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Chemotaxis Inhibitory Protein of *Staphylococcus aureus* Binds Specifically to the C5a and Formylated Peptide Receptor

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Chemotaxis inhibitory protein of *Staphylococcus aureus* (CHIPS) is an exoprotein produced by several strains of *S. aureus*, and a potent inhibitor of neutrophil and monocyte chemotaxis toward C5a and formylated peptides like fMLP. These chemoattractants act on their target cells by binding and activating the C5aR and formylated peptide receptor (FPR), respectively. In the present report, we examined the mechanism by which CHIPS affects both of these receptors. We showed that CHIPS blocked binding of anti-C5aR mAb and formylated peptide to human neutrophils as efficiently at temperatures of 0 and 37°C, implying that it is independent of signal transducing systems. This was confirmed by showing that CHIPS acts completely independently of ATP. Additionally, CHIPS was not internalized upon binding to neutrophils. Furthermore, we showed that CHIPS binds specifically to the C5aR and FPR expressed on U937 cells. This binding was functional in blocking C5a- and fMLP-induced calcium mobilization in these cell lines. These results suggest that CHIPS binds directly to the C5aR and FPR, thereby preventing the natural ligands from activating these receptors. The apparent K_d values of CHIPS for the C5aR and FPR were 1.1 ± 0.2 nM and 35.4 ± 7.7 nM, respectively. Moreover, after screening a wide variety of other G protein-coupled receptors, CHIPS was found to affect exclusively the C5aR and FPR. This selectivity and high-affinity binding with potent antagonistic effects makes CHIPS a promising lead for the development of new anti-inflammatory compounds for diseases in which damage by neutrophils plays a key role. ***The Journal of Immunology*****, 2004, 172: 6994–7001.**

We recently described chemotaxis inhibitory protein of *Staphylococcus aureus* (CHIPS),[2] a 121-residue protein excreted by several strains of *S. aureus*. CHIPS acts as a potent and specific inhibitor of neutrophil and monocyte chemotaxis toward anaphylatoxin C5a and formylated peptides, bacterial excretion products like fMLP (1, 2). Both chemoattractants play a crucial role in the migration of neutrophils from the circulation to the site of infection, an important early event in the nonspecific immune response against invading pathogens. C5a and fMLP signal through their specific cell surface receptors, the C5aR and formylated peptide receptor (FPR), respectively, which both belong to the family of G protein-coupled seven-transmembrane receptors (GPCRs). Receptor ligand interactions result in the activation of intracellular signal transduction pathways in leukocytes, leading to directed motility, phagocytosis, superoxide anion generation, and degranulation responses culminating in the killing and clearance of invading microorganisms (3). Although very effective, this process can lead to local tissue injury (4–6). The premature or excessive activation of neutrophils has been implicated in the pathogenesis of a wide variety of inflammatory diseases including the adult respiratory distress syndrome (7).

Detection of invading bacteria by neutrophils, triggered by excretion of formylated peptides and generation of C5a, is considered as one of the earliest innate recognition events. We hypothesized that CHIPS production by *S. aureus* may prevent this early detection, thereby stalling the immune response. CHIPS, present in the growth medium of *S. aureus* strains, was found to inhibit the binding of anti-C5aR mAb and formylated peptide to the C5aR and FPR, respectively, on neutrophils. Subsequent experiments with recombinant CHIPS proved that it specifically inhibited the C5a- and fMLP-induced calcium mobilization in neutrophils and monocytes. Moreover, fluorescently labeled CHIPS bound to both cell types, with neutrophils showing higher binding than monocytes, coinciding with known C5aR and FPR expression patterns (1). These observations led to the hypothesis that CHIPS might bind directly to the C5aR and FPR, thereby preventing the natural ligands from binding and activating the receptors. However, an indirect effect could not be excluded. In the present report, we used neutrophils to show that CHIPS acts independently of signal transducing systems or internalization. Furthermore, using U937 cells expressing the C5aR or FPR, we show that CHIPS specifically binds the C5aR and FPR, thereby efficiently blocking the C5a- and fMLP-induced calcium responses. Moreover, CHIPS was found to be very selective and might therefore be a promising lead for the development of an agent to control the inflammatory response induced by C5a and/or fMLP.

Materials and Methods

Materials

Fluorescent-formylated peptide (fluorescein conjugate of the hexapeptide *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys) and Fluo-3 acetoxymethyl ester (Fluo-3AM) were purchased from Molecular Probes (Leiden, The Netherlands). FITC-conjugated anti-C5aR (clone S 5/1) was purchased from Serotec (Oxford, U.K.) and PE-conjugated anti-IL-8RB was purchased from

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[2] Abbreviations used in this paper: CHIPS, chemotaxis inhibitory protein of *Staphylococcus aureus*; FPR, formylated peptide receptor; GPCR, G protein-coupled receptor; HSA, human serum albumin; PAF, platelet-activating factor; LTB4, leukotriene B_4; Fluo-3AM, Fluo-3 acetoxymethyl ester.

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R&D Systems (Minneapolis, MN). fMLP and recombinant human C5a were obtained from Sigma-Aldrich (St. Louis, MO). IL-8 (72-aa form) and GRO-α were obtained from PeproTech (London, U.K.). Platelet-activating factor (PAF-16) and purified human C3a were obtained from Calbiochem-Novabiochem (San Diego, CA) and leukotriene B_4 (LTB4) was purchased from Cascade Biochem (Reading, Berkshire, U.K.). The FPR-like 1 specific synthetic peptide MMK-1 (LESIFRSLLFRVM) was synthesized by Sigma-Genosys (Cambridge, U.K.). Recombinant CHIPS was expressed in *Escherichia coli*, isolated, and FITC-labeled (CHIPS-FITC) as described before (1). The purity of CHIPS used in this study was >97% based on Coomassie brilliant blue-stained SDS-PAGE samples. The endotoxin level was 50 pg/μg protein as determined by the *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, MD).

