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Site-specific mutations in the N-terminal region of human C5a that affect interactions of C5a with the neutrophil C5a receptor

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Abstract

C5a is an inflammatory mediator that evokes a variety of immune effector functions including chemotaxis, cell activation, spasmogenesis, and immune modulation. It is well established that the effector site in C5a is located in the C-terminal region, although other regions in C5a also contribute to receptor interaction. We have examined the N-terminal region (NTR) of human C5a by replacing selected residues in the NTR with glycine via site-directed mutagenesis. Mutants of rC5a were expressed as fusion proteins, and rC5a was isolated after factor Xa cleavage. The potency of the mutants was evaluated by measuring both neutrophil chemotaxis and degranulation (β-glucuronidase release). Mutants that contained the single residue substitutions Ile-6 → Gly or Tyr-13 → Gly were reduced in potency to 4–30% compared with wild-type rC5a. Other single-site glycine substitutions at positions Leu-2, Ala-10, Lys-4, Lys-5, Glu-7, Glu-8, and Lys-14 showed little effect on C5a potency. The double mutant, Ile-6 → Gly/Tyr-13 → Gly, was reduced in potency to <0.2%, which correlated with a correspondingly low binding affinity for neutrophil C5a receptors. Circular dichroism studies revealed a 40% reduction in α-helical content for the double mutant, suggesting that the NTR contributes stabilizing interactions that maintain local secondary or tertiary structure of C5a important for receptor interaction. We conclude that the N-terminal region in C5a is involved in receptor binding either through direct interaction with the receptor or by stabilizing a binding site elsewhere in the intact C5a molecule.

Keywords: C5a; complement; inflammation; ligand-receptor interaction; site-directed mutagenesis; structure/function

Human C5a is a potent inflammatory mediator that is generated by proteolytic cleavage of C5 as a consequence of complement activation. In vitro and in vivo studies have demonstrated that C5a evokes a number of biological activities in a variety of cell types (Hugli, 1984). Specific receptors for C5a have been demonstrated on human neutrophils (Fernandez et al., 1978), basophils (Kurimoto et al., 1989), macrophages (Schorlemmer et al., 1976), and mast cells (Grant et al., 1975). Among the physiologically important cellular activities elicited from leukocytes by C5a, chemotaxis and degranulation are perhaps the best characterized (Hugli, 1981), although cellular mobilization and recruitment to local sites of tissue injury may be the primary function of this mediator in the inflammatory response. In addition to these responses, C5a, as well as the other C-derived anaphylatoxins C3a and C4a, enhance vascular permeability and can induce contraction of smooth muscle tissue (Bernauer et al., 1972; Hugli & Muller-Eberhard, 1978; Stimler et al., 1980; Marceau & Hugli, 1984).

The structure–function relationship of C5a has been studied in some detail. It is a glycosylated single polypeptide chain that consists of 74 amino acids (Fernandez & Hugli, 1978). Based on NMR data (Zuiderweg et al., 1989; Williamson & Madison, 1990), the structure of C5a consists of four antiparallel helical bundles. The folded C5a structure is similar to that of C3a as determined previously by X-ray analysis (Huber et al., 1980). Three intrachain disulfide bonds contribute stability to C5a tertiary structure, which is of critical importance for optimal receptor binding (Gerard et al., 1979).

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Abbreviations: CTR, C-terminal region; ELISA, enzyme-linked immunosorbent assay; IPTG, isopropyl-β-D-thiogalactoside; MBP, maltose binding protein; nC5a or rC5a, natural or recombinant C5a; NTR, N-terminal region; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

While the primary effector site in C5a is located in the C-terminal region (Chenoweth et al., 1979; Chenoweth & Hugli, 1980; Stimler et al., 1980), Gerard et al. (1985) reported that the N-terminal region also plays an important functional role. In that study, removal of residues 1–17 from porcine $C5a_{des\ Arg}$ by cyanogen bromide cleavage at Met-17 resulted in a 1,000-fold loss in potency. In a detailed site-directed mutagenesis study later presented by Mollison et al. (1989), several rC5a mutants that contained residue substitutions in the NTR were examined for changes in potency and receptor binding. Only minor decreases in potency and receptor binding were noted for all of the NTR mutants examined, which included substitutions at residues 1–5, 7–9, 11, and 12. Mutants containing substitutions for Ile-6, Ala-10, and Tyr-13 could not be examined due to poor expression in *Escherichia coli*. Thus, although a functional role for the NTR of C5a has been established, the NTR residue(s) critical for optimizing receptor interaction have not been delineated.

In this study, the role of the human C5a NTR in receptor interaction was reexamined by a site-directed mutagenesis approach. An improved expression system is described that resulted in efficient expression of C5a mutants in *E. coli*. Substitution of selected N-terminal hydrophobic residues by glycine resulted in significant reductions in C5a potency, thus presenting evidence that specific residues in the C5a NTR play an important role for optimal receptor–ligand interaction.

Results

Isolation of rC5a

A fusion protein expression system was chosen for production of rC5a that facilitated affinity purification of the recombinant product. The fusion protein construct consisted of maltose binding protein fused to C5a and is preceded by a signal sequence to direct transport of the fusion protein to the periplasmic compartment. The affinity-purified periplasmic fusion protein was recovered primarily in the form of dimers and higher molecular weight aggregates, as assessed by SDS-PAGE (Fig. 1). Under reduced conditions or after denaturation/renaturation, the majority of the fusion protein was observed as a single band on SDS-PAGE with an apparent size of 50 kDa, consistent with a combined size of C5a (8.6 kDa) plus MBP (42 kDa).

