Biochem. J. (1992) 288, 261–266 (Printed in Great Britain)

A recombinant hybrid anaphylatoxin with dual C3a/C5a activity

Wilfried BAUTSCH,* Titus KRETZSCHMAR, Torsten STÜHMER, Axel KOLA, Monica EMDE, Jörg KÖHL, Andreas KLOS and Dieter BITTER-SUERMANN

Institute of Medical Microbiology, Hanover Medical School, Konstanty-Gutschow-Str. 8, 3000 Hannover 61, Germany

By site-directed mutagenesis of a human complement factor C5a cDNA clone, we have designed a hybrid anaphylatoxin in which three amino acid residues in the *C*-terminal sequence of human C5a were exchanged to create the native *C*-terminal human C3a (hC3a) sequence Leu-Gly-Leu-Ala-Arg. This hybrid anaphylatoxin rC5a-(1–69)-LGLAR exhibited true C3a and C5a activity when tested in the guinea pig ileum contraction assay. Quantitative measurements of ATP release from guinea pig platelets revealed about 1% intrinsic C3a activity for this hybrid, while the C5a activity was essentially unchanged. Competitive binding assays confirmed that the rC5a-(1–69)-LGLAR mutant was able to displace radioiodinated rhC5a with a K_i of approx. 40 nM and hC3a with a K_i of approx. 3.7 μM from guinea pig platelets. Since the C-termini of both human C3a and C5a anaphylatoxins are known to interact with their respective receptors, we conclude that the same peptidic sequence, LGLAR, is able to bind to and activate two different receptors, the C3a receptor as well as the C5a receptor. This clone provides a novel tool for the identification of further receptor-binding residues in both anaphylatoxins, since any mutants may be tested for altered C3a and C5a activity simultaneously.

INTRODUCTION

The complement system is one of the major immunological defence mechanisms against foreign substances. It consists of about 20 plasma proteins, their corresponding cellular receptors and several membrane regulatory proteins. Activation of the complement system by, for example, antigen–antibody complexes or bacterial surface structures, triggers an amplification cascade of proteolytic cleavage and protein assembly events of the complement components, which ultimately leads to the destruction and final elimination of the foreign body (for review, see [1]).

The human anaphylatoxins C3a (hC3a) and C5a (hC5a) are small proteins of 77 and 74 amino acids respectively which are generated by proteolytic cleavage of the complement components C3 and C5 during activation of the complement system. They act as potent mediators of inflammatory reactions, leading to smooth muscle contraction, an increase in vascular permeability, and wheal and flare generation when injected into human skin, as well as producing a variety of diverse cellular responses (for review, see [2]). While both proteins are able to release histamine from mast cells [3] and ATP and 5-hydroxytryptamine from guinea pig platelets [4], many differences in the cellular responses are also apparent. Notably, hC3a has an immunosuppressive effect on T-cell proliferation [5], while hC5a has been shown to augment antibody production *in vitro* [6]. In addition, hC5a induces the oxidative burst and lysosomal enzyme release from granulocytes [7] and is a potent chemoattractant [8]. The anaphylatoxins have been implicated as a causative or aggravating factor in the pathogenesis of several inflammatory diseases, such as adult respiratory distress syndrome [2] and rheumatoid arthritis [9]. The two anaphylatoxins are evolutionarily related to each other, sharing about 36% amino acid sequence identity and a very similar structure in solution [10]. The biological functions of the anaphylatoxins are mediated by interactions with two membrane-bound receptors, the C3a receptor and the C5a receptor, which are different from each other and highly specific for their respective ligands [11–13].

Considerable effort has been directed in the past towards elucidating the structural requirements for the observed ligand specificity of the anaphylatoxin receptors. Qualitatively, the C3a receptor seems to select appropriate ligands almost exclusively by their *C*-terminal sequence: enzymic removal of the *C*-terminal arginine from hC3a by serum carboxypeptidase N, the physiological serum inactivator of hC3a, completely abrogates all biological activities [14]. Furthermore, synthetic peptides based on the *C*-terminal sequence of hC3a have been shown to display C3a-specific activities [15–18]. The minimal active peptide sequence has long been known to be the *C*-terminal pentapeptide Leu-Gly-Leu-Ala-Arg [LGLAR; C3a-(73–77)] [19]. This has been shown in the guinea pig ileum contraction assay and also by the assay of ATP release from guinea pig platelets. In the former assay, this peptide exhibited an approx. 1000-fold lower activity on a molar basis than native hC3a [19], while the latter assay revealed a 10000-fold lower activity [16,20].

By extensive site-directed mutagenesis experiments using a recombinant hC5a (rhC5a) clone, at least three different regions in the hC5a molecule have been identified which participate in receptor binding: a region in the *N*-terminus around Lys-19 and Lys-20 [21], the core residue Arg-40, and the *C*-terminal residues Lys-68, Leu-72 and Arg-74 [22]. Again, the *C*-terminus is involved in receptor binding. Furthermore, recent studies with synthetic peptides based on the *C*-terminus of hC5a suggest that the effector site of hC5a is localized in the *C*-terminus [23,24]. However, much higher peptide concentrations are necessary to elicit a C5a-specific response than for the analogous C3a-specific peptides.

These specificity requirements are not mutually exclusive. Introduction of only three amino acid exchanges in the *C*-terminus of human C5a (Met-70 → Leu, Gln-71 → Gly and Gly-73 → Ala) would generate the native hC3a *C*-terminal sequence LGLAR, which should be able to interact with the C3a receptor, while conserving all amino acids known to participate in binding to the C5a receptor. Such a hybrid compound would therefore represent a rigorous test substance for investigating current

Abbreviations used: hC3a, human complement factor C3a; hC5a, human complement factor C5a; rhC5a, recombinant hC5a; IPTG, isopropyl β-D-thiogalactopyranoside; PMSF, phenylmethanesulphonyl fluoride; Tos-Lys-CH_2Cl, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK'); Tos-Phe-CH_2Cl, 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one ('TPCK').

