Complement Component 5a Receptor Oligomerization and Homologous Receptor Down-regulation*

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Most G-protein-coupled receptors (GPCRs) form di(oligomeric structures that constitute signaling and trafficking units and might be essential for receptor functions. Cell responses to complement C5a receptor (C5aR) are tightly controlled by receptor desensitization and internalization. To examine the implication of dimerization in C5aR regulation, we generated an NH2-terminally modified C5aR mutant, unable to bind C5a, and a phosphorylation-deficient mutant. Neither an intact NH2 terminus nor the presence of COOH-terminal phosphorylation sites appeared to be required for the formation of C5aR dimers. Upon C5a stimulation, mutant receptors did not internalize when individually expressed. C5a stimulation of cells that co-expressed wild type C5aR together with either unliganded or phosphorylation-deficient mutant resulted in co-internalization of mutant receptors with C5aR. Unliganded GPCRs can be cross-phosphorylated within a heterologous receptor dimer or by second messenger-activated kinases. C5a stimulation of 32P-labeled cells that co-expressed the unliganded mutant with either C5aR or the phosphorylation-deficient mutant did not induce phosphorylation of the unliganded mutant. We can thus postulate that, in the case of C5aR, the stimulation and phosphorylation of one monomer is enough to lead to dimer internalization. The existence and functional implication of d(oligo)mer formation may be important for an accurate C5aR down-regulation in pathological conditions.

Leukocytes express multiple chemoattractant receptors that are engaged as these cells are recruited into tissues at the site of infection or inflammation. Interaction of these receptors with their cognate ligand results, among other functions, in directional cell movement (chemotaxis) and release of reactive oxygen species and proteolytic enzymes that play a critical role in the clearance of invading pathogens. Activation of the complement system, which is part of the humoral innate immune response, leads to the generation of small fragments of C3, C4, and C5 components that are referred to as anaphylatoxin C3a, C4a, and C5a, respectively. The C5a anaphylatoxin is a potent chemoattractant and proinflammatory mediator that exerts its effect by binding to the C5a receptor (C5aR). 3 This chemoattractant receptor is expressed differently by a variety of cell types (1). The inappropriate activation of the complement system leads to inflammatory disorders and a number of pathologies. For instance, during sepsis, excessive generation of C5a anaphylatoxin and excessive activation of C5aR have detrimental effects (2). Therefore, the accurate down-regulation of the C5a receptor may be crucial in pathological conditions.

C5aR is a member of the rhodopsin family of G protein-coupled receptors (GPCRs). The ligand binding domain of the human C5aR consists of two distinct subsites (3). One of these sites binds the COOH-terminal 8 amino acids of C5a and is as yet undefined, whereas the second site lies in the NH2 terminus of the receptor and interacts with the core of C5a. Upon ligand binding, chemoattractant receptors undergo a conformational change that enables them to interact with the pertussis toxin-sensitive G12 protein (4), thereby triggering the exchange of GDP to GTP in the G protein α subunit and the dissociation of the βγ complex from the α subunit. As a consequence, downstream effectors are activated and engage signaling cascades. Cell responses to chemoattractant factors are tightly controlled and down-regulated by desensitization/internalization (for a review, see Ref. 5). Desensitization occurs when the receptor is occupied by its cognate ligand and phosphorylated by GPCR kinases (GRKs). The phosphorylation sites on the C5aR are restricted to the carboxyl-terminal region (6). The C5a-stimulated receptor is primarily phosphorylated on serine residues (6) and, to a much lower extent, on threonine residues (7). The phosphorylation of the receptor allows the formation of a stable complex with β-arrestins (8). Binding of β-arrestins precludes further G protein activation and links the receptor to the internalization machinery. The receptor-β-arrestin complex is targeted to clathrin-coated pits, traffics in early endosomes, and accumulates in a perinuclear recycling compartment. Phosphorylation-deficient receptors transduce intracellular signaling but are not internalized (9, 10).

