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Novel G Protein-Coupled Responses in Leukocytes Elicited by a Chemotactic Bacteriophage Displaying a Cell Type-Selective Binding Peptide

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Recently, we identified a neutrophil-binding phage displaying a novel peptide motif, GPNLTGRW. It was determined that this peptide, when displayed on bacteriophage (FGP phage), elicits a transient increase in cytosolic calcium. Here, we show that FGP phage stimulate neutrophil chemotaxis and induce a pertussis toxin-sensitive rise in cytosolic calcium in monocytes as well as in neutrophils. In contrast to the calcium response elicited by classical chemotaxtractants fMLP and IL-8, the FGP phage-elicted response in neutrophils is dependent on extracellular calcium and is mediated by receptor-activated, divalent cation channels. Consistent with G protein-coupled receptor signaling, FGP phage effect homologous and reciprocal heterologous desensitization with fMLP- and IL-8-stimulated calcium responses. Like non-G protein-coupled responses, the FGP-elicted calcium transient is abolished with phosphoinositide-3-kinase inactivation. Nonetheless, specific binding of GTP to neutrophil membranes follows stimulation with FGP phage, further supporting involvement of G proteins. However, FGP phage neither bind to nor elicit a calcium response from transfectant cells harboring known candidate G protein-coupled receptors. These data together suggest that the elicited responses are mediated by a novel G protein-coupled receptor or represent novel responses of a known receptor. The Journal of Immunology, 2001, 166: 7250 –7259.

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4 Abbreviations used in this paper: PMN, neutrophils; FPR, formyl peptide receptor; FPR-L1, FPR-like 1; FGP, peptide FGPNLTGRW; [Ca2+]i, cytosolic calcium concentration; GTP·S, guanosine 5'-O-(3-thiotriphosphate; MCP, monocyte chemoattractant protein; MIP-1α, macrophage inflammatory protein 1α; SDF-1, stromal cell-derived factor-1; HBSS(+), HBSS devoid of divalent cations; HBSS(+), HBSS with 1 mM CaCl2 and 1 mM MgCl2; fura-2-AM, fura-2 acetoxymethyl ester; indo-1-AM, indo-1 acetoxymethyl ester; CI, chemotactic index; PI3, phosphoinositide-3-OH; DLV phage, phage bearing peptide DLVTSKLQV.

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Recently, methods have been developed for generating libraries containing vast numbers of peptides from which sequences of interest may be screened for cell type binding or elicitation of selected cell responses. For example, random peptide phage display libraries (5), synthetic Ab phage display libraries (6), and synthetic combinatorial peptide libraries (7) have been used for such purposes. Selection processes have been developed for identification of cell type-specific and biologically active peptide ligands for leukocyte cell surface receptors including IL-8- specific antagonists (8) and formyl-peptide receptor (FPR)-like 1(FPR-L1) receptor agonists (9, 10). In these previous studies, these peptides served as useful tools to study the cell populations to which they bound as well as mechanisms of receptor activation and cell signaling. As key components of the innate immune system, PMN form an important potential target for such selection efforts.

We recently described results of panning experiments in which random peptide phage display libraries were applied directly to live human PMN (11). Panning lead to the discovery of several sets of novel PMN-binding peptide motifs. As displayed in pentameric form on phage particles, one of these peptide sequences, FGPNLTRGW (FGP), was shown to elicit an increase in the concentration of cytosolic calcium ([Ca2+]i) in PMN. This response was abrogated by pretreatment with pertussis toxin and thus consistent with signaling via a Gαi protein-coupled receptor, a feature common to most PMN chemotactic agents. FGP phage binding to PMN was specifically inhibited by a synthetic peptide containing GPNLTGRW. Yet FGP peptide bears no apparent primary amino acid sequence homology to a described protein or peptide based on searches of public databases.

Because of the importance of calcium and G protein-coupled receptor signaling in PMN function (12, 13) and the significance of PMN in disease, we undertook the current studies to better understand the biological significance of FGP. Here, we characterize FGP as a novel PMN chemotactic agent, elucidate the mechanism of calcium signaling, and demonstrate that this activity is independent of known G protein-coupled receptors found on PMN that...
mediate chemotaxis and that otherwise have properties expected of an FGP receptor.

Materials and Methods

Chemicals

Purchased reagents included fMLP, complement component C5a-des arg (C5a), and ATP, GTP, GDP, guanosine 5'-[γ-thio]triphosphate (GTPγS; Sigma, St. Louis, MO), human rFL-8, stromal cell-derived factor-1 (SDF-1), RANTES, macrophage inflammatory protein 1α (MIP-1α), monocyte chemottractant protein (MCP-1; R&D systems, Minneapolis, MN), the competitive fMLP chemotaxis assays as described previously (22). Briefly, FGP phage or control was added to 160 μl of HBSS (−) in upper wells. PMN (1×10⁷ in 20 μl) were added to upper wells to initiate the assay. Cells were allowed to migrate across 0.33-cm² collagen-coated polycarbonate supports (5-μm pore size) for 1.5 h at 37°C. PMN that migrated into the lower chamber were quantified by an enzymatic assay for myeloperoxidase as described (23). Results are presented as the chemotactic indices (CI) representing the fold-increase in the number of migrating cells in response to stimulants over migration in response to medium alone. Checkboard analyses were performed as described (24). Conditions were plated in triplicate.