Cells

Blood was collected from healthy volunteers via vein puncture into tubes containing sodium heparin (Vacuette; Greiner, Alphen a/d Rijn, The Netherlands) as anticoagulants. Neutrophils were isolated as described by Troelstra et al. (8). In short, heparinized blood was diluted 1:1 (v/v) with pyrogen-free PBS and layered onto a gradient of Ficoll (Pharmacia, Peapack, NJ) and Histopaque (density, 1.119; Sigma-Aldrich). After centrifugation for 20 min at 320 $\times$ *g*, the neutrophils were collected from the Histopaque phase. Cells were subjected to a brief hypotonic shock with water, washed, and suspended at 5×10^6 cells/ml in RPMI 1640 containing 0.05% human serum albumin (HSA; CLB, Amsterdam, The Netherlands). U937 cells (human promonocytic cell line) transfected with the C5aR, FPR, IL-8RB, and empty expression vector were generated as previously described (9). Cells were grown in 75-cm^2 cell culture flasks with 2-μm vent caps (Costar, Corning, NY) placed in a 5% CO_2 incubator at 37°C. For maintaining the cell lines, the cells were split 1:10 (v/v) twice a week with RPMI 1640 medium with L-glutamine (Life Technologies, Grand Island, NY), including 1 mM sodium pyruvate (Life Technologies), 2.5 g/L glucose (Riedel-de Hahn, Germany), 10% FCS (Life Technologies), and 10 μg/ml gentamicin (Life Technologies).

CHIPS activity assays using neutrophils

For ATP depletion experiments, neutrophils were ATP depleted as described by Herlin and Borregaard (10). Briefly, neutrophils (5×10^6 cells/ml) were incubated for 30 min at 37°C in HBSS without glucose, with the addition of 5 mM 2-deoxyglucose (Sigma-Aldrich), 1 mM sodium azide (Merck, Darmstadt, Germany), and 5 μg/ml antimycin A (Sigma-Aldrich). Then, part of the cells were frozen in liquid nitrogen and stored at −70°C for determination of ATP levels. As a control, neutrophils incubated in normal HBSS (Life Technologies) were used. The remaining neutrophils (ATP depleted and control cells) were washed in HBSS, resuspended in HBSS/0.05% HSA (2.5×10^6 cells/ml), and incubated with 1 μg/ml CHIPS for 15 min on ice. Subsequently, the cells were incubated with 1×10^{-7} M fluorescent-formylated peptide for 1 h on ice. After incubation, the fluorescent-formylated peptide binding was measured in a FACScan flow cytometer (BD Biosciences, Mountain View, CA). After determination of CHIPS activity, another sample was stored at −70°C for determination of the ATP level. The ATP levels were measured with the ATP Bioluminescence Assay kit HSII (Roche, Mannheim, Germany) according to the manufacturer's description. In other assays, the CHIPS activity was compared at temperatures of 0 and 37°C. Therefore, neutrophils (2.5×10^6 cells/ml) were incubated with a concentration range of CHIPS for 15 min at 0°C (on ice) and 37°C. Subsequently, the cells were incubated with 1×10^{-7} M fluorescent-formylated peptide or 5 μg/ml anti-C5aR-FITC for 45 min on ice. After incubation, the cells were washed once and measured for fluorescent-formylated peptide and anti-C5aR-FITC binding in a flow cytometer.

Internalization of CHIPS-FITC or fluorescent-formylated peptide by neutrophils

Internalization of formylated peptide and CHIPS was measured using trypan blue quenching as described by van Amersfoort and van Strijp (11). Neutrophils at 5×10^6 cells/ml were incubated with 1 μg/ml CHIPS-FITC or 1×10^{-7} M fluorescent-formylated peptide in RPMI 1640/0.05% HSA at 37°C. At different time points, the CHIPS-FITC or fluorescent-formylated peptide binding was measured in a flow cytometer, before and immediately after addition of 62 μg/ml trypan blue (Merck), to discriminate between ligand binding on the outside of the cell and internalized ligand.

Binding of CHIPS-FITC to U937 cells

U937 cells expressing the C5aR, FPR, and IL-8RB (5×10^6 cells/ml) were incubated for 45 min on ice with 1×10^{-7} M fluorescent-formylated peptide, 5 μg/ml anti-C5aR-FITC, and 5 μg/ml anti-IL-8RB-PE, respectively, in RPMI 1640/0.05% HSA to check for receptor expression. CHIPS binding was tested by incubation of these cell lines with 3 μg/ml CHIPS-FITC for 20 min on ice. Control cells were U937 cells transfected with the expression vector only. In separate experiments, U937/C5aR and U937/FPR cells were preincubated for 30 min on ice with different concentrations of unlabeled C5a (1×10^{-10}–1×10^{-5} M) or fMLP (1×10^{-9}–1×10^{-4} M), respectively. Then, 0.1 μg/ml CHIPS-FITC was added for another 20 min. To down-regulate the C5aR and FPR surface expression on U937 transfectants, U937/C5aR and U937/FPR cells were preincubated for 15 min at 37°C with 10^{-6} M of C5a or fMLP, respectively. Then cells were washed twice and incubated with 1×10^{-7} M fluorescent-formylated peptide or 5 μg/ml anti-C5aR-FITC for 45 min and 1 μg/ml CHIPS-FITC for 20 min on ice to compare the percentage of receptor down-regulation with the percentage of decrease in CHIPS-FITC binding. Control cells preincubated on ice were used to make sure that the wash steps removed all of the surface ligand. Cells were analyzed for fluorescent-formylated peptide, mAb, and CHIPS-FITC binding in a FACScan or FACSCalibur flow cytometer (BD Biosciences) gated on forward and side scatter to exclude dead cells and debris.