It was important to acertain the integrity of rC5a mutants containing residue substitutions in the NTR after isolation from *E. coli* because mutations in this region have been shown previously to render C5a highly susceptible to bacterial proteases (Mollison et al., 1989). Utilizing the expression system designed for the current study, degradation appeared minimal by SDS-PAGE analysis for the wild type as well as for selected C5a mutants (Fig. 1). To assess degradation at the essential C-terminal effector region further, an immunoassay was employed. A polyclonal antibody directed against a 14-residue C-terminal C5a peptide (LRANISHKDMQLGR) was highly reactive with both human C5a and the C5a 14-mer used for immunization when tested in an ELISA (Fig. 2). The antibody was unreactive with human C3a or with a C-terminal 21-mer of C3a (residues 57–77), indicating a specificity for C5a (Fig. 2). Antibody binding to the factor Xa-cleaved wild-type rC5a and selected rC5a mutants (see following section) was comparable to or greater than antibody binding to nC5a, suggesting that the C-terminal effector region in these recombinant proteins remained intact (Fig. 2).

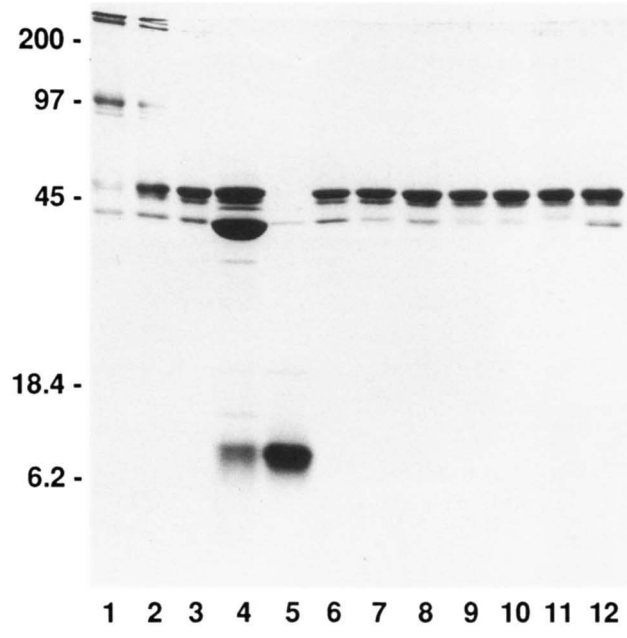

Fig. 1. Affinity-purified MBP–C5a fusion proteins after various treatments were analyzed on a 10–20% gradient SDS–polyacrylamide gel stained with Coomassie blue R250. Wild-type fusion protein without denaturants (lane 1) is a mixture of mostly dimers and high molecular weight forms. In the presence of 2-mercaptoethanol (at a final concentration of 3 mM, lane 2) or (300 mM, lane 3), a 50-kDa monomeric form of the fusion protein was observed. Factor Xa treatment of the fusion protein (lane 4) at an enzyme:substrate ratio of 1:3,500 for 16 h at 22 °C resulted in approximately 50% cleavage to yield isolated MBP (42 kDa) and rC5a (8.6 kDa). Factor Xa-cleaved rC5a purified by gel filtration is shown in lane 5. Mutant fusion proteins after denaturation/renaturation (lanes 7–12) appear as intact as the wild-type protein (lane 6). Residues substituted for glycine in the mutants are Ile-6 (lane 7), Tyr-13 (lane 8), Ile-6 + Tyr-13 (lane 9), Ile-6 + Lys-14 (lane 10), Lys-4 + Lys-5 + Tyr-13 (lane 11), and Glu-7 + Glu-8 + Tyr-13 (lane 12). All lanes contain 2 μg of protein except for lane 4 (6 μg) and lane 5 (4 μg).

Cleavage of fusion protein for isolation of rC5a

The pMAL-p expression vector used in this study was designed with a factor Xa cleavage site immediately 5′ to the codon for residue Thr-1 in the C5a gene. This

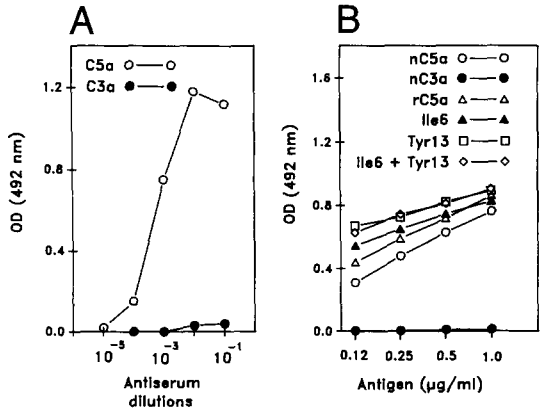

Fig. 2. Assessment of integrity of the C-terminal effector region of rC5a mutants by an ELISA assay. A rabbit antibody was raised against a C-terminal 14-mer of human C5a. The antibody reacted with high specificity to the C5a 14-mer used for immunization, but not to a C-terminal 10-mer of C3a (**A**). **B:** The high reactivity of the antisera (10^{-3} dilution) to nC5a and rC5a, as well as to rC5a mutants can be seen, indicating that the CTR remains intact. Reactivity to nC3a was negligible. Mutants contain specific residues (i.e., Ile-6, Tyr-13) substituted by glycine. Abbreviations nC3a or nC5a refer to natural C3a or C5a; rC5a to recombinant C5a.