To examine the effect of G protein inactivation on FGP-stimulated chemotaxis, cells were preincubated for 2 h with 1 μg/ml pertussis toxin (Sigma, St. Louis, MO) which ADP-ribosylates and inactivates Gi subunits (11). For further experiments, the fold-increase in the number of migrating cells in response to stimulants over migration in response to medium alone. Normal saline/HEPES buffer (NS/HEPES; 150 mM NaCl, 10 mM HEPES, pH 7.4) were determined by standard plaque counting assays (15). The amino acid sequences of the displayed peptides were confirmed for large-scale preparations by sequencing the coding nucleotides in the viral DNA as described (16).

Human blood cell and lymph node cell isolation

Blood cells obtained from healthy volunteers were isolated for experiments by one of two methods by using an institutionally approved protocol. For flow cytometric analyses of blood cells, blood anticoagulated with 3.8% sodium citrate underwent partial RBC lysis, washing, and suspension in modified HBSS, 10 mM HEPES, pH 7.4, devoid of Ca²⁺ and Mg²⁺ (HBSS(−)). For calcium mobilization and desensitization assays, PMN were isolated from whole blood, by a standard Ficoll separation technique (17). The separate mononuclear fraction enriched with monocytes and d-epleted of PMN was taken from Ficoll preparations for some experiments. After isolation, cells were resuspended at 4°C at a concentration of 5×10⁶ cells/ml and used for subsequent experiments. Additional human lymphocytes for flow cytometric analyses had been collected from lymph node biopsy material by physical disaggregation and tested negative for disease by prior morphologic and immunophenotypic evaluations at Emory University Hospital (Atlanta, GA).

Cell lines

Experiments were performed with several stable transfectant cell lines and nontransfected parent lines. These included Chinese hamster ovary cells with FPR (18) and C5a receptors (generous gifts of Dr. P. M. Murphy, National Institute of Allergy and Infectious Diseases, Bethesda, MD) and 30-19 and 30-19 marine pre-B cells expressing chemokine receptors CXCR1, CXCR2, CXCR4, CCR2b, CCR4, and CCR5 (Ref. 20; generous gifts of Dr. M. Bagnoli, University of Berne, Berne, Switzerland). Transfectant cells were grown and passaged in the appropriate selective media as described (18–20).

Flow cytometry

Blood cells and nodule lymphoid cells (1–5×10⁶ cells in 100 μl in HBSS(−), 0.5% BSA) were incubated for 45 min with combinations of 30 μl of phage suspension (3×10¹⁰ phage) and 10 μl of either FITC-, PE- or allophycocyanin-labeled mAbs to the following Ags: CD3, CD4, CD14, CD15, CD19, and CD61 (Becton Dickinson, San Jose, CA). Binding studies with phage and transfectant cell lines were performed identically, except no anti-CD mAbs were added. After washing, cells were fixed with 1.8% paraformaldehyde for 10 min and washed once with HBSS(−), 0.5% BSA. Cell-bound phage were labeled with biotinylated sheep anti-M13 phage polyonal Ab (1:300; 5 Prime-3 Prime, Boulder, CO), washed once and stained with PE-streptavidin or FITC-streptavidin (The Jackson Laboratory, Bar Harbor, ME). Cells were analyzed on a FACSort cytometer (Becton Dickinson). Experiments were performed with different donors, and at least 8000 events were measured. Data acquisition and analysis were accomplished with CellQuest software, version 3.1 (Becton Dickson). Standard criteria were used for placing gates on different hematopoietic cell populations (21).

Chemotaxis assays

Migration of PMN was assessed by using modified Boyden chamber-type chemotaxis assays as described previously (22). Briefly, FGP phage or controls were diluted in NS/HEPES buffer. In the lower wells, 100 μl of FGP phage or control was placed in 900 μl HBSS(−) containing 1 mM CaCl2 and 1 mM MgCl2 (HBSS(+); for some experiments, 20 μl of FGP phage or control was added to 160 μl of HBSS(−)) in upper wells. PMN (1×10⁷ in 20 μl) were added to upper wells to initiate the assay. Cells were allowed to migrate across 0.33-cm² collagen-coated permeable polycarbonate supports (5-μm pore size) for 1.5 h at 37°C. PMN that migrated into the lower chamber were quantified by an enzymatic assay for myeloperoxidase as described (23). Results are presented as the chemotactic indices (CI) representing the fold-increase in the number of migrating cells in response to stimulants over migration in response to medium alone. Checkboard analyses were performed as described (24). Conditions were plated in triplicate.

