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Amino acids 327–350 of the human C5a-receptor are not essential for [^{125}I]C5a binding in COS cells and signal transduction in *Xenopus* oocytes

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Abstract

The anaphylatoxic peptide C5a is an important inflammatory mediator of the complement system. We have generated human C5a-receptor (hC5aR) mutants with truncation of its cytosolic carboxyl-terminus (C-terminus). Both mutants were analysed for C5a-binding in transiently expressing COS cells, and one mutant additionally for GTP-binding regulatory protein (G-protein) coupling in cRNA-injected *Xenopus* oocytes. Our data suggest that (a) amino acids (aa) 314 to 326 as part of the C-terminus are necessary for proper receptor folding or expression and (b) the receptor C-terminus distal from position 327 is not critical for receptor expression, folding, binding and G-protein coupling.

Key words: C5a; C5a-receptor; Anaphylatoxin; Complement; G-protein; *Xenopus*

1. Introduction

The anaphylatoxic peptide C5a as part of the complement system is an important mediator of inflammatory reactions. It causes chemotaxis, activation of neutrophils, smooth muscle contraction and an increase in vascular permeability. After generation by proteolytic cleavage of native C5, the anaphylatoxin can interact with its specific receptor, described on several cells like neutrophils, eosinophils, and dibutyryl-cAMP-differentiated U937 or HL-60 cells (for a review see [1–3]). The hC5aR was recently cloned from these two cell-lines [4,5]. Its identity was confirmed by specific ligand-binding and various functional assays [4–6]. Recently we reported the cloning and functional expression of the recombinant hC5aR in *Xenopus* oocytes and demonstrated its absolute dependence on an additional human factor for functional expression [7]. In this paper we have analysed deletion mutants of the hC5aR truncated after aa-position 326 (tr326) and 313 (tr313), respectively, for ligand-binding in transfected COS cells. After in vitro transcription, we have investigated mutant tr326 for G-protein coupling as C5a-induced whole cell current in cRNA-microinjected *Xenopus laevis* oocytes.

2. Materials and methods

2.1. Chemicals and enzymes

Oligonucleotides for PCR and all sequencing primers were synthesized on a GENE Assembler Plus (Pharmacia) and purified according to manufacturer's instructions. Restriction endonucleases were obtained from Pharmacia, deoxynucleotide-triphosphate solutions from Gibco. All other materials were obtained from Sigma if not otherwise indicated.

2.2. Genetic constructions

As previously described the coding sequence for the hC5aR was recloned by PCR from genomic DNA of peripheral blood leukocytes into the *Hin*dIII site of the pCDM8 (Invitrogen) generating plasmid pCC1 [7]. This wild-type construct was taken as PCR-template using (1) the original primer for the 5'-end (TGT AGA ATT C*AA GCT T*AG CCC AGG AGA CGA G), and (2a) a reverse anti-sense 3'-end primer consisting of part of the hC5aR sequence and an additional **STOP-codon** (bold) behind the nucleotides corresponding to aa position 313 (CTA GTC AGC *AAG CTT* **CTA** TTT CCG CAG TCG GCC CTG), or (2b) corresponding to aa position 326 (GAA TTC *AAG CTT* **CTA** CTC TTC AGT CAA CAC GTT), respectively. Additionally, all primers contained a *Hin*dIII cloning site (italics) for convenient subcloning. Amplification was performed at an annealing temperature of 50°C in 30 cycles using Taq polymerase (Boehringer) according to the manufacturer's instructions. After digestion with *Hin*dIII the PCR fragment was subcloned into the eucaryotic expression vector pCDM8, also suitable for in-vitro transcription, and amplified in the *E. coli* strain XS127/p3 (Invitrogen). Positive clones were completely sequenced using the T7 sequencing kit (Promega). Plasmid preparations for transfection and in vitro transcription were prepared by a commercial kit (Qiagen) according to the manufacturer's specifications.

