytes for which fMLP has low affinity.

Two receptors that interact with fMLP have been identified and molecularly cloned (for review see references 8, 9). The prototype receptor FPR bound fMLP with high

Abbreviations used in this paper: CI, chemotaxis indexes; EC, effective concentration; fMLP, N-formyl-methionyl-leucyl-phenylalanine; HDL, high density lipoprotein; LXA4, lipoxin A4; SAA, serum amyloid A.
affinity and was activated by low nanomolar concentrations of fMLP. The other, a highly homologous variant of FPR, named FPR L1 (also referred to as FPR H2 and LXA4R), was originally cloned as an orphan receptor (10–14) but was subsequently found to bind FPR L1 with high affinity and to increase arachidonic acid production and G protein activation in FPR L1-transfected cells (15). LXA4 inhibits proinflammatory neutrophil responses (15–25) as well as the release of the proinflammatory cytokine, IL-8, by epithelial cells (20, 24). These effects of LXA4 have been attributed to the activation of FPR L1 (or LXA4R) in neutrophils and epithelial cells. Another lipid mediator receptor, the leukotriene B4 receptor, is structurally related to FPR L1 (30.7% amino acid sequence identity; reference 25), and was also reported to be a fusion co-factor for HAV-1 (26), similar to various chemokine receptors (for review see reference 27). This activity has not been reported for FPR L1 (9, 27). Because SAA might use a low affinity fMLP receptor on phagocytes, we further investigated whether FPR L1 could be activated by SAA and demonstrate that SAA uses FPR L1 as a functional receptor.

**Materials and Methods**

Reagents and Cells. rhSAA was purchased from Pepro Tech Inc. with the sequence as follows: M RF S F G E AF GDG D M W R A Y S D M R E A N Y I G Y H F A A K R G P G G V W A A E I N A R E N I Q R F G R GA E S L D A Q A N E W G R S G K D N H F R P A G L E P K Y.

This rhSAA corresponds to SAA-1α, one of the major SAA isoforms in the serum, except for the addition of a methionine at the NH₂ terminus as well as the substitution of aspartic acid for asparagin at position 60, which appears in the SAA 2 isoform (for review see reference 28). rhSAA at concentrations used in the study was negative for endotoxin as assessed by Limulus amebocyte lysate assays (sensitivity: 0.06 IU/ml; BioWhittaker). High density lipoprotein (HDL) was purchased from Sigma Chemical Co. Human peripheral blood enriched in mononuclear cells or neutrophils was obtained from normal donors by leukapheresis (courtesy of the Transfusion Medicine Department, Clinical Center, N ational Institutes of Health, Bethesda, Md.). The blood was centrifuged through Ficoll-Hypaque (Sigma Chemical Co.). The packed cells were washed once with PBS and resuspended in fresh loading buffer. The cells were then transferred into quartz cuvettes (10⁶ cells in 2 ml) that were placed in a luminescence spectrometer LS50 B (Perking-Elmer Limited). Stimulants at different concentrations were added in a volume of 20 μl to the cuvettes at indicated time points. The ratio of fluorescence at 340 and 380 nm wavelengths was calculated using the FL WinLab (Perkin Elmer) program. The assays were performed at least five times and results from representative experiments are shown.

**Ligand Binding Assays.** rhSAA (20 μg) was radioiodinated on tyrosine residues with the chloramine T method and the specific activity of the labeled SAA was 5.8 mCi/mg (courtesy of J. Dobbs, SAIC Frederick, NCI-FCRDC, Frederick, Md.). A constant concentration of 183 mM NaCl, 6 mM KCl, 1 mM CaCl₂, 10 mM Hepes (pH 7.4), 5 mM glucose, and 0.1% BSA with 5 μM Fura-2 (Sigma Chemical Co.) at 37°C for 30 min. The dye-loaded cells were washed and resuspended in fresh loading buffer. The cells were then transferred into quartz cuvettes (10⁶ cells in 2 ml) that were placed in a luminescence spectrometer LS50 B (Perkin-Elmer Limited). Stimulants at different concentrations were added in a volume of 20 μl to the cuvettes at indicated time points. The ratio of fluorescence at 340 and 380 nm wavelengths was calculated using the FL WinLab (Perkin Elmer) program. The assays were performed at least five times and results from representative experiments are shown.