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Calcium mobilization assays

Cells were loaded with the calcium indicator indo-1 acetoxymethyl ester (indo-1-AM) (blood leukocytes) or fura-2 acetoxymethyl ester (fura-2-AM; cell lines; Molecular Probes, Eugene, OR), and [Ca²⁺], was measured as reported (26, 27) with a Hitachi F-4500 spectrofluorometer (Hitachi, Tokyo, Japan). After loading with indicator for 30 min at 37°C and gentle centrifugation, cells were resuspended in HBSS(−). Loaded cells (1×10⁶) were suspended in 960 μl of HBSS(+) for 2–5 min at 37°C and then [Ca²⁺], measured before and after the addition of phage clones or control agonists, all of which were dissolved in NS/HEPES. Purified phage clones were taken from stock solutions (2.5×10¹² PFU/ml) in 150 mM NaCl, 10 mM HEPES, pH 7.4) and added to 1×10⁶ PFU/ml final concentration. For some experiments, cells were additionally preincubated with 25 μM SK&F96365 (1-(β-3-(4-methoxyphenyl)propoxy)-4-methoxyphenethyl)-1H-imidazolide hydrochloride; Sigma, Ref. 28), an inhibitor of receptor-mediated calcium entry, for 5 min before use, or with 100 nM wortmannin (Sigma) or 1 μg/ml pertussis toxin under conditions identical with those described above for chemotaxis studies. For other experiments, loaded cells were placed in HBSS(−), 4 mg EGTA before the addition of test substances to deplete extracellular calcium. Excitation wavelengths monitoring of fluorescence signals and calculations to determine relative cytoplasmic calcium concentrations were as previously detailed (11, 20, 29).

Desensitization assays

To study desensitization of the calcium mobilization response, PMN were loaded with the indicator indo-1-AM as above. The magnitudes of the transient increase in [Ca²⁺], were evaluated as a function of the concentration of phage and control agonists to determine for each the effective concentration yielding a maximal response (EC100) and half-maximal response (EC50) (Ref. 30). For homologous desensitization, with 100 s after an EC100 dose of the first agonist was applied, cells were challenged with an EC100 dose of the same agonist (32) and relative [Ca²⁺], changes were quantified. For heterologous desensitization experiments (32), the ability of an EC100 dose of agonist to induce [Ca²⁺], changes was quantified 80–160 s after exposure of cells to an EC100 dose of an initially applied different agonist.

GTPγS membrane binding assay

PMN membranes were prepared and assays performed as described previously (33) with some modifications. PMN were resuspended in 10 mM HEPES, 100 mM KCl, 10 mM NaCl, 3.5 mM MgCl₂, and 1 mM PMSF, pH 7.4. Membranes were prepared by N2 cavitation at 4°C for 15 min at 400 p.s.i. followed by sequential centrifugation at low speed (1000 x g) to remove nuclei/cellular debris and high speed (100,000 x g for 30 min). Membrane pellets then were resuspended in 100 mM NaCl, 10 mM MgCl₂, and 1 mM PMSF, pH 7.4. Total G protein was quantified (BCA protein assay kit; Pierce, Rockford, IL). [35S]GTPγS assays were performed with 30 μg of membrane protein in 700 μl of membrane buffer. Samples were incubated with or without FGP or control phage (5×10⁸ PFU) in 300 μl of NS/HEPES or control 1 μM fMLP in the presence of 0.5 μM GTPγS for 30 min at 30°C. [35S]GTPγS, a radiolabeled, nonhydrolyzable analog of GTP, was added to tubes (0.01 μCi) with or without 10 μM GTPγS to control for nonspecific [35S]GTPγS binding. Reactions proceeded for 30 min at 30°C and were terminated by...
filtration through 24-mm-diameter Whatman GF/C filters. Filters were washed three times with 2 ml of buffer containing 50 mM Tris-HCl (pH 7.5) and 5 mM MgCl₂. Filters were placed in 6 ml of scintillation fluid and counted in a liquid scintillation counter.

Statistical analysis

All experiments were performed three times except for flow cytometric analyses that were performed twice. Results presented are from representative experiments. The significance of the difference between test and control group means was analyzed with Student’s t test; p values < 0.05 are considered to be significant.

Results

FGP binding to normal human hemolymphoid cells

To obtain a more complete profile of FGP phage binding specificity to normal human hemolymphoid cells, flow cytometric analyses were performed. Experiments were performed with unfractionated human blood cells from different donors, after partial RBC lysis, and lymph node lymphocytes. Fig. 1 displays selective binding to monocytes and PMN. As demonstrated in Fig. 1A, FGP phage binding to PMN reaches above levels of wild-type control phage that lack an additional displayed peptide; gates were placed on PMN in forward by right angle light scatter plots (21). For two-color analyses, FGP phage were combined with a cell type-selective, labeled anti-CD Ab for costaining of cells (Fig. 1, B–E). Wild-type phage served as the control phage and together with isotype-matched Ab was used to set background gates. As displayed in Fig. 1B, monocytes coexpressing CD14 likewise bind to FGP phage avidly. In contrast, CD61-positive blood platelets (Fig. 1C), glycoprotein- expressing RBC (Fig. 1D), and nodal CD19-positive B and CD3-positive T lymphocytes (Fig. 1, E and F) fail to bind to FGP phage above background control levels. CD19- and CD3-positive lymphocyte populations from blood displayed a similar lack of FGP phage staining (data not shown). Thus, FGP phage show a clear selectivity for binding to monocytes and PMN among mature hemolymphoid cells, and indicate a limited pattern of FGP receptor expression.

