Human Complement 5a (C5a) Anaphylatoxin Receptor (CD88) Phosphorylation Sites and Their Specific Role in Receptor Phosphorylation and Attenuation of G Protein-mediated Responses

DESENSITIZATION OF C5a RECEPTOR CONTROLS SUPEROXIDE PRODUCTION BUT NOT RECEPTOR SEQUESTRATION IN HL-60 CELLS*

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Upon agonist binding, the anaphylatoxin human complement 5a receptor (C5aR) has previously been found to be phosphorylated on the six serine residues of its carboxyl-terminal tail (Giannini, E., Brouchon, L., and Boulay, F. (1995) J. Biol. Chem. 270, 19166–19172). To evaluate the precise roles that specific phosphorylation sites may play in receptor signaling, a series of mutants were expressed transiently in COS-7 cells and stably in the physiologically relevant myeloid HL-60 cells. Ser334 was found to be a key residue that controls receptor phosphorylation. Phosphorylation of either of two serine pairs, namely Ser332 and Ser334 or Ser334 and Ser338, was critical for the phosphorylation of C5aR and its subsequent desensitization. Full phosphorylation and desensitization of C5aR were obtained when these serines were replaced by aspartic acid residues. The mutation S338A had no marked effect on the agonist-mediated phosphorylation of C5aR, but it allowed a sustained C5a-evoked calcium mobilization in HL-60 cells. These findings and the ability of the S314A/S317A/S327A/S332A mutant receptor to undergo desensitization indicate that the phosphorylation of Ser334 and Ser338 is critical and sufficient for C5aR desensitization. The lack of phosphorylation was found to result not only in a sustained calcium mobilization and extracellular signal-regulated kinase 2 activity but also in the enhancement of the C5α-mediated respiratory burst in neutrophil-like HL-60 cells. For instance, the nonphosphorylatable S332A/S334A mutant receptor triggered a 1.8–2-fold higher production of superoxide as compared with the wild-type receptor. Interestingly, although the desensitization of this mutant was defective, it was quenched with the same time course and the same efficiency as the wild-type receptor. Thus, in myeloid HL-60 cells, desensitization and sequestration of C5aR appear to occur through divergent molecular mechanisms.

The capacity of phagocytic leukocytes to clear the host from invading microorganisms is dependent on their ability to migrate to sites of inflammation and to release large amounts of proteolytic enzymes and reactive oxygen species. Phagocytes do this in response to a variety of structurally diverse chemotaxants, including bacterial N-formylated peptides, the complement fragment C5a,1 leukotriene B4, platelet-activating factor, and interleukin 8 (reviewed in Ref. 1). Chemotaxants elicit intracellular signaling through specific receptors that are coupled to the pertussis toxin-sensitive heterotrimeric G protein. This latter activator in turn second messenger-generating enzymes, including phospholipases C, D, and A2 (reviewed in Ref. 2), as well as kinases, such as phosphatidylinositol 3-kinase (3) and sphingosine kinase (4). Ultimately, the NADPH-oxidase is activated and releases superoxide anions. This is a crucial bactericidal mechanism, but it is also believed to be a major cause of inflammatory disorders when inappropriately activated.

The activation of the NADPH-oxidase complex responsible for the production of superoxide requires the coordinated action of multiple signaling pathways. The NADPH-oxidase becomes functional through a multistep assembly of several components at the plasma membrane, namely the flavocytochrome bc1, the small G proteins (Rac and Rap), and cytosolic factors (p47phox, p67phox, and p40phox) (reviewed in Ref. 5). The ability of chemotaxants to induce the production of superoxide varies from one chemotaxant to another. The activation of the NADPH-oxidase complex is correlated with the rapid increase in Ca2+ and diacylglycerol, which subsequently activate protein kinase C (PKC), and the phosphorylation of the cytosolic factor p47phox (6, 7). The involvement of PKC in triggering the activation of NADPH oxidase is supported by the ability of phorbol 12-myristate 13-acetate (PMA) to induce a sustained superoxide production and the phosphorylation and translocation of p47phox to the plasma membrane (8).

