Dynamics of Protein Kinase C-mediated Phosphorylation of the Complement C5a Receptor on Serine 334

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Upon agonist binding, the C5a anaphylatoxin receptor (C5aR) is rapidly phosphorylated on phosphorylation sites that are located within the C-terminal domain of the receptor. Previous studies suggested that C5aR phosphorylation proceeds in a hierarchical manner with serine 334 presenting a highly accessible priming site that controls subsequent phosphorylation at other positions. To better understand the dynamics of Ser-334 phosphorylation, we generated site-specific monoclonal antibodies that specifically react with phosphoryserine 334. In differentiated U937 cells, which endogenously express C5aR, stimulation with low C5a concentrations resulted in a very rapid (t \( t_{1/2} \approx 20 \) s), albeit transient, receptor phosphorylation. Whole cell phosphorylation assays with specific inhibitors as well as in vitro phosphorylation assays with recombinant enzymes and peptide substrates revealed that phosphorylation of Ser-334 is regulated by protein kinase C-\( \beta \) and a calmodulin A-sensitive protein phosphatase. Surprisingly, at high concentrations (\( >10 \) \( \mu \)M) of C5a, the protein kinase C-mediated phosphorylation of Ser-334 was essentially blocked. This could be attributed to the even faster (\( t_{1/2} < 5 \) s) binding of \( \beta \)-arrestin to the receptor. Analysis of C5aR Ser/Ala mutants that possess a single intact serine residue either at position 334 or at neighboring positions 327, 332, or 338 revealed functional redundancy of C-terminal phosphorylation sites since all 4 serine residues could individually support C5aR internalization and desensitization. This study is among the first to analyze in a detailed manner, using a non-mutational approach, modifications of a defined phosphorylation site in a G protein-coupled receptor and to correlate these findings with functional parameters of receptor deactivation.

The complement-derived C5a\(^2\) anaphylatoxin and its less active derivative C5a(desArg) are potent chemoattractants and fulfill important roles in host defense and inflammation. Biological activities of C5a are mediated via interaction with the C5a receptor (C5aR; CD88), a member of the subfamily of chemoattractant G protein-coupled receptors (GPCR). The C5aR is highly expressed on cells of myeloid origin such as neutrophils and monocytes and was also detected on non-myeloid cells of the lung and the liver (1, 2). Its expression can be induced on myelo-monocytic cell lines such as U937 or HL-60 by treatment with dibutyryl-cAMP (Bt2cAMP) (3). In these cells, C5a activates a wide range of biological responses that include directed cell migration, granular enzyme release, and activation of the respiratory burst. Many but not all of these processes require pertussis toxin-sensitive heterotrimeric G\(_{12}\) protein activation of phospholipase C. This results in increases of inositol 1,4,5-trisphosphate and diacylglycerol production, an increase in intracellular calcium concentrations, and the activation of protein kinase C.

The cellular response to C5a is rapidly diminished with continued agonist exposure by well established mechanisms (4, 5). According to a current model of GPCR desensitization, agonist-activated receptors are phosphorylated by GPCR kinases (GRKs) on serine and threonine residues that are located in the C terminus or intracellular loops of the receptor. Many GPCR are also substrates for second messenger-activated kinases (PKC or cAMP-dependent protein kinase (PKA)), which are activated downstream of the receptor. The phosphorylated receptors then firmly associate with \( \beta \)-arrestins, which preclude further G protein binding. In addition, \( \beta \)-arrestins interact directly (or indirectly via the \( \beta_{2} \)-adaptin subunit of AP-2) with clathrin heavy chains and thereby target phosphorylated receptors to the endocytic machinery. In addition to terminating classical G protein signaling, \( \beta \)-arrestins can also initiate cellular signaling by acting as adaptor proteins that recruit tyrosine kinases and other proteins into signaling complexes with receptors (6). Apart from this simple paradigm, alternative mechanisms operate in the fine-tuning of GPCR responses. Evidence for this hypothesis derives from the observation that in many GPCR, there is no direct correlation between receptor phosphorylation, \( \beta \)-arrestin binding, desensitization, and internalization (7, 8). This indicates that these adaptive mechanisms are regulated by different conformations or specific phosphorylation patterns of the GPCR C-terminal domain.