* To whom correspondence should be addressed.

views on anaphylatoxin receptor specificities. Notably, such a hybrid molecule would not represent a mere combination of different protein domains known to interact with different receptors, but rather is a 'true' hybrid: the same *C*-terminal sequence LGLAR would participate in binding to two different receptors.

We have tested these projections and describe in the present paper a novel mutant anaphylatoxin, rC5a-(1–69)-LGLAR, which we obtained by site-directed mutagenesis of a hC5a cDNA clone previously cloned and expressed in *Escherichia coli* [25]. We provide conclusive evidence that this mutant protein displays true C5a as well as C3a activity.

Part of this work was presented at the XIV International Complement Workshop, Cambridge, U.K., 1991.

MATERIALS AND METHODS

Materials

Isopropyl β-D-thiogalactopyranoside (IPTG) was obtained from Biomol (Hamburg, Germany), histamine from Merck (Darmstadt, Germany), Iodogen from Pierce (Oud Beijerland, The Netherlands), heavy mineral oil, ampicillin, Tos-Phe-CH_2Cl, phenylmethanesulphonyl fluoride (PMSF) and β-mercaptoethanol from Sigma (St. Louis, MO, U.S.A.), casamino acids from Difco (Detroit, MI, U.S.A.), α_2-macroglobulin and dibutyryl cyclic AMP from Boehringer (Mannheim, Germany), aprotinin and acetylsalicylic acid from Bayer (Leverkusen, Germany), *o*-phenanthroline, Tos-Lys-CH_2Cl and leupeptin from Fluka (Neu-Ulm, Germany) and all enzymes from Pharmacia (Piscataway, NJ, U.S.A.), Life Technologies (Gaithersburg, MD, U.S.A.) or New England Biolabs (Beverly, MA, U.S.A.) as indicated in the text. hC3a was purified from complement-activated human serum as described previously [26]. Guinea pig platelets and ileum were prepared from guinea pigs of both sexes (600–1000 g) from strains C2BB/R^+ (C3a-receptor-positive) and C2BB/R^- (C3a-receptor-negative) from our own breeding colonies [27]. *E. coli* strain JM105 (*thi*, *rpsL*, *endA*, *sbcB*15, *hsdR*4, Δ[*lacproAB*]/*F'traD36*, *proAB*, *lacI*^{q}*ZΔM15*) was used as host for all cloning procedures with plasmid pKK233-2, and *E. coli* Y1089 (Δ*lacU169*, Δ*lon*, *araD139*, *strA*, *hflA150*::*Tn10*, [*pMC9*]) used for all expression procedures. Oligonucleotides P1 (5'-ACGCTGCAAAAGAAGATAGAA-3') and P2 (5'-GCT-GCAGCTATTATTAACGAGCCAGACCCAGGTCTTTAT-GAGAGATAT-3') were synthesized on a Gene Assembler Plus (Pharmacia) and purified by gel chromatography on NAP10 columns (Pharmacia).

The composition of buffers was as follows. M9CA medium: 7.52 g of Na_2HPO_4,$2H_2O$, 3 g of KH_2PO_4, 1 g of NH_4Cl, 0.5 g of NaCl, 3 mg of $CaCl_2$, 0.25 mg of vitamin B1, 0.5 mM-$MgCl_2$, 2.3 ml of glycerol (87%, w/v), 2.5 ml of casamino acids (20%, w/v) and 80 mg of ampicillin/litre. GIT buffer: 6 M-guanidinium hydrochloride and 0.1 M-potassium phosphate buffer, pH 7.6. Tyrode buffer: 7.8 g of NaCl, 0.2 g of KCl, 1.0 g of $NaHCO_3$, 50 mg of NaH_2PO_4, 1 mM-$MgCl_2$, 5 mM-Tris/HCl, pH 7.35–7.45, 0.1 g of glucose and 0.2 g of BSA/litre. For the assay of ATP release from guinea pig platelets, Tyrode buffer was supplemented with 2 mM-$CaCl_2$, and for all binding experiments, 2 mM-dibutyryl cyclic AMP and 15 mM-acetylsalicylic acid were added as aggregation inhibitors. For competitive binding experiments with ^{125}I-hC3a, Tyrode buffer was additionally supplemented with 1 unit of α_2-macroglobulin/ml, 200 kallikrein-inhibitory units of aprotinin/ml, 2 mM-Tos-Lys-CH_2Cl, 2 mM-Tos-Phe-CH_2Cl, 2 mM-leupeptin, 0.5 mM-PMSF and 0.5 mM-*o*-phenanthroline as proteinase inhibitors. All chemicals were of at least pro-analysis quality.

