Internalization and recycling of the C5a anaphylatoxin receptor: evidence that the agonist-mediated internalization is modulated by phosphorylation of the C-terminal domain

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SUMMARY
The C5a anaphylatoxin receptor is a member of the G protein-coupled receptor family involved in chemoattraction and activation of myeloid cells, as well as in host defence against infection by Pseudomonas aeruginosa. Upon challenge by C5a, the C5a receptor undergoes a rapid phosphorylation on serine residues in the carboxy-terminal region. In this study, we used cells stably transfected with either the wild-type C5a receptor, or mutants affected in their capacity to be phosphorylated, to examine the role played by phosphorylation in the intracellular trafficking of the C5a receptor. Upon agonist binding, the wild-type receptor was rapidly internalized into endosomes that cluster near the nucleus after 10 minutes. Internalization of a non-phosphorylatable mutant was severely impaired relative to wild-type receptor, whereas a mutant phosphorylated on serine 327 and/or serine 338, showed a rate of internalization intermediate between that of wild-type receptor and that of the non-phosphorylatable mutant. Under continuous exposure to C5a and in the absence of protein synthesis, the C5a receptor was maintained in a highly phosphorylated state but was not degraded. Confocal microscopy and ligand-binding studies indicated that internalized receptors were recycled to the plasma membrane. During this process, receptors were dephosphorylated with kinetics that correlated with the kinetics of receptor recovery on the cell surface. Altogether, our data suggest that phosphorylation plays a key role in the intracellular trafficking of the C5a receptor. Phosphorylated receptors might be recognized by an adaptor protein that interacts with the endocytic machinery.

Key words: C5a receptor, Phosphorylation, Chemoattractant, Endocytosis, Recycling

INTRODUCTION
The complement-derived C5a anaphylatoxin is a potent chemoattractant and activator of neutrophils and other leukocytes (Füreder et al., 1995), as well as of the differentiated myeloid cell lines HL-60 and U937 (reviewed by Gerard and Gerard, 1994; Ye and Boulay, 1997). In these cells, C5a is known to activate a complex array of cellular responses that result in directed cell migration and release of active oxygen intermediates and proteolytic enzymes (McPhail and Harwath, 1993). Effects of C5a are mediated by binding to a specific surface receptor that belongs to the G protein-coupled receptor family (Boulay et al., 1991; Gerard and Gerard, 1991). Several recent studies have provided evidence that the C5a receptor (C5aR) is also expressed in non-myeloid cells including endothelial cells, vascular smooth muscle cells, bronchial and alveolar epithelial cells and hepatocytes (reviewed by Ye and Boulay, 1997). The broad distribution of C5aR suggests it plays an important role in modulating inflammatory and immune responses. The deletion of C5aR in mice has revealed that the C5a chemoattractant receptor is essential in mice for protection against infection by the bacterium Pseudomonas aeruginosa (Höpken et al., 1996).

As for most G protein-coupled receptors, exposure to agonist rapidly modulates C5aR. Despite the persistent presence of C5a, cells rapidly attenuate their responses (McLeish et al., 1989; Wilde et al., 1989). This ubiquitous phenomenon known as desensitization has been particularly well-studied in the case of the β2-adrenergic receptor. Desensitization may occur primarily as a result of receptor phosphorylation that interferes with coupling to the G protein partner(s) (Dohlman et al., 1991; Hausdorff et al., 1990). An additional mechanism involves the internalization of agonist-occupied receptors into intracellular compartments. Some of the serpentine receptors, including β2-adrenergic (von Zastrow and Kolbika, 1992; von Zastrow et al., 1993), neurokinin 1 (Grady et al., 1995a), bombesin (Grady et al., 1995b; Tseng et al., 1995), thyrotropin-releasing hormone (Ashworth et al., 1995), and cholecystokinin A (Roettger et al., 1995) receptors, are rapidly internalized into early endosomes and subsequently
recycled to the plasma membrane. However, internalization and recycling is not a general process: (1) the β3-adrenergic receptor is not internalized (Liggett et al., 1993); (2) in the case of the hCG/LH receptor, only a very small fraction of the pool of internalized receptor molecules is recycled to the cell surface (Ghinea et al., 1992); and (3) the activated thrombin receptors are internalized but they are largely targeted to lysosomes where they are degraded (Hein et al., 1994; Hoxie et al., 1993). Routing to the degradative pathways leads to a net loss of receptors on the surface and may account for long-term desensitization. In yeast, the pheromone receptors are members of the seven transmembrane segment receptor family. They are subject to a constitutive endocytosis as well as a ligand-mediated internalization. They do not appear to be recycled but are transported through the endocytic pathway to the vacuole, where they are degraded (Schandel and Jennes, 1994). Upon binding mating factor, the receptors show a ligand-dependent phosphorylation (Zanolari et al., 1992) and ubiquitination (Hicke and Riezman, 1996). It is thought that ubiquitination plays an initiating role in both constitutive and ligand-dependent endocytosis of pheromone receptors (Hicke and Riezman, 1996; Roth and Davis, 1996).