Previous studies have established that agonist-activated C5aRs are phosphorylated on C-terminal serine residues by protein kinase C and an additional staurosporine-insensitive kinase (9–11), which may belong to the GRK family (12). Among the six major serine phosphorylation sites on C5aR that were identified through receptor mutagenesis, serine 334 was...
found to be the single most important residue since ligand-induced C5aR phosphorylation was reduced by 50–60% in receptor mutants with alanine exchange of this particular serine (13). Receptor phosphorylation was essentially lost if, in addition, the neighboring serine residues at positions 332 and/or 338 were mutated as well. From these findings, it was concluded that C5aR phosphorylation proceeds in a hierarchical manner with serine 334 presenting a highly accessible priming site that controls subsequent phosphorylation at other positions.

In the present study, we investigate using site-specific phosphopeptide antibodies the kinetics of C5a-induced C5aR-Ser 334 phosphorylation in cells that express receptors at physiological levels. We identify protein kinases and phosphatases as well as adapter proteins that together modulate the phosphorylation status at this particular site upon agonist stimulation. Using receptor mutants that lack all C-terminal serine phosphorylation sites except for Ser-334 (or 3 alternative adjacent serine residues), we provide further insight into regulatory mechanisms that determine C5aR desensitization and internalization.

EXPERIMENTAL PROCEDURES

Materials—Most reagents have been reported (14). Synthetic phospho- peptides and the peptide spots were synthesized by Jerini. Synthetic C-terminal C5aR peptide RIC4 was supplied by Bernhard Schmidt (Department of Biochemistry II, Georg-August-University Göttingen). P12/1 and S5/1 anti-C5aR/CD88 mAb were from Serotec. Recombinant human C5a was generated as described previously (15). Restriction enzymes, T4 ligase, and calf intestine alkaline phosphatase were from MBI Fermentas. pEF1/Myc-His A was from Invitrogen. Accu TaqDNA-polymerase and dibutyryl-cAMP were from Sigma. Keyhole limpet hemocyanin was from Pierce. PKC isozymes and PKC inhibitors bisindolylmaleimide, rottlerin, and G06976 were from Calbiochem. LY333531 was kindly provided by Lilly Pharma.

Cell Culture—U937 cells were cultured at 37 °C in a humidified atmosphere with 5% CO2 in 80:20-10 medium (80 parts RPMI, 20 parts medium 199, supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin). To induce C5aR expression, cells were incubated in the presence of 1 mM Bt2cAMP for 3 days prior to the experiment. Rat basophilic leukemia cells that stably express wild-type C5aR together with CCR5 (RBL-CCR5/RBL-CCR5CT) comprising C5aR with its 25 C-terminal residues substituted by the 23 C-terminal residues of human CCR5 were maintained in RPMI, 20 parts medium 199, supplemented with 100 units/ml penicillin, and 100 μg/ml streptomycin and treated with 1 mM Bt2cAMP for 3 days prior to the experiment. Rat basophilic leukemia cells that stably express wild-type C5aR together with CCR5 (RBL-CCR5/RBL-CCR5CT) comprising C5aR with its 25 C-terminal residues substituted by the 23 C-terminal residues of human CCR5. Thereby, a receptor was obtained in which four of a total of six essential phosphorylation sites were substituted by the homologous region of CCR5, including four critical serine phosphorylation sites. This chimera was generated from the vector C5aR-FLAG-pcDNA3 (12) using a mutagenic 5’ primer containing a BamH1 site and a 3’ oligonucleotide containing 69 bases corresponding to amino acids Glu-330-Leu-352 of CCR5 and a EcoRV site. After cleavage with BamH1 and EcoRV, the PCR fragment was cloned into the vector pEF1/Myc-His A. C5aR variants with replacement of C-terminal serine residues at positions 314, 317, 327, 332, 334, and 338 by alanine in various combinations were generated with mutagenic 3’ oligonucleotides. The nucleotide sequence of the amplified PCR fragments was verified by sequencing. The resulting receptor constructs were stably expressed in RBL-2H3 cells, as described before (17). C5aR expression at the cell surface was quantitated by flow cytometry with anti-CD88 mAb S5/1. All functional assays were performed in two different cell clones per receptor mutant, in parallel, to exclude artifacts resulting from clonal variation.