Cloning of C5a-(1–69)-LGLAR

Plasmid pME10 contains the hC5a DNA coding sequence in the expression vector pKK233-2 (Pharmacia) [25]. We performed PCR-mediated site-directed mutagenesis [28] of pME10 to obtain the cDNA for the rC5a-(1–69)-LGLAR mutant. A 1 ng portion of pME10 was amplified by PCR in a total volume of 100 μl containing 10 mM-Tris/HCl, pH 8.3, 50 mM-KCl, 1.5 mM-$MgCl_2$, 0.01% gelatin, 200 μM of each dNTP (Life Technologies), 1 μM each of primers P1 and P2, and 2.5 units of *TaqI* polymerase (Life Technologies). The reaction mixture was overlaid with heavy mineral oil to prevent evaporation. A total of 25 cycles (2 min at 48 °C, 1 min to 72 °C, 1 min at 72 °C, 1.5 min at 92 °C) were run on a thermocycler constructed at the Hanover Medical School. The PCR fragment was treated with Klenow enzyme (Life Technologies) to remove 3'-overhanging nucleotides [28], phosphorylated with T4 polynucleotide kinase (Life Technologies), digested with *PstI* (Pharmacia) and cloned into the *NcoI*/*PstI* sites of plasmid pKK233-2 (Pharmacia) using standard protocols [29]. To allow for blunt-end ligation of the 5'-end of the PCR fragment into pKK233-2, the *NcoI* site was filled in with Klenow polymerase and dNTPs prior to the *PstI* digestion. Recombinant clones were sequenced (including the promotor region) using the T7 Deaza sequencing kit (Pharmacia). The recombinant plasmid was called pME18.

Expression and purification [25]

Freshly transformed *E. coli* Y1089 were grown in M9CA medium and expression was started by addition of 1 mM-IPTG for about 2 h. Cells were harvested at about $A_{600} = 0.8$, solubilized in GIT buffer supplemented with 100 mM-β-mercaptoethanol and dialysed against 20 mM-potassium phosphate buffer, pH 7.6 [30]. The protein was purified by ion-exchange chromatography on SP Sephadex C-25 (Pharmacia) as described elsewhere [31] and by subsequent reversed-phase f.p.l.c. on a PEP-RPC HR 10/10 column (Pharmacia) by applying a linear gradient of buffer A in buffer B from 30 to 60% (buffer A: 30 mM-ammonium acetate/0.1% trifluoroacetic acid, pH 4; buffer B: 40% buffer A/60% acetonitrile). Positive fractions were pooled, concentrated several times to near-dryness under vacuum and stored in water at −70 °C. The homogeneity of the protein was checked on silver-stained SDS/PAGE gels.

C.d. measurements

C.d. spectra of rhC5a and rC5a-(1–69)-LGLAR were recorded in 50 mM-sodium phosphate buffer, pH 7.2, on an AVIV 62 DS c.d. spectrometer (Lakewood, NJ, U.S.A.) in the far-u.v. region between 180 and 250 nm.

Ileum contraction assay

The guinea pig ileum contraction assay was performed as described elsewhere [32]. The ileum was prepared from the C2BB/R^+ strain and all experiments were performed at least twice.

ATP release assay

Guinea pig platelet preparation and the ATP release assay (activation and desensitization) were performed essentially as described previously [33]. To allow for a quantitative measurement of the inherent C3a activity of the rC5a-(1–69)-LGLAR mutant, activation of guinea pig platelets via the C5a receptor had to be blocked completely by ligand-specific receptor desensitization prior to the C3a measurement. Because of the small concentration difference between the ED_{50} values for activation and desensitization [25], however, a single dose of rhC5a sufficient for complete C5a receptor desensitization would

result in considerable ATP release via C5a receptor activation. To circumvent this problem we employed repeated low-dose desensitization to block the C5a receptor activation. Small amounts of rhC5a (starting at about 1.5 nM) were successively added (five or six steps) at 10 min intervals up to a final concentration of about 15–20 nM. After each addition the amount of desensitization was determined in an aliquot by measurement of ATP release. This procedure resulted in complete C5a receptor desensitization, whereas the C3a responsiveness of the platelets was essentially unchanged. Determination of activation curves was performed at least five times, and desensitization experiments were carried out at least twice in triplicate.

Myeloperoxidase assay

The assay of myeloperoxidase release from human granulocytes was performed as described elsewhere [34]. Granulocytes for the assay were isolated from peripheral venous blood by isopyknic centrifugation using Polyprep (Nycomed, Oslo, Norway) according to the manufacturer's instructions. Each experiment was performed at least three times in quadruplicate.

Competitive binding assays

Competitive binding assays with radioiodinated rhC5a on C2BB/R$^-$ platelets were performed as described previously [33]. Competitive binding assays with radioiodinated hC3a were performed as will be described in detail elsewhere (T. Kretzschmar, M. Pohl, M. Casaretto, M. Przewosny, W. Bautsch, A. Klos, D. Saunders & J. Köhl, unpublished work). Briefly, hC3a was radioiodinated by a slight modification of the Iodogen method [35] with carrier-free ^{125}I (Amersham) to a specific radioactivity of 450 Ci/mmol. About 3.8×10^4 c.p.m. of ^{125}I-hC3a were incubated with 2.9×10^7 guinea pig platelets in 150 μl of Tyrode buffer containing a mixture of proteinase and aggregation inhibitors as described above together with the unlabelled ligand for 30 min at room temperature. Three 45 μl aliquots were centrifuged (12000 g, 6 min, 4 °C) through a 10 % (w/v) sucrose cushion and cell-bound radioactivity was measured (model 5130 γ-radiation counter, Packard). IC_{50} data from the binding curves were converted into K_I values using the Cheng–Prussow equation [36].

Miscellaneous

RhC5a, rC5a-(1–69)-LGLAR and hC3a concentrations were determined by quantitative e.l.i.s.a.s [37].

