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Stabilization of C5a Receptor–G-Protein Interactions Through Ligand Binding

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Abstract Binding of biotin-C5a to the C5a receptor in membrane fragments followed by detergent solubilization *and purification* with streptavidin-agarose affinity chromatography resulted in the isolation of a receptor complex with associated G-proteins. In contrast, when receptor was detergent-solubilized in the absence of C5a and purified by affinity chromatography with Affigel-C5a, G-proteins did not copurify. Since the results indicate that receptor ligation stabilized the receptor–G-protein interaction to allow purification of the complex, the findings emphasize the dynamic nature of the C5a receptor–effector interactions. When biotin-C5a–ligated receptor was purified from a mouse cell line overexpressing recombinant human receptor, both $G_i$alpha$_2$ and $G_i$alpha$_3$ subunits copurified, confirming that multiple transducing systems are linked to the C5a receptor. The method of stabilization of receptor-transducer complexes offers the opportunity to further elaborate the interactions of the C5a receptor with diverse transducing elements and second messenger systems.

Key words: C5a, ligand binding, G-protein, second messenger systems, neutrophils, signal transduction, receptor

The class of receptors referred to as seven-transmembrane spanning receptors, which includes the beta adrenergic and numerous other receptors for peptide and nonpeptide ligands, has been extensively characterized by both protein chemical methods and genetic analysis [5,15,30]. Still, the exact nature by which these receptors interact with transducing proteins and achieve cellular responses is not completely understood. It is not clear, for example, how several second messenger responses emanate from a single receptor, nor how various second messengers act in concert to achieve complex cellular responses. Current theories of transmembrane signaling have focused on the specificity of receptor–guanyl nucleotide regulatory protein (G-protein) interactions in this context [2,9,13,23,28,33].

Abbreviations: biotin-C5a, biotin-xx-succinimidyl ester modified *C5a; C5aR, C5a* receptor; G-protein, a member of the guanyl nucleotide regulator protein family; GppNp, guanylylimidodiphosphate; GTPgammaS, guanosine 5′-O-(3-thiodiphosphate); ^{125}I-C5a, ^{125} I-Bolton-Hunter-labeled C5a.

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The C5a receptor (C5aR) present on human neutrophils is a useful model system for the study of cellular response mechanisms. Its purification [25] and cloning have recently been reported [1,8,10]. This peptide receptor activates various second messenger systems such as calcium release, phosphatidylinositol turnover, and phospholipase D activation [18,27], and these systems lead to a variety of cellular responses including chemotaxis, shape change, adherence, vesicle release, and superoxide production [3,4,17,19,21,34]. Although G-proteins are known to be involved in C5aR-induced responses [26] and human neutrophils are known to be rich in G_{i2} and G_{i3} alpha subunits as well as the hematopoietic cell specific G_{16} subunit [7,14,22], it is not clear which transducing proteins interact with the C5aR and how they are positioned in space and time. Likewise, it is not clear how these G-proteins and other transducing systems transmit receptor information to achieve neutrophil responses.

The work presented here describes a novel method for the isolation of the C5aR which has been used to elucidate the nature and strength of receptor–G-protein associations. The method has revealed the dynamic nature of the interaction of the C5aR with G-proteins and should

facilitate further understanding of how the C5aR interacts with diverse second messenger components during cell activation.

MATERIALS

All chemicals were highest purity available and were obtained from Sigma (St. Louis, MO) unless noted otherwise. Unless noted otherwise, C5a refers to human recombinant C5a(Thr1Met, 1-74). Streptavidin-agarose and 6-{6-biotinoylamino)hexanoylamino}hexanoic acid, succinimidyl ester (biotin-xx-succinimidyl ester) were from Molecular Probes (Eugene, OR). ^{125}I-C5a(1-74) was from New England Nuclear/Dupont (Boston, MA). Digitonin was from Gallard-Schlesinger (Carle Place, NY). Antisera to the C-terminal 23 amino acid sequence of the C5aR were produced in rabbit by Babco (Richmond, CA) using a synthetic peptide conjugate at the N-terminal to KLH. This antibody was purified with a Protein-G HPLC column (Chromatochem, Missoula, MT). The nucleotide regulatory protein antibodies to alpha G_{i3} and to G_{i2} were from CalBiochem (San Diego, CA) or Dupont/NEN; the antibodies had some crossreactivity to G_o and G_{i1}, respectively, as noted by the manufacturer. Similar results were obtained using antibodies from the two suppliers. Guanosine 5′-O-(3-thiodiphosphate) (GTPgammaS) and guanylylimidodiphosphate (GppNp) were from Sigma. SDS-PAGE gels were from Bio-Rad (Hercules, CA) or Daiichi and protein standards from Bio-Rad. C127 and U937 cells were from ATCC (Rockville, MD). RPMI and DMEM media were from GIBCO (Gaithersburg, MD). Preparation of biotin-labeled C5a with the amino-group specific labeling reagent biotin-xx-succinimidyl ester will be described elsewhere [Wennogle et al., in preparation].