Generation of Phosphosite-specific Antibodies—A phosphopeptide (C(ε-aminocaproic acid)SVVRE(pS)K(pS)FTR(pS)TVDTMA) corresponding to 18 C-terminal amino acid residues of the human C5aR with phosphoserine (highlighted in boldface type) incorporated at the three C-terminal serine phosphorylation sites of this receptor were synthesized by standard solid phase methods and purified to >70% purity by reversed phase high pressure liquid chromatography. The peptide was conjugated to keyhole limpet hemocyanin using succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, and BALB/c mice were immunized with the resulting keyhole limpet hemocyanin-phosphopeptide conjugate at four monthly intervals. After the fusion of splenocytes with X63-Ag8.653 myeloma cells, positive hybridoma clones were identified by differential ELISA reactivities toward phosphorylated and nonphosphorylated receptor peptides as well as by their binding of agonist activated C5aR as assessed by immunoblotting.

Immunoblotting—U937 cells were washed once in serum-free 80:20 medium supplemented with 0.2% bovine serum albumin and stimulated for 2 min at 37 °C with 10 nM C5a or 200 nM PMA or left untreated. Cells were washed once with ice-cold phosphate-buffered saline and solubilized in detergent buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.05% SDS in the presence of protease and phosphatase inhibitors (18)) on ice. Cellular lysates were centrifuged at 13,000 rpm at 4 °C for 5 min. Receptors were immunoprecipitated by the incubation (2 h/4 °C) with 10 μg of anti-C5aR S5/1 and protein G-Sepharose. Receptors were dissociated under reducing conditions, electrophoresed, and electrotransferred to nitrocellulose membranes. After blocking with 1% bovine serum albumin dissolved in Tris-buffered saline containing 0.1% Tween 20, the nitrocellulose membrane was incubated with the monoclonal anti-pC5aR antibody 32-G1 followed by reaction with a peroxidase-conjugated secondary antibody. Visualization was performed using ECL detection reagents. Afterward, blot membranes were stripped and reprobed with anti-C5aR mAb P12/1.

Functional Assays—To document C5a-induced changes in cytosolic calcium concentrations, RBL-C5aR cells were loaded with 1 μM Indo-1 AM for 25 min at 30 °C, washed twice with Krebs-Ringer phosphate containing 1 mM CaCl2, and centrifuged at 300 × g for 5 min. Measurement of calcium flux in individual cells was performed by flow cytometry on a LSR II (BD Biosciences). The argon laser was tuned to 357 nm to excite Indo-1, and emissions were collected simultaneously on linear
Analysis of C5aR Phosphorylation

![Graph showing C5aR phosphorylation](image)

**Figure 1. Binding of the anti-pC5aR mAb 32-G1 to activated/phosphorylated C5aR in U937 cells.** U937 cells with or without prior treatment with Bt2cAMP (1 mM for 3 days) were exposed to 10 nM C5a or 200 nM PMA for 2 min or left untreated (M, medium). Receptors were immunoprecipitated from cellular lysates and analyzed by serial immunoblotting with anti-pC5aR mAb 32-G1 (top) and anti-C5aR mAb P12/1 (bottom) to show equal loading of C5aR per lane.

