Articles

Designing Synthetic Superagonists of C3a Anaphylatoxin†

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ABSTRACT: An extensive structure–activity study of synthetic analogues of the C3a anaphylatoxin was conducted. Our goal was to map C3a–C3a receptor interactions by designing synthetic analogue molecules having maximal biologic potency. Nonspecific binding of the polycationic C3a to polyanionic molecules on cellular surfaces often obscures specific binding to the receptor. Less cationic synthetic C3a analogues would be useful tools in identifying and characterizing the various cell types having C3a receptors. These factors should also be useful as pharmacologic probes for mechanism studies, as high-affinity ligands for target cell identification, and for receptor isolation. Attachment of amino-terminal hydrophobic groups such as Fmoc to C3a analogues [as originally introduced by Gerardy-Schahn et al. (1988) *Biochem. J. 255*, 209] markedly enhanced the potency of synthetic C3a peptides. The enhancement effect on potency from introducing hydrophobic groups to C3a analogues was interpreted as possibly being nonspecific. Our systematic search for an optimal peptide length, composition, and N-terminal hydrophobic unit resulted in several superpotent C3a analogues having 200–1500% the potency of natural C3a. One particularly potent C3a peptide was designed by incorporating two tryptophanyl residues at the N-terminal end of a 15-residue C3a analogue. The superpotent peptide W-W-G-K-K-Y-R-A-S-K-L-G-L-A-R has several residues differing (underlined) from the sequence corresponding to positions 63–77 in human C3a, a region that contains the essential functional site of the molecule. This 15-residue model peptide exhibited the greatest biological potency of all peptides tested, being 12–15 times more active than natural C3a. Since an optimal distance was found to exist between the N-terminal hydrophobic unit (W-W) and the C-terminal primary binding site (LGLAR), we concluded that the hydrophobic unit interacts specifically with a secondary binding site on the C3a receptor. The presence of both a primary (effector) and secondary (hydrophobic) binding site on these linear synthetic ligands, which can interact cooperatively with the C3a receptor, presumably accounts for the high relative potency of the analogues. Our design of superpotent analogues of C3a demonstrates the feasibility for constructing small synthetic peptides to mimic natural biologic factors that depend on secondary or tertiary structure for their activity. These synthetic peptide studies demonstrate that a linear array of amino acids (e.g., W-W) can successfully substitute for a conformation-dependent binding site on a bioactive factor.

Activation of the blood complement cascade leads to a proteolytic cleavage of the complement component C3. Cleavage of human C3 by convertases of either the classical or the alternative pathway releases a 77-residue fragment called C3a anaphylatoxin. The C3a fragment is derived from the N-terminal end of the α chain of the C3 molecule (Müller-Eberhard, 1975). C3a is a potent mediator of inflammation capable of contracting smooth muscle tissue, through prostanoid- (Stimler et al., 1983) and histamine-mediated pathways (Dias da Silva & Lepow, 1967), and of increasing vascular permeability (Lepow et al., 1970; Wuepper et al., 1972). Macrophages release interleukin 1 (Becker et al., 1978) and thromboxane A_2 (Zanker et al., 1976) when exposed to C3a. The immunosuppressive effect of C3a on T-cell proliferation is well documented (Morgan et al., 1983, 1985). It has been reported that C3a induces lysosomal enzyme release from leukocytes (Showell et al., 1982; Nagata et al., 1987), histamine release from mast cells (Johnson et al., 1975; Glovsky et al., 1979), and serotonin and ATP release from human and/or guinea pig platelets (Meuer et al., 1981a,b; Polley & Nachman, 1983). This humoral mediator also induces guinea pig platelet aggregation (Grossklaus et al., 1976; Becker et al., 1978; Zanker et al., 1982), a phenomenon that was used to develop a fast, sensitive, and reliable microassay to evaluate the C3a analogues in this study.

In general, only micromolar or submicromolar concentrations of C3a are required to elicit these various biologic effects, suggesting the existence of specific high-affinity receptors on the target cells. These various biologic reactions to C3a, involving numerous target cell types in various tissues, are well-defined functions that have been summarized in several recent reviews (Hugli, 1986, 1989).

The presence of C3a receptors on guinea pig platelets was initially suggested on the basis of the observed biologic responses of these cells (Becker et al., 1978a,b). More recently, homobifunctional cross-linking studies by Fukuoka and Hugli (1988) revealed molecules between 95 and 105 kDa on isolated platelets that appear to be specific receptors for C3a because

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they bind C3a but not $C3a_{des\text{-}Arg}$, the inactivated form of C3a. Scatchard analysis of specific uptake provided a crude estimation of only 1200 C3a binding sites on these cells (Fukuoka & Hugli, 1988). Similar results were later reported by Gerardy-Schahn et al. (1989) showing that a specific heterobifunctional reagent cross-linked C3a molecules to a heterogeneous population of molecules (85–114 kDa) on the guinea pig platelet. These investigators also claimed to have demonstrated both high- (200 binding sites/cell) and low- (500 binding sites/cell) affinity C3a binding sites on platelets on the basis of their ligand uptake studies.

Mast cells in human skin are degranulated by exposure to C3a but not $C3a_{des\text{-}Arg}$ (Wuepper et al., 1972); consequently, this cell type is presumed to bear C3a receptors. Gervasoni et al. (1986) searched for specific C3a receptors on isolated rat mast cells. These investigators failed to identify receptors but observed high levels of background binding and a rapid degradation of C3a by chymase, an enzyme that is exposed on the mast cell surface during degranulation. Specific receptors to C3a were not identified on the isolated rat mast cells by either ligand binding or chemical cross-linking techniques (Fukuoka & Hugli, 1990). It was concluded that the rat mast cell was activated by a nonspecific mechanism involving the polycationic properties of the C3a molecule and unlike human mast cells may not express C3a receptors.

The C-terminal portion of C3a is known to be essential for spasmogenic activity (Caporale et al., 1980). This conclusion is based on earlier observations that removal of the C-terminal amino acid arginine by carboxypeptidase B digestion abolished tissue contractile activity (Bokisch & Müller-Eberhard, 1970). It was also determined that a synthetic 21-residue C-terminal fragment of C3a (i.e., C3a 57–77) expresses biologic activities essentially equipotent to that of the natural molecule (Lu et al., 1983). Shorter C3a analogues have less, but significant potency; and even analogues of the C-terminal pentapeptide, LGLAR,[1] were shown to exhibit measurable biologic potency (Unson et al., 1984).

Circular dichroism (CD) studies (Paques et al., 1980; Lu et al., 1984; Hoeprich et al., 1986) of natural C3a, C3a 57–77, and substituted analogues of peptide C3a 57–77 showed that the C-terminal portion of the natural molecule favors a helical conformation. The C-terminal analogues of C3a, which were shorter than the tridecapeptide C3a 65–77, contained only irregular or β-turn-like conformations in trifluoroethanol while the α helix was observed for the 21-residue analogue C3a 57–77 (Lu et al., 1984). It was concluded that helical conformation contributes to the higher potency of the 21-residue analogue of C3a (C3a 57–77). More recently, NMR studies of human C3a suggest that the C-terminal helical region extends from residues 49 to 65 or 66 and that the remaining 11 to 12 residues assume no regular structure (Chazin et al., 1988; Kalnik et al., 1990).