METHODS

Cell Culture and Isolation

U937 cells were cultured at 37°C in tissue flasks in RPMI 1640 medium containing penicillin-streptomycin and 10% fetal bovine serum. Cells were differentiated by the addition of 1 mM dibutryl-cyclic AMP for 72 h. C127 cells, a mouse mammary tumor cell line, were cultured in DMEM containing penicillin-streptomycin and 10% fetal calf serum and passaged by trypsin treatment. C127-C5aR cells, a stable transfected C127 cell line overexpressing recombinant human C5a receptor, were maintained in the presence of 0.4 mg/ml G418. Human neutrophils were obtained from whole blood of healthy donors [6].

Recombinant Human C5a Receptor Cloning and Expression

A human C5aR cDNA was isolated by PCR from a cDNA library constructed using mRNA from differentiated U937 cells and probes based on the published sequence [10]. C5aR cDNA was subcloned into an expression vector pBPV-MMT which contains 69% of the bovine papillomavirus-1 genome (a BamHI/HindIII fragment), and transcription of the inserted cDNA was driven by the mouse metallothionein 1 gene promoter. This construct was cotransfected with plasmid pSV2neo at a ratio of 20 to 1 into C127 cells. Stable transformants were selected with Geneticin (G418) at a concentration of 0.40 mg/ml. Clones which expressed C5a receptors were then identified from the stable transformants by ^{125}I-C5a binding or by Western blot analysis.

Preparation of Purified C5aR

All manipulations for membrane preparations and receptor purification were carried out in an ice bath in polypropylene tubes. In order to prepare membranes, cells were washed with phosphate buffered saline and then collected by scraping (C127-C5aR cells) or sedimentation (U937 cell or human neutrophils). Receptor isolation buffer (20 mM Hepes, pH 7.4, 1 mM EDTA, 0.1 mM PMSF, 10 μg/ml aprotinin, chymostatin, leupeptin, and pepstatin) was used for all steps of the purification, supplemented as noted below. Cells were suspended to 1×10^8 cells/ml and disrupted by sonication. Membranes were pelleted at 15,000 rpm in a Sorvall SS34 rotor and resuspended to 1×10^8 cell equivalents/ml. Membranes could be frozen indefinitely at −80°C.

Preparation of Soluble Receptor and Biotin-C5a–Solubilized Receptor

To produce biotin-C5a–solubilized receptor, 5 ml of the membrane suspension was added to 5 ml of binding buffer containing 50 μg of biotin-C5a, and the suspension was sonicated. At this concentration, the peptide is present in approximately a fivefold molar excess to the receptor in C127-C5aR cells. After 1 h incubation, the membranes were centrifuged and the pellet resuspended to 5 ml in receptor isolation buffer. Mem-

branes were solubilized by the addition of 1.5 volumes of 1.5% digitonin in receptor isolation buffer supplemented with 5% glycerol and 0.002% lecithin (egg, Avanti). The suspension was sonicated, incubated for 1 h, and centrifuged at 100,000*g* for 60 min, and then the clear supernatant was decanted and used as the solubilized receptor fraction. In the case of C127-C5aR cells containing recombinant human C5aR, detergent-solubilized material contained 12% of the protein content of whole cells. Although this soluble receptor fraction was stable for several days at 4°C, solubilized receptor was made fresh each day and kept at 4°C at all times. For experiments with unligated soluble receptor, a similar protocol was followed, but without the biotin-C5a addition.