Intracellular calcium mobilization in U937 cells

fMLP and C5a-induced intracellular calcium mobilization was measured in a flow cytometer as described before (1). Briefly, U937 cells were loaded with 2 μM Fluo-3AM in RPMI 1640/0.05% HSA for 20 min at room

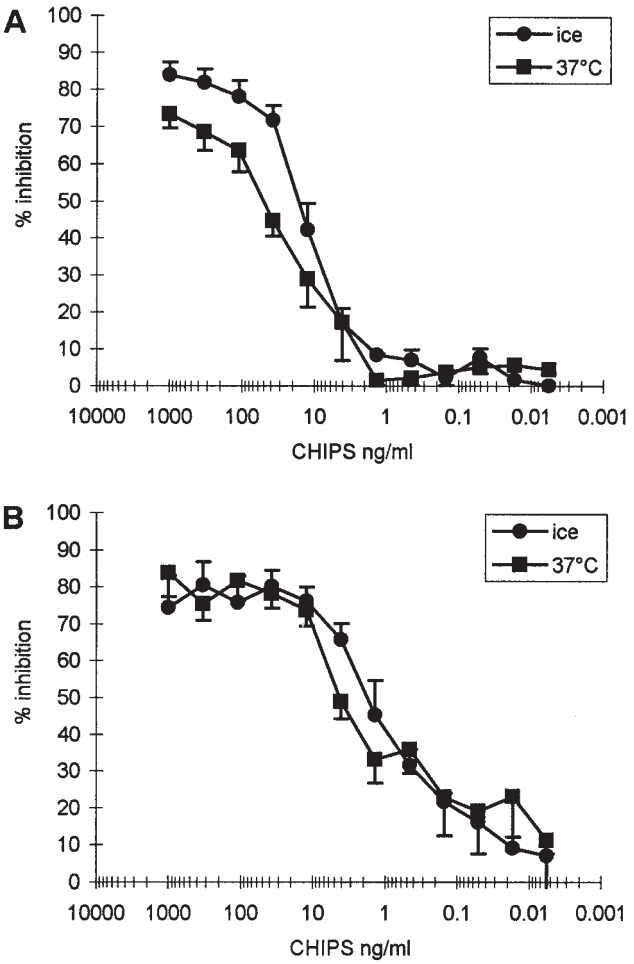

FIGURE 1. CHIPS inhibits the binding of anti-C5aR-FITC and fluorescent-formylated peptide independent of the incubation temperature. Neutrophils were incubated with different concentrations of CHIPS for 15 min on ice and at 37°C. Then the cells were incubated for 45 min with 5 μg/ml anti-C5aR-FITC (*A*) or 1×10^{-7} M fluorescent-formylated peptide (*B*) on ice and, subsequently, binding of anti-C5aR-FITC and fluorescent-formylated peptide was determined in a flow cytometer. Data are expressed as percentage of inhibition compared with neutrophils without CHIPS and are the mean ± SEM of three separate experiments.

temperature under constant agitation, washed twice with buffer, and suspended to 1×10^6 cells/ml in RPMI 1640/0.05% HSA. Subsequently, the cells were preincubated with or without 1 μg/ml CHIPS for 15 min at room temperature. Each sample of cells was first measured for ~10 s to determine the basal fluorescence level. Next, concentrated ligand (final concentrations C5a 10^{-10} M or fMLP 10^{-9} M) was added and rapidly placed back in the sample holder to continue the measurement. Subsequently, ~1 min after the first stimulus, cells were stimulated with ionomycin (10^{-6} M) for a second round of measurements. Cells were analyzed in a FACScan or FACSCalibur flow cytometer gated on forward and side scatter to exclude dead cells and debris.

Iodination of CHIPS

CHIPS was iodinated using the chloramine-T method (12). Briefly, 500 μCi of $Na^{125}I$ was added to 12.5 μg of CHIPS in 0.04 M phosphate buffer (pH 7.4). Chloramine-T (1 μg) was added for 90 s and the reaction was quenched by the addition of 6 μg of sodium thiosulfate for 60 s. Then 2% of HSA was added as carrier and bound ^{125}I was separated from free ^{125}I by chromatography on a PD-10 desalting column (Amersham Pharmacia Biotech, Piscataway, NJ) using RPMI 1640/0.2% HSA. The ^{125}I-CHIPS had a specific activity of 25–55 μCi/μg.

K_d *determinations using U937 cells*

For K_d determinations, binding assays were performed using U937/C5aR or U937/FPR. The assay buffer consisted of RPMI 1640/0.05% HSA. Buffer or 10 μM unlabeled CHIPS, 0.15–150 nM ^{125}I-CHIPS, and U937/C5aR (0.8×10^6/ml) or U937/FPR (1×10^7/ml) were added sequentially to a final volume of 100 μl. After a 90-min incubation at room temperature, 90 μl was applied to a 150-μl mixture of 20% olive oil/80% dibutyl phthalate (Sigma-Aldrich) in a microsediment-winning 0.3-ml tube (Sarstedt, Newton, NC). To separate the cell-bound ^{125}I-CHIPS from free ^{125}I-CHIPS, the tubes were centrifuged for 5 min at $1800 \times g$, after which the tips of the tubes containing the cell pellet were cut and counted in a COBRA gamma counter (Packard Instrument, Meriden, CT) for 5 min. Curves were fit to nonlinear regression analysis and the K_d values were quantitated using the PRISM program (GraphPad, San Diego, CA).

Screening of CHIPS effects on receptor systems

Several receptor systems were screened for the effect of CHIPS. The receptors FPR, CCR1, CCR2, CCR3, and CCR5 were kindly tested by Prof. Jo van Damme (Laboratory of Molecular Immunology, Rega Institute, Leuven, Belgium) by use of a calcium mobilization assay using fura 2-labeled PBMC or HEK 293 cells transfected with the FPR. Calcium mobilization upon stimulation with specific ligands was measured after incubation with or without 10 μg/ml CHIPS. The percentage of inhibition by CHIPS was calculated as = [100 − 100 × (Ca concentration after stimulus with CHIPS/Ca concentration after stimulus without CHIPS)]%. Other receptors were tested by NovaScreen Biosciences (Hanover, MD) via radioligand-binding assay techniques using specific radiolabeled ligands in microplate receptor plates. Values were expressed as the percentage of inhibition of specific binding and represent the average of replicate tubes at each of the concentrations tested. Additional calcium mobilization assays were performed in Fluo-3AM-labeled neutrophils with the following ligands: 10^{-9} M fMLP, 10^{-10} M C5a, 10^{-10} M LTB4, 10^{-10} M IL-8, 3.3×10^{-8} M synthetic MMK-1, 3.3×10^{-9} M C3a, 10^{-10} M PAF, or 3.3×10^{-10} M GRO-α as described above for U937 cells.

