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Selective coupling of the human anaphylatoxin C5a receptor and α_{16} in human kidney 293 cells

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The peptide C5a which is generated during the complement cascade is an important chemotactic factor involved in the inflammatory response. The C5a receptor (C5aR) primary sequence suggests that it has a serpentine structure of seven transmembrane domains which is typical of classical G-protein-coupled receptors. To investigate the signal transduction mechanism of C5a we transiently expressed the C5aR in combination with different G-protein α subunits in human kidney 293 cells and measured the PLC activity induced upon C5a stimulation. Cotransfection of C5aR and α_{16} stimulated PLC while cotransfection of C5aR with either α_q or α_{12} did not.

C5a receptor, $G_{\alpha 16}$; Human kidney 293 cell

1. INTRODUCTION

The inflammatory response is amplified by specific chemotactic factors which direct the accumulation of necessary phagocytic cells. One of the chemotactic factors involved is the 74 amino acid peptide C5a which is liberated by proteolytic cleavage from its precursor C5 during the complement cascade [1]. Chemotactic factors such as C5a, N-formylated peptides and leukotriene B4 bind to cell surface receptors and initiate the chemotactic response [2]. C5a interacts with its receptor (C5aR) which is expressed in hematopoietic cells such as neutrophils and eosinophils. Cloning of the C5aR revealed a 'serpentine' structure of seven transmembrane domains typical of classical G-protein-coupled receptors [3]. The functional C5aR was purified from neutrophils as a receptor–G_i complex [4] suggesting that at least part of the C5aR signal is transmitted by G_i. Previous reports showed that transient expression of C5aR in human kidney 293 cells allowed a prominent increase in intracellular Ca^{2+} induced by stimulation with C5a. Pertussis toxin (PTX) catalyzes the ADP-ribosylation of α-subunits of the G_i-class and blocks the activation of the α-subunit by inhibiting subunit dissociation. Only 70% of the C5a induced rise in intracellular Ca^{2+} could be blocked by PTX-treatment of the cells prior to stimulation indicating that other G-proteins outside the G_i-family must be involved in transmitting the C5aR signal [5]. The rise in intracellular Ca^{2+} might be promoted by Pdt-Ins PLC-β hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2) generating DAG and inositol trisphosphate (IP_3), suggesting a possible involvement of G-proteins from the G_q-family. In order to investigate the signaling of the C5aR we transiently transfected the C5aR alone and in combination with different G protein α-subunits in human kidney 293 cells. Activation of Pdt-Ins PLC-β was obtained by treatment with C5a only by cotransfecting the C5aR with α_{16}. No stimulation of PLC-β was seen with cotransfections of C5aR and either α_q or α_{12}.

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2. MATERIALS AND METHODS

2.1. *Materials*

C5aR cDNA was originally from N. Gerard, Harvard Medical School, hGRPR cDNA was originally from J. Battey, NIH. Recombinant C5a was purchased from Sigma and dissolved in PBS, 0.25% BSA.

2.2. *Transfection*

The M_1 muscarinic receptor cDNA was inserted into the pCD expression vector. hGRPR cDNA, C5aR cDNA as well as all α-subunit cDNAs were cloned into the expression plasmid pCMV5. The α_{12} cDNA was originally provided by R. Reed [6]. The α_q cDNA [7] was PCR cloned and sequenced as previously described [8]. A cDNA for the entire translated sequence of α_{16} [9] was isolated using reverse transcriptase and PCR of poly(A)$^+$ RNA from HL-60 cells. The entire sequence was verified by dideoxy sequencing Human kidney 293 cells were split and seeded into 60 mm dishes 24 h before transfection to obtain 30–40% 293 cells at this density were transfected with 1 5 μg of DNA / 60 mm dish by Ca^{2+} phosphate precipitation method [10]. After incubation with DNA for 6 h the cells were glycerol-shocked for

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1 min and labeled with *myo*-[2-^{3}H]inositol (1 μCi/ml, 23 Ci/mmol). 40 h later the cells were starved in serum-free Dulbecco's modified Eagle's medium containing 0.1% BSA and *myo*-[2-^{3}H]inositol (1 μCi/ml, 23 Ci/mmol) for 12–18 h.