**Calcium Mobilization.** Calcium mobilization was assayed by incubating 10⁷/ml of monocytes, neutrophils, or receptor cDNA transfectants in loading buffer containing 138 mM NaCl, 6 mM KCl, 1 mM CaCl₂, 10 mM Hepes (pH 7.4), 5 mM glucose, and 0.1% BSA with 5 μM Fura-2 (Sigma Chemical Co.) at 37°C for 30 min. The dye-loaded cells were washed and resuspended in fresh loading buffer. The cells were then transferred into quartz cuvettes (10⁶ cells in 2 ml) that were placed in a luminescence spectrometer LS50 B (Perkin-Elmer Limited). Stimulants at different concentrations were added in a volume of 20 μl to the cuvettes at indicated time points. The ratio of fluorescence at 340 and 380 nm wavelengths was calculated using the FL WinLab (Perkin Elmer) program. The assays were performed at least five times and results from representative experiments are shown.

**Chemotaxis.** The migration of human 293 cells expressing FPR (FPR/293) or FPR L1 (FPR L1/293) as well as ETFR cells was assessed by a 48-well microchemotaxis chamber technique (31, 32). A 25-μl aliquot of rhSAA or other reagents diluted in chemotaxis medium (RPMI 1640, 1% BSA, 25 mM Hepes) was placed in the wells of the lower compartment, and 50 μl cell suspension (10⁶ cells/ml in chemotaxis medium) were placed in the wells of the upper compartment of the chamber (N europrobe, Cabin John, Md.). The two compartments were separated by a polycarbonate filter (10 μm pore size, N europrobe) coated with 50 μg/ml collagen type I (GIBCO BR L) for 1 h at 37°C. The chamber was incubated at 37°C for 5 h in humidified air with 5% CO₂. At the end of the incubation, the filter was removed, fixed and stained with Diff-Qwik (H artley, G ibbstown, N J). The number of migrated cells in three high-powered fields (400×) was counted by light microscopy after coding the samples. Results are expressed as the mean (± SD) value of the migration in triplicate samples and are representative of at least five experiments performed. For better illustration, chemotaxis indices (CI) reflecting the fold increase of cell migration in response to stimulant over medium are used. Statistical significance of the difference between numbers of cells migrating in response to stimuli versus baseline (migration toward control medium) was calculated with Student’s t-test and the CI ≥ 2 are statistically significant.

**Results**

Assays of Ca²⁺ mobilization have provided a useful approach to identify ligands for chemoattractant receptors. In
primary cells, cross-desensitization of Ca\(^{2+}\) transients is often due to two agonists acting at the same receptor (33). Since SAA induced Ca\(^{2+}\) mobilization in phagocytes (7), we used cross-desensitization to characterize the molecular nature of SAA receptor(s). In a series of cross-desensitization experiments, SAA at 1 \(\mu\)M did not desensitize the Ca\(^{2+}\) flux in monocytes or neutrophils induced by chemokines such as monocyte chemotactic protein (MCP)-1, RANTES, MCP-3, macrophage inflammatory protein (MIP)-1\(\alpha\), IL-8, and stromal cell–derived factor (SDF)-1\(\alpha\) (data not shown). Therefore, SAA is unlikely to share a receptor with any of the chemokines tested. SAA also did not attenuate the cell response to the bacterial chemotactic N-formylated peptide fMLP when fMLP was used at 100 nM (10\(^{-7}\) M) (Fig. 1 A). However, in reciprocal tests, fMLP at 100 nM showed a partial desensitizing effect on SAA-induced Ca\(^{2+}\) mobilization in monocytes (Fig. 1 B). Furthermore, the cell response to SAA was completely desensitized by higher concentrations of fMLP (10\(^{-3}\) M = 1 mM, Fig. 1 C), suggesting that SAA might use a receptor(s) for which fMLP has low affinity.