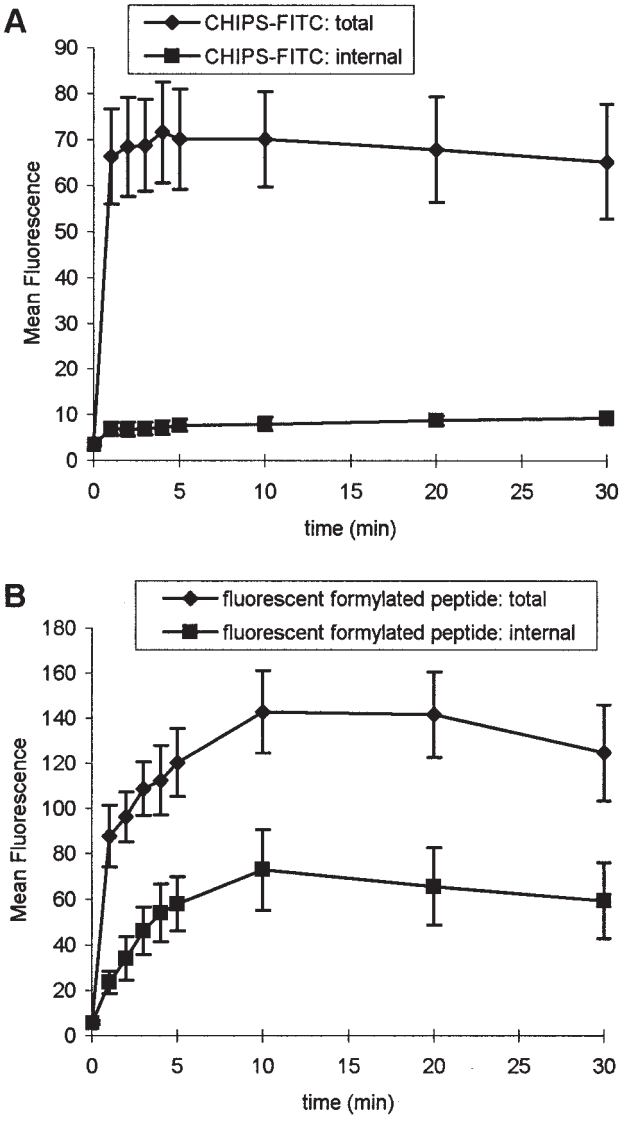

FIGURE 2. CHIPS is not internalized upon binding neutrophils. Neutrophils were incubated with 1 μg/ml CHIPS-FITC (*A*) or 1×10^{-7} M fluorescent-formylated peptide (*B*) in RPMI 1640/0.05% HSA. At different time points, the CHIPS-FITC or fluorescent-formylated peptide binding was measured in a flow cytometer before and immediately after addition of 62 μg/ml trypan blue to discriminate between ligand binding on the outside of the cell and internalized ligand. Data are expressed as fluorescence values and are the mean ± SEM of three separate experiments.

Results

CHIPS is not internalized after binding neutrophils

To elucidate more precisely the mechanism by which CHIPS inhibits C5a- and fMLP-induced cellular responses, we started by

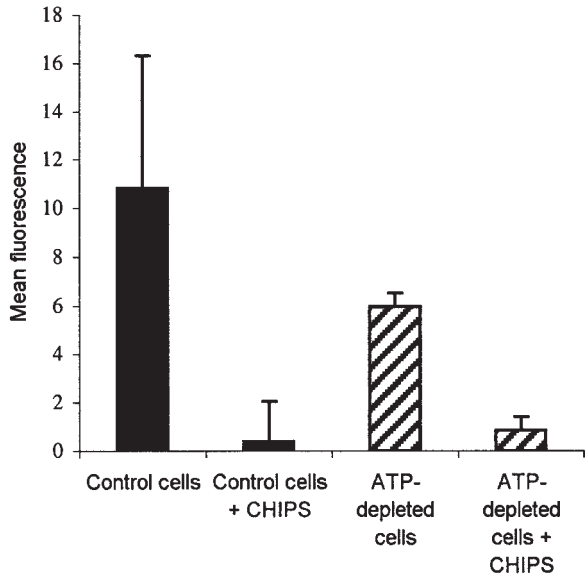

FIGURE 3. CHIPS acts independently of the presence of ATP. ATP-depleted and control neutrophils were incubated with 1 μg/ml CHIPS for 15 min on ice. Subsequently, the cells were incubated with 1×10^{-7} M fluorescent-formylated peptide for 1 h on ice. After incubation, the fluorescent-formylated peptide binding was measured in a flow cytometer. Data are expressed as fluorescence values and are the mean ± SD of two separate experiments.

testing whether CHIPS is also active at a temperature of 0°C. Fig. 1 shows that CHIPS is equally effective in inhibiting the binding of anti-C5aR-FITC and fluorescent-formylated peptide to their receptors on neutrophils at 0 and 37°C. This suggests that CHIPS exerts its function outside the cell, apparently independent of cell signaling events. To further address this, we determined the internalization of CHIPS by neutrophils. Fig. 2 shows that CHIPS-FITC remains outside the cell, whereas fluorescent-formylated peptide, used as a positive control, is internalized. Additionally, to exclude the existence of a separate CHIPS receptor that might be responsible for down-modulation of the C5aR and FPR via intracellular signaling, we tested the effect of CHIPS on formylated peptide binding to neutrophils under ATP-depleted conditions. Fig. 3 shows that CHIPS still inhibited binding of fluorescent-formylated peptide when neutrophils were 95% ATP depleted. Under these conditions, internalization of fluorescent-formylated peptide was blocked, indicating sufficient depletion of ATP (data not shown). Taken together, these results strongly suggest that CHIPS affects the C5aR and FPR directly, independently of signaling events or internalization.