Substituted analogues of C3a that are considerably shorter than the 21-residue peptide C3a 57–77, but more potent than the 21-residue analogue, were recently reported (Gerardy-Schahn et al., 1988; Ambrosius et al., 1989). A 2–10-fold increase in biologic potency, as measured by the guinea pig platelet ATP-release assay, was achieved by attaching an N-terminal Fmoc or Nap group to various 5–13-residue analogues of C3a. The potency-enhancing effect from incorporating hydrophobic groups at the N-termini of C3a peptides was interpreted as promoting nonspecific binding interactions between the ligand and the platelet membrane. These authors suggest that the hydrophobic group inserts into the lipid bilayer, thereby increasing the effective peptide concentration that enhances receptor–ligand association. A 13-residue C3a analogue with an amino-terminal Nap group attached via a 6-aminohexanoic acid spacer group (Nap-Ahx-Y-R-R-G-R-A-A-A-L-G-L-A-R) exhibits a potency 6-fold greater than that of natural C3a (Gerardy-Schahn et al., 1988). This remarkable increase in potency ($\simeq$100-fold over the 13-residue peptide) suggested to us that the hydrophobic interaction may actually be specific for the C3a receptor.

On the basis of sequence and structural requirements for optimal biologic potency of C3a analogues, we conclude that a bulky hydrophobic unit interacts optimally and specifically with a secondary site on the receptor. A conceptual model for the C3a receptor–ligand interaction is presented on the basis of our current knowledge of the folded natural factor and structural requirements for the synthetic analogues.

Materials and Methods

Synthesis of C3a Analogue Peptides. The C3a analogues prepared for this study were synthesized by using standard solid-phase methodology (Merrifield, 1963) and were either assembled with a Fmoc protection scheme and a multireaction vessel manifold (The RAMPS Multiple Peptide System, Du Pont) or assembled with the Boc protection scheme on an automatic peptide synthesizer (430A, Applied Biosystems).

In the case of the Fmoc strategy, Bu^t, OBu^t, Boc (lysine), Mtr (arginine), and Trt (histidine) were used for side-chain protection with polystyrene-based *p*-alkoxybenzyl alcohol resin, which was obtained with the C-terminal residue attached (Du Pont). Couplings were performed in *N*-methylpyrrolidone for 90 min with 2.5 equiv of preformed HOBt esters, and the reaction was monitored by using the qualitative ninhydrin test (Stewart & Young, 1984). Deprotection of the Fmoc group was accomplished in dimethylformamide containing 20% piperidine. The deprotection and cleavage of the peptide from the resin was carried out in TFA/phenol/4-(methylthio)-phenol/ethanedithiol/2-methylindole/H_2O (26:1:1:1:1:1) for 18 h at room temperature, followed by precipitation and washing of the crude peptide with diethyl ether.

In the case of the Boc strategy, Bzl, OBzl, Clz (lysine), Tos (arginine), and Bom (histidine) were used for side-chain protection in connection with the standard protocols (Std 1r) supplied by Applied Biosystems (Foster City, CA). A polystyrene-based [[*p*-(hydroxymethyl)phenyl]acetamido]methyl resin was used that was obtained with the C-terminal residue attached (Applied Biosystems).

The peptides were deprotected of Boc groups and cleaved from the resin by using liquid HF/*m*-cresol (9:1) at 0 °C for 1 h, or in the cases of tryptophan-containing peptides we used the low–high HF procedure (Tam et al., 1983).

All of the peptides were purified by semipreparative RP-HPLC on a 16 mm × 200 mm (5-μm) C-18 column with a linear gradient of water to 60% acetonitrile in 0.2 M $(NH_4)_2SO_4$ at pH 2.7. The peptides were desalted on Sep-Pak cartridges (Millipore) followed by washing with 0.1% TFA and elution with 70% acetonitrile in 0.1% TFA. The eluates were

[1] Abbreviations: ACD, acid citrate dextran; Ahx, 6-aminohexanoic acid; Boc, *tert*-butyloxycarbonyl; Bom, benzyloxymethyl; Bu^t, *tert*-butyl ether; Bzl, benzyl ether; Clz, 2-chlorobenzyloxycarbonyl; CPB, carboxypeptidase B; EBSS, Earle's balanced salt solution; EDTA, ethylenediaminetetraacetic acid; Fmoc, 9-fluorenylmethyloxycarbonyl; For, formyl; GP, guinea pig; HOBt, *N*-hydroxybenzotriazole; HU, human; LGLAR, leucylglycylleucylalanylarginine; Mtr, 4-methoxy-2,3,6-trimethylbenzenesulfonyl; MU, mice; Nap, 2-nitro-4-azidophenyl; NMP, *N*-methylpyrrolidone; OBu^t, *tert*-butyl ester; OBzl, benzyl ester; PG, porcine; PRP, platelet-rich plasma; RT, rat; TFA, trifluoroacetate; TFE, trifluoroethanol; Tos, *p*-toluenesulfonyl; Trt, triphenylmethyl.

diluted 10-fold with water and lyophilized.

The peptides were characterized by RP-HPLC and amino acid analysis. RP-HPLC analysis was performed on C-18 columns with different gradients of acetonitrile in 0.2 M $(NH_4)_2SO_4$ at pH 2.7 and UV detection at 214 nm (specific conditions are given in Table I of the supplementary material). Concentrations and purity of peptides in the stock solutions (10% ethanol in water) used for biological assays were determined by amino acid analysis (Beckman Model 6300). The amino acid compositions of isolated peptides were in good agreement with the expected values, and all peptides were homogeneous according to HPLC analysis (retention times of the peptides are given in Table II of the supplementary material).

Mass Spectra. The FIB positive-ion mass spectra of each peptide were obtained on a VG ZAB-VSE double-focusing mass spectrometer equipped with a cesium ion gun. The mass spectrometer was manually tuned to a resolution of 2000 (10% valley definition) with amplifier and multiplier gains (300 V) of 10^6. A 35-keV cesium ion beam was used as the fast ion beam, and the accelerating voltage for the desorbed ions was set at 8 kV. Spectra were recorded with a Digital VAXstation 3100, and the peaks were automatically centroided. The *m*-nitrobenzyl alcohol matrix compound was obtained from Aldrich at 98% purity. The mass spectra were acquired by scanning the magnet through the mass range of 310–3000 Da with CsI being used to calibrate the instrument; typically 10 spectra were accumulated and averaged (peptide mass determinations presented in Table II of the supplementary material).

Circular Dichroism. CD studies were performed on an AVIV 61DS spectropolarimeter. Spectra were recorded by using peptide solutions of approximately 0.1 mg/mL in 0.01 M phosphate buffer at pH 7.4. The exact peptide concentrations were determined by amino acid analysis. A 1.5-nm bandwidth and a 0.5-nm step size were used to measure the peptide solutions in 2-mm path-length cuvettes at 25 °C. Multiple scans (n = 3 or 5) were averaged for both samples and buffer controls. After correction of the spectrum for solvent contribution, nonlinear regression analysis was used to fit the data. Mean residue ellipticity (θ) was expressed in units of deg cm^2/dmol. Secondary structure parameters were estimated by using the program PROSEC (Aviv Associates, Lakewood, NJ), based on the method of Chang et al. (1978).

Preparation of Des-Arg Peptides. The des-Arg peptides were prepared by incubating 2.5 nmol of peptides with 0.75 unit of carboxypeptidase B (CPB) (Type I from porcine pancreas, Sigma) for 15 min at 37 °C. The reaction was terminated by a 200-fold dilution with ice-cold phosphate buffer. Further dilutions of the peptide were made in performing the aggregation assay. In the case of peptide E7, CPB and peptide des-Arg were separated by gel filtration (Bio-Rad P-10). Treatment by CPB abolished the platelet aggregation activity of all synthetic peptides tested up to 5×10^{-6} M.

Preparation of C3a. Human C3a was prepared by the method of Hugli et al. (1981).