A rapid internalization and recycling process may be of major importance for the receptors whose primary function is to direct cell migration up the chemotactic factor gradient. Among the subfamily of chemoattractant receptors, C5aR is particularly well-suited for delineating the molecular and cellular mechanisms underlying the regulation of these receptors. The kinetics of its agonist-stimulated phosphorylation (Ali et al., 1993; Tardif et al., 1993), as well as the positions of the key serine residues phosphorylated upon exposure to C5a, have been determined (Giannini et al., 1995). In this study, we report results on the dynamic process of internalization and recycling of the C5aR. By replacing serine residues with alanine residues we generated mutant receptors that displayed a reduction in phosphorylation and internalization upon C5a binding. The results presented here suggest that the phosphorylation of C5aR plays a critical role in its agonist-stimulated endocytosis.

MATERIALS AND METHODS

Reagents

Bovine serum albumin fraction V. human recombinant C5a, and 1,4-diazabicyclo(2.2.2)octane (DABCO), and bovine serum albumin (BSA) were purchased from Sigma Chemical. BODIPY-conjugated goat anti-rabbit antibody was from Molecular Probes, Inc. (Eugene, OR). Cycloheximide, okadaic acid and carboxypeptidase B (CbP) were from Boehringer Mannheim. Cyscinorin A (CsA) and FK506 were a gift from the Laboratory of Immunology (DBMS/Nuclear Centre, Grenoble). Carrier-free [32P]orthophosphoric acid was from Amersham Corp. Sepharose 4B-Protein A was from Pharmacia Biotech Inc. Tissue culture media were from Gibco BRL. All chemicals were reagent grade.

Oligonucleotide-directed mutagenesis and stable transformants of RINm5F cells

cDNAs encoding human C5aR was excised from CDM8-C5aR with HindIII and BamHI and subcloned into pSELECT-1 vector (Promega) to generate mutants as described previously (Giannini et al., 1995). Coding sequences of all mutated cDNAs were sequenced after reinsertion into CDM8. cDNAs encoding the wild-type C5aR as well as the non-glycosylated form Asn5 → Gin (wt/gly) (Mery and Boulay, 1993), and the mutant receptors Ser332,334,338 → Ala (A332,334,338) and Ser314,317,322,323 → Ala (A314,317,322,323) were used for the transfection of rat insulinoma cell line RINm5F as described previously (Lang et al., 1993). DNA-mediated gene transfer into RINm5F cells was performed by electroporation. The rat insulinoma RINm5F cell line was chosen on the basis of previous work that showed a conserved functional coupling of chemoattractant receptors to pertussis toxin sensitive G proteins analogous to the mechanism in neutrophils (Lang et al., 1993). Individual colonies resistant to G418 were expanded and cell monolayers were assayed for their ability to bind 125I-labeled recombinant C5a in the presence, or absence, of 250 nM unlabeled C5a as described previously (Mery and Boulay, 1994). Binding parameters were calculated by computer fitting using the iterative nonlinear regression program Ligand (Monson and Rodbard, 1980). The receptors expressed on the surface of the different clones demonstrated appropriate binding affinity with a dissociation constant (Kd) of about 10 nM for wild-type and mutant receptors. All stable cell lines used in this work expressed a similar number of C5a receptors on the cell surface (7.0 ± 105 sites per cell), and they were appropriately coupled to the intracellular signaling machinery as demonstrated by the formation of inositol trisphosphate (F. Boulay, unpublished data).

Metabolic labeling and immunoprecipitation of C5aR

Cells were grown in 35 mm dishes to a confluence of ~80%. When indicated protein biosynthesis was inhibited by preincubating cells with cycloheximide (100 μg/ml) for 3 hours. Cycloheximide was maintained during all subsequent incubations. Cells were metabolically labeled with [32P]orthophosphoric acid (0.3-0.5 mCi/ml) for 3 hours at 37°C, as described previously (Mery and Boulay, 1994). Phosphorylation of C5aR was initiated by addition of 50 nM C5a in RPMI 1640 supplemented with 1% BSA at 37°C. In some experiments a pulse of stimulation was generated by adding a saturating concentration of C5a (50 nM). After 6 minutes, carboxypeptidase B (8 units/ml) was added to inactivate C5a (Giannini and Boulay, 1995). At this concentration of CbP, a saturating concentration of C5a was completely inactivated in 2-3 minutes. At various time points after the addition of CbP, the monolayers were lysed and C5aR was immunoprecipitated as described previously (Tardif et al., 1993). Immunoprecipitates were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (Laemmli, 1970) and autoradiography. Quantification of the amount of radioactivity incorporated in the receptor bands was performed with a Molecular Dynamics PhosphorImager.