**RESULTS**

**Characterization of Anti-pC5aR Ser-334 mAb 32-G1**—To generate phosphosite-specific antibodies that specifically react with activated/phosphorylated C5aR, mice were immunized with a synthetic C5aR C-terminal phosphopeptide that contained phosphoserine instead of serine at position 334 and also at the neighboring phosphorylation sites Ser-332 and Ser-338. The mAb 32-G1 was chosen among several hybridoma clones that were derived from the fusion of splenocytes with myeloma cells and selectively reacted with the C5aR phosphopeptide but not with a non-phosphorylated version of the same peptide. To determine the epitope specificity of this mAb, we tested its binding to overlapping peptides (10- to 11-mers), which contained only one phosphoserine in the context of the C5aR C-terminal sequence. The mAb 32-G1 reacted specifically with a C5aR peptide that contained phosphoserine 334 (supplemental Fig. S1) and also reacted with C5aRs that were isolated from C5a-stimulated, transfected RBL-C5aR cells. It did not recognize a receptor mutant (C5aR-S334A) with alanine replacement of all six C-terminal serine phosphorylation sites (not shown). Together, these findings show that 32-G1 binding to C5aR directly correlated with serine 334 phosphorylation.

To test whether mAb 32-G1 also reacts with C5aR expressed at physiological levels in non-transfected cells, we stimulated promonocytic U937 cells with a membrane-permeable cAMP analogue (Bt2cAMP). This treatment induces a monocyte/macrophage-like phenotype and a concomitant increase in C5aR expression in these cells. As shown by immunoblot analysis scales at 405 and 485 nm with 20- and 22-nm band pass filters, respectively. The base-line fluorescence of unstimulated cells was collected before each cell stimulation. A sheath buffer of phosphate-buffered saline without glucose at pH 7.4 was used. Before fluorescence-activated cell sorter analysis, cells were centrifuged and resuspended in Krebs-Ringer phosphate, 0.5 mM EGTA at a concentration of 2×10⁶ cells/ml. Cells were stimulated with 20 nM C5a for 3 min, and fluorescence was monitored after the addition of 1 mM CaCl₂.

The C5a-stimulated N-acetyl-β-D-glucosaminidase release from RBL-2H3 cells was determined as described (19). Values were expressed as a percentage of total enzyme present in cells after lysis with 0.1% Triton X-100.

**Enzyme Immunoassay**—The ligand-induced phosphorylation of C5aR was determined in differentiated U937 or in RBL-C5aR cells. Cells were stimulated for up to 30 min at 37 °C with the indicated concentrations of C5a in 80:20 medium supplemented with 0.2% bovine serum albumin. After centrifugation (3 min; 150 × g), samples were placed on ice and incubated for 15 min with 900 µl of lysis buffer. Cell debris was removed by centrifugation for 15 min at 4 °C and 13,000 rpm. Supernatants were applied directly into the wells of a microtiter plate precoated with the anti-C5aR mAb S5/1 as the capture antibody and incubated for 2 h at room temperature. Phosphorylated C5aR receptors were recognized by adding the biotinylated anti-pC5aR mAb 32-G1 (1 µg/ml; 2 h at room temperature) followed by 1 h incubation with peroxidase-conjugated streptavidin (1:4000 dilution) and 2.2-azino-di-(3-ethylbenzthiazoline sulfonate) as substrate.

To analyze the effect of PKC inhibitors on C5a-induced phosphorylation of C5aR-S334, RBL-C5aR cells were pre-treated for 30 min at 37 °C with the indicated concentrations of bisindolylmaleimide, rottlerin, LY333531, or Go6976. Thereafter, cells were stimulated with 20 nM C5a for 3 min, and fluorescence was monitored after the addition of 1 mM CaCl₂.

**In Vitro Phosphorylation of a C5aR Peptide by Recombinant PKC**—Protein kinase C activity was assayed essentially as described (20). The reaction mixture (75 µl) contained 100 µM ATP, 1 mM dithiothreitol, 5 mM MgCl₂, 20 mM HEPES (pH 7.4), 20 µg/ml phosphatidylserine (sonicated vesicle suspension), 500 nM PMA, 200 µM CaCl₂ (for PKC-α, -βI, -βII, and -γ), or 0.5 mM EGTA (for PKC-δ, -ε, -ζ, and -η) and 100 µM of a synthetic peptide comprising 45 amino acids (Ala-303–Val-350) of the C5aR C terminus (21). Reactions were started by the addition of human recombinant PKC isoforms (8 nm). After incubation for up to 20 min at 30 °C, the reaction was quenched by the addition of EGTA (25 mM) and further diluted in coating buffer (50 mM carbonate, pH 10.6). Reaction mixtures were then directly coated on a microtiter plate, and phosphorylation was determined by the use of the pC5aR-Ser-334-specific mAb 32-G1 as described above. The assays were calibrated using a phosphorylated C5aR peptide as a standard protein.

