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Streptococcal C5a Peptidase Is a Highly Specific Endopeptidase

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Compositional analysis of streptococcal C5a peptidase (SCPA) cleavage products from a synthetic peptide corresponding to the 20 C-terminal residues of C5a demonstrated that the target cleavage site is His-Lys rather than Lys-Asp, as previously suggested. A C5a peptide analog with Lys replaced by Gln was also subject to cleavage by SCPA. This confirmed that His-Lys rather than Lys-Asp is the scissile bond. Cleavage at histidine is unusual but is the same as that suggested for a peptidase produced by group B streptococci. Native C5 protein was also resistant to SCPA, suggesting that the His-Lys bond is inaccessible prior to proteolytic cleavage by C5 convertase. These experiments showed that the streptococcal C5a peptidase is highly specific for C5a and suggest that its function is not merely to process protein for metabolic consumption but to act primarily to eliminate this chemotactic signal from inflammatory foci.

Recognition that mucosal pathogens depend on highly specific endoproteases to circumvent local immunological defenses began with the discovery of immunoglobulin A proteases (14). More recently, we discovered that virulent strains of *Streptococcus pyogenes* produce a C5a peptidase (SCPA) which is bound to their surface (12, 22). Activation of the complement system by either the classical or alternative pathway generates C5 convertase that proteolytically liberates C5a, a 74-residue chemotactic peptide (7). A chemical gradient of C5a attracts phagocytic polymorphonuclear leukocytes to the site of infection to form the first line of defense against persistent colonization and invasion of deeper tissues. The association of C5a peptidase enzymes with two other human streptococcal pathogens (6, 11) suggests that elimination of this chemotactic signal may be a common mechanism for bacteria to avoid phagocytic detection.

The nucleotide sequence of SCPA was deduced to encode a mature protein (after processing of the signal sequence) with an M_r of 124,814 (3). The signal sequence was predicted to be 31 amino acids in length. At present, there is no indication that SCPA is activated by further processing. Near the carboxy terminus is a sequence rich in glycine and proline, a region thought to pass through the cell wall peptidoglycan (8). Immediately distal to that region is a hydrophobic core of 17 residues which is followed by 10 highly charged residues at the carboxy terminus. A similar hydrophobic region and a 6-amino-acid motif just preceding it are characteristic of M (15) and Fc receptor (5, 10) proteins, also on the surface of *S. pyogenes*. This motif was shown to bind M protein to the cell membrane (8). Despite the lack of sequence similarity between M proteins and SCPA, similar structural topologies at their C termini suggest that they share a mechanism of attachment to the cell surface (15).

Little is known about SCPA substrate specificity. Alignment by functional and structural domains shows significant homology between SCPA and subtilisins in regions containing charge transfer residues and determinants of substrate binding specificity (3, 16). The subtilisins are known to have broad substrate specificity and for this reason have even found use as supplements to laundry detergents. This observation raised a question about SCPA specificity. Has SCPA evolved to specifically eliminate chemotactic signals or does it function merely to process proteins for metabolic consumption?

Previous experiments using carboxypeptidases to sequence the C terminus of a C5a N-terminal fragment suggested that SCPA cleaved C5a between Lys-68 and Asp-69, a position known to be within the polymorphonuclear leukocyte binding domain (20). Because this method is notoriously subject to interpretation, we sought to verify the cleavage position of SCPA by using synthetic peptides as substrate and to confirm that it is indeed an endopeptidase. We also tested a variety of other proteins as potential substrates. The data presented here demonstrate that SCPA is highly specific for C5a and that it cleaves C5a on the carboxyl side of His-67 rather than on that of Lys-68 as originally suggested.

MATERIALS AND METHODS

SCPA. Enzyme was extracted from an M type 49 group A streptococcus, strain CS101, by using the muralytic enzyme phage C lysin extracted from phage-infected group C streptococci (6, 13). Purification to homogeneity was described previously (21). The method involved size-selective ultrafiltration, gel filtration on a P200 column, hydrophobic interaction chromatography on phenyl Sepharose-CL, and finally gel filtration by Sephacryl-300 chromatography. The preparation of enzyme used in these experiments showed a single band on sodium dodecyl sulfate (SDS)-polyacrylamide gels stained with Coomassie blue. Enzyme was stored at −70°C indefinitely.