Although highly controversial up to a few years ago, the idea that GPCRs may form dimers or higher order oligomers and undergo homo- or hetero-oligomerization is largely accepted

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3 The abbreviations used are: C5aR, complement C5a receptor; GPCR, G protein-coupled receptor; GRK, GPCR kinase; EGFP, enhanced green fluorescent protein.
now. Fluorescence resonance energy transfer analyses on C5a receptors tagged with fluorescent proteins demonstrate that C5aR self-assembles into dimers and higher order oligomers in vivo when expressed in Saccharomyces cerevisiae (11). Using a disulfide-trapping strategy to probe intermolecular contact surfaces, Klco et al. (12) have demonstrated cross-linking of C5a receptors in membranes prepared from human neutrophils and stably transfected mammalian cells. C5aR oligomerization appears to be constitutive, as suggested by the observation that binding of the ligand does not affect the fluorescence resonance energy transfer signal (11). Dimerization may be required for an efficient interaction of agonist activated receptors with intracellular partners, such as receptor kinases and β-arrestins, and may play a role in receptor internalization. If a targeted receptor can dimerize with a second, inactive receptor species, then it is possible for both species to be co-internalized. This kind of co-internalization has been observed experimentally for dimers of β2-adrenergic receptor (13), heterodimers of β2-adrenergic receptor and δ-opioid receptors (14), heterodimers of C5aR and CCR5 chemokine receptor (7). In this last case, GRKs can cross-phosphorylate an unliganded receptor if it forms homo- or heterodimer complexes with another receptor that undergoes GRK-mediated phosphorylation. Cross-phosphorylation within hetero-oligomeric complexes and co-internalization of CCR5 together with C5a-occupied C5aR lead to the efficient down-regulation of the CCR5 chemokine receptor.

To explore the implication of C5aR homodimerization in the C5aR down-regulation process, we generated several C5aR mutants and analyzed their ability to form oligomers and to internalize. C5a stimulation of cells that co-expressed wild type C5aR together with either an NH2-terminally modified C5aR mutant that is unable to bind C5a or a COOH-terminally modified, phosphorylation-deficient C5aR mutant resulted in co-internalization of mutant receptors with wild type C5aR. C5a stimulation of cells that co-expressed the unliganded mutant with either wild type C5aR or the phosphorylation-deficient mutant did not induce phosphorylation of the unliganded mutant. Thus, in the case of C5aR, there is no cross-phosphorylation within the homodimer, and the stimulation and phosphorylation of one C5aR monomer is enough to lead to C5aR dimer internalization.

**EXPERIMENTAL PROCEDURES**

**Eukaryotic Expression Constructs**—The entire coding sequence of C5aR, including the 5′- and 3′-untranslated regions has been cloned into the expression vector pCDM8 (16). The construction of a plasmid for the expression of an NH2-terminally modified version of wild type C5aR, in which the 12 NH2-terminal amino acids were replaced by their counterparts in the formyl-peptide receptor, has been previously described (16). This NH2-terminally modified version of C5aR does not bind the complement fragment C5a and is named “mutant A” in this study. To create “C5aR-V5,” the COOH-terminal 12 amino acids of wild type C5aR were replaced by a V5 (GKIPPNPLGLDST) epitope. This COOH-terminally modified C5aR was generated by PCR using pCDM8-C5aR (16) as a template. A 5′-oligonucleotide corresponding to the pCDM8 sequence upstream of the HindIII site (5′- CGACT- CACTATAGGGAGACC-3′) and a 3′-oligonucleotide that deleted the COOH-terminal 12 amino acids of C5aR and introduced a SmaI site (5′-CTT ACC CGG GCC GGA CGT GAA TGA CTT ACC CCT CTC-3′) were used. The resulting PCR product was inserted in frame into the HindIII/SmaI sites of the pN3-V5 expression vector. This vector was derived from the eukaryotic expression vector pEGFP-N3 (Clontech) by introducing a V5 sequence in place of EGFP in pEGFP-N3. Two complementary nucleotides corresponding to the V5 sequence and designed to provide a SmaI site 5′ of the double-stranded DNA fragment (5′-GGG GTA CCC CAG ACC ACA GCC GG-3′) and a 3′-oligonucleotide that deleted the COOH-terminal 12 amino acids of C5aR and introduced a SmaI site (5′-CTT ACC CGG GCC GGA CGT GAA TGA CTT ACC CTC-3′) were hybridized and cloned into the SmaI/NotI sites of pEGFP-N3. To obtain “mutant A-V5,” a COOH-terminally truncated mutant A was generated by polymerase chain reaction using a 5′-oligonucleotide containing a KpnI site and retrieving the 5′-untranslated region of the mutant A construct in pCDM8 (5′-GGG GTA CCC CAG ACC ACA GCC GG-3′) and a 3′-oligonucleotide that deleted the COOH-terminal 12 amino acids of C5aR and introduced a SmaI site (5′-CTT ACC CGG GCC GGA CGT GAA TGA CTT ACC CTC-3′). The resulting PCR product was inserted in frame into the KpnI/SmaI sites of the pN3-V5 expression vector. To construct the phosphorylation-deficient C5aR mutant, named “mutant B-V5,” two complementary oligonucleotides (5′-CCAGGGCTTTCAGGGCGACTCGGGAAGGCCCT- CCCCAGCTCTCCGGAGAAGCTTGGCTGAGAGGC- CTGGTATAGGGAGCGACAAGCATTCCGGCCTGCCG- GCCC-3′ and 5′-GGGGGCCGGCGCGGCGGTGAGAGGAA- CGTGGTATAGGGAGCGACAAGCATTCCGGCCTGCCG- GCCC-3′) were used. The resulting PCR product was inserted in frame into the mutant A/V5 expression vector to create a chimera containing a COOH-terminal 12 amino acid deletion and an NH2-terminal 12 amino acid insertion. The two oligonucleotides were designed to provide an FseI site at the 5′-end of the double-stranded DNA fragment and a NotI site at the 3′-end. The DNA fragment was cloned into the C5aR-V5 expression vector between the FseI and NotI sites. A chimeric mutant named “mutant A/B-V5” was constructed by exchange of the Scal-NotI restriction fragment of the mutant B-V5 plasmid in the mutant A-V5 expression plasmid. The coding sequences of all mutated cDNAs were verified by automated DNA sequencing. The construction of β-arrestin 2-EGFP fusion has been previously described (8). All constructs were under the control of a cytomegalovirus promoter.