Detection of C5aR by immunoblotting

Cell monolayers were lysed in 2-fold Laemmli sample buffer (Laemmli, 1970) supplemented with 10 mM dithiothreitol and briefly sonicated with a microtip. Proteins were then separated by SDS-PAGE and transferred to a 0.22 μm Protran nitrocellulose filter (Schleicher & Schuell). After transfer, the filter was incubated in phosphate-buffered saline (PBS) containing 0.1% Tween-20 and 3% BSA for 1 hour at room temperature and then overnight in the same solution containing antibodies. Affinity-purified anti-C5aR rabbit IgGs were used at a 1:400 dilution. The filter was then extensively washed and bound radioactivity was visualized by autoradiography using Fuji RX film at ~80°C. Quantification was performed with a Molecular Dynamics PhosphorImager.

C5a internalization assay

Cells were seeded in 35 mm plates 48 hours prior to experiments. Cell density was adjusted so that the global amount of receptors was similar in plates seeded with wild-type- or mutant-expressing cells.
Cells were washed twice with Hanks’ balanced salt (HBS) and incubated with a saturating concentration of $^{125}$I-labeled C5a (40 nM) in HBS, 1% BSA, 20 mM Hepes, pH 7.5, at 4°C for 60 minutes. Cells were then rapidly washed three times with ice-cold HBS to remove unbound $^{125}$I-labeled C5a. For wild-type- and mutant-expressing cells, 90-95% of the radioactivity bound at 4°C could be removed by a 10 minute treatment with 1 ml of ice-cold acid wash solution (150 mM NaCl, 200 mM acetic acid, pH 2.5). Internalization was initiated by the addition of 1 ml of RPMI at 37°C. At the indicated time the warm medium was removed and cells were treated for 10 minutes with 1 ml of ice-cold acid wash solution to remove the surface-bound radiogland, thus giving a measure of C5a internalization. A time course of C5a internalization was generated as a ratio of cpm detected in pH 2.5-washed cells to the cpm specifically bound in control plates after 60 minutes at 4°C. The internalization rate was calculated by determining the slope of the initial linear phase of the internalization kinetic plot.

Biochemical assay for C5aR recycling
To follow receptor recycling wild-type-expressing cells were seeded in 35 mm plates two days prior to the experiments. Protein biosynthesis was blocked by preincubating the cells for 3 hours with 100 µg/ml cycloheximide. Cycloheximide was maintained during all subsequent incubations. Recycling was assessed by titrating the C5a binding sites at different time points after a brief wave of C5a-mediated internalization in the absence of protein synthesis. The wave of internalization was generated by briefly stimulating cells with a saturating concentration of C5a (50 nM) in RPMI 1640 containing 1% BSA, at 37°C. To inactivate C5a, all plates (control and experimental) were supplemented with 8 units CpB/ml. After 2-3 minutes, plates were rapidly washed twice with 2 ml of warm RPMI 1640 containing 1% BSA, and further incubated at 37°C. At various time points, plates were placed on ice, and washed twice with ice-cold HBS. The amount of cell surface C5aR at each time point was assayed in duplicate by titrating the C5a binding sites. Cells were incubated with a saturating concentration of $^{125}$I-labeled recombinant C5a (40 nM) in HBS, 1% BSA, 20 mM Hepes, pH 7.5, at 4°C for 30 minutes, washed three times with ice-cold HBS, and lysed in 1 N NaOH/0.1% SDS. Radioactivity was counted by $\gamma$ spectroscopy. A time course of C5aR recycling was generated as the ratio of the cpm bound at 4°C on C5a-treated cells to that bound at 4°C on control cells at each time point.