RESULTS

Design, construction and isolation of rC5a-(1–69)-LGLAR

From the known specificity requirements of human C3a, it appeared possible to introduce C3a activity into a C5a molecule by changing the *C*-terminal sequence of rhC5a into that of native hC3a. Although even the tripeptide LAR has been found by us to induce C3a-specific responses, this did require the addition of potentiating non-peptidic hydrophobic groups to the *N*-terminus [17]. The smallest pure peptide sequence known to display C3a-specific activity is the pentapeptide LGLAR [19]. In general, elongation increases the C3a activity of small synthetic peptides. However, since we wanted to completely conserve C5a activity, this imposed an inherent limit to the sequence length that could be safely exchanged. Sequence comparison of the *C*-termini of hC5a and hC3a clearly showed that exchange of the last five amino acids of hC5a (MQLGR) into those of hC3a (LGLAR) would conserve the residues Leu-72 and Arg-74 that are essential for C5a receptor binding [22]. A seven-residue exchange, however, would change Lys-68, implicated in C5a receptor interaction,

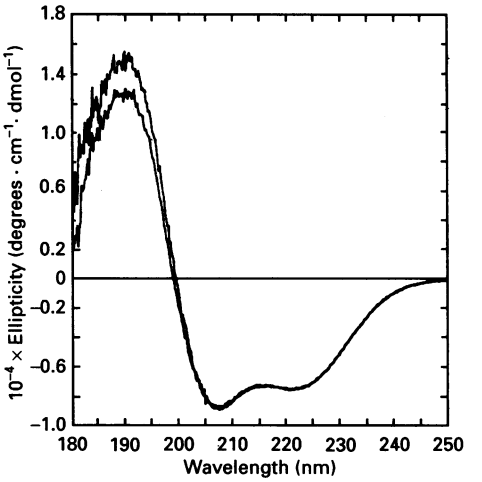

Fig. 1. C.d. spectra of rhC5a and rC5a-(1–69)-LGLAR

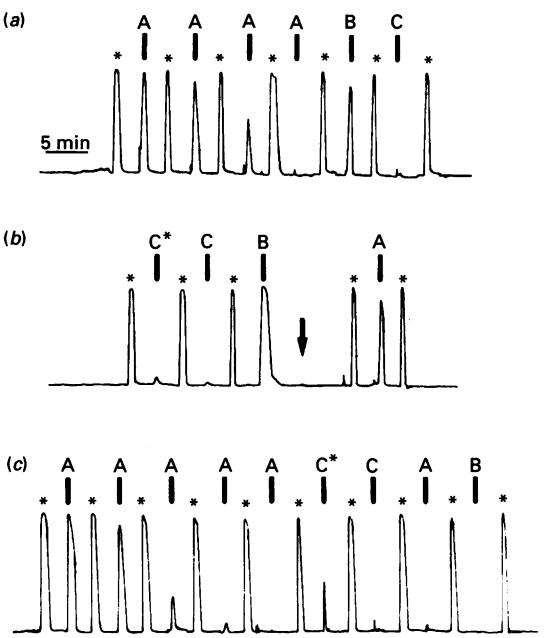

Fig. 2. Functional characterization of rC5a-(1–69)-LGLAR in the guinea pig ileum contraction assay

Isotonic smooth muscle contractions were recorded on the ordinate in response to different stimuli. Histamine (10^{-5} M) was used as a positive control (*) to demonstrate the functional integrity of the smooth muscle throughout the experiments. (*a*) Demonstration of C3a activity. Repeated doses of 100 % stimuli of rhC5a (A) completely desensitize the C5a receptor. Application of rC5a-(1–69)-LGLAR (B) still elicits a contraction which must be mediated via the C3a receptor. A subsequent 100 % stimulus of hC3a (C) fails to elicit a response due to C3a receptor desensitization. (*b*) Demonstration of C5a activity. A single application of a small dose of hC3a (C*) completely desensitizes the C3a receptor to a subsequent 100 % stimulus, a characteristic feature of a C3a-specific response in this assay [32]. Application of rC5a-(1–69)-LGLAR (B) still elicits a strong and prolonged contraction which must be mediated via the C5a receptor. A subsequent 100 % stimulus of rhC5a (A) elicits a decreased response, thus demonstrating some C5a receptor desensitization. The arrow indicates an additional 10 min interval to allow for more efficient C5a receptor desensitization. (*c*) After complete C5a and C3a receptor desensitization, the rC5a-(1–69)-LGLAR mutant fails to elicit any smooth muscle contraction. A, 0.76 μM-rhC5a; B, 5.2 μM-rC5a-(1–69)-LGLAR; C, 1 μM-hC3a; C*, 0.1 μM-hC3a.

Table 1. Biological activity of hC3a, LGLAR [C3a-(73–77)], rC5a-(1–69)-LGLAR and rhC5a in the assay of ATP release from guinea pig platelets

Anaphylatoxic activities were measured by the ability of the substances to release ATP from guinea pig platelets. ATP release was quantified by a luciferin–luciferase reaction with subsequent photon counting [17,33]. For activation curves, about 1.6×10^7 platelets were incubated with various dilutions of the activating peptide in a total volume of 150 μl of Tyrode buffer containing 2 mM-Ca^{2+} for 30 s at 37 °C. A 100 μl portion of Lumit (luciferin–luciferase kit; Tecator) was added and the light emission was counted for 10 s. The ED_{50} value is the concentration leading to half-maximal ATP release. For desensitization curves, about 3.5×10^7 platelets were incubated in 230 μl of Tyrode buffer containing 2 mM-Ca^{2+} with various concentrations of desensitizing peptide for 15 min at 37 °C. The extent of receptor desensitization was quantified by adding 50 μl of a 100% stimulus of hC3a (~ 120 nM) or rhC5a (~ 300 nM) to 100 μl of the desensitized platelet preparation and measuring ATP release as described above. The peptide concentration leading to half-maximal inhibition of ATP release by a 100% stimulus of hC3a or rhC5a is given as the ED_{50} value for C3a receptor or C5a receptor desensitization respectively. Prior to measuring the C3a-specific activation and desensitization curves of the rC5a-(1–69)-LGLAR hybrid, the C5a receptor was completely desensitized by repeated low-dose desensitization with rhC5a as described in the Materials and methods section. Results are means ± S.D. N.D., not determined.