Guinea Pig Platelet Aggregation Assay. Arterial blood was collected from the carotid artery of anesthetized (sodium pentobarbital, Nembutal Abbott Labs.) male Hartley guinea pigs (400–600 g) and mixed in a 1:9 (v/v) ratio with 3.9% sodium citrate at pH 7.3. Platelet-rich plasma (PRP) was prepared by differential centrifugation and diluted to 3×10^8 platelets/mL of 0.39% citrate solution. Peptide samples to be tested were diluted stepwise in 1.5-fold dilutions in EBSS (Gibco) and placed in BSA-coated (BSA, Sigma) 96-well microplates (Corning). A 20-μL peptide sample, 80 μL of prewarmed (37 °C) PRP, and one 3-mm-diameter glass bead were placed in each well for mixing. The plates were placed on a horizontal shaker for 5 min at 110 rpm and 37 °C. The aggregation reaction was visually evaluated by scoring the plates (+ or −) in an inverted microscope at a 40× magnification. The greatest dilution of peptide showing visible aggregates was considered the threshold concentration for activity. An internal standard was included in each series of experiments. The average threshold activity for [Ala^{57}]C3a 57–77 was 0.092 ± 0.013 μM (n = 31). At least three independent duplicate determinations were conducted for every peptide. The results were expressed as percent potency relative to the reference peptide [Ala^{57}]C3a 57–77 with standard deviations.

Vascular Permeability Assay. The skin test to measure enhanced vascular permeability in guinea pig skin followed the procedure of Cochrane and Müller-Eberhard (1968). The peptide samples were diluted in sterile saline and injected intradermally in 50-μL aliquots on the dorsum of anesthetized (Ketalar, Parke-Davis) male Hartley guinea pigs. Two milliliters of a 1% solution of Evans blue dye (Eastman Kodak Co.) in saline was administered by cardiac puncture. The developing wheal and flare reaction was visualized by leakage of Evans blue dye into the skin. After 30 min, the animals were sacrificed and the dorsal skin was removed. Connective tissue was cleared away, the area of dye infiltration was measured, and the skin was photographed for documentation.

Smooth Muscle Contraction Assay. Smooth muscle contraction was measured on guinea pig ileal segments by the methods of Cochrane and Müller-Eberhard (1968) with modifications as described by Caporale et al. (1980) and Stimler et al. (1981), respectively. Each segment of ileum was suspended in Tyrode's solution (2 mL) maintained at 37 °C and with a constant oxygen purge. The tissue segment was stimulated with two consecutive applications of histamine (0.26 μg/mL). After each application the muscle strip was quickly washed twice with fresh Tyrode's solution. An aliquot of either human C3a or synthetic peptide in Tyrode's solution was immediately applied to the lower end of the suspended tissue segment, and the contraction was recorded. Tissue desensitization (tachyphylaxis) was used to measure the specificity of the response. Ileal strips previously exposed to human C3a failed to contract when exposed to synthetic C3a peptides and, vice versa, if the response is specific.

Results

The C-terminal sequences of C3a molecules obtained from different animal species are listed for comparison in Table I. The relative activities given are based on the guinea pig ileal assay. The C3a isolated from various sources show approximately the same potencies, except for rat C3a, which is approximately twice as potent as human C3a. The consensus sequence shows that the C-terminal pentapeptide (LGLAR) has been rigorously conserved. Several positively charged residues (Arg-64 and Arg-69) and certain of the hydrophobic side chains (Tyr-59, Ile-60, and Leu-63) are also apparently conserved. The residue positions are identified on the basis of the human C3a sequence.

Analysis of Peptides. Each of the synthetic peptides used in the study was applied to an analytical RP-HPLC C-18 column and eluted as a single peak whose retention times are reported in Table II of the supplementary material. The amino acid compositions for all peptides synthesized were determined, and the results corresponded to the expected integral values (Table III of the supplementary material). Mass spectral

Table I: Relative Activities of Natural C3a from Various Species

Peptide	Sequence (60 / 70 / 77)	Potency[a] (%)
C3a HU	<-C-N-Y-I-T-E-L-R-R-Q-H-A-R-A-S-H-L-G-L-A-R	100
C3a GP	<-C-T-Y-M-A-Q-L-R-Q-Q-H-R-R-E-Q-H-L-G-L-A-R	100
C3a MU	<-C-N-H-I-T-K-L-R-E-Q-H-R-R-D-H-V-L-G-L-A-R	ND
C3a PG	<-C-E-Y-I-A-K-L-R-Q-Q-H-S-R-N-K-P-L-G-L-A-R	100
C3a RT[b]	<-C-N-Y-I-T-K-L-R-E-Q-H-R-R-D-H-V-L-G-L-A-R	210
CONSENSUS	-C- -Y-I- - -L-R- -Q-H- -R- - - -L-G-L-A-R	

[a] The potency of human (HU), guinea pig (GP), pig (PG), and rat (RT) C3a is based on the ileal assay. ND: the potency of mouse (MU) C3a has not been determined. [b] Rat C3a is 78 residues long and exhibits the greatest potency of the natural factors.

Table II: Point-Substituted Analogues of Human C3a Peptide [Ala[57]]C3a 57–77

Peptide	Sequence (60 / 70 / 77)	Potency relative to Ala[57] C3a 57-77[a] (%)
C3a HU	<-C-N-Y-I-T-E-L-R-R-Q-H-A-R-A-S-H-L-G-L-A-R	150 ± 59
A 1	**A-N-Y-I-T-E-L-R-R-Q-H-A-R-A-S-H-L-G-L-A-R**	**100**
A 2	----------Q----------	220 ± 20
A 3	----------A----------	50 ± 12
A 4	----------A----------	90 ± 18
A 5	----------L----------	100 ± 22
A 6	----------A----------	100 ± 12
A 7	----------A-A----------	150 ± 31
A 8	----------K----------	170 ± 28
A 9	----------K----------	150 ± 21
A10	----------K----------	200 ± 32
A11	----------	40 ± 11

[a] Potencies of peptides A1–A11 were determined on the basis of a guinea pig platelet aggregation assay. The threshold concentration of [Ala[57]]C3a 57–77 required to induce guinea pig platelet aggregation is 92 ± 13 nM (n = 31). The 21-residue C3a analogue [Ala[57]]C3a 57–77 is listed as reference peptide A1 (bold type).

analysis of all peptides agreed within 1 mass unit of the expected value (Table II, supplementary material). One model C3a analogue exhibiting maximal potency underwent sequence analysis, and the results confirmed the expected primary structure as presented in Table IV of the supplementary material.

Single-Point Substitutions in C3a 57–77. Several 21-residue analogues, based on the C-terminal sequence of human C3a, were synthesized with single residue replacements made at internal sites (Table II). The goal of this study was to identify specific residues or local regions in C3a 57–77, other than LGLAR, that are important for biologic potency. Potencies were determined by a platelet aggregation assay and are expressed relative to the synthetic 21-residue peptide (C3a 57–77) (Lu et al., 1984), a fully active fragment that is based on the human C3a sequence.