Purification of C5aR by Affinity Chromatography With Streptavidin-Agarose

For all further steps, the receptor isolation buffer was supplemented with 0.05% digitonin, 5% glycerol, and 0.002% lecithin. One milliliter of streptavidin-agarose beads was washed and resuspended with 12.5 ml of solubilized receptor and incubated from 2 h to overnight with rotation. Care was taken to minimize aeration. The beads were then centrifuged and washed successively two times with 15 ml of receptor isolation buffer containing 5 mM $MgCl_2$, one time with buffer containing 0.25 M NaCl and three times with buffer alone. After the last wash, the beads were suspended in 1.5 ml of 3 M potassium thiocyanate (2 M final) and incubated for 10 min with shaking. The buffer was decanted, and purified receptor was immediately desalted by passing through a PD10 gel filtration column (Pharmacia, Piscataway, NJ, prepacked G25) preequilibrated with receptor isolation buffer supplemented with 5 mM $MgCl_2$. The purified receptor was concentrated to 200 μl using an Amicon Centricon-30. This material was stored at 4°C for 2–3 weeks with less than 50% loss of activity. The affinity purification can also be performed using a column method, but only a minimal increase in purity was noted. Care had to be taken to wash the affinity beads well to avoid contaminating proteins. Due to the minute quantity of receptor material, disposable polypropylene labware, new streptavidin-agarose and new PD10 gel filtration columns were used for each experiment.

Starting with 5×10^8 C127 cells typically 1–2 μg of 42–44,000 dalton band was isolated, as estimated via silver staining of SDS-PAGE gels and comparison to standard proteins. Although solubilization of C5aR from neutrophils or U937 cells occurs routinely with an efficiency of between 35 and 50%, solubilization of receptor from C127 cells was lower than 10%. The reason for this low solubilization efficiency is unclear, but this decreased efficiency lowers the overall yield of receptor protein from these cells. Using the ^{125}I-C5a binding assay to quantitate recovery relative to the quantity of crude solubilized receptor, the yield of purified receptor in five separate experiments was 24.3 ± 5.6%. Two factors contributed to this efficiency of recovery. Typically 25% of the receptor sites remained unoccupied by biotin-C5a after it was incubated with membranes. After loading the affinity column, another 25% of biotin-C5a–C5aR could be found in the column flow through, not binding to the affinity matrix. It is unclear why these steps were not quantitative as excess biotin-C5a and excess streptavidin-agarose were used. Perhaps certain C5a-biotin species were not accessible to streptavidin due to steric hindrance. In addition to the biotin-C5a reagent, a variety of other biotin-C5a reagents were tested in receptor purification, including biotin incorporated into Cysteine-27 via sulfhydryl modification and the use of biotin reagents with smaller—six carbon—spacer lengths. These reagents were successfully used to purify receptor, and recoveries with different reagents were similar to those reported here.

Purification of C5aR by Affinity Chromatography With Affigel-C5a

An alternate method for the isolation of C5aR used C5a linked to Affigel-10. The procedure was similar to that described above, but without ligation of the receptor with biotin-C5a and with the use of Affigel-C5a beads in place of streptavidin-agarose. Affigel-10 (Bio-Rad) was coupled to 1 mg/ml C5a in 0.1 M HEPES, pH 8.0, at 4°C for 2 h; remaining sites on the resin were inactivated with addition of 1 M ethanolamine and continued overnight incubation followed by extensive washings.

Recovery of Receptor From Biotin-C5a–C5aR Complexes

In order to judge the recovery of C5aR from the affinity matrix, receptor not absorbing to the streptavidin-agarose was quantitated by the regeneration of C5a binding activity from biotin-

C5a–solubilized receptor complexes. In order to achieve this regeneration, the material was made 2 M potassium thiocyanate chaotrope and passed through a AcA 34 (LKB) column to remove the biotin-C5a and chaotrope from active receptor eluting in the void volume.

C5a Binding Assay

A binding assay used to quantify soluble receptor employed polyethylene glycol precipitation in the presence of gamma globulin carrier protein and was a variation of that reported by Rollins et al. [24]. The binding buffer contained 50 mM Hepes, pH 7.2, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.5% BSA (Sigma A-7511), 0.1% bacitracin, and 0.1 mM PMSF. In a volume of 250 μl, 30,000 cpm of ^{125}I-C5a (approximately 20 pM) was combined with receptor in polypropylene tubes and incubated at 4°C. After 1 h, 500 μl 0.1% gamma globulin (Cohn fraction II and III) and 500 μl 30% PEG 8000 were added followed by vigorous vortexing. The suspension was centrifuged for 10 min at 10,000 rpm in a Sorvall GSA rotor, the supernatant containing free radioligand removed, and radioactivity in the pellets determined. The assays were performed in triplicate. Nonspecific binding was defined by including 1 μg/ml of unlabeled C5a in the assay, and the resulting nonspecific values were subtracted from total binding to determine specific binding. Specific binding typically represented 85% of the total binding, and triplicate determinations were generally within 5% of one another.