	ED_{50} (nM)			
	C5a-specific		C3a-specific	
Ligand	Activation	Desensitization	Activation	Desensitization
hC3a	None	N.D.	2.9*	0.49*
LGLAR	None†	N.D.	32000†	N.D.
rC5a-(1–69)-LGLAR	4.3 ± 1.4	1.4 ± 0.5	264 ± 42	9.9 ± 5.1
rhC5a	7.7 ± 2.1	3.2 ± 0.8	None	N.D.

* Data from [17].
† Data from [16].

into Ser, thus potentially seriously affecting C5a activity. To minimize the number of simultaneous mutations, we decided to construct a rC5a-(1–69)-LGLAR mutant, requiring only three amino acid exchanges in the *C*-terminus of rhC5a.

The gene for the rC5a-(1–69)-LGLAR mutant was obtained by PCR-mediated site-directed mutagenesis [25] of the recombinant human C5a clone pME10. The mutated PCR fragment was cloned into plasmid pKK233-2 to generate the recombinant plasmid pME18 containing a *trc* promotor (a derivative of the *lac* promotor) and an ATG initiation codon in front of the C5a-(1–69)-LGLAR sequence. The DNA sequence for the first 69 amino acids was identical to the known cDNA sequence of hC5a [38], with the exception of a silent transition in codon 52 (ACT → ACC). This mutation, however, was already present in pME10 [25]. The gene was expressed in *E. coli* Y1089 and purified to homogeneity with a final yield of about 1 mg of purified protein per litre of bacterial culture. The purified protein was homogeneous on silver-stained SDS/PAGE gels, immunoreactive with the anti-C5a monoclonal antibodies 557 and 561 [37] and showed a c.d. spectrum in the far-u.v. region (180–250 nm) identical to those of our rhC5a (Fig. 1), porcine C5a [39] and bovine des-Arg^{74}-C5a [40]. The mutations therefore do not interfere with the correct folding of rC5a-(1–69)-LGLAR, which was to be expected from the known three-dimensional structure in solution of rhC5a, in which the *C*-terminal sequence rhC5a-(64–74) adopts a random coil structure [41].

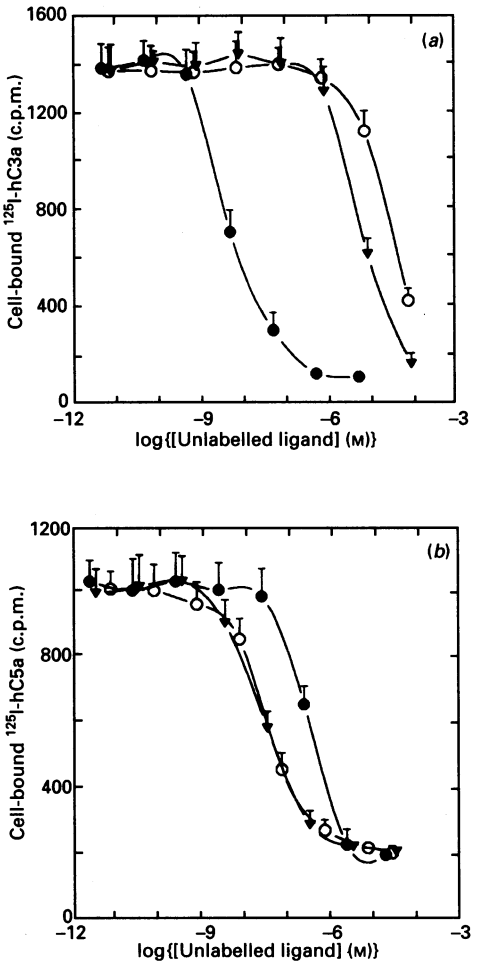

Fig. 3. Competitive binding experiments

(*a*) Inhibition of ^{125}I-hC3a binding to guinea pig platelets by hC3a, rC5a-(1–69)-LGLAR and rhC5a. About 2.9×10^7 guinea pig platelets were incubated with 3.8×10^4 c.p.m. of ^{125}I-hC3a (~ 450 Ci/mmol) and unlabelled ligand (at the concentrations shown on the abscissa) in 150 μl of Tyrode buffer for 30 min at room temperature. Unbound ligand was removed by centrifugation through a 10% sucrose cushion and cell-bound radioactivity was counted. Shown are competitive binding curves (means + S.D.) for hC3a (●, $n = 3$), rC5a-(1–69)-LGLAR (▼, $n = 2$) and rhC5a (○, $n = 3$) from n experiments in triplicate. (*b*) Inhibition of ^{125}I-rhC5a binding to guinea pig platelets by hC3a, rC5a-(1–69)-LGLAR and rhC5a. About 1.4×10^8 guinea pig platelets were incubated with 1.5×10^5 c.p.m. of rhC5a (~ 1700 Ci/mmol) and unlabelled ligand (at the concentrations shown on the abscissa) in 150 μl of Tyrode buffer for 30 min at room temperature. Cell-bound radioactivity was determined as described in the text. Shown are competitive binding curves (means ± S.D.) for hC3a (●, $n = 2$), rC5a-(1–69)-LGLAR (▼, $n = 2$) and rhC5a (○, $n = 3$) of n experiments in triplicate.