construct permits proteolytic cleavage of rC5a from the affinity-purified fusion protein. Surprisingly, the aggregated form of the fusion protein was 40-fold more susceptible to factor Xa cleavage than the denatured/renatured fusion protein (Fig. 3), indicating that the factor Xa site in the aggregated protein is more accessible to the enzyme than the renatured product. The enzyme/substrate ratios required to obtain approximately 50% cleavage of the Leu-2 → Gly and Ala-10 → Gly mutants

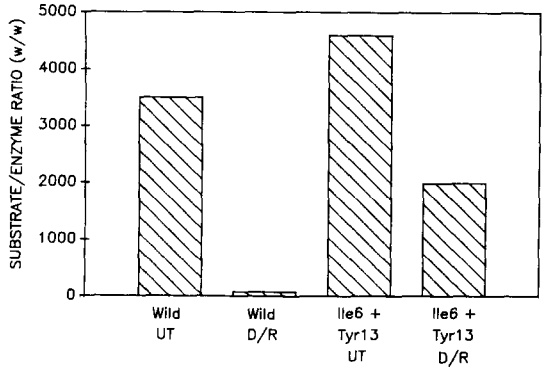

Fig. 3. Factor Xa cleavage of C5a fusion proteins. Affinity-purified fusion proteins (5 mg/mL) were mixed with factor Xa at an enzyme: substrate ratio that resulted in approximately 50% cleavage after 16 h at 22 °C as assessed by SDS-PAGE analysis. After denaturation/renaturation (D/R) the wild-type protein was 40-fold less sensitive to factor Xa than the untreated protein (UT). The double mutant Ile-6 → Gly/Tyr-13 → Gly was 25-fold more sensitive than the wild-type protein after D/R.

of C5a in the fusion product were similar to those required to cleave the wild-type fusion protein. The Ile-6 → Gly and Tyr-13 → Gly mutants required half as much enzyme as the wild-type protein (data not shown). Notably, an Ile-6 → Gly/Tyr-13 → Gly double mutant was 25-fold more sensitive than wild-type rC5a to factor Xa cleavage after denaturation/renaturation (Fig. 3).

Potencies of fusion protein and rC5a mutants

Two functional assays (i.e., neutrophil chemotaxis and β-glucuronidase release) were used to assess the potencies of the rC5a proteins. Potency of the factor Xa-cleaved wild-type rC5a was nearly identical to that of nC5a, having an ED_{50} of about 1 nM in both assays (Fig. 4). Thus, the rC5a proteins prepared by the method used in this study retain full potency. The denatured/renatured wild-type fusion protein was also equal in potency to nC5a in the chemotaxis assay, whereas the untreated fusion protein was only half as potent (Table 1). In the enzyme release assay, the denatured/renatured and untreated wild-type fusion proteins were 5-fold and 60-fold less potent than nC5a, respectively (Table 1). The results of ^{125}I-C5a competitive binding studies indicated 4-fold and 100-fold lower binding affinities for the denatured/renatured and untreated wild-type fusion proteins, respectively, when compared with nC5a (Table 1). One possible explanation for the dissimilarities in potency between the two assay procedures may be related to differences in the kinetic endpoints. The β-glucuronidase is released rapidly from cytochalasin B-treated cells after addition of C5a, with a $t_{1/2}$ of less than 3 min at 37 °C (unpubl.). In contrast, the modified Boyden chamber chemotaxis assay exhibits a $t_{1/2}$ of 30 min (data not shown). Therefore, migratory movement through the filter requires ligand-receptor interactions over a more prolonged period, thus allowing more time for the fusion protein to refold into

Table 1. *Comparison of receptor binding and potencies of wild-type rC5a and MBP-rC5a fusion protein*[a]

	β-Glucuronidase release		Chemotaxis		
	ED_{50} (nM)[b]	% nC5a	ED_{50} (nM)	% nC5a	Binding[c]: % nC5a
nC5a	1.2 ± 0.14	100	0.8 ± 0.17	100	100
rC5a	1.0 ± 0.10	120	0.8 ± 0.20	100	100
Fusion D/R	6.0 ± 0.50	20	1.0 ± 0.15	80	27
Fusion NT	74 ± 5.3	1.6	2.3 ± 0.22	43	0.9

[a] nC5a, natural C5a; rC5a, recombinant factor Xa-cleaved C5a; D/R, denatured/renatured; NT, not treated with denaturants.

[b] ED_{50} values reflect the mean ± SE and were obtained from at least two separate experiments consisting of at least six dilutions of C5a (each performed in triplicate).

[c] Binding for each of the proteins to human neutrophils was determined in an ^{125}I-C5a competition assay as described in the Materials and methods.