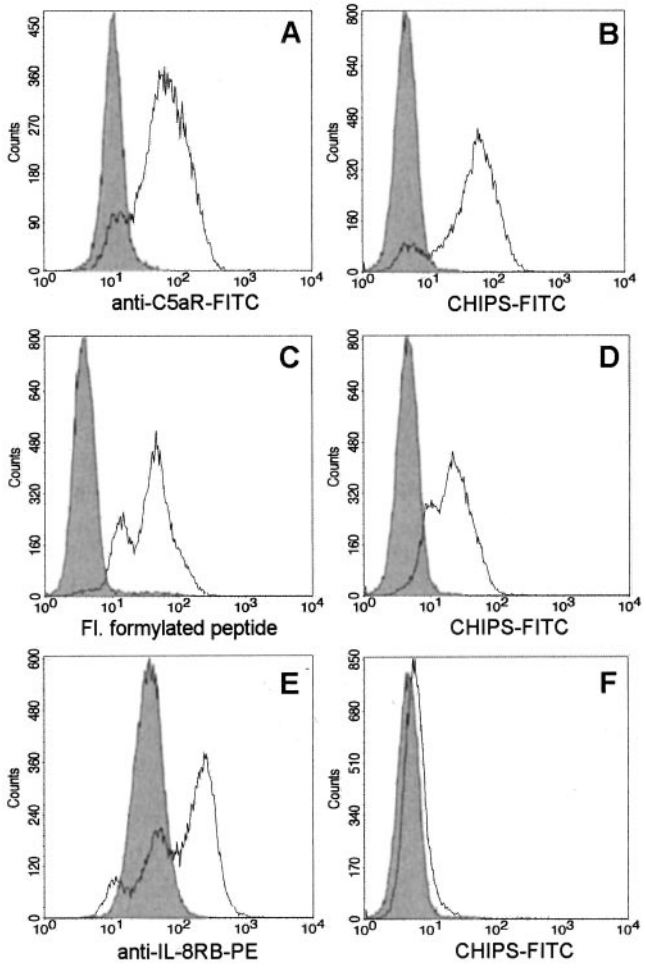

FIGURE 4. CHIPS binds direct to the C5aR and FPR. U937 cells expressing the C5aR (*A* and *B*), FPR (*C* and *D*), and IL-8RB (*E* and *F*) were incubated for 45 min on ice with 5 μg/ml anti-C5aR-FITC, 1×10^{-7} M fluorescent-formylated peptide, and 5 μg/ml anti-IL-8RB-PE, respectively, to check for receptor expression (*A*, *C*, and *E*). CHIPS binding was tested by incubation of these cell lines with 3 μg/ml CHIPS-FITC for 20 min on ice (*B*, *D*, and *F*). Cells were analyzed in a flow cytometer. Shaded histograms represent control cells incubated with medium only.

CHIPS binds directly to the C5aR and FPR

To examine whether CHIPS indeed interacts directly with the C5aR and FPR, we tested CHIPS binding to these specific receptors. Experiments using neutrophils are complicated by the fact that they not only express multiple chemoattractant receptors, including C5aR and FPR, but several other signaling receptors as well. To avoid this problem, we used undifferentiated U937 cells, transfected with the C5aR, FPR, or IL-8RB, which enabled us to determine binding of CHIPS to these receptors individually. IL-8RB is a GPCR closely related to the C5aR and FPR, but is not affected by CHIPS (1). Cells transfected with this receptor were used as a negative control. Flow cytometric data demonstrated the presence of each receptor on the different cell lines by binding of their specific fluorescently labeled ligand or mAb. Only the U937/FPR and U937/C5aR bound CHIPS-FITC, while the U937/IL-8RB did not (Fig. 4). U937 cells transfected with only the expression vector did not bind the fluorescently labeled ligand or mAb and also did not bind CHIPS (data not shown). These results indicate that CHIPS binds directly to the C5aR and FPR. To further verify specific CHIPS binding to the C5aR and FPR, the transfected U937 cells were preincubated with C5a and fMLP, respectively. This resulted in a down-regulation of 63.9 ± 4.5% and 63.0 ± 3.0% of C5aR and FPR, respectively, with a concomitant decrease in CHIPS-FITC binding of 71.0 ± 6.0% and 58.7 ± 1.9%,

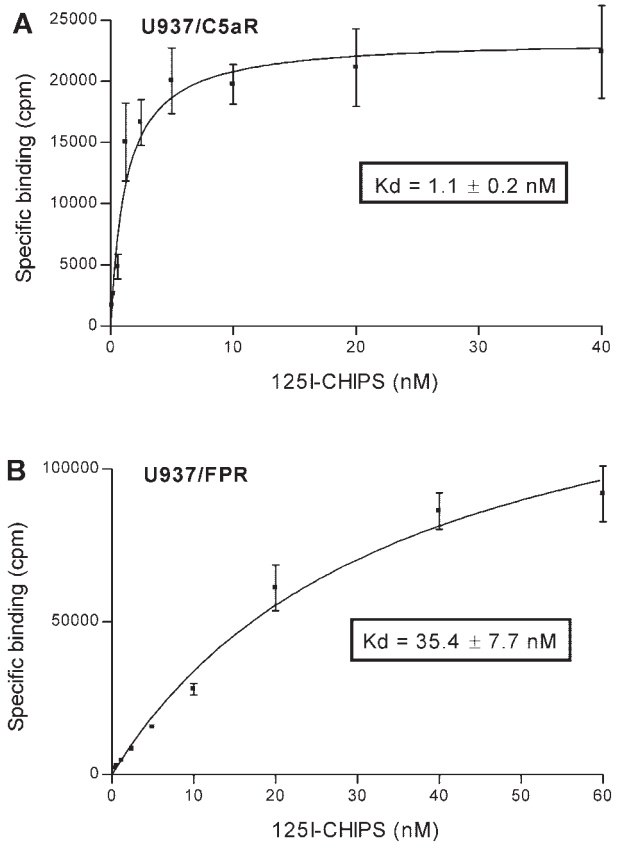

FIGURE 5. CHIPS binds U937/C5aR and U937/FPR cells with K_d values of 1.1 and 35.4 nM, respectively. U937/C5aR (*A*) and U937/FPR (*B*) were incubated with 0.15–150 nM ^{125}I-CHIPS in the presence and absence of 10 μM unlabeled CHIPS for 90 min at room temperature. To separate the cell-bound ^{125}I-CHIPS from free ^{125}I-CHIPS, the cells were centrifuged through a mixture of 20% olive oil/80% dibutyl phthalate in microsediment-winning tubes. Then the tips of the tubes containing the cell pellet were cut and counted in a gamma counter for 5 min. Curves were fit to nonlinear regression analysis and the K_d values were quantitated using the PRISM program.

respectively. This again indicates that CHIPS directly binds the C5aR and FPR. Radioionated CHIPS was used to determine the binding affinities of CHIPS for the C5aR and FPR using U937/C5aR and U937/FPR cells. Fig. 5 shows that CHIPS bound C5aR and FPR with apparent K_d values of 1.1 ± 0.2 and 35.4 ± 7.7 nM, respectively. We also examined the capacity of unlabeled C5a and fMLP to compete for CHIPS binding to C5aR and FPR, respectively. Fig. 6 shows that both C5a and fMLP compete with the binding of CHIPS in accordance to their respective K_d values for the C5aR and FPR (13–16).