**β-Arbretin Translocation Assay**—The C5a-induced translocation of β-arrestin from the cytosol to the plasma membrane was performed as described previously (7). Briefly, Bt₂cAMP-treated U937 cells or RBL-C5aR cells were stimulated with varying concentrations of C5a for up to 30 min at 37 °C. Cells were scraped into buffer A (10 mM PIPES, 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, pH 7.0), and the homogenates were loaded on a discontinuous gradient of 50, 35, and 20% sucrose in the same buffer. After centrifugation (160,000 × g; 2 h), the cytosolic and the membrane fraction were collected, and equivalent amounts of protein were separated by 10% SDS-PAGE. β-Arbretins were detected by immunoblotting with anti-β-arrestin 1/2 mAb.
(Fig. 1), mAb 32-G1 reacted with a 40–45-kDa protein in differentiated and C5a-stimulated U937 cells. This protein corresponds to the phosphorylated C5aR monomer. When Bt2cAMP-U937 cells were treated with PMA, a potent PKC activator, the immunoreactivity of immunoprecipitated C5aR increased significantly. Repropbing of the immunoblot with a mAb (P12/1), which reacts with a C5aR N-terminal epitope in a phosphorylation-independent manner, confirmed receptor expression in Bt2cAMP-treated U937 cells. Pretreatment of cells with pertussis toxin, which inhibits Go1-dependent signal-
conditions of low ATP concentrations. Since kinase inhibitors used in this study were ATP-competitive, inhibitory effects of these compounds depend on the ATP concentrations in the assay, which in living cells are much higher. Together with the potential problem of limited cell permeability, this may explain why higher concentrations were required for efficient PKC inhibition.

Characterization of Phosphatases Responsible for Ser-334 Dephosphorylation—Our earlier work on the regulation of CCR5, a related chemotactic leukocyte receptor, showed that PKC-mediated receptor phosphorylation is tightly controlled by protein phosphatases that rapidly dephosphorylate ligand-activated receptors. To identify the phosphatase(s) responsible for C5aR-Ser-334 dephosphorylation, we examined the effect of calyculin A and okadaic acid, potent cell-permeable inhibitors of protein phosphatase 1 and protein phosphatase 2A, on C5aR-Ser-334 phosphorylation and dephosphorylation in intact cells. Treatment with calyculin A (100 nM) for 15 min prior to C5a stimulation significantly, but not completely, retarded C5aR-Ser-334 dephosphorylation, whereas okadaic acid at concentrations (200 nM) that are efficacious in inhibiting dephosphorylation of the β2-adrenergic receptor (24) had absolutely no effect on C5aR-Ser-334 (Fig. 4). Since both phosphatase inhibitors at low concentrations block protein phosphatase 2A, but only calyculin A in addition blocks protein phosphatase 1 (25), our findings imply a role for protein phosphatase 1 in C5aR-Ser-334 dephosphorylation. Since calyculin A was only partially effective, another as yet ill-defined phosphatase may also participate in C5aR dephosphorylation.

Kinetic Analysis of C5aR-Ser-334 Phosphorylation at Different Agonist Concentrations—Earlier studies on the regulation of C5aR by agonist-induced phosphorylation, which were based on 32P incorporation assays, revealed the presence of fast and slow migrating forms of the phosphorylated receptor in SDS-PAGE analysis (9, 26). In dose-response experiments, these forms were phosphorylated at low or high C5a concentrations, respectively. The electrophoretic shift that accompanies C5aR stimulation at higher agonist concentrations results most likely from GRK-mediated phosphorylation, whereas the faster migrating form corresponds to the PKC phosphorylated receptor (12). These studies indicated that GRK- and PKC-mediated C5aR phosphorylation at different receptor sites proceeds with different kinetics and concentration dependencies. The availability of a PKC site-specific mAb (32-G1) allowed us to test this hypothesis.