Microscopical analysis of internalization and recycling of C5aR
An indirect immunofluorescence microscopy staining technique was used to study ligand-induced internalization and recycling of the receptor. For all the microscopic analyses, cells were seeded on polylysine-coated coverslips 48 hours prior to the experiments. To stop protein synthesis, cells were incubated in RPMI 1640 containing 1% BSA and 100 µg/ml cycloheximide three hours before each experiment. Cells were maintained in the same medium throughout all experiments. To study ligand-induced receptor internalization, cells were treated with 50 nM C5a at 37°C for various time periods ranging from 0 to 60 minutes. Cells not treated with the ligand were used as a control. To follow receptor recycling, a pulse stimulation of 6 minutes was given to the cells with 50 nM C5a. Unbound C5a was inactivated by adding 8 units CpB/ml as mentioned above. Incubation at 37°C was continued and cells were fixed at various time points from 0 to 90 minutes. Finally, cells were permeabilized with acetone at −20°C for 30 seconds.

RESULTS
Characterization of phosphorylation-deficient mutants of C5aR
To examine whether the phosphorylation of the C5aR plays a role in the agonist-stimulated internalization process, we established RINm5F cell lines stably expressing either the wild-type receptor (wt) or the mutant receptors (A32,334,338 and A14,317,322,334). We had previously shown that C5aR was phosphorylated in a hierarchical manner on the six serine residues localized in the C-terminal cytoplasmic portion of the polypeptide chain (Giannini et al., 1995). By replacing serine residues with alanine (Fig. 1A), we generated two mutants that were affected in their ability to be phosphorylated upon agonist binding. The expressed receptors demonstrated appropriate binding affinity with a dissociation constant ($K_D$) of 10 nM for both wild-type and mutant receptors (not shown). As previously reported with differentiated HL-60 cells and stably transfected RBL-2H3 cells (Ali et al., 1993; Tardif et al., 1993), we found a rapid phosphorylation of wild-type C5aR in RINm5F cells. Phosphorylation was half maximal in less than 1 minute and reached a plateau within 4 minutes (not shown).

The phosphorylation of mutant receptors after C5a addition was assessed by metabolic labeling of cells with $^{32}$Porthophosphoric acid followed by immunoprecipitation and SDS-PAGE. As shown in Fig. 1B, the agonist-mediated phosphorylation was dramatically reduced for both mutants. Quantification with the phosphorImager of the amount of radioactivity incorporated in the receptor bands indicated that the quadruple mutant A314,317,322,334 showed a reduced incorporation of phosphate as compared to the wild-type receptor (18±3% of wild-type phosphorylation, n=3). The triple mutant A32,334,338 was not phosphorylated in spite of the fact that serine residues at positions 314, 317, and 327 are still potential phosphorylation sites (Giannini et al., 1995). Immunoprecipitated wild-type and A314,317,322,332 mutant receptors migrated as a major $^{32}$P-labeled band with an apparent molecular mass of 48 kDa and high molecular mass species ranging from 100 kDa to 200 kDa. A band at 200 kDa was weakly detected with mutant A314,317,322,334, and not detected with the phosphorylation-deficient mutant A32,334,338. High molecular mass species are likely to represent oligomers of C5aR that are not solubilized in Laemmli sample buffer at 37°C since they largely disappeared when immunoprecipitates were heated at 60°C for 20
Point mutations in the C-terminal region of C5a

Wild type

A 32P-labeling Western Blot

32P-labeling Western Blot

Fig. 1. (A) Primary amino acid sequence of the carboxy-terminal region of C5aR. It has been previously established that the C5aR can be phosphorylated on serine residues at positions 314, 317, 327, 332, and 338 (Giannini et al., 1995). Alanine substitution mutants used in this study are shown. (B) SDS-PAGE analysis of C5aR immunoprecipitated from cell extracts containing an equal number of wild-type and mutant receptors. Cells were metabolically labeled with [32P]orthophosphoric acid and stimulated with C5a for 10 minutes. Immunoprecipitates were treated with 2-fold Laemmli sample buffer under reducing conditions for 10 minutes (Fig. 2A). Cell surface labeling with radioactive iodine has previously shown that dimers are observed in immunoprecipitates irrespective of C5a binding (Mery and Boulay, 1994). However, it is presently unclear whether dimers of C5aR pre-exist in the plasma membrane or whether they are formed artifically during the immunoprecipitation step. The fact that dimers are not detected by immunoblotting with anti-C5aR serum when cells are directly lysed with Laemmli sample buffer is more consistent with the latter hypothesis (Figs 1C, 2A,B). Moreover, we exclude the possibility that the anti-C5aR serum could not detect dimers and oligomers because when immunoprecipitates were analyzed by SDS-PAGE and transferred to nitrocellulose, dimers and oligomers were detected by immunoblotting with anti-C5aR serum (data not shown).