As shown in Table II, single-residue substitutions at selected internal sites in the peptide, even replacement of conserved internal residues such as arginine 69, histamine 67, or glutamine 66, have only minor effects on potency. In peptide A3, where arginine 64 is replaced by an alanine, the potency is somewhat reduced. In peptide All, where four N-terminal residues are deleted, potency is significantly decreased, indicating that the amino-terminal end of the peptide is important. The deletion analogue All, a 16-residue peptide, has less than half of the potency of the reference peptide C3a 57–77, a result consistent with the C3a peptide series reported previously by

Table III: Relative Potencies of C3a Analogues Containing Fmoc and Other Hydrophobic Amino Acid Groups: Influence of Peptide Length on Activity

Peptide	Sequence (65 / 69 / 73 / 77)	Potency relative to Ala[57] C3a 57-77[a] (%)
C3a Hu	-R-R-Q-H-A-R-A-S-H-L-G-L-A-R	
B 1	L-G-L-A-R	0.2[b]
B 2	R-Y-A-S-K-L-G-L-A-R	7 ± 2
B 3	Y-R-A-S-K-L-G-L-A-R	55 ± 11
B 4	Fmoc-Y-R-A-S-K-L-G-L-A-R	121 ± 13
B 5	W-G-G-Y-R-A-S-K-L-G-L-A-R	99 ± 24
B 6	W-W-G-G-Y-R-A-S-K-L-G-L-A-R	259 ± 60
B 7	W-I-G-G-Y-R-A-S-K-L-G-L-A-R	118 ± 5
B 8	I-I-G-G-Y-R-A-S-K-L-G-L-A-R	50 ± 14
B 9	I-I-G-G-Y-R-K-S-A-L-G-L-A-R	37 ± 27
B10	G-I-G-G-Y-R-K-S-A-L-G-L-A-R	41 ± 37
B11	I-G-G-G-Y-R-K-S-A-L-G-L-A-R	16 ± 3
B12	R-R-Y-A-S-K-L-G-L-A-R	18 ± 3
B13	R-R-R-Y-A-S-K-L-G-L-A-R	50 ± 10
B14	W-R-R-R-Y-A-S-K-L-G-L-A-R	66 ± 4
B15	I-R-R-R-Y-A-S-K-L-G-L-A-R	99 ± 14
B16	**W-W-R-R-R-Y-A-S-K-L-G-L-A-R**	**296 ± 91**
B17	**Fmoc-R-R-R-Y-A-S-K-L-G-L-A-R**	**261 ± 160**
B18	Fmoc-I-R-R-R-Y-A-S-K-L-G-L-A-R	50 ± 3
B19	Fmoc-W-W-R-R-R-Y-A-S-K-L-G-L-A-R	53 ± 3
B20	Fmoc-W-R-R-R-Y-A-S-K-L-G-L-A-R	70 ± 4

[a] Potencies of peptides B2–B20 were determined on the basis of the guinea pig platelet aggregation assay. [b] Based on the concentration of peptide required for guinea pig ileal contraction relative to C3a.

Caporale et al. (1980). These results imply that the amino- and carboxyl-terminal ends of the C3a 21-mer are important for optimal potency but that the internal portion including residues 65–72 is of minor functional consequence.

Effect of Hydrophobicity on C3a Peptide Activity. Previous studies on synthetic C3a analogues by Caporale et al. (1980) demonstrated that increasing peptide chain length progressively increased potency of the C3a peptides. Since the C-terminal pentapeptide LGLAR exhibits only about 0.2% of the potency of native C3a and nearly full potency is attained at the 21-residue stage, it was concluded that the 21-mer contributes the essential elements required for optimizing C3a activity. As shown by Gerardy-Schahn et al. (1988), introduction of a hydrophobic group to the N-terminal end of short analogues of C3a, peptides significantly smaller than the 21-mer of C3a, markedly increased their potency. According to the consensus sequence, hydrophobic groups (Tyr-59, Ile-60, and Leu-63) near the N-terminal end of the 21-mer appear to be conserved (Table I). Therefore, preserving this hydrophobic region appears to be important for maintaining high potency of the analogues. To further investigate this observation, we synthesized analogues of various chain lengths, and different hydrophobic groups were attached at the N-terminal position. The results in Table III show that introduction of hydrophobic groups to the N-terminus indeed increases potency. There is nearly a 10-fold increase in potency from peptides B2 to B3, when the more hydrophobic tyrosyl group is interchanged with arginine at the N-terminal position. Similar effects are noted when the Fmoc group is added (B4), the latter showing approximately a 2-fold increase in potency. Therefore, a decapeptide with an N-terminal Fmoc group exhibits 120% potency compared to the longer 21-residue reference peptide A1 (Table I).

Attachment of different aliphatic or aromatic amino acids at the N-terminal position of dodecapeptides (-G-G-Y-R-A-S-K-L-G-L-A-R or -G-G-Y-R-K-S-A-L-G-L-A-R) indicates a requirement for specific hydrophobic groups. Introduction of tryptophan (B5–B7) resulted in peptides generally more

Table IV: Enhancing Effect of Hydrophobic Groups on Potency of C3a and C5a Analogues

Peptide		Sequence	Potency relative to Ala[57] C3a 57-77 (%)
C 1	C3a analogs[a]	Y-A-S-K-L-G-L-A-R	3.4 ± 3
C 2		Ahx-Y-A-S-K-L-G-L-A-R	13 ± 3
C 3		Fmoc-Ahx-Y-A-S-K-L-G-L-A-R	22 ± 3
C 4		W-Ahx-Y-A-S-K-L-G-L-A-R	22 ± 3
C 5		Fmoc-Ahx-Y-R-A-S-K-L-G-L-A-R	112 ± 58
C 6		W-W-Ahx-Y-R-A-S-K-L-G-L-A-R	603 ± 96
C 7	C5a analogs[b]	Y-S-H-K-G-M-L-L-G-R	0.5 ± 0.10
C 8		Ahx-Y-S-H-K-G-M-L-L-G-R	0.4 ± 0.05
C 9		Fmoc-Ahx-Y-S-H-K-G-M-L-L-G-R	0.8 ± 0.26
C10		W-Ahx-Y-S-H-K-G-M-L-L-G-R	1.2 ± 0.35

[a] The C3a analogue sequence is based on the human C3a sequence. [b] The C5a analogue sequence is based on the rat C5a sequence.

potent than those containing isoleucine or glycine (B8–B11). Similar effects can be seen in the series B12–B17 with the addition of various hydrophobic groups to peptides of different length. Extending the C3a analogues at the N-terminal end by introducing one or more hydrophilic residues (e.g., arginyl) has a similar but less significant effect on enhancing potency, as can be seen by comparing analogue B2 with analogue B12 or B13. The most effective analogues contain either two tryptophanyl or one Fmoc residue at the N-terminal end, perhaps indicating that aromatic groups were preferred over aliphatic groups. Effectiveness of the hydrophobic groups decreases in the following order: W-W ≥ Fmoc > W ≃ W-I > I-I > I-G. However, if the N-terminal hydrophobic group is too bulky, as appears to be the case with peptides B18–B20, having either Fmoc-I, Fmoc-W, or Fmoc-W-W, then the potency actually decreases.

Specific Role of the Hydrophobic Group. Introduction of hydrophobic N-terminal groups to C3a peptides might result in either additional or enhanced specific receptor–ligand interactions, but it may also be the result of nonspecific interactions between the peptide and the plasma membrane bilayer as proposed by Gerardy-Schahn et al. (1989). Table IV shows a 5–10-fold increase in the potency for Ahx-, Fmoc-Ahx-, or W-Ahx-derivatized nonapeptide analogues of C3a (C1–C4), respectively. When a decapeptide (B3 from Table III) was derivatized with a 6-aminohexanoate extension and two tryptophanyl groups were added (C6), the derivative was 10 times more potent than the decapeptide B3. We found that peptides based on the C-terminal sequence of C5a also exhibit weak spasmogenic and platelet-aggregating activity. The C5a molecule is related to C3a by sequence homology (Fernandez & Hugli, 1977) but interacts with a separate and unique set of cellular receptors. The synthetic C5a decapeptide Y-S-H-K-G-M-L-L-G-R is an analogue of the C-terminal peptide of rat C5a (G-S-H-K-G-M-L-L-G-R) (Cui et al., 1985). Therefore, if potency is nonspecifically enhanced because peptides having N-terminal hydrophobic groups are inserted into the membrane bilayer, then introducing Ahx, Fmoc, or W to the C5a analogues should also result in enhanced potency. The attachment of a hydrophobic group to C5a peptides had little effect on the potency of the C5a decapeptides (C6–C9). This result suggests that the influence of the hydrophobic groups on C3a peptides is not just to insert the peptide into the membrane bilayer, resulting in an increase in effective ligand concentration near the receptor, but rather to promote a specific interaction with the C3a receptor.