2.3. *Measurement of Pdt-Ins PLC activity*

Transfected cells labeled with *myo*-[2-^{3}H]inositol were washed three times in serum-free medium and then incubated for 5 min in serum-free medium containing 20 mM LiCl. The cells were stimulated with agonist for 30 min, fixed in ice-cold methanol/HCl (100:1), scraped and placed into a tube containing chloroform (2 ml) and methanol/HCl (final 2 ml). After thorough mixing and centrifugation the aqueous phase was passed over an AGI-X8 (200–400 mesh) formate anion-exchange column to isolate inositol phosphates [11]. Total [^{3}H]inositol incorporation was determined by measuring the radioactivity of the organic phase.

2.4. *Expression analysis of α chains*

For immunoblots 250 μg of membrane protein from transfected 293 cells was resolved on a sodium dodecyl sulphate-polyacrylamide gel (10% acrylamide), transferred to nitrocellulose and probed with either α_{16}, α_q or α_i specific antibodies. Each of the α subunit antibodies are polyclonal rabbit antibodies. Peptides were synthesized corresponding to the carboxyl-terminal 12 amino acids for each α subunit, conjugated to keyhole limpet hemacyanin protein and used to immunize rabbits. The blots were probed with [^{125}I]protein A and autoradiographed as previously described [12,13].

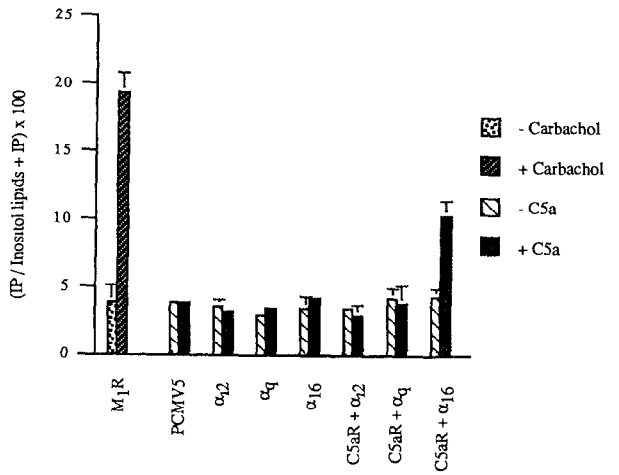

Fig. 1. Inositol phosphate formation was measured as described in section 2 for a 30 min incubation in the presence or absence of 1mM carbachol for 293 cells transfected with the M_1 receptor or for a 30 min incubation in the presence or absence of 100 nM C5a for cells transfected with vector only, α subunits only or cotransfections of C5aR and α subunits. Data points represent the mean ± S.D. of triplicate measurements in a single experiment and are representative of three or more independent experiments.

3. RESULTS AND DISCUSSION

Several lines of evidence suggest that multiple G proteins are involved in C5aR signaling. Functional C5aR was extracted from neutrophils as a C5aR–G_i complex indicating that G_i is involved in C5aR signaling [4]. However when C5aR was expressed in 293 cells only a portion of the C5a-induced rise in intracellular Ca^{2+} was blocked by pertussis toxin suggesting a possible role for additional G proteins outside the G_i family [5]. Additionally when C5a receptor cRNA was microinjected into *Xenopus* oocytes, functional signaling as measured by Ca^{2+} transients was not seen unless additional poly(A)$^+$ RNA from undifferentiated HL-60 cells was introduced suggesting that a second protein was needed for C5aR signaling [14] The G protein Gα_{16} is restricted in its expression to cells of hematopoietic lineage [9].

To investigate the functional coupling of different α-subunits expressed in hematopoietic cells to the C5aR regulated Pdt-Ins PLC activation, 293 cells were used for transient expression analysis. For all experiments either the M_1 muscarinic receptor, which is known to couple to G_q and stimulate PLC [15] or the human gastrin releasing peptide receptor (hGRPR), which stimulates PLC through activation of a pertussis toxin insensitive G protein [16,17], were used as controls. As shown in Fig. 1 neither transient transfection of the C5aR nor any of the α-subunits alone could promote PIP_2 breakdown upon C5a stimulation. We also examined combinations of C5aR and either α_q, α_{12} or α_{16} subunits by transient cotransfection, stimulation with C5a and assayed PLC activity. Only α_{16} in combination with C5aR stimulated PLC activity upon agonist stimulation. No activation of PLC was found when C5aR was cotransfected with either α_q or α_{12} (Fig. 1). To further confirm the requirement for coupling between C5aR and α_{16} for activation of PLC activity the C5aR was coexpressed with $\alpha_{16G211A}$, an inactive mutant α_{16} which cannot transmit a signal from the C5aR. This mutation is analogous to the previously characterized inactive