Radioligated Receptor Stability Studies

^{125}I-C5a–ligated C5aR was produced by incubating membrane preparations in binding buffer with ^{125}I-C5a for 1 h at 4°C followed by washing via centrifugation and solubilization with 1% digitonin, using the method outlined above (Preparation of Soluble Receptor). The stability of this radioligated receptor was tested using two methods to separate receptor-bound ^{125}I-C5a from free ^{125}I-C5a: precipitating the ligated receptor with polyethylene glycol (see C5a Binding Assay, or by a Superose-12 gel filtration column (Pharmacia) run at 1 ml/min in 20 mM Hepes, pH 7.4, 1 mM EDTA, 2 mM $MgCl_2$, 0.05% digitonin.

Miscellaneous Methods

Digitonin was solubilized by heating a 5% suspension with constant mixing to near boiling until the solution cleared, followed by filtration through Whatman #1 paper. This stock was stable for several weeks at 4°C, and minimal clouding occurred. Sonication of samples was performed by three 10 second bursts of a Branson probe sonicator at setting 1, with 1 min of cooling on ice between bursts. Proteins were measured by a coomassie blue protein reagent (Bio-Rad). Tris-Laemmli SDS-PAGE, silver staining, and Western analyses were performed using Bio-Rad products and standard procedures recommended by the manufacturer.

RESULTS

We established a mouse mammary tumor cell line overexpressing human C5a receptors as a source for soluble receptor activity. The line (C127-C5aR) expressed 2×10^6 C5a binding sites per cell, and these sites had an affinity for C5a which was equivalent to that of receptors found on human neutrophils. C5a-induced (^{3}H)phosphatidylinositol release was demonstrated in these cells, indicating proper coupling to signal transduction systems (not shown). Although the absolute magnitude of the C5a-induced phosphatidyl inositol turnover in C127-C5aR cells was lower than that demonstrated with U937 cells (2.0 ± 0.6– vs. 9.9 ± 2.0–fold stimulation over basal, n = 3), C5a stimulated the response with similar potency in both cell types (K_{act} = 1.3 ± 0.5 nM vs. 4.8 ± 0.9 nM, n = 3).

To isolate the C5a receptor, biotin-C5a was first bound to receptor-rich membranes of C127-C5aR cells. After treatment with 1% digitonin, solubilized biotin-C5a–C5aR complexes were enriched by affinity chromatography using streptavidin-agarose. Receptor was dissociated from the streptavidin-bound biotin-C5a at neutral pH with chaotrope (2 M KSCN) and rapidly desalted by gel filtration. The receptor from C127 cells was purified over 2,000-fold from solubilized receptor by the biotin-C5a–streptavidin-agarose method with a yield of 24% relative to soluble receptor. The purified receptor was tested for its ability to bind ^{125}I-C5a, and displacement studies were performed with C5a(1-73) using various receptor fractions (Fig. 1). The displacement curves for C5a(1-73) were identical for the three forms of the C127-C5aR receptor: membrane-bound, crude detergent-solubilized, and purified.

SDS-PAGE analysis of receptor from C127-C5aR cells (Fig. 2A) revealed three major bands