Structural Requirements for C3a Peptides. Our search for an optimally potent C3a analogue led us to investigate further the region between the N-terminal hydrophobic group and the C-terminal LGLAR sequence. A set of nonapeptides were synthesized with the Fmoc group at the N-terminal position and having various internal replacements corresponding to positions 69–72 in C3a. As seen in Table V, systematic placement of an arginine in positions 69–72 (D1–D4) had little effect on potency and there was no significant effect observed when two argininyl residues were introduced in this region (D5), relative potencies of all peptides being between 35% and 80%. In the case of longer analogues, D6–D9, where the influence of residues 66 to 67 was investigated, neither the presence of a bulky charged group (D6, D7), elimination of the side chains (D8), nor insertion of the 6-aminohexanoate spacer group (D9) had a major influence on potency. All of the Fmoc dodecapeptides were more than 100% potent relative to C3a 57–77. These results indicate that residues in positions 66–72 of C3a serve mostly a spacer function.

There is no apparent requirement for helical conformation in these shorter peptides as there was for the 21-residue C3a analogue (Hoeprich & Hugli, 1986). This conclusion is based on a series of replacements made at C3a position 70 (D10–D13 on Table V) in tetradecapeptides where L-alanine is replaced by either lysine, proline, or D-alanine. Both proline and D-alanine were substituted to disrupt a helical conformation, and CD measurement of peptide D12 confirms our prediction indicating that this peptide fails to assume a helix even in TFE (see Table VI). Substitution of proline did not greatly reduce the relative potency of the modified C3a analogue.

Location of the Hydrophobic Group. As already shown from comparing peptides B3 and B4 in Table III, the exact

Table V: Structural Requirements for C3a Analogues Containing an N-Terminal Hydrophobic Group

Peptide	Sequence (64 69 73 77)	Potency relative to Ala[57] C3a 57-77 (%)
D 1	Fmoc-A-A-A-R-L-G-L-A-R	66 ± 1
D 2	Fmoc-A-A-R-A-L-G-L-A-R	41 ± 3
D 3	Fmoc-A-R-A-A-L-G-L-A-R	37 ± 7
D 4	Fmoc-R-A-A-A-L-G-L-A-R	79 ± 9
D 5	Fmoc-R-A-A-R-L-A-L-A-R	61 ± 28
D 6	Fmoc-R-R-Y-R-A-S-K-L-G-L-A-R	176 ± 83
D 7	Fmoc-K-K-Y-R-A-S-K-L-G-L-A-R	279 ± 79
D 8	Fmoc-G-G-Y-R-A-S-K-L-G-L-A-R	227 ± 49
D 9	Fmoc-Ahx-Y-R-A-S-K-L-G-L-A-R	112 ± 58
D10	W-W-G-G-Y-R-A-S-A-L-G-L-A-R	181 ± 69
D11	W-W-G-G-Y-R-K-S-A-L-G-L-A-R	259 ± 54
D12	W-W-G-G-Y-R-P-S-A-L-G-L-A-R	252 ± 10
D13[a]	W-W-G-G-Y-R-a-S-A-L-G-L-A-R	118 ± 6

[a] a denotes D-alanine.

Table VI: Summary of Circular Dichroism Spectroscopy of HU C3a and Analogues A1,[a] D12,[b] and E7[c]

peptide	solvent[d]	estimated[e] percentage of α-helix	β-sheet	β-turn	random coil
C3a Hu	B	55.4	0	10.3	34.3
	B + 25% TFE	70.4	0	0	29.6
A1	B	0	49.5	4.3	46.2
	B + 25% TFE	75.0	0	0	25.0
E7	B	0	48.1	10.7	41.2
	B + 25% TFE	24.9	29.3	13.7	32.1
D12	B	0	81.2	0	18.8
	B + 25% TFE	0	81.2	0	18.8

[a] Sequence of A1: A-N-Y-I-T-E-L-R-R-Q-H-A-R-A-S-H-L-G-L-A-R. [b] Sequence of D12: W-W-G-G-Y-R-P-S-A-L-G-L-A-R. [c] Sequence of E7: W-W-G-K-K-Y-R-A-S-K-L-G-L-A-R. [d] CD measurements were made in 0.01 M phosphate buffer at pH 7.4 (B) or in 0.01 M phosphate buffer at pH 7.4 + 25% TFE (B + 25% TFE). [e] Estimation was made by using the program PROSEC (Aviv Associates, Lakewood, NJ), based on the method of Chang et al. (1978).

Table VII: Potency as a Function of Elongation of C3a Analogues[a]

Peptide	Sequence (65 69 73 77)	Potency relative to Ala[57] C3a 57-77 (%)
E 1	W-W-G-S-K-L-G-L-A-R	120 ± 21
E 2	W-W-G-A-S-K-L-G-L-A-R	65 ± 12
E 3	W-W-G-R-A-S-K-L-G-L-A-R	325 ± 78
E 4	W-W-G-Y-R-A-S-K-L-G-L-A-R	604 ± 171
E 5	W-W-K-K-Y-R-A-S-K-L-G-L-A-R	299 ± 136
E 6	W-W-G-K-Y-R-A-S-K-L-G-L-A-R	1271 ± 730
E 7	W-W-G-K-K-Y-R-A-S-K-L-G-L-A-R	1406 ± 570
E 8	W-W-Ahx-K-K-Y-R-A-S-K-L-G-L-A-R	586 ± 178
E 9	W-W-G-G-K-K-Y-R-A-S-K-L-G-L-A-R	662 ± 196
E10	W-W-G-G-G-K-K-Y-R-A-S-K-L-G-L-A-R	807 ± 135

[a] The elongation series of C3a analogues containing the same N-terminal sequence (W-W-G) is presented in bold type. Their relative potencies are also plotted in Figure 1.

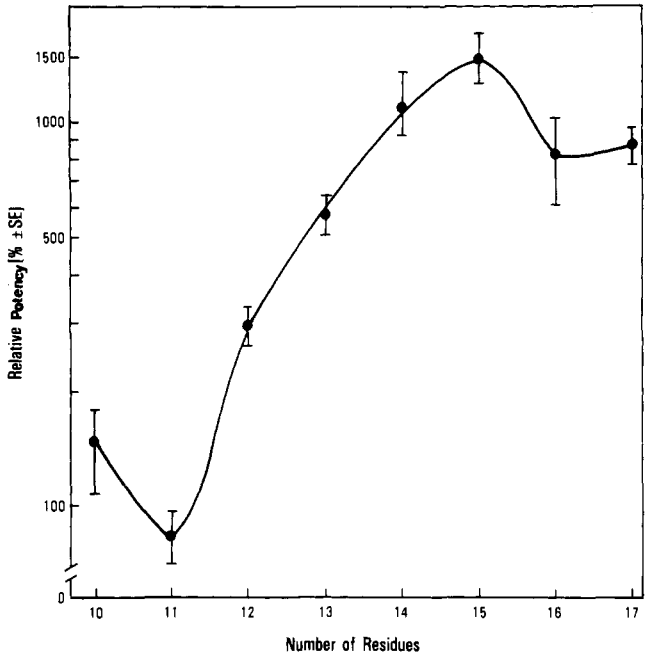

FIGURE 1: Relative potency of C3a analogues of different length. Potencies of these synthetic C3a peptides are expressed relative to the natural 21-residue analogue C3a 57–77. Each member of this series has the same hydrophobic N-terminal sequence: W-W-G-. The W-W-G-S-K-L-G-L-A-R decapeptide was elongated by inserting residues between G and S according to the sequence previously established for the highly active peptide E7. Sequences are shown in Table VII (bold type).