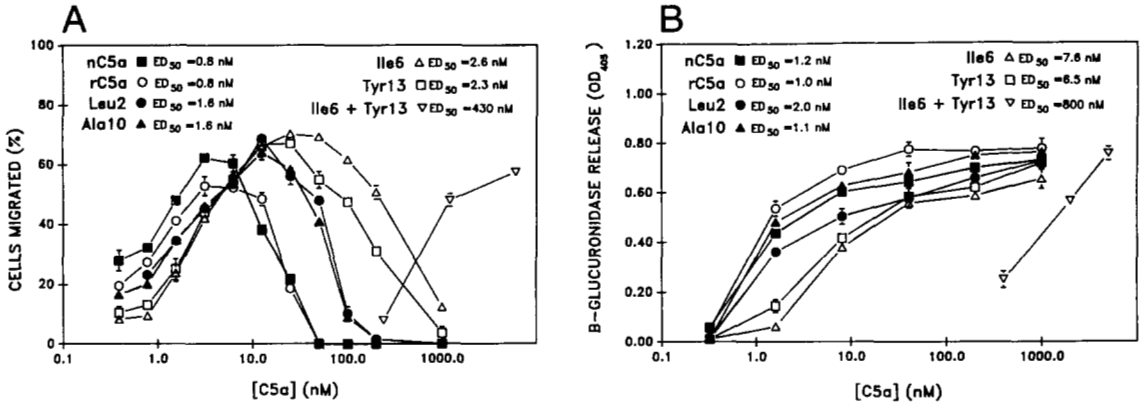

Fig. 4. Potency of factor Xa-cleaved rC5a mutants containing glycine substituted for selected residues in the NTR of C5a. **A:** Chemotaxis assay. Dilutions of the rC5a mutants were placed in the lower chamber of a chemotaxis apparatus. Human neutrophils (5×10^6/mL) were added to the upper chamber that contained an 8-μm pore size membrane. The relative percentage of cells migrating to the lower chamber was measured after 90 min at 37 °C. **B:** β-Glucuronidase release. Dilutions of the rC5a mutants were mixed with cytochalasin B-treated neutrophils and incubated at 37 °C for 60 min. β-Glucuronidase released into the medium was then quantitated by measuring the change in optical density of a chromogenic substrate. The Leu-2 → Gly and Ala-10 → Gly mutants were nearly fully potent compared with wild-type rC5a, whereas the Ile-6 → Gly and Tyr-13 → Gly mutants were less potent. The Ile-6 → Gly/Tyr-13 → Gly double mutant was markedly reduced in potency to 0.1–0.2% of the rC5a potency.

a more favorable (i.e., native) conformation prior to receptor binding.

Recombinant C5a mutants containing glycine in place of selected hydrophobic or charged residues in the NTR were examined for functional potency. The mutants containing hydrophobic residue substitutions were examined both as factor Xa-cleaved rC5a and as denatured/renatured fusion proteins. Of the hydrophobic substitution sites examined, the Ala-10 → Gly and Leu-2 → Gly mutants were 40–100% as potent as the wild-type protein, whereas the Ile-6 → Gly and Tyr-13 → Gly mutants were substantially less potent (4–35%) (Fig. 4; Table 2). To explore the functional contribution of Ile-6 and Tyr-13 further, a double mutant containing glycine in place of both residues was found to be markedly reduced in potency in both assay systems (<0.2%). Binding of the double mutant to neutrophil C5a receptors was also markedly reduced as shown in Figure 5, with an ED_{50} value of only 0.05%

Table 2. *Potencies of denatured/renatured MBP–C5a fusion protein mutants with glycine substituted for selected residues in the NTR of C5a*

Residue classification	Residue(s) substituted	β-Glucuronidase release assay[a]: ED_{50} (nM)	Chemotaxis assay: ED_{50} (nM)
	Wild type	6.5 ± 0.5	1.0 ± 0.15
Hydrophobic	Leu-2	16 ± 1.7	1.72 ± 0.19
	Ile-6	140 ± 34.0	15.1 ± 3.0
	Ala-10	9.5 ± 1.4	0.8 ± 0.20
	Tyr-13	89 ± 17.0	6.7 ± 1.1
Hydrophilic/charged	Lys-4 + Lys-5	11.1 ± 2.0	1.1 ± 0.16
	Glu-7 + Glu-8	8.8 ± 1.7	1.75 ± 0.18
	Lys-14	19.4 ± 1.6	0.93 ± 0.18
Combination	Ile-6 + Lys-14	770 ± 51	350 ± 14.0
	Lys-4/5 + Tyr-13	680 ± 70	250 ± 25.0
	Glu-7/8 + Tyr-13	770 ± 37	425 ± 45.0
	Ile-6 + Tyr-13	20,000 ± 600	15,000 ± 200

[a] ED_{50} values reflect the mean ± SE and were obtained from at least two separate experiments consisting of at least six fusion protein dilutions (each performed in triplicate).

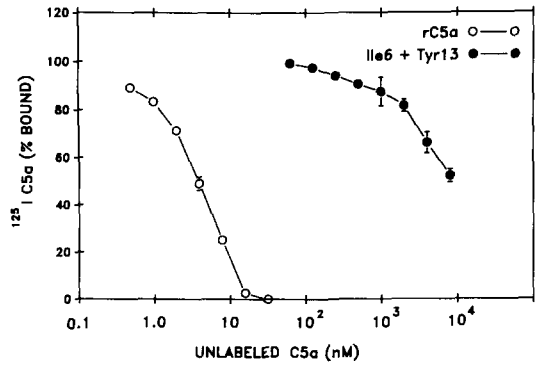

Fig. 5. Competitive binding of the rC5a Ile-6 → Gly/Tyr-13 → Gly double mutant to human neutrophils. The ^{125}I-C5a (3 nM) was mixed with various dilutions of rC5a or rC5a containing glycine in place of Ile-6 and Tyr-13. Neutrophils (1×10^6/0.2 mL, final concentration) were added and the mixtures were incubated on ice for 60 min. Specific binding of ^{125}I-C5a to the cells was then determined. Binding of the double mutant was 0.05% compared with rC5a.

compared with wild-type rC5a. To assess whether the loss in potency of the double mutant was due to alterations in secondary structure, CD measurements were obtained. An α-helical content of 45% was obtained for the wild-type C5a (Fig. 6), in good agreement with estimates obtained for natural C5a (Hugli, 1981). The α-helical content for the Ile-6 → Gly/Tyr-13 → Gly double mutant decreased to 25%, and the curve shifted to the left, indicating increased irregular structure (Fig. 6).