**Cell Culture and Transfection**—HEK293 cells were obtained from the American Type Culture Collection. These cells do not express C5aR. Cells were cultured at 37°C in a humidified atmosphere with 5% CO2 in Dulbecco’s modified Eagle’s/F-12 GlutaMAX I medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum. Culture medium, serum, and antibiotics were obtained from Invitrogen. Lipofectamine 2000® (Invitrogen) was used.
for transient DNA transfections. Cells were grown to reach ~90% confluence, and transfections were performed according to the manufacturer’s recommendations.

**Immunoprecipitation and Immunoblotting**—HEK293 cells were plated in polylysine-coated 6-well plates and transiently transfected with the appropriate plasmids. Two days after transfection, cells were lysed in non-denaturing ice-cold buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 15% glycerol) containing protease and phosphatase inhibitors (protease inhibitor mixture and phosphatase inhibitor mixture; Sigma). Following centrifugation (12,000 × g for 10 min), receptors were immunoprecipitated. Immunoprecipitation of C5aR and mutant A was performed with affinity-purified polyclonal anti-C5aR immunoglobulins (rabbit polyclonal antibody generated against the last 11 amino acid residues of C5aR (6)). Insoluble material was removed by centrifugation at 4 °C for 5 min at 15,000 × g. Immunoprecipitation of V5-tagged receptors was performed with anti-V5 antibody (Invitrogen). Lysates were incubated with 5 µl of antibody for 1 h at 4 °C prior to the addition of 20 µl of protein A/G plus agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and further incubation for 2 h at 4 °C. Immunoprecipitates were resolved by SDS-PAGE under reducing conditions (17) and transferred to nitrocellulose membranes for Western blotting. C5aR and mutant A were detected with polyclonal anti-C5aR antibodies. V5-tagged receptors were detected with monoclonal anti-V5 antibodies. Membranes were incubated with species-specific secondary antibodies conjugated to horseradish peroxidase, and immunoblots were developed using the ECL detection system (Amer sham Biosciences).

**Immunofluorescence Microscopy**—HEK293 cells were plated into polylysine-coated 8-chamber glass LAB-TEK® (Nalge Nunc International, Hereford, UK) and transiently transfected. Two days after transfection, cells were stimulated with 100 nM C5a Receptor Homodimer Endocytosis fragment C5a and, consequently, is unable to undergo the necessary conformational shift leading to both activation of signaling cascades and receptor phosphorylation. Substitution of the last 12 COOH-terminal amino acids of mutant A by a V5 tag sequence yielded mutant A-V5. To generate mutant B-V5, all serines and threonines of the COOH-terminal region of mutant A-V5 for that of mutant B-V5. The amino acid sequences of the NH2-terminal and COOH-terminal regions of wild type C5aR and mutants A/B-V5 are represented. The anti-C5aR epitope is underlined.