location of a hydrophobic unit plays a critical role in C3a peptide potency. To further investigate the function of peptide length, a series of C3a peptides were synthesized with W-W groups at the N-terminal end (Table VII). Elongation of the C3a peptide series from a decapeptide to a pentadecapeptide resulted in a 10–15-fold enhancement of the potency (E1–E7). The pentadecapeptide (E7) exhibits maximal potency, and most of the peptides in this series (E3–E10) are superpotent. The pentadecapeptide represents the most potent synthetic analogue that has been designed for C3a. Further elongation of the peptide chain actually resulted in decreased potency (E8–E10). On the basis of the above analogues with lengths varying from a decapeptide to a septadecapeptide, we conclude that the optimal structure depends on a proper distance or separation between the hydrophobic group or groups and the essential sequence LGLAR. Figure 1 contains a plot of the relative potencies of C3a analogues as a function of peptide length. There is a slight decrease in potency at the nonapeptide level (E2) compared to the decapeptide (E1), followed by a

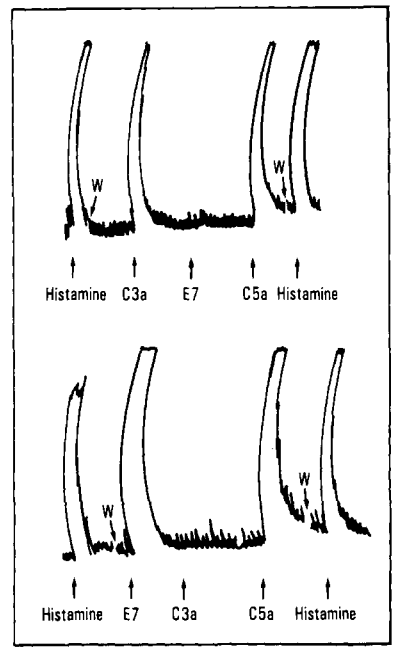

FIGURE 2: Contractile effects of C3a and peptide E7 (W-W-G-K-K-Y-R-A-S-K-L-G-L-A-R) observed with guinea pig ileal strips. Cross-desensitization (tachyphylaxis) between C3a and peptide E7 demonstrates an identity in specificity. C5a is a closely related anaphylatoxin having a specificity unique from C3a. The smooth muscle contractile effect of C5a is not inhibited by pretreatment with C3a and E7, indicating that the analogue E7 interacts exclusively with the C3a receptor. Arrows indicate administration of histamine, C3a, peptide E7, and C5a (W indicates washes between treatments).

sharp stepwise increase until a plateau is reached at the tetradeca- (E6) and pentadecapeptide level (E7). Further elongation of these peptides resulted in moderate but significant decreases in potency.

Other Biologic Assays. Biologic specificity of the C3a analogues was demonstrated by using carboxypeptidase B digestion to inactivate several of the synthetic C3a peptides. Removal of the C-terminal arginine abolishes the biological activity of native C3a (Bokisch et al., 1970). A panel of C3a analogues (A1, C6, D7, and E7) were tested for guinea pig platelet aggregation activity after CPB digestion. Removal of the C-terminal arginine abolished activity of these synthetic C3a analogues. The des-Arg derivative of peptide E7 produced by CPB digestion showed no guinea pig platelet aggregating activity up to a 600-fold molar excess of the effective concentration for intact peptide E7 (data not shown).

Specificity of the analogues has also been shown on guinea pig ileal contraction. Results from representative experiments (Figure 2) show that peptide E7 induces ileal contraction and desensitizes (tachyphylaxis) this tissue to human C3a, but not to C5a. We conclude from the biologic data that peptide E7 interacts selectively with the C3a receptor.

The guinea pig skin assay has been routinely used to evalute the effects of C3a and other spasmogens on vascular permeability. Serial dilutions of equimolar concentrations of human C3a and the peptide E7 (shown in Table VII) were injected intradermally into guinea pig skin on the dorsal surface. Levels from 1×10^{-5} to 1.25×10^{-6} M (see Figure 3) produce a marked increase in vascular permeability. Approximately 8 times less peptide E7 than native C3a is needed to induce the same size of skin wheal and flare reaction. These results show that peptide E7 exhibits greater potency in vivo than C3a. We conclude that the model C3a peptide E7 is decidedly more potent on a molar basis than natural C3a, under both in vivo and in vitro conditions.

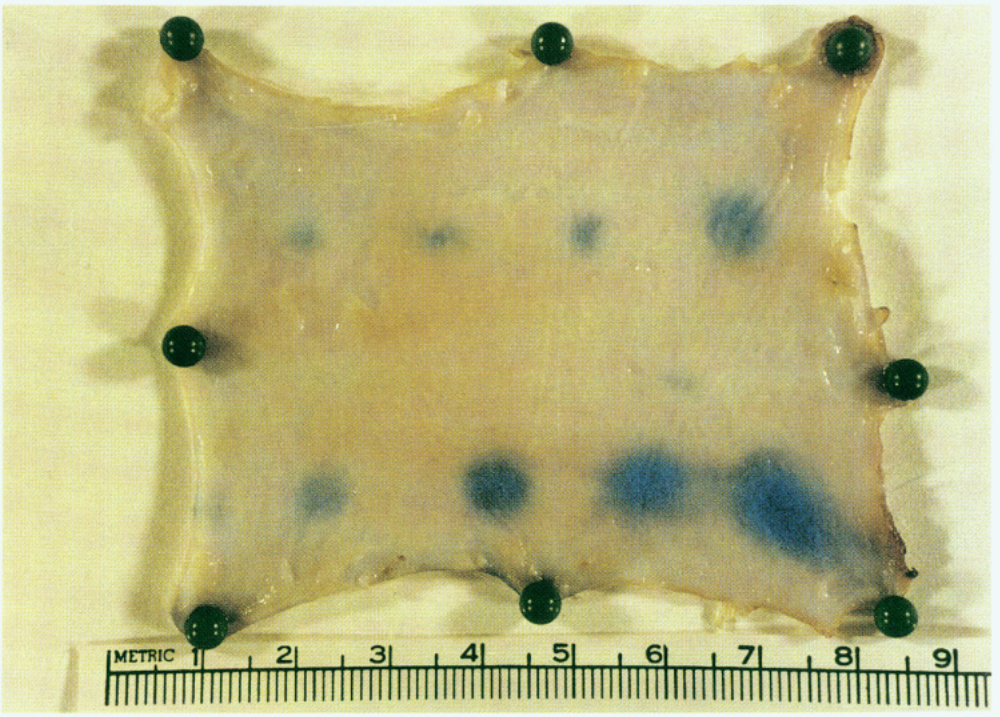

FIGURE 3: Vascular permeability induced in guinea pig skin by equimolar quantities of C3a (on the left) or peptide (E7) W-W-G-K-K-Y-R-A-S-K-L-G-L-A-R (on the right). From top to bottom, 0.5, 0.25, 0.125, and 0.062 nmol of C3a or peptide E7 was injected. As shown, approximately 8 times less peptide E7 is required to induce a wheal reaction the same size as C3a.