Since fMLP is known to induce Ca\(^{2+}\) mobilization in phagocytes through at least two seven-transmembrane, G protein–coupled receptors, FPR and FPRL1 (10, 11, 13, 29), we tested the effect of SAA using cells transfected to express these receptors that originally were not responsive to fMLP stimulation. fMLP in a wide range of concentrations induced Ca\(^{2+}\) mobilization in FPRL1-transfected rat basophil leukemia cell line (ETFR cells), with an EC\(_{50}\) of 10 pM (data not shown). In contrast, the EC\(_{50}\) for fMLP to induce Ca\(^{2+}\) mobilization in FPRL1 transfected cells (FPRL1/293 cells) was much higher at 10 \(\mu\)M (Fig. 2 A). These results confirmed the previous observation that FPR is a high affinity receptor for fMLP, whereas FPRL1 has a much lower affinity (10, 11, 13, 29). rhSAA induced Ca\(^{2+}\) mobilization in cells transfected with FPRL1 (FPRL1/293 cells; Fig. 2 B), but not in FPR-expressing cells or mock-transfected 293 cells (Fig. 2 C and D). The EC\(_{50}\) of rhSAA on FPRL1 transfected cells was 250 nM, suggesting that SAA activates FPRL1 with higher efficacy than fMLP. This was supported by studies of cross-desensitization of Ca\(^{2+}\) flux between SAA and fMLP in FPRL1/293 cells. As shown in Fig. 2 E, although sequential stimulation of FPRL1/293 cells with SAA and fMLP resulted in bidirectional desensitization, SAA was able to desensitize the cell response to a 100-fold excess of fMLP. In contrast, fMLP at 100-fold excess of SAA only partially desensitized the effect of SAA (Fig. 2 E).

Leukocyte infiltration in vivo is considered to be based on migration of cells toward a gradient of locally produced chemoattractant(s). This process can be emulated by in vitro assays of chemotaxis, which provides a very sensitive and biologically relevant means of evaluating the function of cloned chemoattractant receptors (32–35). Since SAA has been shown in our previous studies to induce leukocyte infiltration in vivo and chemotaxis in vitro (5, 6), we next investigated whether SAA could induce cell migration via FPRL1. FPRL1/293 cells showed a potent migratory response to SAA with an EC\(_{50}\) of 200 nM (Fig. 3 A), but these cells failed to migrate in response to a wide range of concentrations of fMLP (Fig. 3 B). In contrast, fMLP induced migration of ETFR cells at nanomolar range concentrations, whereas the same cells did not migrate in response to SAA (Fig. 3 C). The chemotaxis experiments indicate that fMLP is only a partial agonist for FPRL1 since it did not induce cell migration through FPRL1. On the other hand, SAA showed full agonist activity on FPRL1. Both SAA-induced Ca\(^{2+}\) mobilization and chemotaxis in FPRL1/293 cells were inhibited by pretreatment of the cells with pertussis toxin but not cholera toxin (data not shown) in correlation with the observation in native cells (5–7), suggesting activation of G protein of the Gi type is required for SAA signaling through FPRL1. In addition, since SAA can form complexes with HDL, which acts as a natural inhibitor of SAA (5, 6), we examined the effect of HDL on the chemotactic activity of SAA for FPRL1/293 cells. Fig. 3 D shows that HDL, whether preincubated with SAA or simultaneously added to SAA, completely abolished SAA-induced FPRL1/293 cell migration. In contrast, the same concentration of HDL did not affect migration of FPR-expressing ETFR cells induced by fMLP (data not shown). These results confirmed that HDL specifically inhibited the agonist activity of SAA on FPRL1.
To further verify the usage of FPRL1 by SAA, we performed ligand binding experiments. Fig. 4 shows that radio-iodinated SAA specifically bound to FPRL1/293 cells with an estimated $K_d$ at 64 nM and 42,000 binding sites per cell (Fig. 4 A). $^{125}\text{I}$-labeled SAA also specifically bound to monocytes (Fig. 4 B) and neutrophils ($K_d$ 545 nM, $R = 6,700$/cell) with $K_d$ values comparable to those achieved with FPRL1/293 cells. In the displacement assay, unlabelled SAA in a dose-dependent manner inhibited its own binding to monocytes (Fig. 4 C), neutrophils (data not shown) and FPRL1/293 (Fig. 4 D) with an IC$_{50}$ at $\approx 50$ nM. In contrast, unlabelled fMLP at high concentrations ($\approx 10 \mu M$) only partially competed with $^{125}\text{I}$-SAA for binding. These results confirm SAA to be a far more efficient agonist for FPRL1 than fMLP.