FGP stimulates directional migration of PMN

Because FGP phage bind selectively to motile cell types (PMN and monocytes) and elicit a pertussis toxin-sensitive calcium transient like many leukocyte chemoattractants, we hypothesized that FGP may promote directional cell migration. To test this hypothesis, modified Boyden chamber-type chemotaxis assays were performed in which PMN were added to the upper well and phage placed into the lower well. As displayed in Fig. 2, PMN migrate preferentially in response to FGP phage over either phage bearing DLVTSKLQV (DLV phage) that bind PMN (11) or wild-type control phage. The concentration of phage promoting maximal migration was determined to be \( \sim 1 \times 10^{12} \) PFU/ml. The observed migratory response to FGP phage was \( \sim 50\% \) of that promoted by the well characterized chemoattractant fMLP (10 nM). To differentiate chemotaxis from chemokinetics, varying concentrations of FGP phage were added to the upper and lower wells of the chemotaxis apparatus. As shown in the checkerboard analysis in Table I, dose-dependent migration is observed with increasing phage concentrations in the lower well. This finding suggests a small chemokinetic effect, the degree of which is similar to that observed for other well characterized chemoattractants such as fMLP when applied in the same type of assay (24).

FGP elicits a calcium signal in monocytes that is pertussis toxin-sensitive

The cell type-selective pattern of binding and chemotaxis results suggested that monocytes like PMN might display a calcium mobilization response to FGP phage. For these studies, Ficoll-prepared mononuclear cells were loaded with the indicator indo-1-AM and the calcium mobilization responses measured. Fig. 3A shows elicitation of the transient rise in \([Ca^{2+}]_i\) specifically by FGP phage (1 \( \times 10^{11} \) PFU/ml). The magnitude of the response is approximately one-third that of an EC\(_{100}\) dose (1 \( \mu \)M) of the control agonist fMLP. In contrast, control phage bearing peptide WDWLPW fail to elicit a calcium mobilization response, although

![FIGURE 1. Pattern of FGP binding to mature hemolymphoid cells in flow cytometric analyses: selective binding to PMN granulocytes and monocytes. For these studies, cell-bound phage were detected with a biotinylated anti-M13 phage Ab and PE-labeled avidin (plots A and C–E) or FITC- labeled avidin (plot B). Histogram plot A shows FGP staining (solid) compared with wild-type control phage staining (transparent) of PMN granulocytes. Granulocytes were selectively gated with a forward by right angle light scatter plot. For two-color flow cytometric analyses (plots B–E), gates along the abscissas were set with fluor-labeled isotype-matched control Ab staining levels (unpublished observations). Gates along the ordinate (labeled “Phage”) were set with wild-type control staining levels. Arrows indicate the salient cell population (described above the plots) that are positively stained with the anti-CD Ab. Abs were labeled as follows: PE-anti-CD14, FITC- anti-CD61, FITC- anti-glycophrin, allophycocyanin-anti-CD19, allophycocyanin-anti-CD3. For plots A–D, blood cells were used; for plots E and F, nodal lymphocytes were used. Fluorescence intensities are displayed on a 4-decade logarithmic scale.](http://www.jimmunol.org/ftp/f9752296.html)
these phage bind avidly to monocytes in flow cytometric analyses (D.L.J., L.M., and C.A.P., unpublished results). Cells exposed to WDWLPW phage are still capable of responding to an EC100 dose (D.L.J., L.M., and C.A.P., unpublished results). fMLP (10 nM) served as a positive control in a separate run or after addition of control phage where it was added at 120 s. B and C, Indo-1-AM-loaded cells were preincubated with or without 1 μg/ml pertussis toxin (PTx) before undergoing calcium testing with agonists, FGP phage (1 × 10^{11} PFU/ml), and controls ATP (1 mM) and fMLP (10 nM). The tracings represent relative changes in [Ca^{2+}].

The FGP-elicted response is dependent on extracellular calcium and uses receptor-activated, divalent cation channels

Experiments were performed to further define the mechanism by which calcium signaling is generated in PMN by FGP. To test for use of extracellular calcium, EGTA (4 mM) was added to divalent cation-free buffer, HBSS(−), for use as the extracellular buffer in calcium mobilization assays. Fig. 4A shows that chelation of extracellular calcium ablates the FGP-induced, transient increase in [Ca^{2+}]. This effect is not attributable merely to failure of the ligand to bind the cells because FGP phage bind in the absence of extracellular calcium (Fig. 1). As displayed in Fig. 4B, the calcium increase in response to control fMLP, shown previously to be largely independent of extracellular sources (34), is unchanged in the presence of EGTA. A similar pattern also was demonstrable for the response to control IL-8 (data not shown), in accord with results reported by others (28). These data indicated use of extracellular calcium, rather than intracellular stores, in generation of the calcium transient and thereby suggested that cell surface channels may be activated in response to binding of FGP phage. Therefore,

\[ \text{Table I. Checkerboard assay of PMN migration toward FGP phage} \]