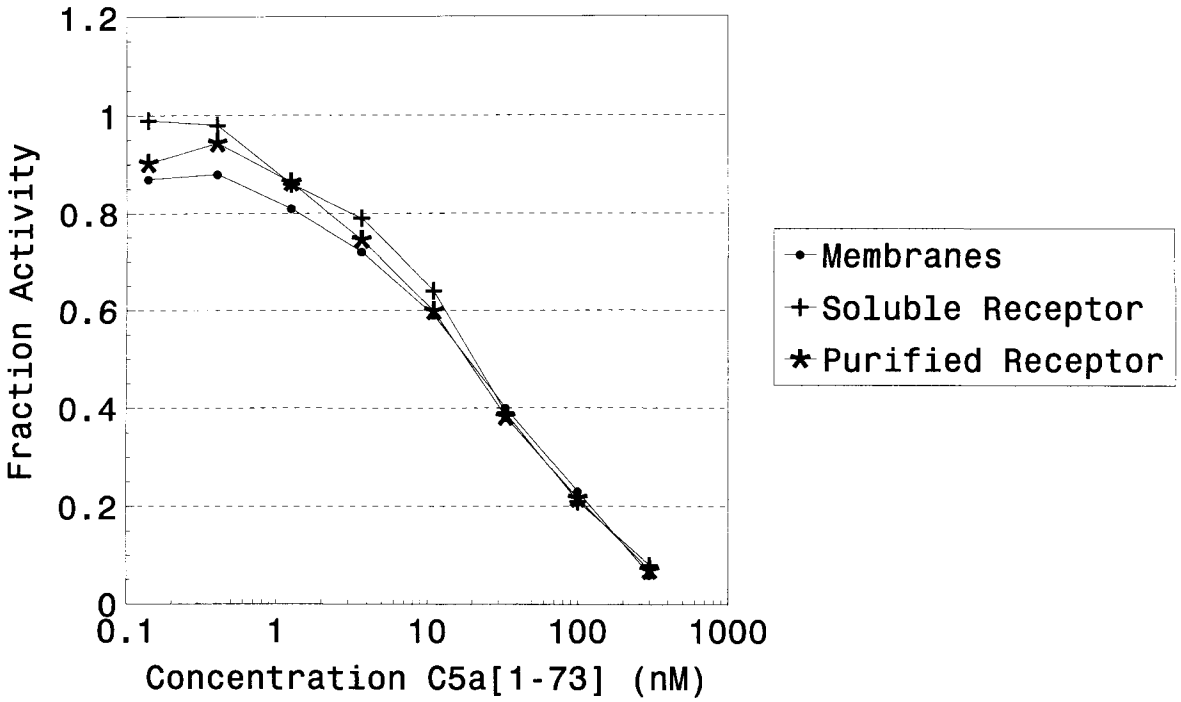

Fig. 1. Displacement of ^{125}I-C5a binding to various receptor preparations. Binding of ^{125}I-C5a(1-74) to receptor preparations from C127-C5aR membranes was determined with the indicated concentrations of unlabeled C5a(1-73) as displacer.

that typically represented 40–90% of the protein loaded on the gel. A broad band was detected in the range of 42–44 kDa, consistent with the reported mobility of the C5a receptor containing heterogeneous N-linked carbohydrate. Two sharp bands were found at about 35 and 40 kDa. Densitometry scanning indicated that the three bands were present in roughly equal amounts, although in some preparations less 35,000 dalton band was detected. Similar gel profiles as those shown in Figure 2 and similar recoveries were found when the preparation was repeated six independent times.

Western analysis of the gels using either antibodies against the C-terminal peptide of the receptor or antisera against unique sequences of G-protein alpha and beta subunits (Fig. 2B) showed that the 42–44 kDa protein crossreacted with antireceptor antibody, the 35 kDa protein crossreacted with G-protein beta antiserum, and the 40,000 dalton band crossreacted with both alpha G_{i3} and G_{i2} antisera. The 40 kDa band therefore represents an alpha subunit and the 35 kDa band a beta subunit of nucleotide regulatory proteins which copurify with the C5a receptor, represented by a band of 42–44 kDa.

When C5aR was purified by the previously reported method [25] of C5a-Affigel affinity chromatography (Fig. 3), in which the receptor was not complexed to C5a before digitonin solubilization, only the 42–44 kDa receptor band was detected, indicating that G-protein did not copurify with receptor under these conditions. Similar results were obtained in six independent experiments. In addition to this Affigel-C5a method, where C5a was coupled to the matrix via random lysine amine groups, other affinity methods were similarly tested including supports with C5a immobilized through Cys-27 or with C5a linked to the N-terminal Met. In these cases receptor-binding activity was isolated but no G-protein was detected (not shown).

Numerous attempts were made to demonstrate an effect of guanyl nucleotides on C5a binding to detergent-solubilized C5aR. In contrast to previous reports [25] little or no effect of GTPgammaS was found on the binding of C5a to crude (solubilized) or to purified nonligated receptor (Fig. 4). Preincubating receptor at room temperature or 37°C prior to the binding assay or performing the binding assays at room temperature had no effect on these results. GTP and GppNp gave similar results as GTPgammaS. A likely interpretation of this data is that G-proteins are not firmly associated with unligated C5aR or that the C5aR–G-protein interac-