**RESULTS**

**Design of the C5aR Mutants**—To examine the significance of receptor oligomerization for homologous down-regulation of C5aR, we made use of several C5aR mutants that are schematically depicted in Fig. 1. The mutant receptor, referred to as mutant A, was generated by replacing the 12 NH2-terminal amino acids by their counterpart in the formyl-peptide receptor. This mutant is unable to bind the complement fragment C5a. Substitution of the last 12 COOH-terminal amino acids of mutant A by a V5 tag sequence yields mutant A-V5. To generate a phosphorylation-deficient mutant (mutant B-V5) all serine and threonine of C5aR Phosphorylation Assays—HEK293 cells were plated in polylysine-coated 6-well plates and transiently transfected with the appropriate plasmids. Three days after transfection, the cells were washed twice with phosphate-free buffer and metabolically labeled with [32P]orthophosphoric acid (0.3–0.5 mCi/ml), as previously described (18). Phosphorylation of C5aR was initiated with 50 nM C5a. After 15 min of incubation at 37 °C, the cells were lysed in 500 µl of ice-cold buffer (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 1% SDS) supplemented with 1 µg/ml each aprotinin, leupeptin, and pepstatin, 1 mM benzamidine, 10 mM NaF, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hypochloride, 0.1 µM okadaic acid, and 1 mM sodium orthovanadate. Insoluble material was removed by centrifugation at 4 °C for 5 min at 15,000 × g. Immunoprecipitation assays were performed by incubating cell lysates with 5 µl of affinity-purified anti-C5aR or anti-V5 monoclonal antibody that were preadsorbed on 20 µl of protein A/G plus agarose. The immunoprecipitates were extensively washed and analyzed by SDS-PAGE under reducing conditions and autoradiography.
the COOH-terminal domain downstream of the seventh transmembrane domain were replaced by alanine, and a V5 tag sequence replaced the last 12 amino acids. According to previous reports (6, 7), this phosphorylation-deficient C5aR mutant binds C5a, undergoes conformational changes, and transduces intracellular signals but is not phosphorylated.

Finally, a chimeric mutant referred to as mutant A/B-V5 was constructed by exchanging the COOH-terminal region of mutant A-V5 for that of mutant B-V5. This mutant is unable to bind the ligand and transduce intracellular signals through the heterotrimeric G protein. In addition, it behaves as a phosphorylation-deficient receptor in that it cannot be phosphorylated. All DNA constructs were under the control of the same cytomegalovirus promoter to ensure a similar level of expression of the different proteins.

Formation of Wild Type and Mutant C5aR Heterodimers—Previous studies based on a disulfide trapping strategy in mammalian cells (12) and bioluminescence resonance energy transfer technology in yeast (11) have provided evidence that C5a receptors form homo-oligomers. By co-immunoprecipitation analysis, Huttenrauch et al. (7) have shown that C5aR also associates as a heterodimer with a related chemotactic receptor (CCR5). Since it is well established that C5aR can form homo- and hetero-oligomers in living cells, we controlled the formation of heterodimers between wild type C5aR and the mutant receptors by co-immunoprecipitation analysis. We used antibodies that specifically reacted with COOH-terminal epitopes on the respective receptor constructs. An anti-C5aR, directed against the last 11 amino acid residues of C5aR (6), recognized wild type C5aR and mutant A, and a commercially available anti-V5 recognized V5-tagged C5aR variants.

In a first experiment, wild type C5aR and a C5aR construct in which the 12 terminal amino acids have been replaced by a V5 epitope (C5aR-V5) were co-expressed in HEK293 cells. Two days after transfection, cells were lysed by treatment with detergent-containing buffer under non-denaturing conditions. Receptors were immunoprecipitated with antibodies against one receptor epitope (i.e. immunoprecipitation of C5aR was performed with polyclonal anti-C5aR antibodies, and C5aR-V5 was immunoprecipitated with monoclonal anti-V5 antibodies). The immunoprecipitates were subjected to SDS-PAGE under reducing conditions and immunoblotted with antibodies against the other receptor. As shown in Fig. 2, immunodetection of C5aR-V5 with anti-V5 antibodies, following C5aR immunoprecipitation using anti-C5aR antibodies, indicated that C5aR-V5 was co-immunoprecipitated with C5aR. Conversely, C5aR was successfully co-precipitated from the same lysate using anti-V5 antibodies. In contrast, no receptors were detectable in immunoprecipitates prepared under the same conditions from untransfected cells or cells that express either receptor alone (not shown). This indicates that heterodimers (oligomers) preexisted in cells that co-expressed both receptors and that the formation of C5aR dimers was not impaired by the replacement of the 12 COOH-terminal amino acids by a V5 epitope.