The electrophoretic shift accompanying C5a binding results from phosphorylation

Interestingly, immunoblotting experiments revealed that the migration of wild-type and A314,317,332,334 receptors was slightly retarded when receptors were extracted from cells stimulated with C5a. In contrast, the migration of the non-phosphorylated mutant A332,334,338 was unchanged (Fig. 1C). The modification of the electrophoretic mobility was most conveniently observed with cells expressing the non-glycosylated form of C5aR (wt/gly-). This non-glycosylated form of C5aR responded as the wild-type receptor as far as agonist binding, signal transduction, and C5a-stimulated phosphorylation and internalization were concerned. In the absence of C5a, wt/gly- C5aR migrated as a 40 kDa band, whereas after the addition of C5a, 80±5% (n=8) of the receptor migrated with a slower electrophoretic mobility (Fig. 2B). The same pattern was observed for stimulation times ranging from 1 to 15 minutes (not shown). Immunoprecipitation of C5aR from 32P-labeled

Fig. 2. Phosphorylation of C5aR induces a mobility shift. (A) High molecular mass species largely disappeared at 60°C with a concomitant increase of both the 43 kDa and 75-90 kDa bands. The non-glycosylated C5aR (wt/gly-) was immunoprecipitated from 32P metabolically-labeled cells before and after 10 minutes stimulation with C5a. Immunoprecipitates were treated with 2-fold Laemmli sample buffer under reducing conditions either for 10 minutes at 37°C (left) or for 20 minutes at 60°C (right), and analyzed by SDS-PAGE. (B) Companion immunoblot of non-labeled cell extracts before and after C5a addition revealed with anti-C5aR serum. (C) Detection of C5aR by immunoblotting with anti-C5aR serum. Cells were directly lysed in Laemmli sample buffer under reducing conditions, at 37°C, before and after 10 minutes stimulation with C5a.
cells yielded a major phosphorylated band with an apparent molecular mass of 43 kDa that coincided with the retarded band detected by immunoblotting (Fig. 2A). To ascertain that the mobility shift was due to phosphorylation, we examined by immunoblotting analysis whether protein phosphatases present in cell extracts could promote the collapse of the 43 kDa band into a single band of 40 kDa. As shown in Fig. 2B, the absence of protein phosphatase inhibitors allowed the conversion of the 43 kDa band to a 40 kDa species after 45 minutes, whereas the addition of protein phosphatase inhibitors totally blocked this conversion.

Taken together, the data indicate that the retarded mobility of C5aR after C5a binding results from the phosphorylation of C5aR. This conclusion is strengthened by the observation that the non-phosphorylated mutant A332,334,338 presents no alteration of its electrophoretic mobility. The retarded migration of the quadruple mutant A314,317,332,334 after C5a addition suggests that phosphorylation of Ser327 and/or Ser338 is per se sufficient to stabilize a conformational change and to induce a slower electrophoretic mobility.

**Agonist-mediated internalization of wild-type C5aR**

The agonist-stimulated endocytosis of wild-type C5aR was first examined by confocal fluorescence microscopy in permeabilized cells with affinity-purified anti-C5aR IgGs. Three hours before and during the incubation with C5a, cells were treated with 100 μg/ml cycloheximide to prevent protein biosynthesis. In the absence of agonist, the C5aR was visualized in the plasmalemma, as indicated by a thin bright fluorescent ring (Fig. 3A). Addition of C5a initially led to the formation of small receptor-containing vesicles in the subcortical area, which were subsequently transported deep into cell cytoplasm (Fig. 3B-D). By 6 minutes, fluorescent vesicles had moved from the subcortical region to compartments deep within the cell but were still scattered throughout the cytoplasm (Fig. 3C). After 10 minutes, the fluorescence was seen as a bright patch localized at one pole of the nucleus; the cell periphery and cytoplasm were free of fluorescence spots (Fig. 3D). At 20 minutes, this perinuclear region was still brightly fluorescent but punctate fluorescence was again visible in the cytoplasm and near the plasma membrane (Fig. 3E). Upon further incubation, the fluorescence staining was less polarized in the perinuclear region while more fluorescent vesicles were visible throughout the cytoplasm and at/near the plasma membrane (Fig. 3F).

**The phosphorylation of C5aR plays a role in its agonist-mediated endocytosis**

To have a more precise measurement of the internalization kinetics, we assayed the ability of each C5aR-expressing cell
line to internalize radiolabeled C5a. Fig. 4 represents the internalization kinetics of each mutant compared with that of the wild-type receptor. Cells expressing the non-phosphorylated mutant A332,334,338 showed a rate of internalization of radiolabeled C5a 10-fold slower than wild-type-expressing cells. The rate of C5a internalization was less severely affected for cells expressing A314,317,332,334 but it was reproducibly slower than in wild-type cells (2.5-fold). With the wild-type receptor we consistently observed a decrease in internalized radioactivity after 10 minutes. The reason for this decrease is presently not clearly explained.