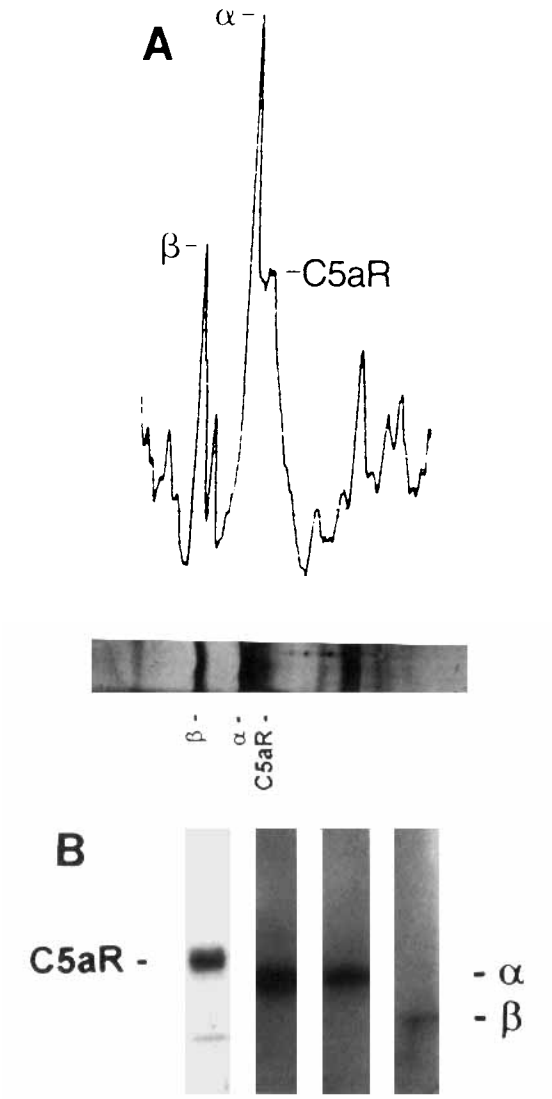

Fig. 2. SDS-polyacrylamide electrophoresis of purified C5aR. Purified C5aR from C127-C5aR cells was separated on 12.5% Tris/Leammli polyacrylamide gels. **A:** The gel was stained with silver stain and photographed **(bottom).** The tracing **(top)** is of a purified receptor preparation obtained from a different experiment which shows the approximate stoichiometries of the three main bands. In this preparation several additional contaminating bands of higher molecular weight are evident. **B:** Western analysis was performed with PVDF membranes using antibodies to (from left to right) C5aR, $Gi_{alpha}3$, $Gi_{alpha}2$, and G_{beta}. Detection was accomplished by autoradiography using ^{125}I-goat-antirabbit IgG second antibody. Positions for the alpha (α) and beta (β) subunits are marked.

tions are unable to survive after detergent solubilization. On the other hand, receptor prelabeled with C5a while in the membrane (ligated receptor) and subsequently solubilized by digitonin demonstrated a dissociation which was accelerated by GppNp (Fig. 5). As it is likely that the GTP effect on dissociation is mediated by a G-protein, the results indicate that under conditions of ligation prior to detergent solubilization, a C5aR–G-protein complex was formed which was stable enough to survive detergent solubilization.

DISCUSSION

Among many seven-transmembrane receptors which have been extensively characterized, the majority are known to assemble into stable G-protein complexes after ligand binding. There are several receptors, however, such as the A1 adrenergic receptor [20,29] and certain muscarinic subclasses [16], which are thought to exist as preformed G-protein complexes, because purification of these receptors yields ligand-binding subunits in association with G-proteins. For the C5a receptor, initial reports suggested that it exists as a preformed G-protein–receptor complex [25]. Copurification of G-protein subunits with the receptor, as demonstrated here, confirms the association of G-proteins with the C5a-ligated C5a receptor. However, it is apparent from this study that the stability of the interaction between C5aR and G-proteins varies depending upon ligand binding.

Two observations indicate that the C5aR associates with G-proteins in a dynamic fashion. First, no effect of GTP was observed on the binding of C5a to digitonin-solubilized or purified C5aR from a variety of cell types including human neutrophils. This implies that the G-protein association with unligated receptor was less stable than originally suggested. Second, purification of receptor solubilized from membranes without bound C5a using a variety of C5a-affinity methods, which were all successful in receptor isolation, never yielded G-protein alpha and beta subunits in the purified material. Only the 42,000 dalton C5a-binding subunit was found. A possible explanation for these observations was that unligated receptor had a relatively weak affinity for G-proteins and that this association was lost upon receptor solubilization. The fact that good binding activity was achieved with receptor solubilized and purified by these alternate techniques—where no G-protein associations were evident—implied that G-proteins were not essential for C5a(1-74) binding to receptor.