Discussion

The goal of the present investigation was to develop highly potent synthetic analogues of C3a for pharmacologic and ligand–receptor studies. A 21-residue C-terminal fragment of C3a (C3a 57–77) possesses biologic activities identical with those of the natural 77-residue C3a molecule and exhibits nearly equal potency (Lu et al., 1984; Huey et al., 1983). The potency of C3a 57–77 has been explained on the basis of CD studies indicating that the 21-residue C-terminal fragment of C3a can readily assume an α-helical conformation (Hoeprich & Hugli, 1986) similar to that in the C-terminal region of the natural molecule based on crystallographic analysis (Paques et al., 1980). It has concluded that the folded peptide structure in C3a 57–77 mimics the conformation of the C-terminal region in intact C3a and thereby provides the various ligand–receptor interactions necessary for initiating optimal cellular responses. Existence of a secondary binding site in C3a 57–77 is suggested by the fact that C3a 57–77 is 500 times as potent as the smaller active peptide C3 73–77 (LGLAR). The recent observation that relative potencies of C-terminal analogues of C3a, peptides considerably shorter than 21 residues, could be enhanced by adding a bulky aromatic group to the N-terminal end of these short analogues was unexpected (Gerardy-Schahn et al., 1988). The bulky aromatic groups were hydrophobic in nature, and we questioned how these added hydrophobic groups could substitute for the sizable contribution to potency made by the N-terminal end of C3a 57–77. It was established that the secondary conformation of C3a 57–77 contributed significantly to its potency (Lu et al., 1984; Hoeprich & Hugli, 1986). We, therefore, explored both the nature of the hydrophobic groups and the primary and secondary structural requirements for enhancing potency of short C3a analogues.

Rather than incorporating bulky organic groups, such as Fmoc or NAP at the N-terminus, we substituted amino acid residues having hydrophobic side chains (see Table II). The heterocyclic indole ring of tryptophan, like Fmoc, proved to be the most effective enhancer of potency. The overall shape or bulk of the total hydrophobic unit introduced at the N-termini of the C3a analogues was apparently important since adding another hydrophobic group adjacent to the Fmoc group actually caused potency to decrease (Table III, B17–B19). When tryptophans were used as the hydrophobic unit, optimal potency was obtained by introducing two tryptophanyl residues (see Table III, B6 and B16).

The influence of residues connecting the hydrophobic unit (Fmoc or W-W) to the essential LGLAR sequence appears to have minimal effect except as spacers (Table V). In a series of nonapeptides containing an N-terminal Fmoc group, the connecting residues were individually interchanged between arginine and alanine with minimal effect on potency. When a decapeptide was used as the basic unit and the Fmoc group was attached via a dipeptide bridge to the N-terminus, little difference in potency was seen whether the bridging groups were Arg-Arg or Gly-Gly, again suggesting these residues were primarily connectors. However, dodecapeptides with an Fmoc group attached (D6–D9) were 3–4-fold more active than the nonapeptide series (D1–D5), indicating the importance of peptide length.

In a tetradecapeptide series (D10–D13) the seventh position was substituted with either proline or D-alanine to disrupt regular structure, and these substitutions caused only a minor decrease in potency (see Table V). Our CD results for peptide D12 indicate that this peptide did not assume a helical conformation as was demonstrated in C3a and for the 21-residue analogue A1 (see Table VI). The short peptides in this series were not expected to assume stable helical structures in solution, and the CD data for peptide D12 support this view. The results suggest that these superpotent peptides exist as extended structures in free solution.

Certainly the best evidence that specific interactions exist between the hydrophobic unit on the C3a analogues and the cell receptor was provided by the results in Table VII. Potency of the analogues increased dramatically as the peptide was

elongated from 10 to 15 residues. A second parameter that was important for optimizing potency of the synthetic analogues was the residue adjacent to the N-terminal hydrophobic unit. An optimal connecting residue was glycine, presumably because it permits free rotation of the hydrophobic unit. The 6-aminohexanoyl group is also a flexible linking group, but it apparently contributes to the total unit bulk and/or hydrophobicity.

A plot of the data in Table VII is shown in Figure 1 for a series of peptides having Trp-Trp linked by a glycyl residue to the essential C-terminal unit LGLAR of the C3a analogue. We conclude that the hydrophobic unit on the C3a analogue interacts with a hydrophobic secondary (noneffector) binding site on the C3a receptor in a specific manner and is cooperative with LGLAR binding; thus the distance between the hydrophobic units and the effector group (LGLAR) is critical for optimizing potency of these peptides. The fact that peptides of 14 to 15 residues in length exhibit maximal potency and that the longer peptides in this series were less potent supports our contention that a secondary binding site exists on the C3a receptor.

If the hydrophobic unit was interacting only with the cell membrane lipid layer, as proposed by Gerardy-Schahn et al. (1988), then the length of the peptide should have less influence than the nature of the hydrophobic unit. C5a shares homology with C3a, and the C-terminal fragment of C5a, like C3a, exhibits weak spasmogenic (Erickson et al., 1986) and platelet-aggregating activities, as demonstrated in Table III. Further evidence that the hydrophobic unit on C3a analogues interacts specifically with the cell receptor was provided by the lack of any enhancing effect by introducing hydrophobic groups to C5a analogues. Addition of Ahx, Fmoc, or Trp groups had little, if any, influence on the potency of C5a peptides.

Although the studies of Gerardy-Schahn et al. (1988) identified a hydrophobic group as an important parameter in the functional expression of C3a analogues, the nature of the contribution of the hydrophobic unit remained unexplored. When the C-terminal portions of natural C3a molecules from several species are compared, the C-terminal pentapeptide LGLAR is rigorously conserved as were several of the nearby hydrophobic residues (i.e., Tyr 59, Ile 60, and Leu 63; see Table I). We know from studies summarized in Table II that many of the other residues in this region may be replaced, without significantly effecting potency, including residues that appear to have been conserved such as Arg 64, Gln 66, His 67, and Arg 69. The region including residues 59–63 is helical in C3a (Paques et al., 1980; Kalnik et al., 1990), and three hydrophobic residues (i.e., Tyr 59, Ile 60, and Leu 63) are oriented to one side of the helical surface. The C-terminal region of C3a whose solution structure is known can now be compared to that of the optimally potent synthetic analogue E7 (see Figure 4). Our proposed structural comparison of C3a with the C3a analogue E7 might explain how the synthetic C3a molecule mimics that of the C-terminal region of natural C3a. Recent NMR data for C3a (Chazin et al., 1988; Kalnik et al., 1990) indicate that the helix at the C-terminus extends only to residues 64 and 65 rather than residues 69 and 70, as demonstrated for crystalline C3a by x-ray analysis (Paques et al., 1980). Therefore, if the C-terminal portion of C3a is irregular or extended from residues 64 or 65 to 77, this region in C3a could compare with the flexible synthetic 14- to 15-residue analogues (E6 to E7). This proposed model for the C3a–C3a receptor interaction would support the argument that the synthetic analogue E7 accurately mimics the essential

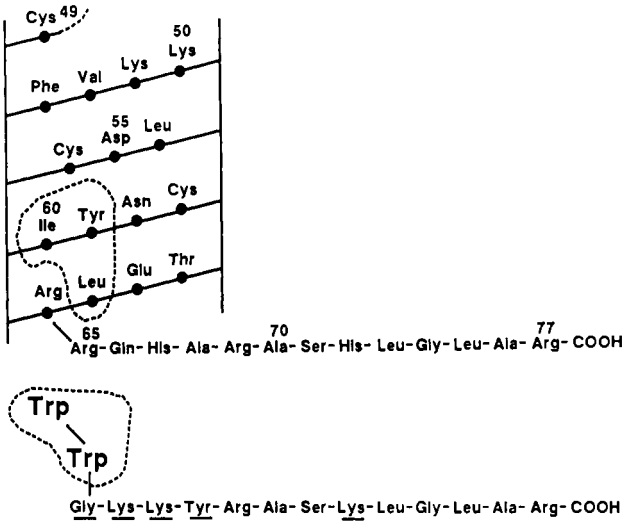

FIGURE 4: A cylindrical projection of the C-terminal region of human C3a (residues 49–77) illustrating the hydrophobic site on the helical surface. This planar projection of the C-terminal region of C3a includes the helical portion represented by residues 49–65 and the irregular portion containing residues 66–77 as indicated by NMR results. The synthetic superagonist analogue of C3a, E7 (below), contains a hydrophobic site (Trp-Trp) and the C-terminal effector pentapeptide LGLAR. Therefore, E7 resembles C3a in the spacial organization of putative binding sites.

binding sites of the natural factor and that the hydrophobic unit represents a secondary interaction site. This concept is further supported by evidence that proper positioning of the hydrophobic unit relative to the LGLAR effector site has a significant influence on potency (see Figure 1).