**Discussion**

In this study, we demonstrate that SAA uses FPRL1, a seven-transmembrane, G protein-coupled receptor expressed on phagocytes as a chemotactic receptor, suggesting a molecular basis for our previous observations that SAA is a potent chemoattractant and activator for human peripheral blood monocytes and neutrophils (5-7). In addition to SAA, FPRL1 has previously been shown to be a low affinity receptor for fMLP (11, 13) and a high affinity receptor for the lipid metabolite LXA4 and its analogues (15-24). Our data suggest that fMLP is a partial agonist incapable of inducing chemotaxis via FPRL1 in this model system. Analysis of LXA4 induction of chemotaxis via FPRL1 has not been reported. Thus, SAA is the first chemotactic agonist identified for FPRL1.

FPRL1 was identified and molecularly cloned from human phagocytic cells by low stringency hybridization of the cDNA library with the FPR sequence and initially was defined as an orphan receptor (10, 11, 13, 14). The cloning of the same receptor termed FPR H2 from a genomic library was described by Bao et al. (12). FPRL1 possesses 69% identity at the amino acid level to FPR, the prototype receptor for synthetic and bacterium-derived formylated peptides (8, 9). Both FPR and FPRL1 are expressed by monocytes and neutrophils and are clustered on human chromosome 19q13 (12, 36). Although fMLP is a high affinity agonist for FPR, it interacts with FPRL1 and transduces signals in response to fMLP only at high concentrations (Fig. 2 and references 11, 13, 36). SAA, on the other hand, selectively bound and activated only FPRL1 at physiologically relevant concentrations, which under inflammatory stimulation could reach 80 $\mu M$ in the serum (1-4). FPRL1 is mainly expressed in monocytes and neutrophils. However, cells other than phagocytes, such as hepatocytes, have also been shown to express FPRL1 (8). Recently, the expression of this receptor (also termed LXA4R) has been reported to be highly inducible in epithelial cells by specific cytokines (20). Our previous study showed that CD3$^+$ human peripheral blood T lymphocytes were induced by SAA to migrate and adhere to endothelial cell monolayers (6), suggesting that T lymphocytes may also express a receptor(s) for SAA. In fact, we detected specific binding sites for $^{125}\text{I}$-labeled SAA on human peripheral blood CD3$^+$ T lymphocytes ($K_d = 300$ nM, $R = 2,200$ sites/cell). However, whether these binding sites on T lymphocytes represent FPRL1 or an additional receptor(s) for SAA is not yet known.

Despite the fact that the chemotactic formyl peptide fMLP has been shown to be a low efficiency agonist for FPRL1, a lipid metabolite LXA4 has been reported to be a high affinity ligand and potent agonist for this receptor.
LXA4 is an eicosanoid generated during a number of host reactions such as inflammation, thrombosis, and atherosclerosis (22), and was initially discovered as an inhibitor of immune response (for review see reference 37). LXA4 was subsequently reported to inhibit neutrophil chemotaxis (38) and transepithelial migration induced by chemotactic agents (23). A seven-transmembrane, G protein–coupled receptor identical to FPRL1 was recently identified for LXA4 (15, 16, 22). LXA4 bound to CHO cells transfected with this receptor with high affinity and increased GTPase activity and the release of esterified arachidonate (15). Thus, LXA4 has been proposed to be an endogenously
produced ligand for FPR L1 (15, 16). Although LXA4 has not been documented to induce Ca\(^{2+}\) mobilization in neutrophils or FPR L1-transfected cells (15), it was reported to induce Ca\(^{2+}\) flux and chemotaxis in monocytes, presumably through FPR L1 (17, 22). Thus, differential activation of second messengers in monocytes versus neutrophils by LXA4 was postulated. In our study, we did not detect significant induction of Ca\(^{2+}\) flux or chemotaxis in FPR L1-transfected cells by a commercially available LXA4 (Biomol, Plymouth Meeting, PA), nor did we observe inhibition of SAA signaling or binding by this LXA4 in either phagocytes or FPR L1/293 cells. Further study, beyond the scope of this report, will be needed to compare the interaction of FPR L1 with its peptide ligands, SAA and fMLP, versus its lipid ligand, LXA4, to clarify these results.