The slow rate of agonist-mediated endocytosis of A323,334,338 was further confirmed by confocal microscopy. Unlike the wild-type receptor (Fig. 5C,D) this mutant did not accumulate in the perinuclear region when exposed to C5a for 20 minutes (Fig. 5A,B). The immunostaining remained restricted to the plasmalemma. Shorter or longer exposures to C5a gave essentially the same pattern of fluorescence. Two independent cell clones were tested that yielded similar results. Therefore, it is unlikely that the lack of C5a-mediated endocytosis could result from the selection of a clone defective in receptor endocytosis. When the mutant A314,317,332,334 was examined by immunofluorescence, we observed the presence of fluorescent vesicles in the cytoplasm after the addition of C5a and the overall pattern of fluorescence was not significantly different from that obtained with wild-type cells (data not shown).

Taken together the results suggest that phosphorylation on multiple serine residues is required to signal the rapid endocytosis of C5aR. The observation that the mutant A314,317,332,334, which can be phosphorylated only on Ser327 and/or Ser338, is internalized at a rate faster than the non-phosphorylated mutant A332,334,338 suggests that the degree of phosphorylation modulates the interaction of the agonist-occupied receptor with the endocytic machinery and, thereby, the rate of internalization of the receptor. It has not been possible to determine the relative importance of Ser327 and Ser338 since the replacement of Ser327 together with Ser332 and Ser334 yielded a receptor that was not transported to the cell surface (E. Giannini and F. Boulay, unpublished data).

**Internalized C5a receptors are not degraded but recycled to the plasma membrane**

The recycling of C5aR was conveniently observed by immunofluorescence microscopy after the generation of a brief wave of agonist-mediated receptor internalization in the absence of protein biosynthesis. Immediately after the pulse of C5a, fluorescent vesicles were detected on or near the cell surface. A few cells showed an internalized fluorescence that was either polarized or dispersed. By 10 minutes after C5a quenching, most of the cells showed a polarized fluorescence in the perinuclear region (Fig. 6B). After 20 minutes, this bright fluorescent staining was no longer visible in a majority of cells; receptors were concentrated at, or near, the cell surface, as evidenced by the bright fluorescent staining of the plasma membrane and the subcortical region (Fig. 6C). After 30 minutes, all cells showed a fluorescent staining of the plasma membrane (Fig. 6D). The pattern of fluorescence was not significantly modified by further incubation. This suggested that C5aR was recycled to the cell surface. However, it was not possible to establish by this approach whether a fraction of receptor had been degraded, or whether receptors that had returned to the plasma membrane were again available for C5a binding.

To demonstrate that C5aR was not degraded but was recycled to the plasma membrane in the absence of protein synthesis, we first performed immunoblotting of cell extracts either after a pulse of C5a, or at different times in the continuous presence of C5a. While the 43 kDa phosphorylated band was rapidly converted into a 40 kDa band after a pulse of C5a, the continuous presence of ligand maintained 70% to 80% of C5aR in a phosphorylated state as evidenced by the persistent presence of the 43 kDa species (Fig. 7A). As protein biosynthesis was blocked by treating cells with 100 μg/ml cycloheximide for 3 hours before the addition of C5a and during all subsequent incubations, a persistent phosphorylation could only occur if the plasma membrane was continuously replenished with receptors that had regained the ability to bind C5a. Quantification of the amount of C5aR present in cells was performed by immunoblot analysis of cell lysates as described in the experimental procedure. The amount of C5aR was not significantly different before or after a pulse of C5a and decreased by less than 10% after 90 minutes of continuous exposure to C5a. This suggests that little, if any, receptor was degraded in lysosomes. With the exception of a 30 kDa band that is already present before C5a binding, we never observed other degradation products.