Wild type C5aR was co-expressed with mutant A-V5, mutant B-V5, or mutant A/B-V5. Mutant A was co-expressed with mutant B-V5. As shown in Fig. 3, wild type C5aR can form dimers with all forms of mutants. In addition, the NH2-terminally modified mutant (mutant A) can form dimers with the phosphorylation-deficient mutant (mutant B-V5). These results indicate that neither an intact NH2 terminus nor the presence of COOH-terminal phosphorylation sites appear to be required for the formation of C5aR homo-oligomers.
**C5a Receptor Homodimer Endocytosis**

Agonist-induced Endocytosis of Wild Type C5aR and Mutant Receptors Individually Expressed in HEK293 Cells—We observed previously that C5aR expressed in RINm5F cells is internalized in a rapid, β-arrestin-dependent manner and that the receptor and β-arrestin 1 and 2 co-localized in perinuclear recycling endosomes (8). In contrast, phosphorylation-deficient receptors do not internalize and do not associate with β-arrestins upon agonist stimulation.

Wild type C5aR and V5-tagged C5aR mutant A were individually co-expressed in HEK293 cells with a β-arrestin 1-EGFP fusion protein (β-Arr1-EGFP). As shown in Fig. 4, A and B, by immunofluorescence microscopy (as described under “Experimental Procedures”), in unstimulated cells, both receptors were detected at the plasma membrane (red fluorescence). The immunodetections of C5aR and mutant A-V5 were performed with polyclonal anti-C5aR antibodies and monoclonal anti-V5 antibodies, respectively, and red fluorescent Alexa 568-conjugated goat anti-rabbit antibody or anti-mouse antibody. β-Arr1-EGFP was evenly distributed in the cytoplasm (green fluorescence). After exposure to 100 nM C5a for 30 min, wild type C5aR was internalized in perinuclear endosomes, where it colocalized with β-arrestin (Fig. 4A). Mutant A-V5 did not internalize (Fig. 4B), and there was no relocalization of β-arrestin.

Mutant B-V5 and mutant A/B-V5 are phosphorylation-deficient mutants. Since a co-expression with β-arrestin was not relevant, they were expressed individually in HEK293 cells. As seen in Fig. 4, C and D, both mutants were detected at the plasma membrane in unstimulated cells, and, as expected, they did not internalize when cells were stimulated with C5a.

**Agonist-induced Endocytosis of Wild Type and Mutant C5aR Heterodimers**—The cDNAs encoding wild type C5aR and mutant receptors are under the control of the same cytomegalovirus promoter in the expression plasmids. In the co-transfection experiments, the same concentration of each plasmid was used. We could thus expect that, when two different receptors are expressed, their levels of expression would be similar. The extent of heterodimer versus homodimer formation could thus be expected to be roughly 50%.

As illustrated by immunofluorescence microscopy in Fig. 5A, when wild type C5aR and V5-tagged mutant A were co-expressed, both C5aR (in green) and V5 tagged mutant A (in red) were present at the cell surface of unstimulated cells (Fig. 5A). After exposure to 100 nM C5a for 30 min, C5aR and V5-tagged mutant A were both internalized and co-localized in perinuclear endosomes, as indicated by the yellow color in the overlay. Similarly, when wild type C5aR and V5-tagged mutant B were co-expressed, both receptors were present at the cell surface of unstimulated cells (Fig. 5B), whereas, after exposure to 100 nM C5a for 30 min, C5aR (in green) and mutant B-V5 (in red) were both internalized and co-localized in perinuclear endosomes as evidenced by the yellow color in the overlay.

The V5-tagged C5aR chimeric mutant A/B was also co-expressed with wild type C5aR in HEK293 cells. As seen in Fig. 5C, both receptors were present at the cell surface in unstimulated cells. After exposure to 100 nM C5a for 30 min, C5aR and mutant A/B-V5 were both internalized and co-localized in perinuclear endosomes, as indicated by the yellow color in the overlay.