Only minor differences in potency were observed for mutants that contained glycine substituted for selected charged residues in the NTR when tested in both cellular assays (Table 2). These data imply that charged residues do not directly contribute to the receptor interaction.

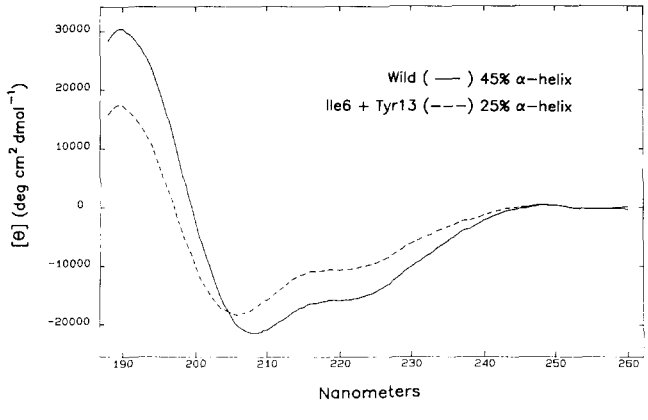

Fig. 6. CD measurements were obtained on rC5a solutions of 0.25 mg/mL in 0.05 mM phosphate buffer, pH 8.0. The data from at least four scans for each protein sample were averaged and fit by nonlinear regression analysis. The α-helical content was estimated using the program PROSEC.

However, combination mutants prepared by replacing glycine for selected charged residues plus either Ile-6 or Tyr-13 were 30–400-fold less potent than substitution of Ile-6 or Tyr-13 alone, but 40-fold more potent than the Ile-6/Tyr-13 combination mutant (Table 2).

Discussion

The functional importance of the C5a NTR was first described in a report that showed a significant reduction in potency after removal of the first 17 residues from porcine $C5a_{des\ Arg}$ (Gerard et al., 1985). In a site-directed mutagenesis study designed to locate receptor binding sites in C5a (Mollison et al., 1989), single residue substitutions in the NTR at residues 1–5, 7–9, 11, and 12 had no significant effect on C5a potency or receptor binding. Interestingly, C5a mutants containing substitutions at Ile-6, Ala-10, or Tyr-13 could not be obtained with the expression system used in that study. Poor expression yield may be the result of altered folding in the mutants and a subsequent increased susceptibility to proteolysis in *E. coli*. This reasoning is based on the proposal by Greer (1985) that residues 6, 10, and 13 contribute stabilizing forces that maintain a stabilized NTR structure, to be discussed further below.

The rational for selection of NTR residues for mutation in the current study was based in part on the potentially important role of selected hydrophobic NTR residues in stabilizing NTR structure, as first proposed by Greer (1985) and later confirmed by C5a NMR data (Zuiderweg et al., 1989; Williamson & Madison, 1990). It has been proposed that the NTR interacts with the disulfide-linked (i.e., core) region and the C-terminal helical region (CTR) via stabilizing forces contributed by hydrophobic residue side chains (Greer, 1985) (see Fig. 7A; Kinemage 1). Edalji et al. (1987) found that enzymatic removal of the NTR affected the NMR structure of the core region, implying that the NTR may serve a role in stabilizing other regions in C5a. Helical wheel arrangement of NTR residues demonstrates a distinct hydrophobic patch comprised of residues 2, 6, 10, and 13 that faces inward toward the main body of the C5a molecule. Thus, site-specific mutations of these residues were examined for effects of C5a potency. Selected hydrophilic NTR residues were also mutated to determine the specificity of the hydrophobic residue substitutions on C5a potency.

It was previously reported that rC5a was relatively unstable when expressed in *E. coli* (Mandecki et al., 1986), and that rC5a mutants varied considerably in their susceptibility to bacterial proteases (Mollison et al., 1989). In an attempt to produce stable rC5a mutants for this study, rC5a fusion proteins were directed to the periplasm via a signal sequence, thus limiting exposure of rC5a to bacterial cytoplasmic proteases. This approach was successfully used previously for production of wild-type rC5a, although mutant rC5a stability was not investigated