CHIPS inhibits the C5a- and fMLP-induced calcium mobilization in U937 cell lines

Ligand binding to GPCRs initiates a signal that leads to a rapid mobilization of intracellular calcium. To examine whether binding of CHIPS to the C5aR and FPR is functional in blocking C5a- and fMLP-induced responses, we tested the calcium mobilization in these cell lines, with and without preincubation with CHIPS. Flow cytometric data show that U937/C5aR and U937/FPR exhibit a ligand-specific calcium mobilization upon stimulation with C5a or fMLP, respectively. CHIPS completely inhibited this C5a- and fMLP-induced calcium mobilization in each respective cell line (Fig. 7). These experiments clearly show that CHIPS binding to the C5aR and FPR is functional in blocking agonist-dependent activation. The ability of the CHIPS-treated cells to respond normally to other stimuli is shown by a rechallenge of the same cells with ionomycin, which bypasses the inhibition by CHIPS and induces a calcium response (Fig. 7). CHIPS itself did not induce a calcium mobilization in any of the cell lines tested (data not shown). These results indicate that the C5aR and FPR are blocked individually and no "cross-talk" is responsible for inhibition of either receptor.

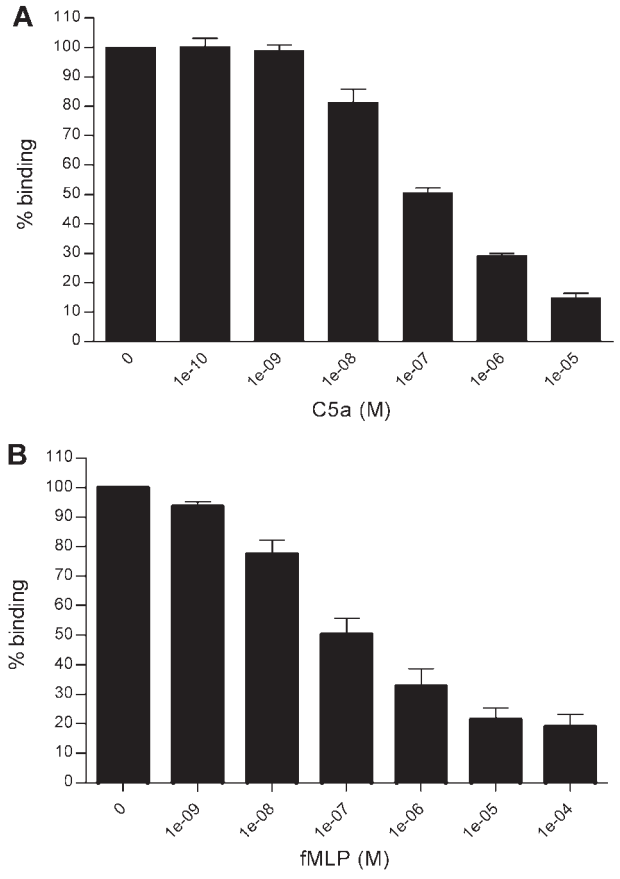

FIGURE 6. C5a and fMLP compete for the binding of CHIPS to C5aR and FPR. U937/C5aR (*A*) and U937/FPR (*B*) were preincubated for 30 min on ice with different concentrations of unlabeled C5a or fMLP, respectively. Then 0.1 μg/ml CHIPS-FITC was added for another 20 min, after which the CHIPS-FITC binding was analyzed in a flow cytometer. Data are expressed as percentage of binding compared with control cells where no C5a or fMLP was added and are the mean ± SEM of three separate experiments.

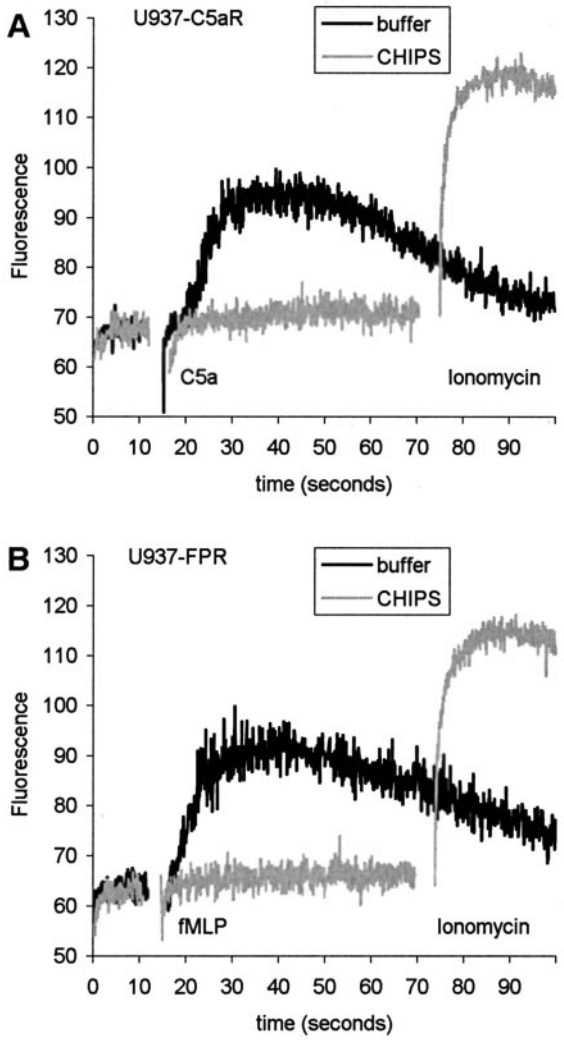

FIGURE 7. CHIPS inhibits the C5a- and fMLP-induced calcium mobilization in U937/C5aR and U937/FPR cells. Fluo-3AM-loaded U937 cells, expressing the C5aR (*A*) and FPR (*B*), were preincubated with or without 1 μg/ml CHIPS for 15 min at room temperature. Each sample of cells was first measured for ~10 s to determine the basal fluorescence level. Next, C5a 10^{-10} M (*A*) or fMLP 10^{-9} M (*B*) was added and rapidly placed back in the sample holder to continue the measurement. Subsequently, ~1 min after the first stimulus, cells were stimulated with ionomycin (10^{-6} M) for a second round of measurements. Cells were analyzed in a flow cytometer.