Despite the persistent presence of chemotaxants, the intracellular signaling events are transient, with a time course in the minute range. This attenuated responsiveness is thought to result from the desensitization of receptors through their phosphorylation and rapid sequestration (reviewed in Refs. 9 and 10). The current concept for desensitization of G protein-coupled receptors, which is supported by research grants from the CNRS, the Commissariat à l’Energie Atomique, and the Université Joseph-Fourier–Faculté de Médecine. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: C5a, human complement 5a; C5aR, C5a receptor; GPCR, G protein-coupled receptor; PKC, protein kinase C; MLPK, N-formyl-Met-Leu-Phe-Lys-OH; Bt2cAMP, Na2O-2'-dibutyryl adenosine 3',5' cyclic monophosphate; Erk, extracellular signal-regulated kinase; MAP, mitogen-activated protein; PMA, phorbol 12-myristate 13-acetate.
pled receptors (GPCRs), largely extrapolated from numerous studies with rhodopsin and the β2-adrenergic receptor, is that arrestin proteins bind to the phosphorylated receptors and sterically prevent its interaction with the G protein (11–13). In addition, β-arrestin acts as a clathrin adapter that targets the agonist-occupied β2-adrenergic receptor to the endocytic pathway via clathrin-coated pits (14, 15). Expression of a GTPase defective dynamin mutant (K44A) (16) specifically inhibits the clathrin-dependent endocytic pathway and the agonist-mediated internalization of GPCRs is inhibited. However, mounting evidence indicates that not all GPCRs are internalized through this pathway. For instance, internalization of the angiotensin II 1A and m2 muscarinic acetylcholine receptors in HEK293 cells is independent of β-arrestin and is not inhibited when the clathrin-coated vesicle pathway is blocked by overexpression of a dominant negative form of dynamin (17–19).

Although receptor desensitization is thought to be a key regulatory mechanism controlling the inflammatory potential of phagocytic cells, the impact of an altered chemoattractant receptor desensitization on intracellular signaling events and the release of reactive oxygen species has never been investigated in leukocytes. The C5a receptor (C5aR) is particularly well suited to examine this issue because previous studies have shown the rapid agonist-mediated phosphorylation of this receptor on serine residues in differentiated HL-60 cells and after heterologous expression (20–22). In transfected COS-7 cells, the six serine residues of the carboxy-terminal domain, at positions 314, 317, 327, 332, 334, and 338, were identified as the major phosphorylation sites, but it is not known whether a stoichiometry of 6 phosphate groups per receptor is required for receptor desensitization (22).

In this paper, we show that phosphorylation of either of the two serine pairs (Ser332/Ser334 and Ser334/Ser338) is a prerequisite for receptor phosphorylation. This strongly supports the notion that C5aR is sequentially phosphorylated upon agonist binding. However, phosphorylation of the first four serine residues was found to be dispensable for receptor desensitization. In stably transfected HL-60 cells, receptor sequestration was found to be independent of receptor desensitization. In addition, the results show for the first time that a nonphosphorylatable receptor is able to support sustained intracellular signaling events that result in a significant increase in the production of superoxide by neutrophil-like differentiated HL-60 cells. This indicates that cell adaptation to a persistent stimulus is to some extent dependent on receptor phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Materials—**PMA, N-formyl-Met-Leu-Phe-Lys-OH (FMLFk), N6, O2'-dibutyryl adenosine 3',5' cyclic monophosphate (Br2cAMP), human recombinant C5a, bovine serum albumin, myelin basic protein, leupeptin, benzamidine, pepstatin, aprotonin, phenylmethylsulfon fluoride, p-nitrophenylphosphate, pertussis toxin, cytochrome c, and phosphate-free RPMI medium were obtained from Sigma Chemical Co. Restriction enzymes and 4-(2-aminoethyl)-benzenesulfon fluoride hydrochloride were from Roche Molecular Biochemicals. Protein A-Sepharose was purchased from Amersham Pharmacia Biotech. Cell culture media, fetal calf serum, and Genetecin (G418) were from Life Technologies, Inc. [32P]Orthophosphoric acid and Na125I were purchased from Amersham Pharmacia Biotech.

**Cell Culture and Differentiation—**Promyelocytic HL-60 cells and COS-7 cells were cultured in RPMI 1640 medium and Dulbecco’s modified Eagle’s medium with GlutaMAX 1, respectively. Differentiation of HL-60 cells was initiated with 1 μM Br2cAMP for 3 days as described (23).

**Oligonucleotide-directed Mutagenesis—**To construct serine to alanine replacement mutants, the cDNA encoding wild-type C5aR was excised from pCDM8-C5aR plasmid with HindIII and BamHI and subcloned into pSELECT-1 vector to generate a single-stranded DNA. The Promega pSELECT-1 mutagenesis protocol was followed to carry out mutagenesis reactions. The mutated cDNAs were excised from pSELECT-1 with HindIII and BstEII and subcloned into pCDNA3.1-C5aR after digestion with HindIII and BstEII. Mutagenesis used to generate serine to aspartic acid replacement mutants was performed on pCDNA3.1-C5αR with the QuickChange method of Stratagene, according to the manufacturer’s instructions. Sequencing of all mutated cDNAs were verified by sequencing.