Stimulation of Bt2cAMP-U937 cells with low concentrations (0.2 nM) of C5a resulted in the phosphorylation of C5aR-Ser-334, which was maximal after 30 s, and then the receptor was rapidly dephosphorylated within the next 2 min (Fig. 5). Treatment of cells with up to 6 nM C5a resulted in a dose-dependent increase in maximal levels of C5aR-Ser-334 phosphorylation. Unexpectedly, at high concentrations (>20 nM C5a), the initial peak was markedly suppressed, and C5aR-Ser-
Analysis of C5aR Phosphorylation

![Diagram of C5aR phosphorylation analysis](image)

FIGURE 6. Homologous and heterologous phosphorylation of CCR5 and C5aR expressed in RBL cells. A–C, RBL-CCR5/C5aR cells that co-express CCR5 and C5aR were stimulated for 3 min with the indicated concentrations of C5a (A and B) or CCL5 (C). Cell lysates from the same experiments were analyzed for PKC-mediated homologous or heterologous phosphorylation on C5aR-Ser-334 (A and C) or CCR5-Ser337 (B) by different ELISA procedures. r.u., relative unit. D, homologous GRK-mediated phosphorylation of a C5aR/CCR5CT chimeric receptor on CCR5-S349 following C5a stimulation of RBL-C5aR/CCR5CT cells.

334 phosphorylation was detectable only at much lower levels, albeit for prolonged periods of time.

Inhibition of PKC-mediated C5aR-Ser-334 phosphorylation could be, in principle, due to impaired activity of the kinase at high concentrations of the agonist or reduced access of the kinase to its substrate. To discriminate between these possibilities, we analyzed C5aR phosphorylation in RBL cells that co-express C5aR together with CCR5, a related chemotactic receptor. We confirmed in transfected RBL-C5aR/CCR5 cells that C5aR-Ser-334 phosphorylation is inhibited after homologous receptor stimulation with C5a concentrations higher than 3 nM (Fig. 6A). Analysis of the C5a-induced cross-phosphorylation of CCR5-Ser-337, a known PKC site (14), in the same cellular lysates showed that PKC was fully active at high C5a concentrations (Fig. 6B). At the same time, heterologous PKC-mediated phosphorylation of C5aR-Ser-334 following CCR5 stimulation was also not inhibited. Assuming that the same PKC isoforms are involved in C5aR/CCR5 phosphorylation after homologous and heterologous stimulation, these results show that kinase activity is not impaired at high agonist concentrations. Next, we asked whether GRK-mediated C5aR phosphorylation is also inhibited at high concentrations of C5a. Since no antibody with specificity for a GRK site on C5aR was available, we generated a functional C5aR/CCR5CT chimeric receptor that contains a well characterized GRK site (CCR5-Ser349) and E11/19 (anti-pCCR5) epitope (14). GRK-mediated C5aR phosphorylation was readily detectable at high C5a concentrations that were equal to those at which PKC site phosphorylation was inhibited. The difference in dose-dependent C5aR phosphorylation by the two receptor kinases is probably due to significant amplification of the signal downstream of C5aR, which leads to PKC activation, whereas GRK phosphorylation directly follows ligand occupancy of the receptor.