**Stimulation of the Phosphorylation-deficient Mutant B Does Not Induce Internalization of the NH2-terminally Modified Mutant A**—The NH2-terminally modified mutant A, which cannot undergo ligand-induced conformational change and phosphorylation, was...
co-expressed with the V5-tagged phosphorylation-deficient mutant B that, like wild type C5aR, can bind the C5a anaphylatoxin. Both receptors were present at the cell surface of unstimulated cells, as illustrated in Fig. 5D. Agonist stimulation of mutant B did not induce internalization of mutant A/mutant B-V5 heterodimers, both receptors remaining at the cell surface after 30 min of stimulation by C5a.

Absence of Cross-phosphorylation within the C5aR Dimers—According to a classical model of monomeric GPCR regulation, only ligand-occupied receptors are phosphorylated by GRKs. However, receptors can be phosphorylated by second messenger-activated kinases, such as PKC, in a heterologous ligand-independent manner. By using a functional complementation approach, Huttenrauch et al. (7) have demonstrated that a CCR5 NH2-terminally truncated mutant, deficient in ligand binding, undergoes GRK-mediated phosphorylation if this receptor is co-expressed together with a different mutant that can recruit receptor kinases to the CCR5 homo-oligomer at the plasma membrane. In addition, in cells co-expressing C5aR and CCR5, GRKs have been shown to phosphorylate unliganded receptors within C5a activated C5aR/CCR5 hetero-oligomers.

Substitution of the last 10 COOH-terminal amino acids of the C5aR by a V5 tag destroys the two last threonines of the COOH-terminal domain. The GD sequence immediately upstream of the V5 epitope, due to the introduction of a Small site into the cDNA, destroys an additional threonine. None of these residues has been found to be a phosphoacceptor site or to play a role in C5aR phosphorylation (6, 10). Nevertheless, we first determined whether the modifications generated by the introduction of the V5 tag could interfere with the receptor phosphorylation by assessing the C5a-mediated phosphorylation of C5aR-V5 in HEK293 cells metabolically labeled with [32P]orthophosphoric acid. As seen in the autoradiogram presented in Fig. 6A, stimulation with C5a induced an efficient 32P incorporation into C5aR-V5.

To further examine the significance of dimer formation for a possible receptor cross-phosphorylation, mutant A-V5, which is unable to bind C5a but can potentially be phosphorylated, was co-expressed with wild type C5aR in HEK293 cells. Cells were labeled with [32P]orthophosphoric acid and stimulated with 100 nM C5a for 30 min. The disruption of cell membranes by lysis buffer in the presence of 1% SDS resulted in the dissociation of receptor complexes and thus allowed us to selectively document phosphorylation of the resulting receptor monomers. Monomers (C5aR or mutant A-V5) were immunoprecipitated with the appropriate antibody, and phosphorylation was detected by autoradiography. As shown in Fig. 6B, C5aR was efficiently phosphorylated, but there was no 32P incorporation in mutant A-V5. No trace of phosphorylated C5aR was visible, indicating that dimer disruption occurred in the solubilization step. In parallel, the phosphorylation-deficient mutant B-V5, which is able to bind C5a but lacks COOH-terminal phosphorylation sites, was co-expressed with the NH2-terminally modified mutant A, which does not bind the ligand but has intact phosphorylation sites. As previously, monomers were immunoprecipitated with the appropriate antibodies, and phosphorylated receptors were detected by autoradiography. As shown in Fig. 6C, there was no 32P incorporation in mutant A.

These results indicate that an unliganded C5aR cannot undergo phosphorylation even if this receptor is in close proximity to another C5a-occupied receptor at the plasma membrane. The absence of cross-phosphorylation also explains why the stimulation of mutant B did not induce internalization of mutant A/mutant B-V5 heterodimers. In conclusion, we can postulate that the stimulation and phosphorylation of one monomer is enough to lead to C5aR dimer internalization and that the second monomer is carried along with the first one.