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**Table:**

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<th>Receptors</th>
<th>Internalization rate (fraction of wild type)</th>
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<tr>
<td>△ Wild type</td>
<td>1</td>
</tr>
<tr>
<td>● A314,317,332,334</td>
<td>0.4</td>
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<td>○ A332,334,338</td>
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**Figure 4:** C5a internalization is severely slowed down in the absence of receptor phosphorylation. The ability of wild-type, or mutant, receptor-expressing cells to internalize 125I-labeled C5a was assayed as described in Materials and Methods. The data are presented as the ratio of internalized radioactivity at each time point to the specific cell-associated radioactivity before internalization was initiated. Each data point represents the average (± s.d.) of three independent sets of experiments in which wild-type and mutant receptor-expressing cells were simultaneously examined: wild-type receptor (open triangles), mutant receptor A314,317,332,334 (closed circles) and mutant receptor A332,334,338 (open circles). The internalization rate was estimated by determining the slope of the initial linear phase of the internalization time course plot. Inset: relative rate of internalization of mutants expressed as percent of the wild-type kinetics.
Fig. 5. Phosphorylation-deficient mutant A_{32,334,338} does not accumulate in the perinuclear compartment upon C5a addition. Confocal immunomicroscopy of cells expressing either the mutant A_{32,334,338} or the wild-type receptor before C5a addition (A and C, respectively) and after C5a addition (C and D, respectively). Photomicrographs represent the medial plane of the cells and are representative of two separate experiments with two different clones expressing the mutant receptor. Bar, 20 μm.

Fig. 6. Recycling of C5aR. The receptor distribution was examined by confocal immunomicroscopy after a 6 minute-pulse of C5a as indicated in Materials and Methods. Protein synthesis was blocked by pretreating cells with 100 μg/ml of cycloheximide for 3 hours. Cycloheximide was maintained during all subsequent incubations. Immediately after C5a wash-out, the C5aR was distributed at or near the plasma membrane (A). After 10 minutes, C5aR was seen as a brightly fluorescing aggregate in the perinuclear region (B). By 20 minutes and 30 minutes, the plasma membrane and the subcortical regions were again brightly stained (C and D, respectively). Confocal photomicrographs represent the medial plane of the cells and are representative of four independent experiments. Bar, 20 μm.
To assess more precisely the kinetics of receptor recycling, we performed pulse-chase experiments in the absence of protein biosynthesis. The time course of receptor recycling was determined by titrating cell surface receptors with $^{125}$I-labeled C5a at different time points after a 6 minute-pulse with a saturating concentration of C5a (Fig. 7B). A precise titration of binding sites immediately after the pulse was not possible because it takes 2-3 minutes to completely quench C5a by carboxypeptidase B and the washing steps require one additional minute. Based on the kinetics of internalization in the continuous presence of a saturating concentration of radio-labeled C5a we, however, estimated that 60 to 70% of the cell surface-bound C5a was internalized after 6 minutes of contact with C5a. This was consistent with the percentage of receptors (40%) able to bind radiolabeled C5a 4-5 minutes after unlabeled C5a had been washed out. By 20 minutes after C5a wash-out, 50% of the internalized receptor pool had returned to the cell surface and about 85% was recovered on the cell surface after 70 minutes. The determination of the time course of receptor dephosphorylation under the same circumstances indicated that the kinetics of recovery of functional C5aRs on the cell surface paralleled the kinetics of dephosphorylation. This suggests that the dephosphorylation process may have conditioned the recovery of functional receptors.

**DISCUSSION**

This study has shown that when C5aR-expressing cells were exposed to C5a, the receptors were rapidly recovered into vesicles that clustered in the perinuclear region within 10 minutes. Based on confocal and biochemical studies, internalized receptors were rapidly and efficiently recycled to the plasma membrane, with less than 10% of C5aR degraded after 90 minutes of continuous stimulation with C5a. Judging from the kinetics of recovery of $^{125}$I C5a-binding sites (Fig. 7B), a full cycle is complete in approximately 80-90 minutes. We observed an apparent absence of C5a-mediated internalization and clustering of the triple mutant A332,334,338 by confocal immunomicroscopy, although the radiolabeled C5a internalization assay indicated that slow internalization did occur. This apparent discrepancy most likely results from the fact that the internalization and recycling rates are in the same range for this mutant. The correlation between the kinetics of receptor recovery and the kinetics of receptor dephosphorylation suggests that recycling and dephosphorylation are linked. However, more direct evidence is required to ascertain this hypothesis. Although previous reports have demonstrated that a type 2A-like protein phosphatase dephosphorylates rhodopsin, $\beta_2$-adrenergic and cholecystokinin receptors in vitro (Lutz et al., 1993; Palczewski et al., 1989; Yang et al., 1988), we have been unable to block C5aR dephosphorylation in vivo with okadaic acid (Giannini and Boulay, 1995; N. Naik and F. Boulay, unpublished data).