Few examples exist in the literature for the design of short peptide superagonists based on proteins the size of C3a that have a definite tertiary structural requirement for activity (Lu et al., 1984). However, numerous examples of superagonists of smaller bioactive factors exist where secondary and tertiary structural influences are minimized. For example, when luteinizing hormone-releasing hormone (LH-RH) is substituted at position Gly 6 by various hydrophobic residues, the analogues exhibited superpotency (Vale et al., 1976; Coy et al., 1976; Nestor, 1984). Until direct receptor-binding studies are performed with our C3a analogues, it is not possible to confirm that activity enhancement correlates directly with binding affinity to the C3a receptor.

The ability of the synthetic C3a peptides to exhibit superagonist activity is best explained by affinity enhancement from cooperative secondary interactions between the receptor and the ligand. We conclude that the Trp-Trp moiety of E7 binds to the secondary C3a receptor site with greater affinity than does the hydrophobic cluster on the helical surface of the natural ligand (see Figure 4). Therefore, the design of a superagonist for C3a requires three properties: (1) an essential LGLAR sequence at the C-terminal end of the molecule for receptor specificity, (2) a bulky hydrophobic unit that substitutes for the hydrophobic cluster on the C-terminal helix of C3a and contributes cooperatively to the binding interactions of LGLAR and, (3) a proper spacing of these two binding sites on the ligand for optimal mating with the cellular C3a receptor. The critical parameter that appears to confirm superactivity on C3a analogues is the nature of the hydrophobic unit. Bulky hydrophobic groups such as NAP, Fmoc, or Trp-Trp apparently either provide a better fit or bind more strongly to the hydrophobic site on the receptor than does the corresponding site on the natural ligand, thus enhancing activity of the analogue over that of the natural factor. We do

not yet know whether aromaticity or stereoelectronic forces are also important, but aromatic groups have proven more effective than aliphatic groups.

In summary, we propose that a superpotent peptide agonist has been designed utilizing a linear arrangement of amino acids to mimic a conformation-dependent binding site on the natural factor. This synthetic peptide approach may find greater success and wider application to folded protein structures as localized binding sites become more readily identified via mutagenesis and expression technologies.

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Supplementary Material Available

Four tables listing conditions used for RP-HPLC peptide isolation and results of analytical measurements performed to assess peptide purity of C3a analogues, with Table I providing the conditions used for RP-HPLC analysis of synthetic peptides, Table II giving a cumulative list of all peptides used in the studies, with HPLC retention times and molecular mass estimates as determined by mass spectroscopy, Table III containing the amino acid compositions for C3a analogues, and Table IV presenting sequence analysis results for C3a analogue E7 (5 pages). Ordering information is given on any current masthead page.

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Autonomous Folding and Coenzyme Binding of the Excised Pyridoxal 5′-Phosphate Binding Domain of Aspartate Aminotransferase from *Escherichia coli*†

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ABSTRACT: The coenzyme (PLP) binding domain (residues 47-329) of the dimeric aspartate aminotransferase from *Escherichia coli* was produced separately by recombinant DNA methods. It folded autonomously both in vivo and in vitro, that is, independently of the native N- and C-terminal extensions that combine to form the small domain of eAAT. The PLP-domain had one binding site for PLP of relatively high affinity involving a covalent bond to the protein. It was monomeric, although the major subunit–subunit interface at the 2-fold symmetry axis remained unchanged. This effect appears to be due mainly to the absence of the N-terminal extension that contains hydrophobic residues, which interact with the PLP-domain of the second subunit in the wild-type dimer. Judged by circular dichroism, fluorescence, and HPLC gel filtration at increasing concentrations of guanidinium chloride, the PLP-domain underwent a three-state unfolding transition ($M' \rightleftarrows M'^* \rightleftarrows U'$) involving a compact intermediate M'^*. This behavior parallels the unfolding of the dissociated native monomer of eAAT.

The three-dimensional structures of many protein monomers consist of two or more compact tertiary substructures or domains that comprise contiguous subregions of the polypeptide chain (Wetlaufer, 1981). Their compact structure implies that they are autonomous units of protein folding, assembly, and sometimes also function. They might have played an important role as modules during protein evolution. However, there is no unequivocal theoretical approach for identifying the boundaries of domains.

The cleavage of an oligopeptide loop at the surface of a native protein can define domain boundaries operationally. If the separated proteolytic fragments either refold autonomously and retain some vestige of function (for example, the Klenow fragment of DNA polymerase; Jacobsen et al., 1974) or reassemble stoichiometrically, it is reasonable to conclude that cleavage occurred in the connector between structural domains. However, even proteins that appear to consist of a single structural domain can be separated proteolytically into autonomously folding and reassembling subdomains (e.g., the α subunit of tryptophan synthase; Hyde et al., 1988; Miles, 1991). Moreover, proteolytic separation into autonomously folding and reassembling domains can occur in loops that are not in the connector defined by protein crystallography (e.g., the β subunit of tryptophan synthase; Högberg-Raibaud & Goldberg, 1977a,b; Hyde et al., 1988).

Recombinant DNA technology is an alternative and more versatile approach for probing the boundaries of putative structural domains. By using the known structure of the protein as a guide, the separation of protein fragments can be performed at the level of the gene, independent of whether the putative connectors are at the surface of the protein or not. Moreover, the subcistrons can be expressed in homogeneous form and in large amounts in transformed microorganisms [e.g., elongation factor Tu (Parmeggiani et al., 1987) and phosphoglycerate kinase (Minard et al., 1989)].

In this work we describe the production and properties of the pyridoxal 5′-phosphate (PLP)[1] binding domain (P-domain) of aspartate aminotransferase from *Escherichia coli* (eAAT). Each of the two identical subunits of eAAT (Figure 1) consists of the P-domain and a second domain formed by its N- and C-terminal extensions. The large P-domain comprises the region from residue 47 to 329 (numbering as for pig cytosolic AAT, cf. Figure 1) and includes all side chains that are important for the binding of PLP (Ford et al., 1980; Jäger et al., 1989). The small domain, which comprises residues 5-46 and 330-409, moves, roughly speaking, as a unit relative to the P-domain during each catalytic cycle (Birchmeyer & Christen, 1971; Jansonius & Vincent, 1987; Picot et al., 1991).

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[1] Abbreviations: eAAT, aspartate aminotransferase from *Escherichia coli* (EC 2.6.1.1); P-domain, genetically isolated coenzyme binding domain of eAAT, comprising residues 47-329; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; DTE, dithioerythritol; GuCl, guanidinium chloride; Hepes, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; CD, circular dichroism; IPTG, isopropyl thiogalactoside; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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