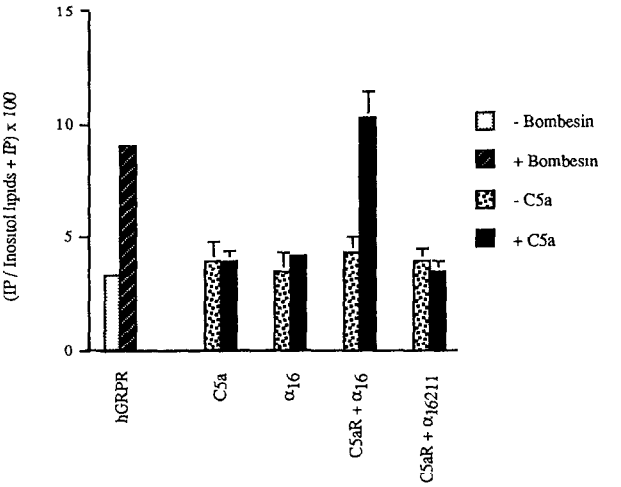

Fig. 2. Inositol phosphate formation was measured as described in section 2 for a 30 min incubation in the presence or absence of 50 nM bombesin for 293 cells transfected with hGRPR or for a 30 min incubation in the presence or absence of 100 nM C5a for cells transfected with C5aR only, α subunits only or cotransfections of C5aR and α subunits. Data points represent the mean ± S.D. of triplicate measurements in a single experiment and are representative of three or more independent experiments.

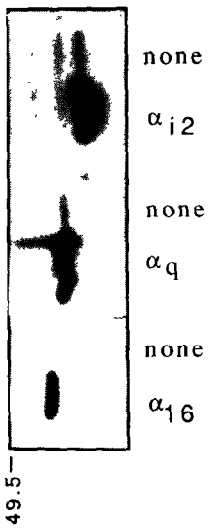

Fig. 3. Immunoblots showing overexpression of α subunits in transiently transfected 293 cell membranes were prepared as described in section 2. None represents 293 cells transfected with only vector DNA and no receptor or α subunit DNA.

$G_{asG226A}$ which cannot signal because it is unable to dissociate from $\beta\gamma$ [18]. In contrast to the stimulation seen with C5aR and α_{16} the combination of C5aR and $\alpha_{16G211A}$ did not promote IP_3 breakdown (Fig. 2). The results seen in Figs. 1 and 2 clearly demonstrate that α_{16} selectively interacts with the C5aR and is able to activate Pdt-Ins PLC-β activity in 293 cells. $G_{\alpha i2}$ or α_q overexpression was unable to confer C5aR regulation of Pdt-Ins PLC activation.

Immunoblotting with specific α subunit antibodies revealed that 293 cells have α_{i2} and α_q but no α_{16} present endogenously. Immunoblotting also showed that each of the α-subunits was overexpressed in transiently transfected 293 cells (Fig. 3). $\alpha_{16G211A}$ was also overexpressed to similar levels as α_{16} in transiently transfected 293 cells (not shown). No change in endogenous $\beta\gamma$ subunits is observed during the transient expression assay (not shown). A fraction of the cDNA expressed is able to associate with endogenous $\beta\gamma$ subunits allowing coupling to the C5aR [8,12,18].

Our results indicate that the C5aR can couple to more than one G protein. In mature neutrophils the C5aR appears to predominantly couple to G_i [5]. However, it is clear that the C5aR can also efficiently couple to G_{16}. Thus, the signaling pathway utilized by the C5aR can vary depending on the relative expression of G_{16} and G_i proteins. During HL-60 differentiation evidence suggests that C5aR's are induced while expression of α_{16} is suppressed [9,19]. The expression of α_{i2} is not significantly altered during HL-60 differentiation [9]. This suggests that in mature differentiated cells C5aR regulates PLC-β_2 activity predominantly by $\beta\gamma$ subunit complexes [20,21]. The role of α_{i2} in C5aR signaling is unclear, but might for example regulate the MAP kinase pathway [22]. During early phases of differentiation C5aR signaling is predicted to also involve G_{16} regulation of Pdt-Ins PLC activity. Thus, signal pathways regulated by serpentine receptors can couple to different G proteins. The ability of the C5aR to couple to two G proteins could confer significantly different second messenger responses during differentiation of cell types such as the neutrophil. The differential regulation of G protein α, β and γ subunit expression may be a significant regulatory mechanism during the differentiation of specific cell types.

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