Multiple events are involved in the early steps of internalization of plasma membrane proteins. The involvement of the ubiquitin-conjugating enzymes system has been described in the agonist-mediated internalization and degradation of several plasma membrane proteins. Ubiquitination of the cytoplasmic tail of the growth hormone receptor appears to represent the molecular event that triggers agonist-mediated receptor endocytosis in CHO cells (Strous et al., 1996). In yeast, it has been suggested that ubiquitin works as a signal for endocytosis and degradation of the G protein-coupled receptors for the $\alpha$ and $\alpha$ mating factors via the lysosomal pathway (Hicke and Riezman, 1996; Roth and Davis, 1996). The intramolecular mechanisms that trigger the internalization process of G protein-coupled receptors in mammalian cells are still not well understood. It is presently unclear whether ubiquitination is a general signal regulating the degradative turnover and the agonist-stimulated internalization of G
protein-coupled receptors. In the case of the C5aR, the pattern of C5aR bands detected by immunoblotting analysis of cell extracts before and after C5a addition is not consistent with a mono- or polyubiquitination of the receptor (Figs 1B, 2B,C). If polyubiquitinated C5aR species were generated upon C5a addition, one should have observed a ladder of C5aR bands by immunoblotting analysis of cell extracts. However, one cannot exclude the possibility that ubiquitination is very transient and at a too low level to be detected. Alternatively, ubiquitinated species may not be recognized by the anti-C5aR serum or deubiquitination may continue when cells are lysed in Laemmli sample buffer.

In constitutively internalized and recycled receptors, such as low density lipoprotein, asialoglycoprotein and transferrin receptors, it is clear that the tyrosine-containing sequence (NPXY or YXRF) play an important role in the constitutive internalization process via clathrin coated pits (reviewed by Trowbridge et al., 1993). The signals involved in the agonist-mediated internalization of G protein-coupled receptors are not as clearly defined. A similar sequence (NPXXY) that is highly conserved in the seventh transmembrane domain of almost all G protein-coupled receptors, including C5aR, appears to be an important signal for agonist-mediated sequestration of the β2-adrenergic receptor (Barak et al., 1994). However, this motif is apparently not a sufficient criterion for agonist-mediated internalization for the following reasons: (i) it is present in the β2-adrenergic receptor that is not internalized (Barak et al., 1994), (ii) it is not an internalization signal for the AT1 receptor (Humayd et al., 1995), and (iii) it is not essential for internalization of gastrin-releasing peptide receptor (Slice et al., 1994). Additional structural motifs are likely to control the internalization process but thus far no recognition consensus motif crucial for endocytosis has emerged. A common feature in almost all G protein-coupled receptors is the high proportion of serine and threonine residues in the carboxyl-terminal tail and/or the third cytoplasmic loop. However, the relationship between the phosphorylation of G protein-coupled receptors on serine/threonine residues and their internalization is still a matter of conflict. While it has been recently observed that the sequestration of the β2-adrenergic receptor and the m2 muscarinic acetylcholine receptor is greatly facilitated by β-adrenergic kinase 1-mediated phosphorylation (Ferguson et al., 1995; Tsuga et al., 1994), the internalization of the secretin receptor is apparently independent of its phosphorylation (Holtmann et al., 1996).

The time course of previously-observed C5aR phosphorylation events suggests that this covalent modification occurs before the receptor is internalized (Ali et al., 1993; Tardif et al., 1993). The reduced agonist-mediated internalization of A332,334,338 supports the idea that phosphorylation is a key step in the agonist-mediated internalization of C5aR or, alternatively, that the mutated serine residues are critical for the interaction of the agonist-occupied C5aR with the endocytic machinery. Our results are consistent with the recent observation that mutations of Ser/thr residues in the carboxy-tail of chemokine receptor CCR2b prevent agonist-mediated receptor internalization (Franci et al., 1996). Phosphorylation may stabilize the agonist-occupied receptor in a conformation with high affinity for an adaptor protein of the endocytic machinery. The degree of phosphorylation would modulate the affinity of the adaptor protein for the receptor. This latter hypothesis is supported by the observation that the mutant receptor A314,317,332,334, which is phosphorylated on Ser327 and/or Ser-338, has a phenotype of internalization intermediate between the wild-type receptor and the non-phosphorylable mutant A332,334,338. In this context, it will be of great interest to determine whether phosphorylated C5aR binds a member of the arrestin family and whether the reduced agonist-mediated internalization of the mutant receptors A332,334,338 and A314,317,332,334 may result from a loose interaction with an arrestin-like molecule. Indeed, β-arrestin has an affinity in the nanomolar range for phosphorylated β-adrenergic receptors (Söthemann et al., 1995), and it has been recently suggested that β-arrestin acts as an adaptor for phosphorylated serpentine receptors and the endocytic machinery (Ferguson et al., 1996). This model has recently received strong support from the observation that β-arrestin and arrestin-3 can bind stoichiometrically and with high affinity to clathrin (Goodman et al., 1996).

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