Since C5aR phosphorylation at different C-terminal sites proceeds in a sequential and hierarchical manner (11), it is conceivable that phosphorylation at a different C5aR site by GRKs could interfere with subsequent PKC-mediated phosphorylation of C5aR-Ser-334. Alternatively, C5a-induced translocation of cytosolic β-arrestins to the receptor may block access of the kinase to its substrate by steric hindrance. To test the latter hypothesis, we analyzed in parallel the C5a-induced membrane recruitment of β-arrestins to the membrane and C5aR-Ser-334 phosphorylation in Bt2cAMP-U937 cells (Fig. 7). Analogous to the results obtained with transfected RBL-C5aR cells, both β-arrestin isoforms very rapidly translocated to the cell membrane within 5 s after C5a stimulation. β-Arrestin translocation required similar agonist concentrations (EC50 ~ 5 nM C5a), which were necessary to induce GRK-mediated receptor phosphorylation. Quantitative analysis of these phenomena revealed an opposite effect of cellular stimulation with high concentrations of C5a on the PKC-mediated phosphorylation of C5aR-Ser-334 and β-arrestin translocation.

Functional Significance of Ser-334 Phosphorylation—Previous studies have established the significance of the phosphorylated C-terminal domain of C5aR for rapid agonist-induced receptor desensitization and endocytosis (13, 26, 27), but the role of discrete serine phosphorylation sites for these processes is not fully understood. To analyze the functional significance specifically of Ser-334 phosphorylation, a C5aR-Ser-Ala mutant with alanine replacement of all C-terminal serine residues except for Ser-334 was generated and expressed in RBL-2H3 cells. This myeloid cell line of rat origin was previously found to express high endogenous levels of GRK2/3 and β-arrestins-1 and -2 and is therefore well suited for the analysis of chemotactic leukocyte receptor phosphorylation and internal-
DISCUSSION

This study extends earlier work on the identification and functional characterization of C-terminal phosphorylation sites on the human C5a receptor. Using a mutagenesis approach, Christophe et al. (13) identified Ser-334 as a primary phosphoacceptor site that controls subsequent receptor phosphorylation on neighboring serine residues. To better understand the mechanisms that regulate phosphorylation of this particular serine residue, we generated monoclonal antibodies that selectively react with a C-terminal C5aR phosphopeptide where Ser-334 as well as neighboring serine residues at positions 332 and 338 were present as phosphoserines. The resulting mAb 32-G1 showed strong reactivity only with phosphopeptides containing phosphoserine at position 334 but not phosphoserine 332 or 338. Thus, mAb 32-G1 specifically reacts with C5aR phosphoserine 334, independent of the phosphorylation state of the neighboring serine residues. Our studies with Bt,cAMP-treated U937 cells show that mAb 32-G1 specifically reacts with ligand-activated C5aR at endogenous receptor expression levels but only weakly recognizes C5aR isolated from unstimulated cells.

In this work, we demonstrate that PKC-βII specifically phosphorylates C5aR-Ser-334 in myeloid cells. This conclusion is based on a combination of in vitro peptide phosphorylation studies using recombinant PKC isoforms and PKC inhibitors with distinct inhibitor profiles. Several different PKC isoforms are expressed in RBL-2H3 cells, as well as in neutrophilic granulocytes (22, 23). Inhibitor studies revealed that serine 334 phosphorylation is strictly controlled by a calcineurin A-sensitive phosphatase that is resistant to okadaic acid. This phosphatase very efficiently and rapidly dephosphorylates serine 334 within 2–5 min. The characteristics of this receptor phosphatase differ from the previously described G protein-coupled receptor phosphatase, a protein phosphatase 2A-like membrane-associated phosphatase (24). Apparently, two different phosphatases control rapid dephosphorylation of GPCRs, which were modified either by heterologous PKC-mediated phosphorylation at the plasma membrane or by homologous GRK-mediated phosphorylation within perinuclear recycling endosomes (14).

Our study of the kinetics and dose dependence of C5a-induced C5aR phosphorylation revealed a new regulatory effect of β-arrestins on PKC-mediated receptor phosphorylation. The data strongly suggest that β-arrestins that rapidly bind to agonist-activated C5aR sterically interfere with further PKC-
mediated phosphorylation at serine 334 and also protect phosphorylated residues from dephosphorylation by protein phosphatases. Although the inhibition of PKC-mediated C5aRSer-334 phosphorylation at high agonist concentrations could also be explained by interference with GRK binding to C5aR, we do not favor this hypothesis since GRKs do not firmly associate with GPCR for prolonged periods of time.