<table>
<thead>
<tr>
<th>Phage Concentration in the Lower Well</th>
<th>0</th>
<th>2.5 × 10^{11}</th>
<th>5 × 10^{11}</th>
<th>1 × 10^{12}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.0 ± 0.7</td>
<td>2.9 ± 1.4</td>
<td>2.7 ± 1.9</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>2.5 × 10^{11}</td>
<td>16.3 ± 4.3</td>
<td>14.1 ± 3.6</td>
<td>—</td>
<td>11.2 ± 4.9</td>
</tr>
<tr>
<td>5 × 10^{11}</td>
<td>21.3 ± 1.2</td>
<td>10.5 ± 1.6</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1 × 10^{12}</td>
<td>36.4 ± 3.7</td>
<td>18.2 ± 5.1</td>
<td>—</td>
<td>18.4 ± 1.6</td>
</tr>
</tbody>
</table>

* Different concentrations of phage were placed in the lower wells and/or upper wells of the chemotaxis chamber as shown; PMN suspensions were placed in the upper wells. The upper and lower wells were separated by polycarbonate filters. After incubation, the cells that migrated across the filters were detected using a myeloperoxidase assay as described in Materials and Methods. Phage concentrations are shown in PFU/ml. Values represent chemotactic indices ± SD for conditions in triplicate.
we investigated whether cell surface, receptor-activated, divalent cation channels are used for the FGP-elicited response by using a selective inhibitor of this mode of calcium entry, SK&F 96365 (28, 35). As shown in Fig. 5A, PMN preincubated with 25 μM SK&F 96365 demonstrated a markedly diminished calcium response to FGP phage, losing the initial sharp rise in [Ca$^{2+}$]. However, as can be seen, a small magnitude increase in [Ca$^{2+}$]$_i$, occurs several seconds later, suggesting that channels that are insensitive to the inhibitor may be activated as well. In contrast, the response to controls fMLP (Fig. 5B) and IL-8 (Fig. 5C) were unaffected by the inhibitor. Thus, receptor-activated, divalent cation channels account for a major component of the calcium signal induced by FGP phage.

FGP elicits a PI3 kinase-dependent calcium signal in PMN

Because PI3 kinase activity is important in several key signaling pathways that govern PMN function (36–38), we investigated whether the FGP-induced calcium signal may occur downstream of PI3 kinase activity by using the fungal-derived, PI3 kinase inhibitor, wortmannin (39). Results displayed in Fig. 6A show that PMN preincubated with the inhibitor fail to manifest the FGP calcium transient. However, the calcium signal generated by control fMLP is unaffected by the inhibitor (Fig. 6B). Thus, PMN activated in response to FGP phage manifest a calcium transient by signaling through PI3 kinase, in contrast to the calcium signaling produced by fMLP binding.

We have shown that FGP stimulation of PMN leads to a calcium response that is sensitive to both pertussis toxin and wortmannin. We also assessed the sensitivity of the PMN chemotactic response to these inhibitors. As displayed in Fig. 7, results of chemotaxis assays in which PMN were preincubated with either pertussis toxin or wortmannin show that these inhibitors significantly diminished migratory responses to FGP phage. Likewise, PMN pretreated with these inhibitors show a significant decrement in migratory response to control fMLP, as expected (22, 38). Corroborating the calcium response findings, these data suggest that FGP signaling that culminates in the PMN chemotactic response requires activation of both G$_{	ext{ai}}$ protein and PI3 kinase.

Homologous desensitization of the FGP-elicited calcium response in PMN and reciprocal heterologous desensitization with the fMLP and IL-8 signals

Experiments were performed to further investigate the regulation of the FGP calcium signal in PMN and whether the signal is consistent with G protein mediation by manifesting the phenomenon of desensitization (30, 40). We first examined whether the response to FGP phage manifests homologous desensitization (32). Phage were titrated to determine the EC$_{100}$ (yielding maximal increase in [Ca$^{2+}$]$_i$) and EC$_{50}$ (yielding half maximal increase in [Ca$^{2+}$]$_i$) doses of FGP phage for calcium responses (data not shown). As displayed in Fig. 8A, the addition of an EC$_{100}$ dose of FGP phage (agonist 1; 1 × 10$^{11}$ PFU/ml) abrogated the calcium response completely to a second EC$_{50}$ dose of FGP phage (agonist 2; 2 × 10$^{10}$ PFU/ml) after a return to baseline [Ca$^{2+}$]$_i$, indicating homologous desensitization. Likewise, the expected homologous desensitization to an EC$_{50}$ dose of control IL-8 (agonist 2; 5 pg/ml) after exposure to an initial EC$_{100}$ dose (agonist 1; 5 ng/ml) is displayed in Fig. 8B.

To begin to define the relationship of the FGP-mediated calcium response to that produced through the characterized G protein-coupled receptors for fMLP and IL-8 (41, 42), heterologous desensitization experiments were undertaken in which the ability of

![FIGURE 4](http://www.jimmunol.org/)

FGP stimulates a transient calcium signal in PMN that depends on the presence of extracellular calcium. Indo-1-AM-loaded PMN were stimulated at 60 s in the presence or absence of 4 mM EGTA in the extracellular buffer with 1 × 10$^{11}$ PFU/ml FGP phage (A) or with fMLP (10 nM) (B).