**Transfection of Cells—**COS-7 cells were transiently transfected by electroporation with wild-type or mutant C5aR in pCDNA3.1 expression vector as described previously (24). Thirty millimeter plates were seeded with 3 × 10⁶ electroporated cells. After 60–72 h, cells were washed twice and further metabolic labeling with [32P]orthophosphoric acid or 125I-labeled C5a binding. The pErk1e expression plasmid (25) was used to stably express mutant C5αR in HL-60 cells as described previously (23). For each clone of HL-60 transfomant, receptor expression was assessed by 125I-labeled C5a binding. A level of surface expression ranging from 25,000 to 35,000 receptors per cell was found for wild-type and most mutant receptors, except the S323A/S324/A/S338A mutant receptor, for which the level of expression did not exceed 10,000 receptors per cell. For all mutant receptors the affinity for 125I-labeled C5a was similar to that found with the wild-type receptor (KD, ~10 nM) (data not shown).

**Radioligand Binding Assays—**125I-labeled C5a was prepared by the chloramine T method (22). All binding studies were carried out at 4 °C using an electroporomed C5αR-transfected COS-7 monolayers 3 days after transfection or with stably transfected HL-60 as described (26).

**Metabolic Labeling and Immunoprecipitation—**Transiently transfected COS-7 cells were metabolically labeled with [32P]orthophosphoric acid (0.3–0.5 mCi/ml) and phosphorylated C5αR was immunoprecipitated as described (22). To be able to compare the level of phosphorylation of the different mutants, the volume of cell lysates withdrawn for immunoprecipitation was adjusted to immunoprecipitate the same amount of surface-expressed receptors.

**Cytosolic Ca2+ Measurements—**HL-60 cells were washed with phosphate-buffered saline, resuspended at a density of 2 × 10⁶ cells/ml in RPMI medium with 0.1% bovine serum albumin and without phenol red. Cells were loaded with 2 μM Fura-2/AM for 30 min at 37 °C and then diluted in 2 volumes of RPMI medium without bovine serum albumin and centrifuged. Cell pellets were washed once with Krebs-Ringer phosphate buffer supplemented with 1.2 mM CaCl2, 5 mM NaHCO3, and 20 mM Hepes at pH 7.5 (KRG buffer). Cells were then resuspended in RPMI medium without phenol red, at a density of 2 × 10⁶ cells/ml. Calcium measurements were performed on 5 × 10⁶ cells in KRG buffer with a SPEX Fluoromax fluorescence spectrophotometer with an excitation wavelength of 340 nm, an emission wavelength of 505 nm, and slit widths of 5 and 10 nm, respectively. In the presence of extracellular calcium, maximal and minimal fluorescence levels were determined in the presence of 0.04% Triton X-100 followed by the addition of 5 mM EGTA plus 30 mM Tris-HCl, pH 7.4, respectively. For measurement of intracellular calcium mobilization in the absence of extracellular calcium, 1 mM EGTA was added into the cuvette before the addition of C5a in order to complex extracellular calcium. Minimal fluorescence was determined as above, and maximal fluorescence was then measured by adding a saturating concentration of CaCl2. Intraocular free calcium concentrations were calculated using the following formula: [Ca2+]i = Kd( F – Fmin)/(Fmax – F) – F, where Kd = 224 nM.

**Immunoprecipitation of Erk2 and Immunocomplex Erk2 Activity Assay—**Differentiated HL-60 cells were resuspended in RPMI medium (33 × 10⁶ cells/ml). Six hundred microliters of cell suspension were treated with C5α (25 μM) for various periods of time. Erk2 was immunoprecipitated and kinase activity was assayed as described previously (23). Aliquots corresponding to 1% of washed complexes were kept for Western blot analysis of immunoprecipitated MAP kinase. For Western blot, the first antibody was the same as that used during the immunoprecipitation step (dilution, 1:1000), and detection of MAP kinase was performed with 125I-labeled protein A.

**Superoxide Production Assay—**Differentiated HL-60 cells expressing either wild-type or mutant C5αR were washed with phosphate-buffered saline and resuspended at a concentration of 4 × 10⁶ cells/ml in phosphate-buffered saline containing 0.5 mM CaCl2, 1 mM MgCl2, and 30 mM glucose (Buffer A). Fifty microliters of cell suspension, kept at 15 °C, were added to 1 ml of Buffer A containing 200 μM of ferricytochrome c. Maximal superoxide production was determined in the presence of FMLFk (10 nM) plus PMA (1 μg/ml) (23). Ferricytochrome c reduction was continuously monitored at 550 nm.