Our work also provides insight into the significance of C5aR phosphorylation for arrestin-mediated receptor desensitization and endocytosis. Earlier studies, which were based on extensive receptor mutagenesis experiments, identified several pairs of phosphorylatable C-terminal serine residues in various combinations that were sufficient for intact desensitization and arrestin-mediated C5aR endocytosis (13, 27). However, in the different receptor mutants, no obvious correlation existed between their abilities to promote β-arrestin binding and to undergo desensitization or internalization. This suggested that these mechanisms are connected to another in a more complex manner. Similar results were recently obtained with the related formyl peptide receptor (FPR) (8). Here, extensive mutagenesis experiments led the authors to propose the existence of specific FPR phosphorylation patterns that differentially affect various receptor functions.

In the present study, we asked whether PKC-mediated phosphorylation of Ser-334 alone restores C5aR internalization and desensitization and whether this also applies to adjacent phosphorylation sites. We therefore generated several C5aR mutants with single intact C-terminal serine residues. Functional characteristics of these mutants were compared with fully phosphorylation-deficient or -competent C5aR. We found that β-arrestins bound to ligand-activated C5aR even in the absence of phosphorylatable serine residues. Moreover, the kinetics and dose dependence of β-arrestin translocation to wild-type C5aR at the plasma membrane also suggest that β-arrestins translocate to the receptor independent of PKC or GRK phosphorylation. These receptor kinases phosphorylate GPCR much slower and at lower agonist concentrations (14). Our findings can be explained with a current model of arrestin-
Analysis of C5aR Phosphorylation

GPCR interaction. This model proposes separate domains on arrestin that either bind to regions of the receptor exposed following receptor activation or bind to phosphorylated residues (28). Only when both the activation and the phosphorylation recognition domain are engaged, arrestin undergoes a conformational change that facilitates its interaction with the endocytic machinery. The relative contributions of receptor conformation and phosphorylation may vary for different receptors. For example, the LTB4 receptor BLT1 associates with β-arrestin and internalizes completely independent of phosphorylation (29). In the case of C5aR, transition of β-arrestins into the secondary conformation, which initiates receptor endocytosis, requires the presence of at least one intact phosphorylation site on the C5aR C terminus.

Although the exact position of this serine phosphorylation site appears to be less critical for endocytosis, we observed significant differences in the desensitization of the C5a-induced calcium response between the various receptor mutants. None of the mutants behaved like wild-type C5aR. This result suggests alternative mechanisms for C5aR desensitization, such as those described for FPR. In this receptor, the phosphorylation of certain serine residues on the FPR alone in the absence of β-arrestin appears to be sufficient to decrease the affinity of G proteins for this particular receptor (30). An alternative explanation relates to the hypothesis that β-arrestin, which associates with the C terminus of the receptor, may adopt functionally distinct conformations, depending on the specific phosphorylation patterns that are generated by different receptor kinases. Evidence for this hypothesis derives from the observation that the selective inhibition of distinct GRKs differentially affects either β-arrestin-mediated ERK1/2 activation or β-arrestin-mediated endocytosis following cellular stimulation via angiotensin II or V2 vasopressin receptors (31, 32). Thus, depending on the different GRK expression levels in various tissues, receptor phosphorylation at distinct sites by different kinases may induce specific conformational changes in β-arrestins and eventually result in different functional outcomes. This same paradigm may well extend to other functions of β-arrestins as well as other receptor kinases besides GRKs, such as PKC. Validation of this concept will require the identification of specific phosphorylation sites for individual receptor kinases on a given receptor and correlation with different parameters of β-arrestin functions. Our study is the first to identify using a non-mutational approach the relevant kinases, phosphatases, and adapter proteins that together determine phosphorylation of a single GPCR site within its native cellular environment. Additional experimental analysis is required to identify the underlying mechanisms and functional consequences of C5aR phosphorylation at other receptor sites.

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