![FIGURE 5](http://www.jimmunol.org/)

FIGURE 5. FGP stimulates a calcium transient in PMN that is dependent on use of receptor-activated, divalent cation channels. Indo-1-AM-loaded cells were stimulated at 60 s with 1 × 10$^{11}$ PFU/ml FGP phage (A) or controls fMLP (10 nM; B) or IL-8 (5 ng/ml; C) in the presence or absence of a specific inhibitor of receptor activated divalent cation entry, SK&F 96365 (25 μM). Inhibitor was added 5 min before calcium measurements.

![FIGURE 6](http://www.jimmunol.org/)

FIGURE 6. FGP elicits a PI3 kinase-dependent calcium signal in PMN. Indo-1-AM-loaded cells were stimulated at 60 s with 1 × 10$^{11}$ PFU/ml FGP phage (A) or control fMLP (10 nM; B) with or without preexposure to the selective PI3 kinase inhibitor, wortmannin (100 nM).
FGP specifically stimulates binding of GTPγS to PMN membranes

Although the pertussis toxin inhibition data, chemotaxis findings, and desensitization studies provide indirect evidence that FGP phage is signaling via a G protein-coupled receptor, we sought more direct evidence for use of membrane-associated G protein. Because G protein-coupled receptors stimulate exchange of GDP for GTP through membrane-associated G protein, we examined whether specific binding to PMN membranes of a nonhydrolyzable, radiolabeled GTP analog [35S]GTPγS was measurable after stimulation in the absence or presence of FGP phage. As shown in Fig. 9, FGP phage (1 × 10^{11} PFU/ml) stimulated ~40% of the specific incorporation of [35S]GTPγS as control fMLP (1 μM). In contrast, two types of control phage, DLV phage and wild-type phage, elicited 8% and 6%, respectively. There was no statistically significant difference between the two control phage responses (p = 0.46), although the response evoked by FGP phage was significantly higher in comparison to DLV phage (p < 0.02). Interestingly, the relative pattern of label incorporation in Fig. 9 in these experiments approximates the pattern encountered for chemotactic indices in Fig. 2. This similarity suggests that the degree of chemotaxis elicited by FGP is proportional to the degree of G protein stimulation. Thus, FGP phage stimulate specific binding of GTP to PMN membranes at levels significantly above controls, consistent with FGP signaling through a G protein-coupled receptor.
FGP phage do not bind to or elicit calcium responses in cells transfected with known G protein-coupled receptors

Results of the foregoing studies provided criteria for selection of candidate FGP receptors. These candidate receptors couple to pertussis toxin-sensitive G proteins to elicit calcium signals and chemotaxis and are selectively expressed on PMN and/or monocytes but not on quiescent lymphocytes, RBC, or platelets. A number of known chemokine and chemotactant receptors selectively expressed on PMN and/or monocytes meet these criteria. Hence, we used a panel of characterized transfectants that express these receptors to determine whether FGP phage might bind to and yield calcium signaling through one or more of them, given the possibility of FGP phage engaging more than one receptor.

For calcium signaling studies, transfector cell lines were loaded with the indicator dye fura-2-AM as described previously (19, 20, 22), and the ability of FGP phage to elicit calcium responses was measured. To confirm expression of functional receptor, native ligand controls were applied after the addition of phage. In separate experiments, heterotropic natural ligands were used to control for the specificity of the response. A typical response curve is shown in Fig. 10 for 300-19 murine, pre-B leukemia transfectants expressing the human IL-8 receptor, CXCR1. In Fig. 10A, cells were challenged with FGP phage (agonist 1; 5 \times 10^{11} PFU/ml) and manifested no increase in [Ca^{2+}], although they responded to a subsequent challenge with IL-8 (agonist 2; 5 ng/ml). Fig. 10B displays results of flow cytometric binding studies in which FGP phage are shown to not bind to CXCR1 transfectants at levels above wild-type phage binding. Control MIP-1, a chemokine that does not bind to CXCR1, failed to stimulate a calcium signal in these transfectants, as shown in Table II, confirming the selectivity of the response to IL-8. Data obtained for other tested transfectants that harbor candidate receptors are summarized in Table II. As can be seen, although transfectants responded appropriately to native ligands and not to control ligands, no calcium signals were detected in response to FGP phage (5 \times 10^{11} PFU/ml) for any of the cell transfectants tested. Furthermore, flow cytometry studies failed to demonstrate any FGP phage binding above wild-type control phage levels. CXCR4, which also tested negative, is more broadly expressed on leukocytes and thus was selected as a specificity control.

To exclude the possibility that contaminating bacterial products in the phage preparations such as formylated peptides might contribute to observed FGP responses and because the FPR has a binding pocket capable of accommodating peptides of various compositions, we used a second method to assess in PMN whether FGP might signal via the FPR. The ability of the competitive fMLP inhibitor, N-t-butoxycarbonyl-met-leu-phe, to abrogate the FGP-induced calcium response was investigated. We found that although the inhibitor, as expected, eliminated the calcium response to fMLP, it demonstrated no effect on the FGP-induced response (D.L.J. and C.A.P., unpublished results), confirming the findings with FPR transfectants (Table II). In aggregate, this last set of studies shows that FGP phage neither bind to nor elicit a calcium signal through any of the characterized G protein-coupled chemotactant receptors that are found on PMN and monocytes, suggesting use of either a novel receptor or novel use of a known receptor by FGP phage.