**Receptor Internalization—**Internalization of C5αR was evaluated as the intracellular accumulation of 125I-labeled C5α as follows. C5αR-transfected HL-60 cells were centrifuged and washed once in phos-
phosphate-buffered saline and resuspended at a density of \( 3 \times 10^7 \) cells/ml in ice-cold KRG buffer. Surface-expressed receptors were saturated with \( ^{125}\text{I} \)-labeled C5a (100 nM) for 60 min, at 4 °C. Control cells were incubated on ice with an excess of unlabeled C5a to determine nonspecific binding. Internalization was initiated by diluting cells in 10 volumes of KRG buffer at 37 °C. At various time points, aliquots (6 \( \times 10^6 \) cells) were withdrawn and added to 5 volumes of ice-cold buffer containing 0.15 M NaCl and 0.2 M acetic acid at pH 2.5 for 10 min on ice. This incubation removes any \( ^{125}\text{I} \)-labeled C5a bound to cell surface but has no effect on ligand internalized into cells. After centrifugation, cell pellets were resuspended in 0.5 ml of ice-cold acetic acid buffer and loaded on a cushion of ice-cold KRG buffer (0.5 ml) containing 8% sucrose to separate free ligand from cell-associated ligand. Results are expressed as the percentage of saturaﬁably bound \( ^{125}\text{I} \)-labeled C5a that is internalized as follows: cpm resistant to acid wash after warming/ (cpm specifically bound at 4 °C under saturating conditions).

Statistics—Statistical signiﬁcance was analyzed by Student's t test.

RESULTS

Sequential Phosphorylation of the C5aR—Mutants with individual or combined amino acid replacements were constructed (Fig. 1), and their ability to be phosphorylated was assayed after transient expression in COS-7 cells. Agonist-induced phosphorylation of exogenous C5aR was not systematically assayed in stably transfected HL-60 cells because an important variability was observed in this system, most likely due to a much lower expression level in these cells. In order to immunoprecipitate the same amount of receptor, the amount of surface-expressed receptors was titrated in companion plates. The agonist-induced phosphorylation of C5aR was not markedly reduced by the mutation S327A, S332A, or S338A (Fig. 2A). This reduction was consistent with the disappearance of a single phosphorylation site. In contrast, the replacement of Ser334 by an alanine severely reduced the level of agonist-deﬁned phosphorylation (50–60% reduction), suggesting that phosphorylation of Ser334 is a key step in a sequential process.

The reduction of phosphorylation induced by the mutation S334A was strongly reinforced by a serine to alanine replacement either at position 332 or at position 338. Indeed, whereas the single point mutation S332A or S338A resulted only in a weak reduction of the agonist-induced phosphorylation, combined mutations at positions 332 and 334, at positions 334 and 338, or at all three positions yielded mutants exhibiting a very weak capacity to incorporate radioactive phosphate in response to C5a binding (about 10% compared with wild-type C5aR) (Fig. 2B). Interestingly, the S314A/S317A/S327A/S332A mutant receptor was phosphorylated. The low level of phosphate incorporation in this mutant is most likely due to the existence of only two potential phosphoacceptor sites at positions 334 and 338 (Fig. 2B, right lane).

The high level of the double mutants S332A/S334A and S334A/S338A and the triple mutant S332A/S334A/S338A to be phosphorylated prompted us to examine whether aspartic acid residues at these positions would yield mutants with a restored capacity to be phosphorylated. Judging by the level of radioactivity incorporated in the S332D/S334D and S332D/S334D/S338D mutant receptors, negative charges at these positions are required to confer to C5aR the ability to be fully phosphorylated (Fig. 2C).

As shown in Fig. 2A, the basal phosphorylation of S334A was completely abolished, whereas that of S332A was reduced by about 50%. Likewise, the S332D/S334D mutant receptor showed absolutely no phosphate incorporation in the absence of agonist, providing further support to the idea that Ser332 and Ser334 may be accessible to kinase(s) when the receptor is in a resting state. To test whether the basal phosphorylation of C5aR takes place at the plasma membrane or occurs during the transport to the plasma membrane, metabolic labeling was performed after inhibition of protein synthesis with cycloheximide. As a basal phosphorylation was still observed (not shown), it is likely that Ser332 and Ser334 are phosphorylated after C5aR has reached the plasma membrane. In the absence of C5a, surface-expressed receptors may wobble between a resting state and a conformation that allows the exposure and the phosphorylation of these two serine residues by cytoplasmic kinase(s). However, the low level of radioactive phosphate incorporated during the metabolic labeling with \( ^{32}\text{P} \) orthophosphoric acid suggests that only a small fraction of surface-expressed receptors undergoes such a conformational change. Alternatively, the low basal incorporation of radioactive phosphate may result from the fact that C5aR is already phosphorylated. Consequently, the incorporation of radioactive phosphate is limited by the rate of phosphorylation/dephosphorylation of C5aR in the

FIG. 1. Schematic representation of point mutations in the carboxyl-terminal domain of C5aR. WT, wild-type. Mutation positions are shown at the left with the single-letter code of the replacement amino acid.