2.3. Cell culture conditions and transient transfection in COS.M6 cells

COS.M6 cells (ATCC) were grown at 37°C in a humidified atmosphere with 5% CO_2 in DMEM (Gibco BRL) medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, and penicillin (50 U/ml)/streptomycin (50 μg/ml). COS cells were transfected by the DEAE-Dextran method with slight modifications: 1×10^7 cells attached to a 175 cm^2-cell culture flask were incubated with a solution containing among others [8,9] 10 μg plasmid DNA, 188 μg/ml DEAE-Dextran (Sigma) and 100 μM chloroquin. After 4 h this solution was removed and cells were shocked with 10% DMSO-PBS for 2 min. After 2 × washing with PBS cells were incubated for 48 h with supplemented DMEM and harvested with EDTA-PBS (2 mM) solution for further analyses in binding studies.

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2.4. Purity of the stimulus recombinant human C5a (rhC5a)

For radiolabeling commercially available rhC5a was used (Sigma). For all other purposes rhC5a was prepared and analysed from our own *E. coli* strain as described previously [10].

2.5. [^{125}I]C5a binding studies

Radiolabelling of C5a: iodination of recombinant C5a (Sigma) was performed as described previously [11]. The recovery of biologically active peptide was approx. 15% for [^{125}I]rhC5a as assayed by the ATP-release assay with guinea-pig platelets [11,12]. The average specific activity obtained was 1700 Ci/mmol [^{125}I]rhC5a.

For binding kinetics 1 ml of a cell suspension was prepared in HBSS buffer at 4°C, 22°C, and 37°C. The experiment was started by the addition of [^{125}I]rhC5a. At different time points three aliquots of 45 μl (0.79 nM [^{125}I]rhC5a equivalent to 10,500 cpm per aliquot) were removed to determine bound radioactivity by separation of free and cell-bound [^{125}I]rhC5a through a sucrose cushion as described [13].

For competitive binding studies a mixture 30 μl of 0.09 nM [^{125}I]rhC5a was mixed with 90 μl of cell suspension. After 30 min at 22°C three aliquots of 45 were removed and bound radioactivity was determined as described above [11]. Iterative curve fitting of the binding data was performed on the bases of one and two-site models using LIGAND [14].

2.6. Preparation of cRNA

cRNA for the human hC5aR and receptor mutant tr326 were prepared from the T7 promoter of the linearized (*Xba*I) pCDM8-receptor constructs using the mCAP mRNA capping kit (Stratagene). cRNA was quantitated by densitometric evaluation from ethidium bromide-stained agarose gels against a known RNA standard using the CS-1 system (Cybertech). To supply the cells with the mRNA for the additional yet uncharacterized human factor [7] total RNA of non-differentiated U937 cells (ATCC) grown in RPMI 1640 medium, (Gibco) supplemented as described for DMEM medium, was prepared by the guanidinium isothiocyanate method [15] and stored at −70°C in water.

2.7. Oocyte expression experiments

Preparation of defolliculated *Xenopus laevis* oocytes (Kähler, Hamburg, Germany) and electrophysiological recordings were performed as described previously [7]. 46 nl RNA per oocyte corresponding to 23 ng cRNA and 23 ng total RNA of non-differentiated U937 cells, were injected by a motor-driven microinjector (Drummond) through glass capillaries broken to an outer diameter of 12–25 μm. Two days later functional activity was tested by current recording of voltage-clamped oocytes (−70 mV) exposed to frog Ringer solution (ND96) containing 20 nM rhC5a.

3. Results

3.1. [^{125}I]rhC5a binding-studies

3.1.1. Kinetics and temperature-dependence of [^{125}I]rhC5a binding. First binding kinetics were performed at 4°C, 22°C, and 37°C. Binding equilibrium was achieved best at 22°C after 30 min (data not shown), and all further competitive binding studies were performed at these conditions.