Discussion

In the current work, we have investigated the biological and signaling properties of a cell type-selective binding peptide, FGP, as displayed on bacteriophage. We previously identified the FGP phage from a linear 9-mer random peptide phage display library by panning on live human PMN (11). We now show that FGP phage bind selectively to normal human monocytes as well as PMN and that these phage represent a novel chemotactic agent for PMN. We
FGP phage are pertussis toxin-sensitive and that they display typical agonist, it is distinct from these former examples in that protein (19). However, although FGP phage clearly represent a synthetic combinatorial peptide library (10), and serum amyloid A protein (44), a synthetic peptide, WKYMVd-M, derived from a example, diverse ligands have been ascribed to the FPR-L1, including an oligopeptide derived from the HIV gp120 envelope proteins that have been found to additionally possess chemotactic activities. Some of these agents have been found to bind to and elicit chemotaxis. Furthermore, this study is the first demonstration that cell type-specific binding phage selected from a random-peptide phage display library may be used to study signal transduction pathways.

FGP phage join a growing list of novel PMN chemoattractants that have been reported recently (10, 19, 44–46). These chemoattractants include sequences selected from synthetic peptide libraries, viral components, and previously described endogenous proteins that have been found to additionally possess chemotactic activities. Some of these agents have been found to bind to and activate the same receptors despite having diverse structures. For example, diverse ligands have been ascribed to the FPR-L1, including an oligopeptide derived from the HIV gp120 envelope protein (44), a synthetic peptide, WKYMv-M, derived from a synthetic combinatorial peptide library (10), and serum amyloid A protein (19). However, although FGP phage clearly represent a complex agonist, it is distinct from these former examples in that it does not appear to bind to any member of a large battery of known candidate G protein-coupled receptors.

In addition, the bell-shaped dose-response curve observed for most chemoattractants was not seen with FGP phage in these studies. For soluble chemoattractants, this phenomenon represents the effects of receptor desensitization to increasing chemoattractant concentration. Because phage are particles, the maximal concentration that can be obtained before these particles precipitate out of solution is $\leq 1 \times 10^{13}$ PFU/ml, which is approximately in the nanomolar range. When used as agonists in the chemotaxis assays, the maximal concentration obtained is $\leq 1 \times 10^{13}$ PFU/ml after phage particles are diluted into appropriate buffer. This is the concentration at which we have observed maximal chemotactic activity. Thus, were the solubility of phage particles not a limiting factor, it is certainly conceivable that a decrement in chemotaxis might be observed at higher concentrations.

The primary amino acid sequence of the displayed peptide, FGP, and related sequences that share the GPNLTFGRW motif that was identified in several sequences by phage screening on PMN (11), bear no significant homology with known chemokines, chemoattractants or other proteins based on searches of public databases. Thus, these peptides may represent mimetics of a known molecule or an undiscovered, naturally occurring ligand such as an endogenous molecule or a portion of a viral coat protein. Complicating this question is the well documented ability of a linear peptide to represent an interactive site formed by discontinuous regions of a protein (15, 47) such that primary amino acid sequence comparisons may not reveal any significant similarities.

A further layer of complexity lies in the inability of monovalent synthetic peptide, GGPNLTFGRW, which acts as a specific competitive inhibitor of FGP phage binding (11) to elicit chemotaxis or calcium signaling (D.L.J. and C.A.P., unpublished results). This suggests several hypotheses. First, because the phage that we use display five copies of the peptide on their surfaces (5), the higher avidity of the phage-based interaction with receptor may be required to attain receptor activation. Second, the multivalent interaction of phage may facilitate functional responses through induction of FGP receptor dimerization or oligomerization. This type of receptor activation would be analogous to that observed for the Fcγ receptor that requires ligand multivalency for calcium signaling, although it is not a G protein-coupled receptor (26). Induction of dimerization of other G protein-coupled, chemoattractant receptors has been shown to occur, for example, when chemokine MCP-1 engages it receptor, CCR2b (48). However, ligand multivalency has not been shown to be a prerequisite for chemokine receptor activation. Third, although the peptide may dictate a binding interaction with the receptor, a second contact site may be provided by the phage that engages the receptor to yield signaling. This third model holds true for the interaction of IL-8 with its