FIG. 2. C5a-induced phosphorylation of wild-type and mutant C5aRs in COS-7 cells. Cells were transfected by electroporation with wild-type (WT) or mutant (denoted by single-letter code and position number) C5aR cDNAs in pcDNA3.1 or CDMS. Three days after transfection, cells were loaded with \( ^{32}\text{P} \) orthophosphoric acid and treated with or without 50 nM C5a for 15 min, at 37 °C. After lysis, C5aR was immunoprecipitated as described under "Experimental Procedures." Two identical experiments were performed after inhibition of protein synthesis with cycloheximide.
absence of agonist. Although a constitutive phosphorylation is speculative, it is clear from the present results that the presence of negative charges at positions 332 and 334 favors the C5a-mediated phosphorylation process.

Altogether, the results indicate that Ser334 and probably Ser332 are the most accessible phosphoacceptor groups and that the agonist-mediated phosphorylation of C5aR is sequential. Phosphorylation of Ser314, Ser317, and Ser327 requires the presence of either phosphoseryl residues or aspartic acid residues at positions 332 and 334 or positions 334 and 338.

Phosphorylation of Key Serine Residues Is Sufficient for C5aR Desensitization—If specific phosphorylation sites are important for the desensitization process, one would predict that their mutation into alanine should result in prolonged intracellular signaling events. Conversely, when a wild-type phenotype is observed when only a few serine residues are conserved, one can predict that these serine residues play a key role in the desensitization process. In order to evaluate the precise roles that specific phosphorylation sites may play in signal transduction, we stably expressed wild-type and mutant receptors in promyelocytic HL-60 cells, a myeloid cell line of physiological relevance that does not express the C5aR unless differentiated into neutrophil-like cells. To exclude clonal artifacts, C5a-induced calcium mobilization was analyzed with at least three independent clones expressing either wild-type C5aR or different mutant receptors (n = 3 for each clone). The calcium decays from the peak height to basal calcium level could be fitted to an exponential $A(e^{-\tau}) + B$. The data shown represent the mean ± S.E. (n ≥ 9) time constant $\tau$ for the return of intracellular free calcium concentration to basal level after C5a stimulation. *p < 0.001 as compared with cells expressing exogenous wild-type C5aR.

![Figure 3](https://example.com/figure3.png)

**Fig. 3.** C5a-evoked intracellular calcium mobilization in HL-60 cells transfected with wild-type or mutant C5aRs. Transfected HL-60 cells were loaded with Fura-2, and calcium mobilization was assayed in the presence of extracellular calcium after addition of a saturating dose of C5a (12.5 nM). A, representative traces of calcium mobilization for each mutant (solid line) compared with the wild-type receptor (dotted line). Mutations are denoted by single-letter code and position number. B, a detailed kinetic analysis was performed on at least three independent clones expressing either wild-type C5aR or different mutant receptors (n ≥ 3 for each clone). The calcium decays from 80% of the peak height to basal calcium level could be fitted to an exponential $A(e^{-\tau}) + B$. The data shown represent the mean ± S.E. (n ≥ 9) time constant $\tau$ for the return of intracellular free calcium concentration to basal level after C5a stimulation. *p < 0.001 as compared with cells expressing exogenous wild-type C5aR.
After stimulation with 25 nM C5a for various periods of time, the Erk2 isoform was immunoprecipitated from lysates of HL-60 cells expressing either wild-type (WT), S338A (A338), or S332A/S334A (A332-334) C5aR. Western blot analyses were systematically performed to check that the same amount of kinase has been immunoprecipitated (data not shown). The myelin basic protein (MBP) phosphorylation assay was conducted as described under “Experimental Procedures.” The figure is representative of two independent experiments.

A single exponential, which allowed us to define a time constant for calcium decay (see legend to Fig. 3B). The mutants S332A, S334A, S314A/S317A, S314A/S317A/S327A/S332A, S332D/S334D, and S332D/S334D/S338D were found to have a time constant that was, on average, similar to that of the wild-type C5aR (Fig. 3B). In contrast, the mutants S332A/S334A, S334A/S338A, and S332A/S334A/S338A were characterized by a time constant that was 3–4-fold higher, which was consistent with the incapacity of these latter to be phosphorylated. Interestingly, although the S338A mutant receptor exhibited a robust phosphorylation in both COS-7 cells (Fig. 2A) and HL-60 cells (not shown), it had the ability to induce a sustained calcium response. The time constant for calcium decay with this mutant was about 4-fold higher than that with wild-type C5aR (Fig. 3B).

Two mutants, namely S338A and S332A/S334A, were further analyzed with respect to the activation of the MAP kinase pathway in HL-60 cells. In neutrophils, C5a is known to activate the MAP kinase pathway through the activation of G_{12} (27). Whereas in wild-type C5aR-expressing cells, Erk2 activity returned to basal level within 90–120 s after the application of C5a, a sustained activation of Erk2 was observed in cells transfected with the nonphosphorylated S332A/S334A mutant receptor (Fig. 4). Erk2 activity was still half-maximal 180 s after C5a application on S332A/S334A receptor-expressing cells. A sustained activation of Erk2 was also found with the S334A/S338A receptors (not shown). Although the S338A mutant receptor demonstrated a defective desensitization of the C5a-induced calcium response, the effect on the activation of Erk2 remained modest, with a return to basal level of Erk2 activity within 180 s (see Fig. 4).