DISCUSSION

Until recently, GPCRs were believed to exist and to function as monomeric entities. However, many findings have indicated that most GPCRs do not exist as monomers but rather as
C5a Receptor Homodimer Endocytosis

dimers or potentially higher order oligomers (19, 20). The recognition that GPCRs may exhibit either dimeric or oligomeric structures is based upon a large body of biochemical and biophysical evidence (reviewed in Refs. 21 and 22). Western blotting and immunodetection identified molecular species of higher molecular mass than expected for receptor monomers. More specific techniques involved the co-immunoprecipitation of differentially epitope-tagged and co-expressed forms of the same receptor. More recently, a variety of biophysical techniques, such as bioluminescence resonance energy transfer and fluorescence resonance energy transfer, have been used to detect the proximity of GPCR monomers (23). Class C GPCRs are known to be obligatory dimers. In the case of the GABA<sub>B</sub> receptor, oligomerization is required for receptor trafficking to the cell surface and G protein activation (24–27). For several GPCRs of the rhodopsin family (class A receptors), including the C5a receptor (11) and the angiotensin II type I receptor (28), plasma membrane targeting has been attributed to oligomeric structures that constitute the trafficking unit of the receptors. Oligomerization appears essential for receptor folding and for further processing as well as for transport to the plasma membrane. C5αR oligomerization occurs early in the biosynthesis of the receptor, and constitutive oligomers transit through the secretory pathway (11). Oligomerization may thus be important in the terminal glycosylation and proper cellular targeting of the receptor. In this paper, we confirmed, by co-immunoprecipitation of differentially epitope-tagged C5α receptors co-expressed in HEK293 cells, that C5αR can form dimers or higher order oligomers. These structures were stable in the detergent-containing lysis buffer (1% Nonidet P-40) without the need to employ cross-linking methods to trap complexes during immunoprecipitation. Wild type C5αR formed dimers either with the NH<sub>2</sub>-terminally modified mutant A or with the phosphorylation-deficient mutant B. In addition, the latter formed dimers with the NH<sub>2</sub>-terminally modified mutant A. Therefore, neither an intact NH<sub>2</sub> terminus nor the phosphorylation of the COOH-terminal cytoplasmic domain is required for the formation of C5αR homo-oligomers. A disulfide trapping strategy has revealed potential contact surfaces in C5αR dimers (12). The molecular architecture of C5αR is likely to be very similar to that of bovine rhodopsin, which is the first GPCR whose crystal structure has been elucidated (29) and has been shown to exist as arrays of dimers (30, 31). Mutagenesis analysis in the second and fourth transmembrane domains of C5αR has allowed the identification of essential residues that may be involved in contact interfaces. These residues map into positions in rhodopsin that could mediate di(oligo)mer interactions (12). The disulfide trapping results suggest two possible helical orientations, a transmembrane helix 4-symmetric C5αR dimer or a transmembrane helix 1,2-symmetric C5αR dimer, and the likely formation of higher order oligomers.

Many GPCRs exhibit physical oligomeric interactions (21, 32, 33). GPCR oligomerization has been proposed to play a role in receptor biosynthesis. Oligomeric structures might also constitute the functional unit of the receptors and be essential for the function and regulation of the receptors, once they are brought to the cell surface. This question is a matter of debate (34, 35). Agonist binding is associated with conformational changes in the tertiary structure of the receptor that are recognized by the associated intracellular partners, in particular the heterotrimeric G-proteins (36, 37). Monomeric rhodopsin (38, 39) and β<sub>2</sub>-adrenergic receptor (40), at very low detergent concentration or in lipids, efficiently activate their G protein. Studies on obligatory dimer class C receptors suggest that only one receptor within a dimer binds the G protein. For example, it has been shown that a single subunit, within the homodimeric glutamate receptors, reaches an active conformation upon activation (41, 42). For class A receptors, many reports consider separate stimulation of receptor homo- or heterodimers to be sufficient for the activation of heterotrimeric G protein with no need for both subunits to be turned on. It has been shown for the leukotriene B4 receptor that the activation of one receptor in a dimer is sufficient for G protein coupling (43, 44). Purified leukotriene B4 receptor forms a stoichiometrically defined complex consisting of two receptors and one G protein (45). In addition, heterodimerization between receptors that bind to distinct G proteins quite often leaves their coupling selectivity unaltered. This indicates an asymmetric functioning of GPCR dimers (43, 46). On the contrary, other studies point to the receptor dimer as the minimal structural configuration necessary for receptor function. For example, paired activation of two receptor monomers within muscarinic M3 dimers is required to stimulate ERK1/2 phosphorylation and for the recruitment of β-arrestin 1 to the site of the activated receptor (47, 48). A similar debate has been launched about the stoichiometry of receptor-β-arrestin binding. From the known structures of rhodopsin (29) and β-arrestin (49), a model in which one β-arrestin binds two receptors has been proposed (31). However, the stoichiometry of the arrestin-rhodopsin complex has been recently determined in in vitro experiments with purified proteins (50) and is more consistent with β-arrestin binding to one receptor monomer.