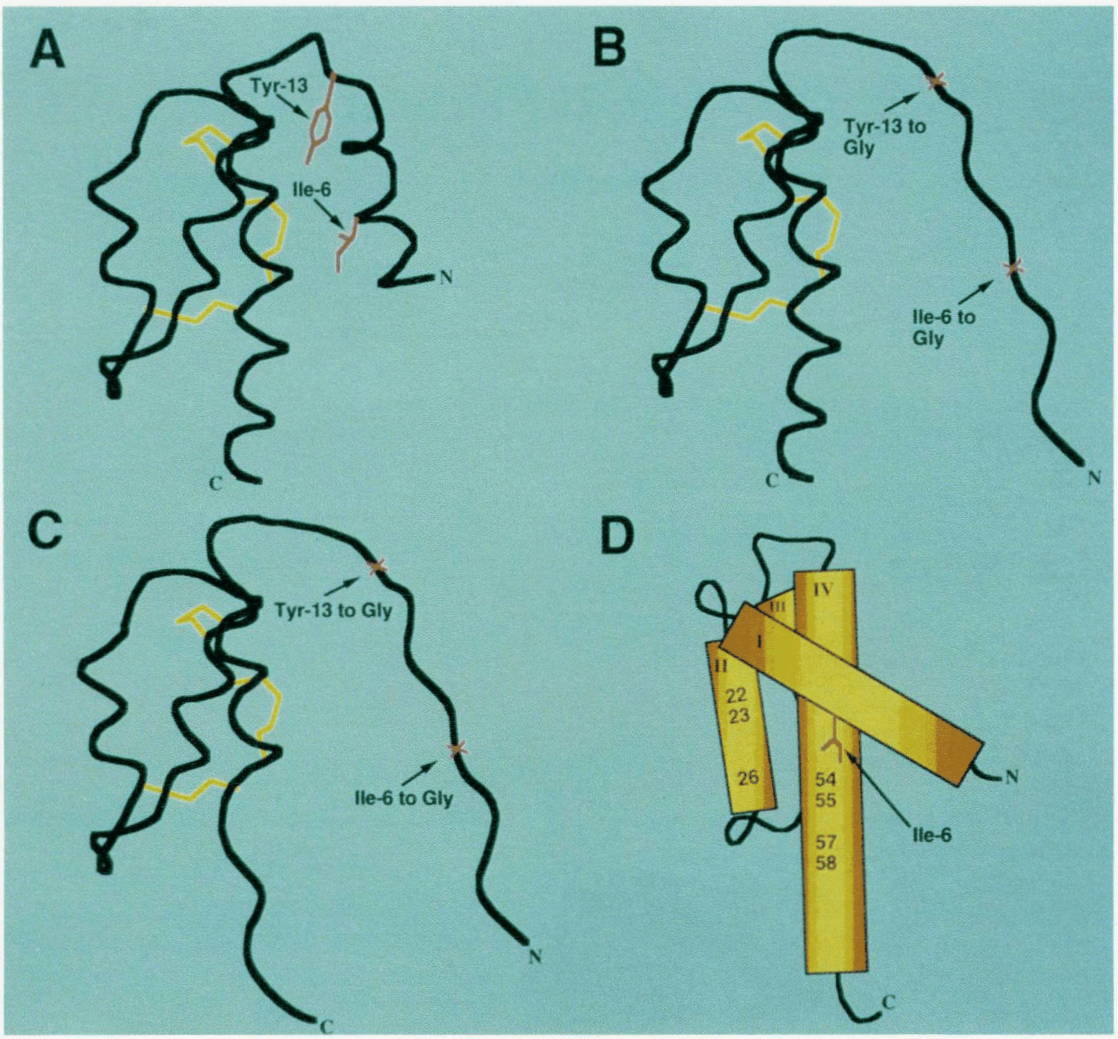

Fig. 7. Proposed effect of substituting glycine for Ile-6 and Tyr-13 in the C5a NTR region. **A:** Illustrated is the backbone structure of porcine C5a, as derived from the NMR data of Williamson and Madison (1990). Relative positions of the Ile-6 and Tyr-13 side chains are indicated in red. The yellow lines within the core region indicate locations of disulfide bonds. The C-terminus ends at Gln-65 because the remaining nine residues assume irregular structure in solution. **B:** Hypothetical effect of the Ile-6 → Gly and Tyr-13 → Gly substitutions on the secondary structure of the NTR. Forces that stabilize docking of the NTR to other regions in C5a, as well as stabilize the α-helical structure of the NTR, are disrupted when Ile-6 and Tyr-13 are replaced by glycine. **C:** Removal of putative stabilizing hydrophobic interactions between the NTR and CTR, as occurs in the Ile-6 → Gly/Tyr-13 → Gly double mutant, may disrupt helical structure in both N and C regions. **D:** The NTR (helix I) was oriented directly in front of helix IV, and helix IV and was rotated to a 0° tilt along the z-axis. This orientation projects the side chain of Ile-6 (red) directly along the axis of the CTR making interactions with residue side chains of Cys-54, Cys-55, Val-57, and Ala 58 possible. Ile-6 may also interact with side chains of Cys-22, Tyr-23, Gly-25, and Ala-26 located in the core region.

(Gerard & Gerard, 1990). The MBP-C5a fusion proteins stored within the periplasmic compartment were obtained in an aggregated but soluble form, and degradation of the rC5a portion of the fusion protein was minimal during the 4-h expression period (Figs. 1, 2). Whereas fusion protein mutants harvested from the periplasm were as stable as the wild-type protein, fusion protein mutants harvested from the cytoplasm instead of the periplasm underwent substantially greater degradation (data not shown). Thus the periplasmic compartment either lacks the degradative proteases or stores the C5a fusion product in a protease-resistant form.

Of the C5a NTR mutants examined in the present study that contained single residue substitutions, only the Ile-6 → Gly and Tyr-13 → Gly mutants showed more than a twofold decrease in potency when examined either as fusion proteins or as factor Xa-cleaved C5a (Fig. 4; Table 2). Substitution of both residues 6 and 13 for glycine resulted in a marked reduction in potency, further substantiating a functional role for these two residues. Inter-

estingly, potency of the double mutant was reduced to a much greater extent than a simple additive effect, which for the factor Xa-cleaved proteins would calculate to a potency of 2–16% compared to our observed value of 0.1–0.2% (Fig. 4). Combination mutants containing glycine substitutions for either Ile-6 or Tyr-13 plus selected hydrophilic residues were also markedly reduced in potency (Table 2), suggesting that Ile-6 and Tyr-13 are critical for maintaining the integrity of the NTR and/or the overall conformational stability of C5a.