Thus, altogether, the results clearly indicate that the desensitization of C5aR is a phosphorylation-dependent process. However, although C5aR is potentially phosphorylated on the six serine residues of its carboxyl-terminal tail (22), a stoichiometry of 6 phosphoserine residues per mole of receptor is not required for the attenuation of signal transduction. The observation that the mutation S338A yields a mutant with a reduced ability to desensitize the C5a-mediated calcium response despite a strong capacity to be phosphorylated suggests that Ser^{338} plays an important role in agonist-induced desensitization of the C5aR.

**Effect of Receptor Phosphorylation on Superoxide Production**—We next asked whether a receptor deficient in its ability to desensitize had an effect on a distal cellular response, such as the generation of superoxide anions, which is also known as the respiratory burst. To accomplish this, HL-60 cells were differentiated into neutrophil-like cells with dibutyryl cyclic AMP. After differentiation, transfected cells expressed endogenous receptors for C5a as well as exogenous wild-type or mutant C5aR. Differentiated cells expressed around 40,000–50,000 receptors per cell, but the proportion of mutant receptors was uncertain because the level of expression of exogenous receptors may be altered in differentiated cells.

Using S338A and S332A/S334A C5aR-expressing cells, we first examined whether exogenous mutant still conferred a mutant phenotype with respect to calcium mobilization in the presence of extracellular calcium. For each type of HL-60 transformant, i.e., expressing wild-type, S338A or S332A/S334A C5aR, three independent clones were analyzed to avoid clonal artifacts. The mean values ± S.E. of agonist-elicited calcium mobilization are shown in Fig. 5A. In the presence of extracellular calcium, the S338A receptors reproducibly exhibited a sustained calcium response for about 30 s, followed by a rapid decay to basal calcium level. Thus, in differentiated HL-60 cells, the S338A receptors appears to have a mutant phenotype for only a short period of time and then becomes desensitized as a wild-type receptor. In the presence of extracellular calcium, the S332A/S334A mutant receptor demonstrated a more sustained calcium response than the wild-type receptor. The duration (measured at half-maximal response after C5a addition) for wild-type and S332A/S334A mutant receptors was around 50 and 150 s, respectively (Fig. 5A). In contrast, in the absence of extracellular calcium, the mean duration of calcium elevation was about 25 s with no significant difference between the response mediated by the mutant and that mediated by the wild-type receptor (Fig. 5B).

To examine whether the S332A/S334A mutant receptor had a higher capacity than the wild-type receptor to trigger superoxide release, we measured the C5a-evoked superoxide response of each HL-60 transformant in the presence or absence of extracellular calcium. For each transformant the maximal superoxide release was achieved by costimulating cells with PMA and fMLFK as described previously (23). The ratio between C5a-induced and maximal superoxide responses is indicative of the ability of C5aRs to trigger the respiratory burst in the different HL-60 transformants. It takes into account possible variations in cell counts and in the amount of functional NADPH-oxidase complex expressed in the different preparations of differentiated HL-60 cells. As illustrated by the dose-response curves shown in Fig. 6, in the presence of extracellular calcium, the activation of the S332A/S334A mutant receptor stimulated a robust superoxide release that was about 1.8–2-fold higher than that following activation of wild-type C5aR-expressing cells. The S338A mutant receptor triggered an oxidative response similar to that yielded by the wild-type receptor (not shown). In the absence of extracellular calcium, the C5a-induced superoxide response was reduced by about 60–70%, whereas the maximal response, i.e. in the presence of PMA and N-formyl peptide, was not affected. This reduction is consistent with previous studies indicating that calcium influx is required for a maximal chemotractant-induced superoxide generation (28, 29). In the absence of extracellular calcium, the C5a-evoked calcium responses by wild-type and S332A/S334A mutant receptors were very similar, but more superoxide was still released by S332A/S334A C5aR-expressing cells (Fig. 6).

Altogether, the results suggest that a defect in chemotractant receptor desensitization can enhance the production of reactive oxygen species by neutrophil granulocytes.