Ligation of C5a to receptor prior to solubilization led to formation of a stable complex between receptor and G-protein that survived purification. Consistent with this scheme, receptor

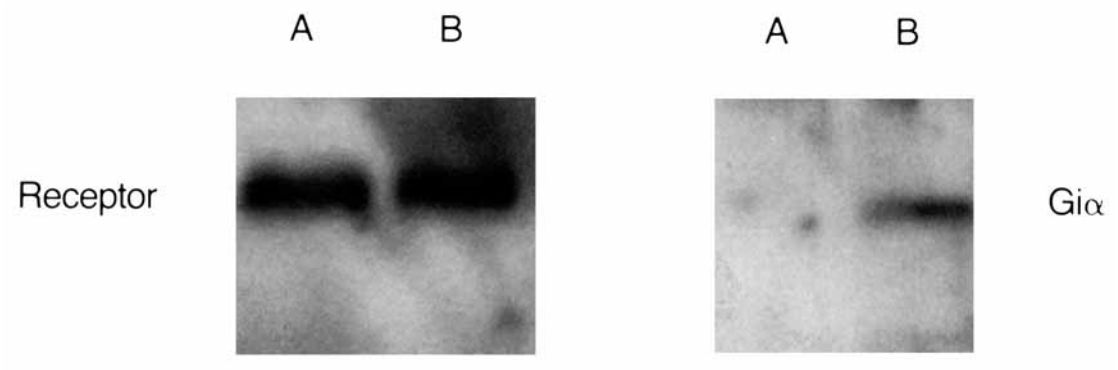

Fig. 3. Comparison of receptor isolated by two different techniques. Receptor was isolated from C127-C5aR cells either with the biotin-C5a technique **(lane B)** or with the traditional affinity chromatography using C5a-Affigel 10 **(lane A).** After SDS-PAGE and blot transfer, Western analysis was performed with antireceptor antibody or G-protein common alpha antibody as indicated.

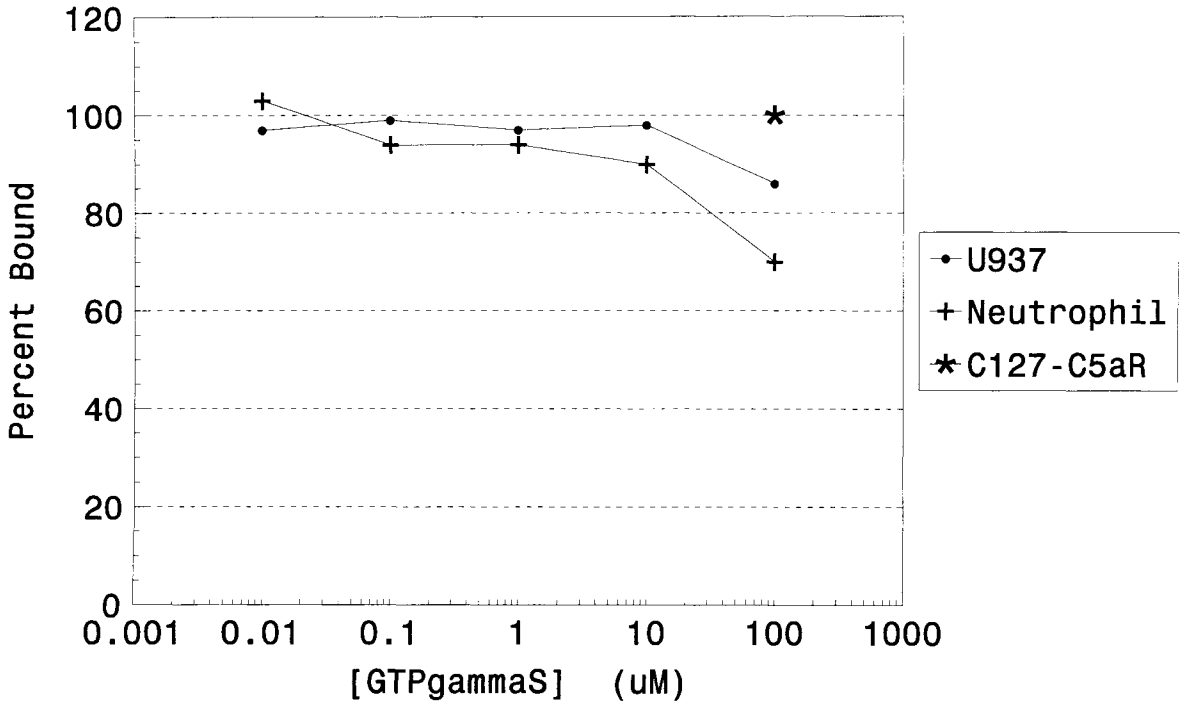

Fig. 4. Effect of GTPgammaS on binding of ^{125}I-C5a to solubilized C5aR. Binding assays were performed in the presence of increasing concentrations of GTPgammaS using digitonin solubilized receptor from human neutrophil or U937 cell membrane preparations or to receptor purified from C127-C5aR cell membranes using the Affigel-10-C5a method.

solubilized from membranes after formation of a ligated-receptor complex was sensitive to GTP when studied in a dissociation protocol. Elution of active receptor from the affinity column with 2 M chaotrope at neutral pH left three proteins in the purified receptor preparation, consistent with previous reports [25]. These proteins included a diffuse receptor-binding subunit together with nucleotide regulatory G-protein alpha and beta subunits. Both G_{i2} and G_{i3} alpha subunits were detected by Western analysis indicating that the receptor is promiscuous in terms of its G-protein partner (see also [11,31,32]).