CD measurements of the Ile-6 → Gly/Tyr-13 → Gly double mutant revealed a significant loss of α-helical content when compared with wild-type rC5a (Fig. 6). From these data it is hypothesized that glycine substitution of Ile-6 and Tyr-13 results in the loss of stabilizing forces between the NTR and the body of the C5a molecule with concomitant loss of NTR helical content (see Fig. 7B; see also Kinemage 1). Destabilization of the NTR helix alone cannot entirely account for the magnitude of the CD changes, indicating that other regions in C5a must also be structurally altered in this double mutant. It is unlikely that significant changes in secondary structure occur in the disulfide-stabilized core region by substitution of residues outside this region. It is therefore suggested that, in addition to the NTR, the CTR is also conformationally affected by the Ile-6 → Gly/Tyr-13 → Gly substitutions that disrupt putative NTR–CTR interactions (see Fig. 7C; see also Kinemage 1). A substantial change in NTR secondary structure in the Ile-6 → Gly/Tyr-13 → Gly double mutant is also supported by the finding that the factor Xa cleavage site, located adjacent to the N-terminus, is 25 times more sensitive to factor Xa cleavage for the double mutant than for the wild-type protein (Fig. 3). This increased sensitivity to factor Xa is likely due to greater exposure of cleavage sites as a consequence of altered folding within or in proximity of the NTR. Confirmation that these structural changes proposed to occur in the C5a double mutant account for the effect on potency will ultimately require NMR analysis.

Modeling studies revealed that the side chains of Ile-6 and Tyr-13 appear to have a direct role in stabilizing interactions between the NTR and other regions of C5a (Greer, 1985; Zuiderweg et al., 1989). Stabilizing effects contributed by Tyr-13 probably occur via hydrophobic interactions with neighboring residue side chains located primarily in the core region (Zuiderweg et al., 1989) (Fig. 7A; see also Kinemage 1). Of particular interest is Ile-6, due to its close proximity (i.e., <5 Å) to a number of hydrophobic residues in the core region (i.e., Cys-22, Tyr-23, Gly-25, Ala-26) and in the CTR (i.e., Cys-54, Cys-55, Val-57, Ala 58). Based on coordinates derived from C5a NMR data (Zuiderweg et al., 1989; Williamson & Madison, 1990), Ile-6 is located proximal to the crossover point of contact between the NTR and the CTR (Fig. 7D; see also Kinemage 1). Thus, Ile-6 is ideally situated for participation in putative NTR–CTR interactions.

As discussed above, it is possible that destabilizing forces resulting from site-specific mutations in the NTR can have a major influence not only on stabilization of the NTR, but also stabilization of the CTR as well. Thus it is likely that the marked loss in C5a potency observed for the Ile-6 → Gly/Tyr-13 → Gly double mutant may reflect conformational alterations in either or both of these regions. In view of the fact that the C-terminal effector site is directly linked to the CTR, it is tempting to suggest that the conformation of the CTR directly influences the C-terminal effector site. Whether or not the NTR directly influences the C-terminal effector site is difficult to predict because the last nine residues of C5a define no ordered structure in solution (Zuiderweg et al., 1989; Williamson & Madison, 1990). Alternatively, it cannot be ruled out that one or more conformationally dependent receptor binding subsites exist in the NTR and/or the CTR of C5a. In fact, studies with synthetic analogue C3a peptides have substantiated the possible existence of a receptor binding subsite in the CTR of C3a (Ember et al., 1991). Site-specific mutations have identified residues in the NTR of C5a important for maintaining the structural and functional integrity of the C5a molecule. Further structure/function studies will be required to delineate the interrelationship between the NTR, CTR, and C-terminal effector region in C5a and sites of interaction with the C5a receptor.

Materials and methods

Construction of expression vector carrying the C5a gene

A synthetic C5a gene (Frank et al., 1988), kindly provided by Dr. C. Gerard (Children's Hospital, Boston, Massachusetts), was inserted in a pMAL-p fusion protein expression vector (New England Biolabs) and oriented such that the first residue of C5a (Thr-1) followed immediately after a factor Xa cleavage site designed in the vector. For site-directed mutagenesis studies, the C5a gene was subcloned into *Eco*RI and *Bam*HI sites of M13mp19. A T7 mutagenesis kit (New England Biolabs) was used for the preparation of mutants.

C5a expression in E. coli

The C5a–pMAL-p construct expresses C5a in the form of a fusion protein consisting of MBP fused to the N-terminus of C5a. The fusion protein under the control of a *tac* promoter was induced with IPTG. Wild-type and mutant C5a–pMAL-p constructs transformed into JM101 *E. coli* were grown to log phase at 37 °C in a shaking incubator after addition of 1/10 volume of saturated bacterial culture to LB medium containing 2% glucose and 100 μg/mL ampicillin. The IPTG was then added to a final concentration of 0.3 mM. Fusion protein transported

to the periplasmic compartment was harvested by a modification of the T7 mutagenesis kit method. After 4 h at 37 °C, the cultures were centrifuged at 4,000 × *g* for 10 min. The pellets from each 250 mL of culture were resuspended in 80 mL of buffer consisting of 30 mM Tris-HCl, pH 8.0, and 20% sucrose. The EDTA was then added to a final concentration of 1 mM followed by mixing at room temperature for 10 min. After centrifuging at 8,000 × *g* for 10 min at 4 °C, the pellets were subjected to cold osmotic shock in ice-cold 5 mM $MgSO_4$ to release the fusion protein contained in the periplasmic space. After mixing for 10 min on ice, the suspension was centrifuged at 8,000 × *g* for 20 min at 4 °C. Potassium phosphate buffer, pH 7.4, and Tween 20 were added to the osmotic shock supernatant at final concentrations of 20 mM and 0.2%, respectively. The osmotic shock fluid was mixed in 50-mL aliquots with 2 mL of amylose resin (New England Biolabs) on ice for 10 min. The resin was then applied to a column and washed with buffer containing 10 mM potassium phosphate, pH 7.4, 30 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 0.25% Tween 20, followed by washing buffer without Tween. The fusion protein was eluted with washing buffer containing 10 mM maltose (Sigma), concentrated to 5 mg/mL with Centricon-30 concentrators (Amicon), and stored at −70 °C. The average yield of purified fusion protein was 10 mg/L of bacterial culture.