**Sequestration of C5aR in HL-60 Cells Is Independent of Its Desensitization**—The heterologous expression of phosphorylation-deficient mutants of C5aR in pancreatic β cells has revealed that the agonist-mediated internalization of C5aR is facilitated by the phosphorylation of its carboxyl-terminal tail.
Expression of mutants, truncated after Ser327 or Ser334, in the rat basophilic RBL2H3 cell line has indicated that the carboxyl-terminal tail of C5aR is required for normal ligand dependent receptor internalization (31). However, the mechanisms of internalization may vary from cell to cell depending on the relative abundance of adapter proteins that interact with the receptor. We therefore assayed the capacity of wild-type and S332A/S334A C5aR-expressing HL-60 cells to internalize 125I-labeled C5a. As illustrated in Fig. 7, wild-type and S332A/S334A C5aRs were found to be equally efficient at internalizing 125I-labeled C5a in HL-60 cells. The time courses of internalization were, on average, not significantly different. No internalization of 125I-labeled C5a was observed with mock-transfected cells, indicating that the accumulation of radioactivity in C5aR-expressing cells was not due to pinocytosis. Thus, although the mutations S332A and S334A impair the ability of the receptor to undergo desensitization, they do not impair its ability to undergo agonist-mediated internalization.

**DISCUSSION**

Sequential Agonist-dependent Phosphorylation of C5aR—In this study, we examined the specific role of the different serine residues of the carboxyl-terminal tail of C5aR with respect to agonist-mediated phosphorylation and signal transduction. The observation that the mutation S334A yields a mutant presenting a reduced agonist-mediated phosphorylation and a complete lack of basal phosphorylation suggests that Ser334 is a key determinant for C5a-dependent phosphorylation and also one of the most accessible hydroxyl residue in the absence of agonist. The mutations S332A and S338A have individually only a limited effect on the phosphorylation of C5aR, but either
of them, in conjunction with the mutation S334A, dramatically reduces the ability of the receptor to undergo agonist-mediated phosphorylation (Fig. 2). This suggests that the phosphorylation of the other serine residues is dictated by the initial phosphorylation of either of these two pairs of serine residues (Ser\textsuperscript{332}/Ser\textsuperscript{334} or Ser\textsuperscript{334}/Ser\textsuperscript{338}). This hypothesis is supported by the observation that aspartic acid residues can substitute for phosphorylated seryl residues at positions 332 and 334. Negative charges at these two positions may have a “priming” function for the recognition of the carboxyl-terminal region by acidic residue-directed kinases. Alternatively, negative charges may induce a conformational change that unmasks the other serine residues. In this respect, a recent NMR study with the carboxyl-terminal tail of the G protein-coupled receptor rhodopsin has revealed that a major structural change occurs upon phosphorylation of a first residue (32). This conformational change is further stabilized by phosphorylation of additional residues. It is worth noting here that the negative charges brought by the replacement of Ser\textsuperscript{332} and Ser\textsuperscript{334} by aspartic acid residues are not sufficient to promote the phosphorylation of the remaining serine residues in the absence of C5a binding. The results presented here support the notion that the C5a-dependent phosphorylation proceeds sequentially, starting on Ser\textsuperscript{334} followed by the phosphorylation of Ser\textsuperscript{332} and/or Ser\textsuperscript{338}, and then by the phosphorylation of the other serine residues. A similar hierarchical mechanism of phosphorylation has been previously found in the case of rhodopsin (33), but it is not a general rule. There is no apparent preference for any single serine residue in the case of the α<sub>1</sub>B-adrenergic receptor (34) and the CC-chemokine receptor CCR5 (35).

The Essential Role of Ser\textsuperscript{334} and Ser\textsuperscript{338} in the Desensitization Process—The calcium mobilization assay with HL-60 transformants indicates that the different serine residues do not have the same functional role. A phosphoserine at position 314, 317, 327, or 332 is not essential for the desensitization of C5aR. Although an alanine residue at position 334 results in a marked reduction of phosphorylation, the S334A mutant continues to transduce signal with the characteristics of a wild-type receptor, suggesting that a key residue is still phosphorylated. This key residue is likely to be Ser\textsuperscript{338}. Indeed, despite an efficient phosphorylation, the S338A mutant receptor has a reduced ability to desensitize the C5a-induced calcium mobilization in undifferentiated cells. Moreover, compared with the C5a-mediated Erk2 activity in wild-type C5aR-expressing cells, the return to basal Erk2 activity in S338A C5aR-expressing cells is delayed by 40–60 s. The pivotal role of Ser\textsuperscript{338} in the desensitization process is further supported by the observation that the S314A/S317A/S327A/S332A mutant receptor is desensitized as efficiently as the wild-type receptor. Thus, the phosphorylation of Ser\textsuperscript{334} and Ser\textsuperscript{338} is apparently sufficient to confer to the receptor the ability to undergo desensitization.