Following activation, GPCRs are specifically phosphorylated by GRKs. The activated receptor acts as both GRK activator and GRK substrate. Interaction of Gβγ with GRK represents a mechanism for targeting the kinase at the plasma membrane for an efficient phosphorylation of agonist-occupied receptors (51). It has been shown that rhodopsin kinase, upon phosphorylating an activated receptor, trans-phosphorylates nearby nonactivated rhodopsin (52). Using rhospho-site-specific antibodies, it has been shown that stimulation with CCL5 of cells that co-expressed the chemokine receptor CCR5 together with an NH<sub>2</sub>-terminally truncated CCR5 mutant resulted in both second messenger-activated protein kinase C- and GRK-mediated cross-phosphorylation of the unliganded mutant receptor (7). Furthermore, both protein kinase C and GRK cross-phosphorylate CCR5, in a heterologous manner, after C5α stimulation of cells co-expressing the chemokine receptor CCR5 and the C5αR (7). One can thus assume that, if co-expressed receptors form dimeric structures, the activation of one monomer would result in the phosphorylation of the other. Moreover, elimination of protein kinase C and/or GRK phosphorylation sites on CCR5 does not inhibit C5α-induced co-internalization of CCR5 in cells where CCR5 and C5αR were co-expressed (7). This suggests that co-internalization of CCR5 after heterologous stimulation does not require cross-phosphorylation but
rather depends on the physical interaction between the two receptors.

In our study, the co-expression of wild type C5aR with the NH2-terminally modified mutant A led to the formation of heterodimers. The same was true when mutant A was co-expressed with the phosphorylation-deficient mutant B. In both cases, after C5a stimulation of the cells, we did not observe any cross-phosphorylation of the nonactivable mutant A (Fig. 6). Since the presence of a V5 tag did not affect the ability of the C5aR to be phosphorylated (Fig. 6A), we can conclude that, in the case of the co-expression of C5aR and V5-tagged mutant A, there was no cross-phosphorylation within the heterodimer. The same was true when both mutant receptor A and B were co-expressed.

Although the nonphosphorylatable mutant B binds C5a and is able to undergo the necessary conformational changes for G protein activation, cross-phosphorylation of mutant A was not observed, although intact phosphorylation sites are present in its COOH-terminal tail and despite the proximity of the two mutant receptors within heterodimers. Although mutant A was unable to bind C5a and was refractory to cross-phosphorylation, it did co-internalize with wild type C5aR upon C5a stimulation, as shown in Fig. 5. Likewise, the phosphorylation-deficient mutant B was co-internalized with wild type C5aR. This suggests that neither the phosphorylation of the two monomers within the heterodimer nor the conformational change induced by the binding of C5a is required for receptor internalization. The early steps of the internalization process involve the formation of a complex at the plasma membrane between phosphorylated agonist-occupied receptor and β-arrestin, which, in turn, recruits components of the internalization machinery, namely clathrin and AP2. The results presented herein revealed that the phosphorylation of only one agonist-occupied monomer is sufficient to engage the internalization of the C5aR di(oligo)meric structures. In contrast, mutant A was not internalized when co-expressed with the phosphorylation-deficient mutant B, most probably because neither of the two monomers that form the heterodimers is able to be phosphorylated and to interact with β-arrestin. This result contrasts with the study performed by Huttenrauch et al. (7), where it was observed that a phosphorylation-deficient mutant of CCR5 co-internalized with a NH2-terminally truncated CCR5-ΔNT mutant deficient in ligand binding. This difference can be explained by the fact that the CCR5-ΔNT mutant was cross-phosphorylated by GRK and PKC following agonist-mediated activation of the phosphorylation-deficient CCR5 mutant.

In conclusion, we provide evidence that, in the case of C5aR homodimers, the activation and phosphorylation of one monomer is sufficient to lead to dimer internalization. One major consequence of this is that, at a low concentration of C5a, C5aR is efficiently internalized, provided it is part of a dimeric/oligomeric complex where one monomer has bound C5a and is phosphorylated. The existence of such a mechanism may be important for an accurate C5aR down-regulation in pathological conditions.

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C5a Receptor Homodimer Endocytosis

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