Factor Xa cleavage of C5a from fusion protein

Sodium chloride and sodium azide were added to aliquots of concentrated fusion protein to a final concentration of 100 mM and 0.05% (w/v), respectively. Sequencing grade factor Xa (Boehringer Mannheim) was added at a concentration that resulted in approximately 50% proteolytic cleavage of the fusion protein at the factor Xa site (IEGR) after 16 h at room temperature, as assessed by SDS-PAGE analysis. The factor Xa-cleaved proteins were denatured in 0.1 M potassium phosphate buffer, pH 8.0, and containing 6 M guanidinium hydrochloride and 0.1 M β-mercaptoethanol, then separated on a Trisacryl Plus GF4-M column (Sigma) equilibrated in the denaturing buffer. Fractions containing purified rC5a were pooled and dialyzed in 20 mM phosphate buffer, pH 8.0. The proteins were then concentrated in Centricon-3 concentrators to >0.5 mg/mL and stored in aliquots at −70 °C.

Chemotaxis assay

The chemotactic activity of the rC5a and fusion proteins was determined by a modification of the Boyden chamber method as described (Ember & Hugli, 1989). Human neutrophils were isolated from whole blood collected in 10 mM EDTA using Mono-Poly Resolving Medium (ICN Flow) and resuspended in assay buffer consisting of sterile Earle's balanced salt solution (GIBCO) containing 20 mM HEPES, pH 7.4, and 1% final bovine serum albumin. The C5a was diluted in assay buffer (0.8 mL, final volume) and added to the lower chemotaxis chamber (1.5 × 3 cm) (ADAPS, Dedham, Massachusetts). The upper chamber (1 × 2.5 cm), which contained a cellulose nitrate membrane (8 μm pore size; Sartorius), was inserted into the lower chamber. Neutrophils (1×10^6 in 0.2 mL) were then added to the upper chamber. After 90 min at 37 °C, 50 μL of 0.5 M EDTA was added to the upper and lower chambers, followed by brief mixing to dislodge any cells attached to the membrane. The relative number of cells migrating to the lower chamber was assayed by measuring total cell β-glucuronidase based on the procedure of Schroder et al. (1987). Triton-X 100 (0.1% final concentration) was added to lyse the cells that migrated to the lower chamber. The lysate was pipetted (50 μL) into microtiter wells in triplicate. Fifty microliters of 10 mM *p*-phenyl-β-D-glucuronide in 0.1 M sodium acetate buffer, pH 4.0, was then added to the wells. The microtiter plates were incubated at room temperature in the dark for 16 h, and then 100 μL of 0.4 M glycine buffer, pH 10.0, was added to the wells. The optical density was measured at 405 nm, and the results were expressed as percentage of total cells that migrated.

β-Glucuronidase release

Human neutrophils were suspended in the assay buffer used for the chemotaxis assay (1×10^7/mL) and treated with cytochalasin B (5 μL of a 1-mg/mL stock solution in dimethyl sulfoxide per milliliter of cells). After incubating the cells at 37 °C for 30 min, 100 μL of cells were mixed with 100 μL of diluted C5a followed by incubation at 37 °C for 1 h. The supernatants were assayed for release of β-glucuronidase as described above.

Assessment of proteolytic degradation at the C-terminus of the rC5a mutants

Expressed proteins in bacteria are frequently subject to proteolytic degradation by bacterial proteases (Wetzel & Goeddel, 1983). Proteolytic degradation at the C-terminal region of rC5a was assessed using an ELISA assay and a rabbit polyclonal anti-peptide antibody directed against the C-terminal tetradecapeptide, LRANISHKDMQLGR (14-mer), of human C5a (Ember et al., 1992). The antigen was prepared by glutaraldehyde conjugation of the C-terminal peptide to keyhole limpet hemocyanin (Harlow & Lane, 1988). Antibody was raised in rabbits according to standard methods.

Competitive ligand binding assay

Highly purified human C5a prepared from plasma as described by Hugli et al. (1981) was radiolabeled with ^{125}I according to the method of Melewicz et al. (1982) to a

specific activity of 100 cpm/fmol. Human neutrophils (2×10^6) were incubated with 3 nM radiolabeled C5a in the presence of increasing concentrations of competing unlabeled rC5a. Incubation was carried out on ice for 30 min at a final concentration of 0.2 mL. An aliquot of the cell suspension was layered over oil and centrifuged at $12,000 \times g$ for 3 min. The radioactivity in the pellet was measured.

CD

The CD measurements were performed on an Aviv model 61DS spectropolarimeter. Spectra were recorded by using protein solutions of 0.25 mg/mL in 0.05 mM phosphate buffer, pH 8.0. Amino analysis and protein concentrations were determined on a Beckman System 6300 amino acid analyzer. A 1.5-nm bandwidth and a 0.5-nm step-size were used to measure the protein solutions in 1-mm-pathlength cuvettes at 25 °C. At least four scans were determined for each protein sample and averaged. 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, New Jersey).

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