Screening of receptor systems

To determine the selectivity of CHIPS for the C5aR and FPR, other receptors, including many GPCRs, were screened for an effect of CHIPS using a calcium mobilization assay or specific radioligand-binding assays. Table I shows that, beside the C5aR and FPR, none of the receptor systems screened was affected by CHIPS. Additionally, Fig. 8 shows that, of several tested chemoattractants, only the responses induced by C5a and fMLP are inhibited by CHIPS using a calcium mobilization assay in neutrophils. These results indicate that CHIPS specifically affects the C5aR and FPR.

Table I. *CHIPS is specific for the C5aR and FPR*[a]

Receptor Specificity	Assay	Inhibition by CHIPS (%)
Neurotransmitter related		
Adrenergic, α1, nonselective	Specific binding	1
Dopamine, nonselective	Specific binding	−5
γ-Aminobutyric acid A, agonist site	Specific binding	5
Glutamate, AMPA site	Specific binding	−12
Glutamate, NMDA site	Specific binding	2
Histamine, H1	Specific binding	8
Histamine, H2	Specific binding	0
Muscarinic, nonselective, central	Specific binding	20
Muscarinic, nonselective, peripheral	Specific binding	−14
Nicotinic (α-bungarotoxin insensitive)	Specific binding	2
Opiate, nonselective	Specific binding	22
Serotonin, nonselective	Specific binding	−1
Chemoattractants/chemokines		
fMLP (FPR)	Calcium mobilization	100
MMK-1 (FPRL1)	Calcium mobilization	0
MIP1α,[b] LDL78β, MCP-3, RANTES (CCR1)	Calcium mobilization	0
MCP-3 (CCR2)	Calcium mobilization	0
MCP-3, RANTES (CCR3)	Calcium mobilization	0
MIP1α, LDL78β, RANTES (CCR5)	Calcium mobilization	0
IL-8 (CXCR1)	Calcium mobilization	0
GRO-α (CXCR2)	Calcium mobilization	0
C5a (C5aR)	Specific binding	106
C3a (C3aR)	Calcium mobilization	0
Ion channels		
Potassium channel, ATP sensitive	Specific binding	12
Potassium channel, Ca^{2+} activated, VI	Specific binding	14
Potassium channel, Ca^{2+} activated, VS	Specific binding	21
Sodium, site 2	Specific binding	0
Second messengers		
Inositol triphosphate, IP3	Specific binding	−6
Protein kinase C, phorbol dibutyrate	Specific binding	−11
Regulatory sites	Specific binding	
γ-Aminobutyric acid A, BDZ, α1, central	Specific binding	2
Prostaglandins		
LTB4	Calcium mobilization	0
LTD4	Specific binding	4
Thromboxane A_2	Specific binding	11
Growth factors/hormones		
Corticotropin-releasing factor, nonselective	Specific binding	−19
PAF	Calcium mobilization	0
Brain/gut peptides		
Bradykinin, BK2	Specific binding	−10
Vasoactive intestinal peptide, nonselective	Specific binding	1
Enzymes		
NOS (constitutive-neuronal)	Specific binding	−5

[a] Several receptor systems were screened for the effect of CHIPS via calcium mobilization assays or radioligand-binding assays, as indicated. Values are expressed as the percentage of inhibition of calcium mobilization or specific binding and represent the average of replicate tubes at each of the concentrations tested.

[b] MIP1α, macrophage-inflammatory protein 1α; MCP-3, monocyte chemoattractant protein 3.

Discussion

Several microorganisms have been described to modulate the chemotactic response of neutrophils by affecting GPCRs. *Bordetella pertussis*, for example, produces pertussis toxin that inhibits GPCRs by ADP-ribosylation of the G protein (17) and *Porphyromonas gingivalis* produces a proteinase, which cleaves the C5aR (18). Furthermore, different cell wall components of *Cryptococcus neoformans* are described to inhibit neutrophil chemotaxis toward IL-8, fMLP, and C5a (19, 20), whereas endotoxins of Gram-negative bacteria down-regulate CXC and CC chemokine receptor expression (21, 22).

Recently, we described the exoprotein CHIPS, produced by several strains of *S. aureus*, able to inhibit both the C5a- and fMLP-induced chemotaxis of neutrophils (1, 2). In the present report, we show that CHIPS binds directly to the C5aR and FPR on the extracellular surface, thereby preventing interaction with their natural ligands and activation. The CHIPS effect was independent of the temperature and the presence of ATP and the protein was not internalized after binding its receptors. CHIPS bound both receptors with apparent K_d values of 1 and 35 nM for the C5aR and FPR, respectively. These K_d values are in the same range as those described for their natural ligands (13–16). Furthermore, CHIPS was found to be very selective, since it did not affect a broad selection of other receptors, including other chemoattractant receptors present on neutrophils, like the FPR-like 1, C3aR, IL-8RA and -B, LTB4 receptor, and PAF receptor (3).