Characterization of rC5a-(1–69)-LGLAR

Functional characterization of the rC5a-(1–69)-LGLAR protein in the assay of myeloperoxidase release from human granulocytes revealed full C5a activity [ED_{50} of 1.0 ± 0.2 nM for rC5a-(1–69)-LGLAR, compared with an ED_{50} of 0.8 ± 0.1 nM for rhC5a]. However, human granulocytes do not allow for functional testing of C3a activity. We therefore used two assays which allow direct comparison of C3a versus C5a activity: the guinea pig ileum contraction assay and the assay of ATP release from guinea pig platelets. The results of the former are shown in Fig. 2. They convincingly demonstrate the C3a as well as the C5a activity of the rC5a-(1–69)-LGLAR protein. The fact that this

hybrid anaphylatoxin did not elicit a full C3a-specific contraction even at 5.2 μM (Fig. 2*a*) suggests that its inherent C3a activity is lower than that of hC3a itself. However, the guinea pig ileum contraction assay only allows a semi-quantitative estimation of anaphylatoxic activities [42] unless proper dose–response curves are recorded [32]. This, however, would require excessively large amounts of substance. We therefore used measured ATP release from guinea pig platelets to quantify the inherent C3a and C5a activities (Table 1). In this test system, the hybrid displayed true C3a activity as well as C5a activity. The C5a activity was essentially unaltered in comparison with rhC5a; it even seemed to be slightly (though not significantly) enhanced (almost 2-fold). The inherent C3a activity was about 1 % of that of native hC3a, i.e. about 100-fold more active on a molar basis than the pentapeptide LGLAR alone. As a comparison, hC4a has about 3 % hC3a activity when tested in the assay of 5-hydroxytryptamine release from guinea pig platelets [43]. Furthermore, the hybrid was able to induce receptor-specific desensitization, thus once more proving its dual activity.

The results of competitive binding experiments with radioiodinated hC3a and rhC5a on guinea pig platelets are shown in Figs. 3(*a*) and 3(*b*). As expected, the hybrid anaphylatoxin was able to displace ^{125}I-hC3a with a K_I of $\sim 3.7 \pm 2.2$ μM (compared with a K_D of $\sim 3.0 \pm 1.5$ nM for hC3a itself). ^{125}I-rhC5a was displaced with a K_I of $\sim 40 \pm 20$ nM, indistinguishable from that of rhC5a itself (K_D 25 ± 10 nM). Interestingly, however, we observed cross-displacement of the anaphylatoxins, with hC3a being able to displace ^{125}I-rhC5a with a K_I of 290 ± 120 nM, and rhC5a being able to displace ^{125}I-hC3a with a K_I of $\sim 20 \pm 7.5$ μM. However, no functional cross-activation could be demonstrated (Table 1).

DISCUSSION

The construction of functionally active hybrid molecules is a well-known technique. Different active protein modules or domains are fused in-frame, a technically simple procedure which is exploited in several vector systems to express and purify gene products as fusion proteins with marker proteins such as β-galactosidase [44], glutathione *S*-transferase [45] and maltose-binding protein [46,47]. This approach fails, however, if both proteins to be fused simultaneously require a free *N*- or *C*-terminus for full activity, as is the case with the anaphylatoxins hC3a and hC5a. A thorough examination of the sequence determinants essential for activity is then necessary in order to decide whether construction of a functionally active hybrid protein is feasible or whether the sequence requirements are incompatible with each other.

In previous studies with short synthetic peptides we have determined the minimal peptide sequence necessary for C3a receptor activation [17]. Our results suggested that a hybrid C3a/C5a anaphylatoxin should be feasible, as explained in the Results section. It is, however, always problematic to extrapolate results obtained with short synthetic peptides to the much more complex situation of a whole protein. Additional receptor sites contributing little or nothing to the overall affinity might well be crucially important in excluding other ligand binding, e.g. by steric hindrance.

To test our hypothesis, we have previously cloned and expressed the gene coding for human C5a in *E. coli* [25] and subsequently constructed the rC5a-(1–69)-LGLAR mutant by site-directed mutagenesis of this cDNA clone. Our expression system allowed us to obtain milligram quantities of both proteins in a structurally and functionally fully active form.

The three amino acid exchanges introduced into rhC5a to create the rC5a-(1–69)-LGLAR mutant did, indeed, suffice to confer an additional, qualitatively new, C3a activity to this molecule. All of these exchanges are located in a short 5-amino acid stretch at the *C*-terminus which is involved in binding to both the C3a and the C5a receptors. We could thus assign in detail the function of these residues in the binding to and activation of the anaphylatoxin receptors.

Our binding data (Figs. 3*a* and 3*b*) clearly show that all anaphylatoxins are able to bind to the guinea pig C3a receptor as well as to the C5a receptor. This suggests a close similarity of the respective ligand-binding pockets of the anaphylatoxin receptors into which the (structurally very similar) anaphylatoxins fit. However, this effect may also be due to unspecific ionic interactions of the quite basic anaphylatoxins with their receptors. This interpretation is supported by the observation that protamine sulphate, a highly basic protein, is able to bind competitively to the anaphylatoxin receptors and to inhibit anaphylatoxic activities *in vitro* ([40]; T. Kretzschmar, unpublished work). A large contribution of ionic interactions to the overall binding affinity would explain the rather high affinity of the very basic hC3a (isoelectric point > 9 as compared with 8.5 for rhC5a) for the C5a receptor (with an apparent affinity only about 10-fold lower than that of hC5a itself; see Fig. 3*b*).