Our previous studies showed that both SAA-induced leukocyte chemotaxis and activation were inhibited by pertussis toxin (6, 7). This study also showed that the signaling of SAA through FPR L1 was sensitive to pertussis toxin. Thus, although the signal transduction pathways triggered by SAA in FPR L1 requires further investigation, the high level homology of FPR L1 to FPR, its sensitivity to pertussis toxin, and its mediation of potent phagocyte activation by SAA suggest that FPR L1 may share major biochemical events with FPR. It is well known that binding of FPR by bacterium-derived or synthetic peptide agonists results in a G protein-mediated signaling cascade leading to phagocytic cell adhesion, chemotaxis, release of oxygen intermediates, enhanced phagocytosis, and bacterial killing, as well as gene transcription (8, 9). Activation of FPR by its agonists can also result in heterologous desensitization of the subsequent cell response to other G protein receptor ligands (39, 40), including chemokines. This “desensitizing” effect of FPR activation may also be seen with FPR L1, although more studies are needed to elucidate the mechanism(s) involved. For instance, SAA was initially reported as an inhibitor of neutrophil response to fMLP (41). In these experiments, neutrophils preincubated with SAA showed reduced superoxide release in response to fMLP (41). Our previous study also showed that preincubation of monocytes and neutrophils with SAA reduced cell response to a number of chemoattractants, including fMLP and chemokines (7), suggesting that FPR L1 is capable of transducing intracellular biochemical events leading to desensitization of other G protein-coupled receptors.

The pathophysiological significance of use of FPR L1 by SAA requires more in-depth investigation. The optimal concentrations for SAA to induce leukocyte migration, adhesion, and tissue infiltration ranged from 0.8 to 4 \(\mu M\) (5–7), which are higher than the SAA levels present in normal serum but well below the concentration seen during a systemic acute phase response (1–4). Increased serum levels of SAA have been observed in a number of inflammatory and infectious diseases as well as after organ transplantation (4).
The SAA concentrations required for activating FPR L1 are well within the range in which native cells are activated, as shown in this study. The overproduction of SAA by hepatocytes can be induced by inflammatory stimuli such as LPS, IL-1, IL-6, and TNF-α (1–4). M acrophages have also been reported as an extra-hepatic source of SAA during inflammation (42) and may produce relatively high concentrations in microcompartments. The expression of SAA mRNA in human atherosclerotic lesions and the induction of SAA by oxidized low density lipoproteins strengthen the hypothesis that SAA may play an important role in vascular inflammation (42) and may produce relatively high concentrations in microcompartments. The expression of SAA mRNA in human atherosclerotic lesions and the induction of SAA by oxidized low density lipoproteins strengthen the hypothesis that SAA may play an important role in vascular injury and atherosclerosis (4). Under normal conditions, most serum SAA will be associated with HDL, which acts as a natural inhibitor of the chemotactic activity of SAA (references 5, 6 and Fig. 3 D). However, since SAA binds to HDL at equimolar ratios (43), a rapid increase in concentration of locally produced SAA could establish a gradient of free active SAA with consequent recruitment of leukocytes into inflammatory sites. Therefore, it is possible that at local inflammatory sites elevated SAA can attract and activate leukocytes for the clearance of pathogenic agents. This process may also cause tissue injury. Furthermore, signals triggered by activated FPR L1, a functional receptor of SAA, could eventually result in unresponsiveness of leukocytes to additional stimulation, thus immobilizing the cells and limiting the degree of inflammation.

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