After binding the C5aR or FPR, CHIPS might behave in several ways: 1) CHIPS may act as a partial agonist, binding to the receptor, but eliciting a submaximal response of the receptor system. Our group showed, however, that addition of CHIPS directly to

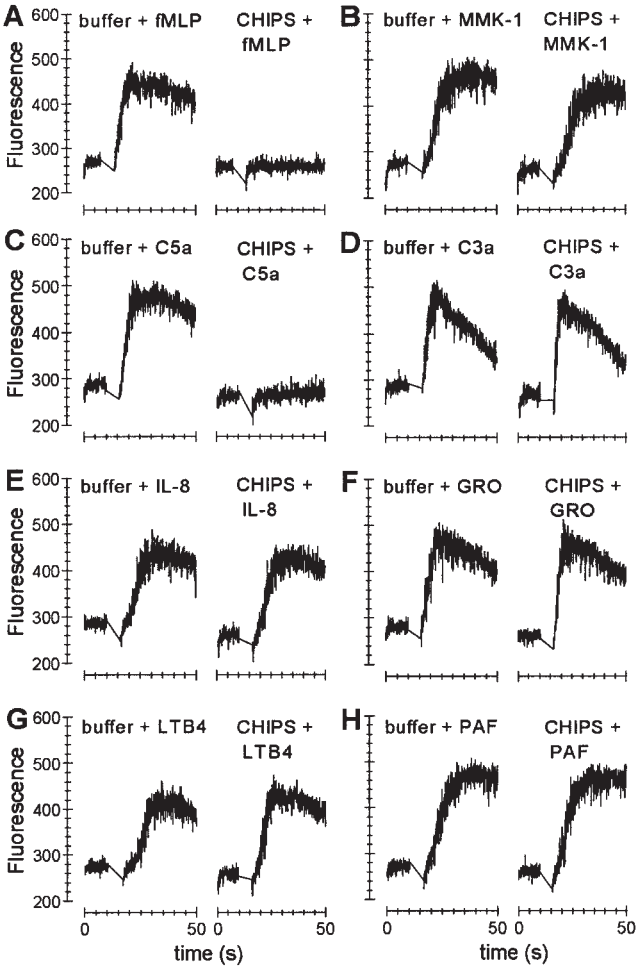

FIGURE 8. CHIPS is specific for the C5aR and FPR. Fluo-3AM-loaded neutrophils were preincubated with or without 1 μg/ml CHIPS for 15 min at room temperature. Each sample of cells was first measured for ~10 s to determine the basal fluorescence level. Next, 10^{-9} M fMLP (*A*), 3.3×10^{-8} M synthetic MMK-1 (*B*), 10^{-10} M C5a (*C*), 3.3×10^{-9} M C3a (*D*), 10^{-10} M IL-8 (*E*), 3.3×10^{-10} M GRO-α (*F*), 10^{-10} M LTB4 (*G*), or 10^{-10} M PAF (*H*) (final concentrations) was added and rapidly placed back in the sample holder to continue the measurement. Cells were analyzed in a flow cytometer.

neutrophils as a ligand did not result in a calcium response (1). 2) CHIPS might behave as a neutral antagonist. 3) CHIPS may act as an inverse agonist, regarding the two-state model of receptor activation (23–25). According to this model GPCRs exist in an inactive ("R") state or an active ("R*") state. Agonist binding shifts the equilibrium to R*, but GPCRs can also isomerize from R to R* in the absence of agonist. Inverse agonists have the capacity to recruit and stabilize these so-called constitutively active GPCRs in an inactive conformation (26). The FPR was the first member of the chemoattractant receptor family identified as being constitutively active (27). Cyclosporin H, a metabolite of the fungus imperfectus *Tolyplacodium inflatum Gams* inhibits, like CHIPS, agonist binding to the FPR, which at the time was interpreted as antagonism (28, 29). Later studies, however, demonstrated that cyclosporin H was an inverse agonist rather than an antagonist (27). Recently, the C5aR was shown to exhibit constitutive activity as well (30). It will be interesting to determine whether CHIPS behaves as a neutral antagonist or an inverse agonist acting on the C5aR and FPR. This may have implications for its biological function, since the constitutive activity of GPCRs is probably of importance in vivo (27). Future experiments are needed to answer these questions.

It is also intriguing that one protein is able to bind and functionally inhibit two different GPCRs. An important question regarding the mechanism of CHIPS is whether it uses similar regions for binding the C5aR and FPR or whether distinctive sites within the molecule have evolved for interaction with each of these receptors. One would expect CHIPS to bind a region that is conserved in both the C5aR and FPR, since these receptors share 34% aa homology (31). In contrast, the amino acid homologies of the C5aR and FPR compared with other CHIPS-unaffected GPCRs, as for example the IL-8RA and -B or the C3aR, are in the same range (26–31%). The question is, however, whether the mechanism of CHIPS inhibiting C5aR and FPR activation is similar, since both ligands and their binding are quite different. C5a, a 74-residue glycoprotein, binds its receptor predominantly through a two-site binding of charge-charge interactions: the C5a N-terminal core binds the receptors N-terminal extracellular domain while its tail interacts with residues in the transmembrane bundle, the latter interaction being essential for activation of the receptor (32, 33). In contrast, the small tripeptide fMLP binds its receptor in the interhelical region and probably has additional points of interaction in the transmembrane domains, thereby entering the transmembrane binding crevice (34, 35). Another observation that favors the hypothesis of two distinct binding sites on the CHIPS molecule is the difference in affinity of CHIPS for C5aR and FPR. The structure of CHIPS has not yet been determined, but since it is a 121-residue protein, we would expect CHIPS to behave more like C5a or other high molecular mass protein ligands of GPCRs, which bind predominantly to the extracellular helices and N-termini of their receptors.

To date, there is considerable interest in receptor blockers to control the inflammatory response induced by C5a and/or fMLP, in particular that generated by C5a, which is thought to be involved in conditions like adult respiratory distress syndrome, rheumatoid arthritis, and reperfusion injury (7, 36–38). The antagonists reported for C5aR and FPR are, among others, modified structures of their ligands and, in the case of C5aR, mimic peptides of the C-terminal tail of C5a, yielding molecules that presumably bind competitively to these receptors. CHIPS exhibits no obvious structural similarity to C5a or fMLP, despite its potent antagonistic properties. Proteins like CHIPS are susceptible to degradation by proteases in serum or the gastrointestinal tract, can be immunogenic, and generally display poor pharmacokinetic properties. However, with its high C5aR and FPR binding affinity and potent antagonistic effects, this bacterial exoprotein might be a lead for development of novel small molecular compounds that inhibit the response to C5a and/or fMLP. Furthermore, this protein, which is carefully selected by evolution, might provide us with new information about mechanisms of blocking GPCRs.

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