In contrast, activation of the anaphylatoxin receptors is strictly ligand-specific (Table 1), being solely dependent on the presence of the appropriate effector sites. The guinea pig C3a receptor seems to become activated only by ligands containing the *C*-terminal sequence LGLAR. *N*-Terminal addition of large foreign sequences such as C5a-(1–69) to the activating LGLAR peptide, thus creating the rC5a-(1–69)-LGLAR hybrid, is well accepted and even has increased activity when compared with LGLAR alone (Table 1). However, binding to the receptor site for LGLAR in the C3a receptor does not contribute very much to the overall affinity of activating ligands, since hC5a and rC5a-(1–69)-LGLAR display very similar binding behaviour (Fig. 3*a*), but only the latter is able to activate the C3a receptor. Since the rC5a-(1–69)-LGLAR mutant displays a 100–1000-fold lower affinity and activity towards the C3a receptor than native hC3a, additional binding sites for hC3a must be present in the C3a receptor to account for the difference. On the basis of results obtained with synthetic C3a analogue peptides, a hydrophobic binding site in the C3a receptor has been postulated which has rather speculatively been attributed to the residues Tyr-59, Ile-60 and Leu-63 of the native hC3a sequence [18]. However, the affinity difference between hC3a and rC5a-(1–69)-LGLAR cannot readily be attributed to this hypothetical binding site, since a similar cluster of hydrophobic residues (Val-56, Val-57, Ala-58 and Leu-61) is also present in the hC5a and rC5a-(1–69)-LGLAR molecules. Therefore other, as yet unidentified, binding sites, possibly involving ionic interactions (see above), must exist.

The C5a activity of the hybrid anaphylatoxin is not affected by the three amino acid exchanges in the *C*-terminus (Met-70 → Leu, Gln-71 → Gly, Gly-73 → Ala). This is in accordance with the results obtained with recombinant hC5a mutants. Single amino acid exchanges at these three sites did not show any negative influence on C5a receptor activation [22]. However, it must be pointed out that these three amino acid positions in human C5a contribute to the receptor specificity: their sequence is critically important to prevent cross-activation of the C3a receptor. The two remaining residues in the *C*-terminal pentapeptide of rC5a-(1–69)-LGLAR, i.e. Leu-72 and Arg-74, are known to be essential for hC3a as well as hC5a activity. They must, therefore, be directly involved in binding to and activation of both the C3a and the C5a receptors.

The *C*-terminal sequence LGLAR in the rC5a-(1–69)-LGLAR

mutant is recognized by the guinea pig C3a and C5a receptors. This suggests a close similarity of the binding sites in the two receptors. However, differences in the discrimination pattern between the anaphylatoxin receptors are also apparent: the binding site in the C5a receptor is less sequence-restricted than that of the C3a receptor, since MQLGR [C5a-(71–74)] and LGLAR [C3a-(73–77)] are equally well accepted. The discrimination between C3a and C5a must be accomplished by additional binding sites, since neither hC3a nor the peptide LGLAR, which should be able to bind to this site, is able to activate the C5a receptor (Table 1). Indeed, it appears from recent studies with synthetic C5a analogue peptides that the minimum peptide length necessary to activate the C5a receptor encompasses at least eight amino acids from the *C*-terminus [23].

Apart from its implications for our understanding of how the anaphylatoxin receptors discriminate between the C3a and C5a ligands, the hybrid clone rC5a-(1–69)-LGLAR provides an important novel tool for future mutagenesis experiments. Mutant hybrid anaphylatoxins in which C5a-specific residues have been replaced by the respective C3a-specific ones may be created by site-directed mutagenesis and tested for decreased C5a and/or increased C3a activity simultaneously. This approach will considerably speed up the process of identifying all receptor-binding sites of both anaphylatoxins.

We thank Miss K. Rech for expert technical assistance in protein purification, and the members of the Department of Biochemistry, RWTH Aachen, for performing the c.d. spectroscopic analyses and many helpful discussions.