In contrast to the mutations S332A/S334A, which confer to the receptor a dominant mutant phenotype with respect to calcium mobilization in differentiated cells, the mutation S338A has only a partial effect in differentiated cells. Indeed, after a latency of about 30 s, the concentration of intracellular calcium rapidly returns to basal level as if the S338A mutant had acquired the features of a wild-type receptor after this lag period. One possible scenario that could explain this behavior is that the lack of phosphoseryl residue at position 338 is compensated by the delayed phosphorylation of the neighboring threonine residue, Thr\textsuperscript{339}, by a kinase that is not expressed or is expressed at a lower level in undifferentiated cells.

Calcium Flux and Superoxide Production in Differentiated HL-60 Cells Expressing Desensitization-deficient C5aR—In the presence of extracellular calcium, the stimulation of the S332A/S334A mutant receptor expressed in differentiated HL-60 cells leads to a prolonged calcium elevation as compared with the calcium response triggered by activation of the wild-type C5aR. The inability of S332A/S334A C5aR-expressing cells to rapidly attenuate the C5a-induced calcium response is likely to result from the deficient phosphorylation of the S332A/S334A mutant receptor inasmuch as all independent clones tested have the same mutant phenotype. The observation that the duration of the C5a-induced calcium response is similar for both wild-type and S332A/S334A mutant receptors in the absence of extracellular calcium indicates that the sustained calcium response induced by the S332A/S334A C5aR in the presence of extracellular calcium is due to a prolonged activation of calcium influx.

An important observation of this study is that the expression of the desensitization defective S332A/S334A mutant in differentiated HL-60 cells leads to a significantly higher C5a-mediated production of reactive oxygen species as compared with cells expressing only wild-type C5aRs. In the presence of extracellular calcium, the effect of the S332A/S334A mutant receptor on superoxide release is correlated with a sustained calcium influx. This is consistent with previous studies indicating that calcium influx is predominantly responsible for the activation of the respiratory burst initiated by chemoattractant receptors (28, 29). However, additional mechanisms are likely to control NADPH-oxidase activation because, in the absence of extracellular calcium, cells expressing the S332A/S334A mutant still release more superoxide despite an intracellular calcium mobilization similar to that triggered by the wild-type C5aR. In addition to the prolonged activation of Erk2, desensitization defective mutant receptors are likely to support a sustained activation of several enzymes that are essential for NADPH-oxidase activation (e.g. phospholipase A<sub>2</sub>, phospholipase D, or PI 3-kinase).

Thus, in contrast to the chemotactic response, for which phosphorylation of chemoattractant receptors is not required (36–38), the cytotoxic activity of phagocytic cells is to some extent regulated by the ability of chemoattractant receptors to be phosphorylated and desensitized. The extent of the increase in superoxide production remains nevertheless limited, indicating that myeloid HL-60 cells are still able to adapt to a persistent stimulation when receptor desensitization is defective. A phosphorylation-independent mechanism of adaptation has been recently described for the termination of G-protein-mediated responses in the case of Dictyostelium discoideum (38). A phosphorylation-independent adaptation of the cytotoxic activity of neutrophils is likely because the chemoattractant-mediated activation of the NADPH-oxidase involves the synergistic action of several signaling pathways that can be regulated at multiple levels by inhibitory feedback loops (5, 23, 28).

In view of the results presented here and based on previous studies (31, 39), one can predict that nonsense mutations that would truncate the cytoplasmic domain of C5aR after Ser\textsuperscript{327} will yield desensitization-deficient receptors. The C5a-mediated activation of leukocytes expressing such mutants should result in an enhancement of the respiratory burst and possibly in a prolonged induction and release of proinflammatory mediators, such as interleukins 1, 6, and 8, and tumor necrosis factor α (40–43). Whether this could lead to pathophysiological disorders under circumstances of complement activation is presently not known. So far, no naturally occurring mutations in the cytoplasmic tail of C5aR have been reported, nor have inflammatory diseases been shown to be associated with deficiencies in C5aR desensitization.

Defective Desensitization and Normal Sequestration of C5aR: a Paradox with Respect to the Current Model of GPCR Regula-
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Conclusions—In summary, the present study reveals that the agonist-mediated phosphorylation of C5aR is sequential and requires the initial phosphorylation of two serine pairs (Ser332/Ser334 or Ser333/Ser338). Although all serine residues of the carboxyl-terminal tail are phosphorylated upon C5a addition, only two of these serine residues, namely Ser334 and Ser338, are sufficient for receptor desensitization. To our knowledge, this is the first demonstration that the chemoaatractant-induced NADPH-oxidase activity is to some extent controlled by the capacity of chemoattractant receptors to rapidly desensitize. This may explain why chemoattractant receptors have variable ability to trigger superoxide production despite a coupling to the same pool of heterotrimeric G\(\beta\) proteins. In addition to a higher production of superoxide, a nondesensitized C5aR is likely to induce an increased synthesis and release of inflammatory mediators. It is therefore reasonable to hypothesize that defects in chemoattractant receptor desensitization can have pathophysiological consequences.

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