3.1.2. Competitive binding studies. The specific C5a-binding sites of wild-type hC5aR and the both receptor mutants were analysed in transfected COS.M6 cells using a constant concentration of [^{125}I]rhC5a and increasing concentrations (in triplicates) of unlabeled rhC5a (Fig. 1). The data of three independent binding-experiments were analysed by iterative curve fitting to one- and two-site models: for the wild-type hC5aR we

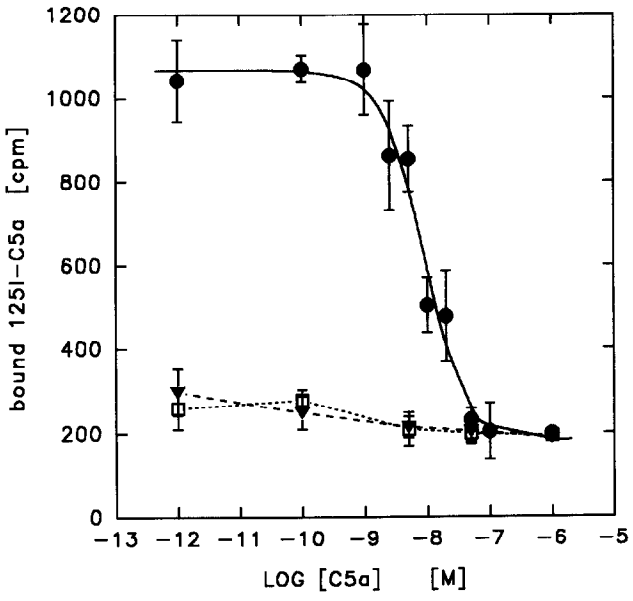

Fig. 1. One representative competitive binding study of COS.M6-cells (1×10^7/ml), transiently transfected with pCDM8-tr326 (●), pCDM8-tr313 (▼), pCDM8 without insert (□) ($n = 3$). A constant concentration of [^{125}I]rhC5a was displaced by increasing concentrations of rhC5a. A total of 14,800 cpm per sample was used. Binding parameters were calculated by iterative curve fitting from three such experiments.

could detect 370,000 ± 160,000 receptors/cell with a dissociation constant (K_d) of 7.0 ± 0.4 (nM). For tr326 we found 70,000 receptors/cell with a K_d of 6.0 ± 2.1 (nM). In contrast, no specific binding for the transfected receptor-mutant tr313 could be detected, in comparison to COS cells transfected with pCDM8 vector alone (Fig. 1).

3.2. Oocyte expression experiments

To analyse the receptor mutant tr326 for functional G-protein coupling we microinjected *Xenopus laevis* oocytes with in vitro transcribed cRNA of hC5aR wild type and mutant tr326 and determined whole cell current during stimulation with 20 nM rhC5a. Mutant tr313 was not further analysed. The experiment was performed in duplicate. Fig. 2 depicts representative responses for oocytes microinjected with mRNA/cRNA (a) without receptor construct, (b) with wild type receptor, and (c) with mutant tr326. In one experiment all 5 tr326 injected oocytes showed positive responses with maximal currents between 50 and 100 nA. In a second independent experiment 5 out of 6 tr326-oocytes responded with maximal currents between 35 and 75 nA. Oocytes without receptor did not respond ($n > 10$) whereas wild type-injected oocytes as positive-controls ($n = 3$) showed maximal currents between 100 and 250 nA.

4. Discussion

The hC5aR is thought to belong to the family of G-

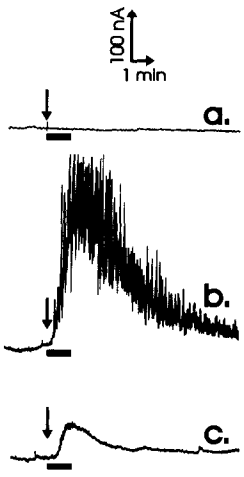

Fig. 2. Representative whole cell currents determined by voltage clamped *Xenopus laevis* oocytes microinjected with supplementing factor plus: (a) dH_2O, (b) cRNA from in-vitro transcribed hC5aR wild type, and (c) cRNA from tr326. The oocytes were stimulated with 20 nM rhC5a (↓) for 1 min (▬).

protein coupled receptors with 7 membrane-spanning regions, an extracellular amino- and an intracellular carboxyl-terminus. Beside its homology to members of these family and the prediction of 7 α-helical hydrophobic putative transmembranous regions, broad experimental evidence has accumulated for this assumption: pertussis-toxin sensitivity of C5a-response [16–18], C5a-dependent guanosine 5′-*O*(3-thiotriphosphate (GTPγS)-binding [6], co-purification of receptor and G-protein [19], and co-transfection experiments with hC5aR and G-α_{16} [20,21]. For other receptors of this family, in particular the adrenergic receptors, there exists a thorough understanding of structure/function relationship using cells transfected with mutants of these receptors for functional studies: simplified, transmembranous regions and/or extracellular parts of the receptor seem to be involved in ligand binding, whereas the intracellular loops and part of the cytosolic C-terminus are described as important for G-protein coupling (for a review see [22]).