REFERENCES

1. Müller-Eberhard, H. J. (1988) Annu. Rev. Biochem. **57**, 321–347
2. Bitter-Suermann, D. (1988) in The Complement System (Rother, K. & Till, G. O., eds.), pp. 367–395, Springer Verlag, Berlin
3. Johnson, A. R., Hugli, T. E. & Müller-Eberhard, H. J. (1975) Immunology **28**, 1067–1087
4. Meuer, S., Ecker, U., Hadding, U. & Bitter-Suermann, D. (1981) J. Immunol. **126**, 1506–1509
5. Morgan, E. L., Thoman, M. L., Weigle, W. O. & Hugli, T. E. (1985) J. Immunol. **134**, 51–57
6. Morgan, E. L., Thoman, M. L., Weigle, W. O. & Hugli, T. E. (1983) J. Immunol. **130**, 1257–1261
7. Webster, R. O., Hong, S. R., Johnston, R. B., Jr. & Henson, P. M. (1980) Immunopharmacology **2**, 201–219
8. Fernandez, H. N., Henson, P. M., Otani, A. & Hugli, T. E. (1978) J. Immunol. **120**, 102–108
9. Haslett, C., Jose, P. J., Giclas, P. C., Williams, T. J. & Henson, P. M. (1989) J. Immunol. **142**, 3510–3517
10. Nettesheim, D. G., Edalji, R. P., Mollison, K. W., Greer, J. & Zuiderweg, E. R. P. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 5036–5040
11. Chenoweth, D. E. & Hugli, T. E. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 3943–3947
12. Fukuoka, Y. & Hugli, T. E. (1988) J. Immunol. **140**, 3496–3501
13. Gerardy-Schahn, R., Ambrosius, D., Saunders, D., Casaretto, M., Mittler, C., Karwath, G., Görgen, S. & Bitter-Suermann, D. (1989) Eur. J. Immunol. **19**, 1095–1102
14. Plummer, H. T. & Hurwitz, M. Y. (1978) J. Biol. Chem. **253**, 3907–3912
15. Hugli, T. E. & Erickson, B. W. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 1826–1830
16. Gerardy-Schahn, R., Ambrosius, D., Casaretto, M., Grötzinger, J., Saunders, D., Wollmer, A., Brandenburg, D. & Bitter-Suermann, D. (1988) Biochem. J. **255**, 209–216
17. Köhl, J., Casaretto, M., Gier, M., Karwath, G., Gietz, C., Bautsch, W., Saunders, D. & Bitter-Suermann, D. (1990) Eur. J. Immunol. **20**, 1463–1468
18. Ember, J. A., Johanson, N. L. & Hugli, T. E. (1991) Biochemistry **30**, 3603–3612
19. Caporale, L. H., Tippet, P. S., Erickson, B. W. & Hugli, T. E. (1980) J. Biol. Chem. **255**, 10758–10763
20. Meuer, S., Hadding, U., Andreatta, R. & Bitter-Suermann, D. (1981) Immunopharmacology **3**, 275–280
21. Mollison, K. W., Fey, T. A., Krause, R. A., Miller, L., Edalji, R. P., Conway, R. G., Mandecki, W., Shallcross, M. A., Kawai, M., Or, Y. S., Lane, B. & Carter, G. W. (1991) Agents Actions **35** (suppl.), 17–21
22. Mollison, K. W., Mandecki, W., Zuiderweg, E. R. P., Fayer, L., Fey, T. A., Krause, R. A., Conway, R. G., Miller, L., Edalji, R. P., Shallcross, M. A., Lane, B., Fox, J. L., Greer, J. & Carter, G. W. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 292–296
23. Kawai, M., Quincy, D. A., Lane, B., Mollison, K. W., Or, Y.-S., Luly, J. R. & Carter, G. W. (1992) J. Med. Chem. **35**, 220–223
24. Or, Y. S., Clark, R. F., Lane, B., Mollison, K. W., Carter, G. W. & Luly, J. R. (1992) J. Med. Chem. **35**, 402–406
25. Bautsch, W., Emde, M., Kretzschmar, T., Köhl, J., Suckau, D. & Bitter-Suermann, D. (1992) Immunobiology, **185**, 41–52
26. Hoffmann, T., Böttger, E. C., Baum, H. P., Messner, M., Hadding, U. & Bitter-Suermann, D. (1988) Clin. Exp. Immunol. **71**, 486–492
27. Bitter-Suermann, D. & Burger, R. (1986) Prog. Allergy **39**, 134–158
28. Hemsley, A., Arnheim, N., Tonay, M. D., Cortopassi, G. & Galas, D. J. (1989) Nucleic Acids Res. **17**, 6545–6551
29. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor
30. Mollison, K. W., Fey, T. A., Krause, R. A., Mandecki, W., Fox, J. L. & Carter, G. W. (1987) Agents Actions **21**, 366–370
31. Franke, A. E., Andrews, G. C., Stimler-Gerard, N. P., Gerard, C. & Showell, H. J. (1988) Methods Enzymol. **162**, 653–668
32. Kola, A., Klos, A., Bautsch, W., Kretzschmar, T. & Köhl, J. (1992) Clin. Exp. Immunol. **88**, 368–372
33. Kretzschmar, T., Kahl, K., Rech, K., Bautsch, W., Köhl, J. & Bitter-Suermann, D. (1991) Immunobiology **183**, 418–432
34. Gerard, N. P., Hodges, M. K., Drazen, J. M., Weller, P. F. & Gerard, C. (1989) J. Biol. Chem. **264**, 1760–1766
35. Fraker, P. J. & Speck, J. C. (1978) Biochem. Biophys. Res. Commun. **80**, 849–857
36. Cheng, Y.-C. & Prussow, W. H. (1973) Biochem. Pharmacol. **22**, 3099–3108
37. Klos, A., Ihrig, V., Messner, M., Grabbe, J. & Bitter-Suermann, D. (1988) J. Immunol. Methods **111**, 241–252
38. Lundwall, A. B., Wetsel, R. A., Kristensen, T., Whitehead, A. S., Woods, D. E., Ogden, R. C., Colten, H. R. & Tack, B. F. (1985) J. Biol. Chem. **260**, 2108–2112
39. Morgan, W. T., Vallota, E. H. & Müller-Eberhard, H. J. (1974) Biochem. Biophys. Res. Commun. **57**, 572–577
40. Gennaro, R., Simonic, T., Negri, A., Mottola, C., Secchi, C., Ronchi, S. & Romeo, D. (1986) Eur. J. Immunol. **155**, 77–86
41. Zuiderweg, E. R. P., Nettesheim, D. G., Mollison, K. W. & Carter, G. W. (1989) Biochemistry **28**, 172–185
42. Hammerschmidt, D. E. (1986) Complement **3**, 166–176
43. Meuer, S., Hugli, T. E., Andreatta, R. H., Hadding, U. & Bitter-Suermann, D. (1981) Inflammation **5**, 263–273
44. Rüther, U. & Müller-Hill, B. (1983) EMBO J. **2**, 1791–1794
45. Smith, D. B. & Johnson, K. S. (1988) Gene **67**, 31–40
46. Guan, C., Li, P., Riggs, P. D. & Inouye, H. (1988) Gene **67**, 21–30
47. Maina, C. V., Riggs, P. D., Grandea, A. G., III, Slatko, B. E., Moran, L. S., Tagliamonte, L. S., McReynolds, L. A. & Guan, C. (1988) Gene **74**, 365–373
48. Olsen, U. B., Selmer, J. & Kahl, J.-U. (1988) Complement **5**, 153–162

Received 27 March 1992/1 June 1992; accepted 9 June 1992