### Table II. Binding and calcium mobilization studies with transfectant cells expressing FGP candidate G protein-coupled receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Transfectant</th>
<th>Native Ligand (Ca&lt;sup&gt;2+&lt;/sup&gt;)</th>
<th>Control Ligand (Ca&lt;sup&gt;2+&lt;/sup&gt;)</th>
<th>FGP Phage (Ca&lt;sup&gt;2+&lt;/sup&gt;)</th>
<th>FGP Phage Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPR&lt;sup&gt;b&lt;/sup&gt;</td>
<td>IMLP (+)</td>
<td>C5a (−)</td>
<td>(−)</td>
<td>(−)</td>
<td></td>
</tr>
<tr>
<td>FPR&lt;sup&gt;c&lt;/sup&gt;</td>
<td>IMLP (+)</td>
<td>C5a (−)</td>
<td>(−)</td>
<td>(−)</td>
<td></td>
</tr>
<tr>
<td>FPR-L1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>IMLP (+)</td>
<td>C5a (−)</td>
<td>(−)</td>
<td>(−)</td>
<td></td>
</tr>
<tr>
<td>C5α&lt;sup&gt;b&lt;/sup&gt;</td>
<td>C5α (+)</td>
<td>IMLP (−)</td>
<td>(−)</td>
<td>(−)</td>
<td></td>
</tr>
<tr>
<td>CXCR1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>IL-8 (−)</td>
<td>MIP-1α (−)</td>
<td>(−)</td>
<td>(−)</td>
<td></td>
</tr>
<tr>
<td>CXCR2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>IL-8 (−)</td>
<td>MIP-1α (−)</td>
<td>(−)</td>
<td>(−)</td>
<td></td>
</tr>
<tr>
<td>CCR1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>RANTES (−)</td>
<td>IL-8 (−)</td>
<td>(−)</td>
<td>(−)</td>
<td></td>
</tr>
<tr>
<td>CCR2b&lt;sup&gt;d&lt;/sup&gt;</td>
<td>MCP-1 (−)</td>
<td>IL-8 (−)</td>
<td>(−)</td>
<td>(−)</td>
<td></td>
</tr>
<tr>
<td>CCR5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>RANTES (−)</td>
<td>IL-8 (−)</td>
<td>(−)</td>
<td>(−)</td>
<td></td>
</tr>
<tr>
<td>CXCR4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>SDF-1 (−)</td>
<td>IMLP (−)</td>
<td>(−)</td>
<td>(−)</td>
<td></td>
</tr>
</tbody>
</table>

* The candidate receptors tested here are found on PMN and/or monocytes, transduce calcium signals through pertussis toxin-sensitive G proteins, and mediate chemotactic responses, all characteristics expected for an FGP receptor. Native and negative control ligands and FGP phage ($5 \times 10^{11}$ PFU/ml) were added in separate runs to Fura-2 loaded cells and changes in $[Ca^{2+}]$ were monitored as described in Materials and Methods. Ligands were added at the following concentrations: IMLP (10 nM); C5α (2.5 μg/ml); IL-8 (5 ng/ml); RANTES (1 μg/ml); MCP-1 (1 μg/ml); and SDF-1 (1 μg/ml). Results displayed as (+) indicate that an increase in $[Ca^{2+}]$ was noted, whereas a (−) indicates no increase above baseline. Flow cytometric binding studies with FGP phage were performed as described in Materials and Methods. Results displayed as (−) indicate no binding above wild-type control. FPR is the formulated peptide receptor, and FPR-L1 is the formulated peptide receptor-like 1.

<sup>a</sup> Chinese hamster ovary cell stable transfectants.

<sup>b</sup> 293 cell stable transfectants.

<sup>c</sup> 300-19 cell stable transfectants.
receptors in which discrete binding and activation sites exist (49, 50). We are actively investigating these possibilities.

Some peptides, such as mastoparan, activate G proteins through a direct binding interaction, thereby mimicking ligand-bound receptors (51). There are several reasons to suspect that FGP phage do not similarly bind and directly activate G proteins. In our previous manuscript in which the FGP phage were initially described, we performed experiments in which neutrophil-bound phage were visualized by immunofluorescence microscopy (11). In these experiments, phage were allowed to bind to live neutrophils at room temperature followed by detection with an anti-phage Ab after cells had been permeabilized with Triton X-100 detergent. By microscopy, phage particles were identified only at the cell surface. Because no phage were found on the cytoplasmic aspect, direct activation of G proteins after internalization of phage particles is highly unlikely. As we report in the manuscript, synthetic, soluble FGP peptide does not elicit a calcium mobilization response, and therefore is unlikely to bind directly to G proteins. In addition, structural features of the FGP phage do not support a direct G protein-binding hypothesis. In particular, for the FGP peptide to bind to a G protein directly, the peptide-displaying phage would have to convey the peptide across the membrane. However, the pH3 coat protein on which the FGP peptide is displayed (projected away from the body of the phage in 5 identical copies) and the surface aspects of the other phage coat proteins are hydrophilic (5). Thus, the phage particle itself would not readily mediate transmembrane projection of the FGP peptide. If the phage or peptide were nonetheless able to access G protein directly, then any cell type with pertussis toxin-sensitive G protein should bind to and be activated by FGP phage. Yet, this possibility is not borne out by our findings. Thus, we favor a model by which FGP phage act only at the cell surface through interaction with a G protein–coupled receptor, and not through direct G protein activation.

In contrast to the calcium responses signaled through typical chemokcartactant and chemokine receptors on PMN (52), the calcium transient in response to FGP phage is dependent on PI3 kinase activation. However, for the FPR, PI3 kinase activation, in particular the γ subtype, does appear to be required both for superoxide production and chemotaxis of PMN (36). The involvement of other specific PI3 kinase isoforms in these responses remains uncertain (38). By contrast, the calcium signal in response to high-valency immune complexes generated by Fc receptors and proteins displayed on filamentous phage. Methods Enzymol. 217:228.