Up to now only limited information is available about structure/function relationship for the hC5aR, which only concerns the extracellular N-terminus and its involvement in ligand binding [23,24]. Thus far no data have been published obtained with recombinant hC5aR mutants, except for one article concerning hC5aR/human formyl peptide receptor (hFMLP-R) chimeras which were only assayed for the specific binding of formyl peptides but not for the ligand C5a [25]. We have analysed two mutants of the hC5aR with deleted intracellular C-terminus for ligand-binding in transiently transfected COS-cells. The C-terminal end of the last membrane-spanning segment of the hC5aR is predicted for aa 302 (PC-GEN-Software, [26,27]) or 305, respectively [28]. The one-deletion mutant, tr326, showed an essentially unchanged K_d compared to the wild type receptor, in good agreement with data obtained with rhC5aR-transfected COS-cells by others (1.4 nM [4]); 1.5–2 nM = $K_{\text{d-high affinity}}$ and 20–25 nM = $K_{\text{d-low affinity}}$ [5]). Mutant tr326 was further analysed for functional G-protein coupling as measured by rhC5a-inducable whole cell current in *Xenopus* oocytes after microinjection of cRNA. We found essentially the same response for this mutant and the wild type receptor with a tendency to lower amplitudes of the current for the mutant. This observation matches with the lower receptor number found in the binding studies with tr326-transfected COS-cells. These differences could be due to decreased receptor-expression or -stability of this mutant. The other more complete deletion mutant tr313 did not show any specific [^{125}I]rhC5a binding. We conclude that (a) aa 314 to 326 as part of the C-terminus are necessary for proper receptor folding or expression, and (b) that the receptor C-terminus distal from position 327 is not critical for expression, folding, binding and G-protein coupling. To our knowledge, only a few such truncations of other G-protein coupled receptors have been studied before: the β_2-adrenergic receptor [29] as the classical example, and recently the luteinizing hormone receptor [30], gastrin-releasing peptide receptor [31] and thyrotropin-releasing hormone receptor [32] giving similar evidence that the distal C-terminus is not essential for G-protein coupling.

So far, the most related cloned receptor to the hC5aR is the hFMLP-R, also a chemoattractant factor receptor, with a 34% sequence homology [33] which similarly couples to a Pertussis toxin sensitive G-protein using similar signal-transduction pathways [6,34]. For the hFMLP-R it was demonstrated by point mutations that the third cytosolic loop, contrary to other receptors, does not play a critical role in the functional coupling to G-proteins [35]. Recently Bommakanti et al. showed that only one site-specific synthetic peptide representing part of the cytosolic hFMLP-R C-terminus (aa position 322–336 of 350) competed with its receptor for binding of bovine G_i, whereas peptides representing the intracellular loops II and III failed [36]. Assuming a similar signal transduction mechanism for the hC5aR one might propose the proximal part of the hC5aR-R cytosolic C-terminus (aa 302–326) as being important for G-protein coupling.

Additionally, for future studies it is valuable to know that mutant tr326 shows essentially normal C5a-ligand-binding and G-protein coupling because it can now be considered as a good candidate for future desensitization or phosphorylation analysis: This mutant is lacking almost all serines and threonines of the cytosolic receptor C-terminus which are discussed as being involved in the recently demonstrated fast receptor desensitization (detectable as C5a-induced decrease in [^{32}P]γ-GTP binding in transfected RBL.2H3 cells) and phosphorylation by a proposed 'β-adrenergic receptor-kinase-like' receptor ki-

nase [6,37,38], (for review of β-adrenergic receptor-kinase function see [22]).

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