The intimate association of C5a receptor with nucleotide regulatory proteins is felt to be only part of complex homeostatic regulatory systems which control leukocyte responses. These cells are able to carefully control C5a responses by priming, feedback, and desensitization mechanisms and ultimately by influencing transcrip-

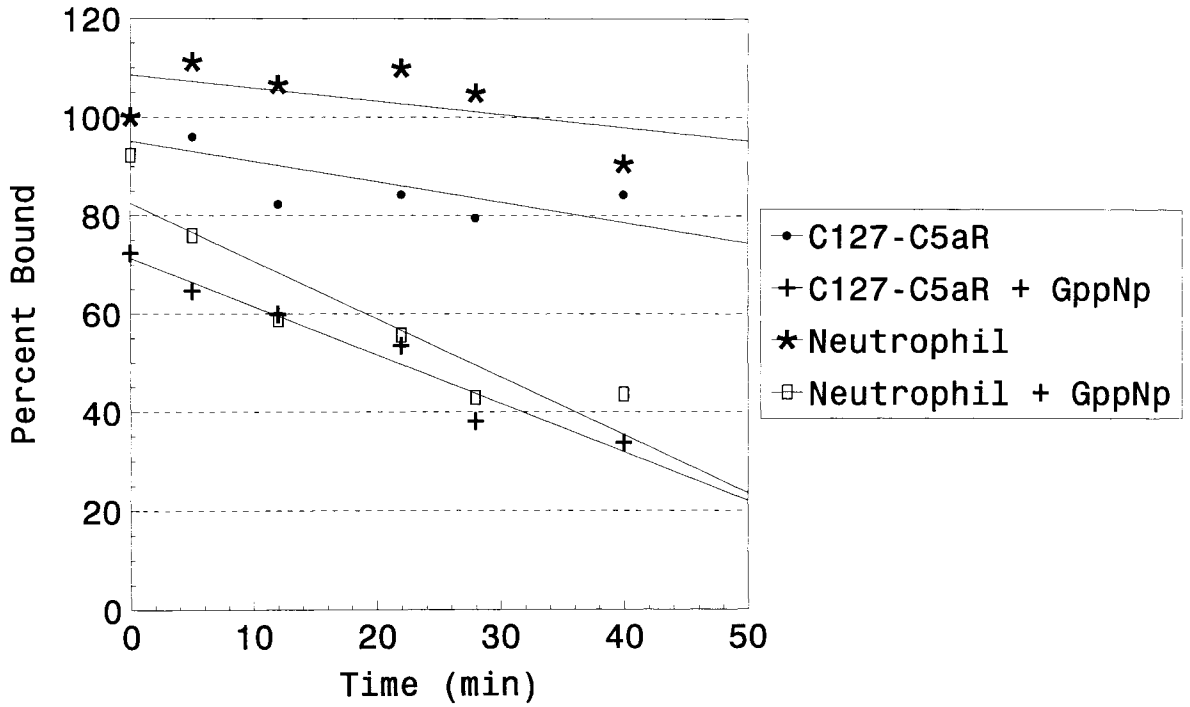

Fig. 5. Effect of GppNp on dissociation of ^{125}I-C5a from the ^{125}I-C5a–C5aR complex. GppNp (100 μM) or buffer, both containing 1 μg/ml unlabeled C5a, was added to ^{125}I-C5a–C5aR (see Methods). The samples were brought to room temperature and incubated for various times and then returned to 4°C. Individual samples were passed through a Superose-12 column, and 1 ml fractions were counted. Samples were passed through the column within 4 h of their return to 4°C. The experiment gave similar results when performed on three separate occasions.

tion and translation of specific genes. The high affinity interaction of G-proteins with ligated C5a-C5aR would be an initial step in these complex processes. Presumably, the techniques described can be extended to provide insight into interactions with other second messenger systems and ancillary components which translate into these complex response and regulatory systems.

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