Synthetic peptides. The C5a peptide (C5apep) corresponds

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	55	56	57	58	59	60	61	62	63	64	65	66	67		68	69	70	71	72	73	74
C5a pep	H_2N-Cys	Val	Val	Ala	Ser	Gln	Leu	Arg	Ala	Asn	Ile	Ser	His		Lys	Asp	Met	Gln	Leu	Gly	Arg-COOH
17C	H_2N-Cys	Val	Val	Ala	Ser	Gln	Leu	Arg	Ala	Asn	Ile	-	His		Gln	Asp	Met	Gln	Leu	Gly	Arg-COOH
17B	H_2N-Cys	Val	Val	Ala	Ser	Gln	Leu	Arg	Ala	Asn	Ile	-	His	Pro	Lys	Asp	Met	Gln	Leu	Gly	Arg-COOH

FIG. 1. Amino acid sequence of synthetic peptides tested as substrates for SCPA. C5apep represents the last 20 amino acids of the complement protein C5a. Peptides 17C and 17B are analogs of C5apep.

to the 20 C-terminal residues of native C5a reported by Fernandez et al. (7). This peptide was synthesized by the solid-phase method (1). C5apep analogs were manually synthesized by Merrifield's solid-phase method (17). Peptides were purified by reversed-phase high-performance liquid chromatography (HPLC) on a Waters 501 system (Waters, Inc., Milford, Mass.) by using an Aquapore octyl Brownlee Prep 10-cartridge column (25 by 1 cm) (Chrom Tech Inc., Apple Valley, Minn.). Composition analyses of peptides 17B and 17C and their putative cleavage products indicated that addition of serine during synthesis was incomplete because only one of the expected two serine residues was detected. Peptide 17C was confirmed to lack Ser-66 by Edman sequencing.

Peptidase digestion conditions. Standard digestion mixtures included 75 μg of peptide and 3 μg of purified SCPA in 100 μl of Hanks balanced salt solution (GIBCO, Gaithersburg, Md.). Reaction mixtures were incubated for 3.5 h at 37°C before they were diluted 1:1 in buffer A (0.1% trifluoroacetic acid in H_2O) and injected for reversed-phase HPLC analysis on an Aquapore octyl Brownlee cartridge column (30 by 4.6 mm; model RP300). Peptide fragments eluted from the column during a linear gradient of 95% buffer A–5% buffer B (0.1% trifluoroacetic acid in acetonitrile) to 70% buffer A–30% buffer B at 1.5 ml/min. Peptide peaks were collected and concentrated. Amino acid compositions were determined after hydrolysis on a Beckman 6300 amino acid analyzer (Beckman Instruments, Fullerton, Calif.). Conditions did not permit measurement of cysteine. Peptides, C5apep, 17C, and the C-terminal digestion products from C5apep were sequenced by the Edman method with an Applied Biosystems automatic sequencer.

RESULTS

SCPA endoproteolytic cleavage of C5apep. Cleavage of C5a by SCPA was previously suggested to be located between Lys-68 and Asp-69 on the basis of the release of free amino acids by carboxypeptidases A and B from an N-terminal fragment of that protein (20). To confirm this putative cleavage position and to establish that SCPA is in fact an endopeptidase, the C5apep synthetic peptide corresponding to 20 residues at the C-terminal sequence of C5a (Fig. 1) was tested as substrate. After incubation of C5apep for 3.5 h with purified SCPA, peptide fragments were identified and purified by HPLC as described in Materials and Methods. Two new peptide peaks were produced, peaks B and C, which were less hydrophobic than the original C5apep, peak A (Fig. 2). Peak A is the C5apep incubated for 3.5 h without SCPA. As expected, peak A was eliminated when exposed to SCPA. The most-hydrophobic peaks (retention time, 25 min) in both Fig. 2 and 3 were present variably and corresponded to residual SCPA protein which persisted through successive gradients. The peptide fragment in each peak was isolated, and its amino acid composition was determined (Table 1). The compositions of peaks B and C correspond to the 13 N-terminal residues and the 7 C-terminal residues, respectively, of C5apep. A very important and unexpected finding was the presence of histidine and lysine in separate peptides, peaks B and C, respectively. Two completely separate digestions were analyzed, and each showed histidine in the N-terminal fragment and lysine in the C-terminal fragment of C5apep. To confirm composition analyses, the amino acid sequence of peptide C was determined by Edman degradation to be the expected sequence, i.e., Lys Asp Met Gln Leu Gly Arg.

Because the above data are contrary to those of our previous study (20), we further tested whether Lys-68 contributed to the position of the scissile bond. Two analog peptides were synthesized and tested as substrates for SCPA (Fig. 1). During manual synthesis of peptides 17B and 17C, coupling of Ser to His was problematic and did not go to completion. The sequence of purified 17C showed that Ser-66 was absent. Amino acid composition analysis suggested that Ser-66 was also missing from peptide 17B. Although glutamine replaced Lys-68 in peptide 17C, this

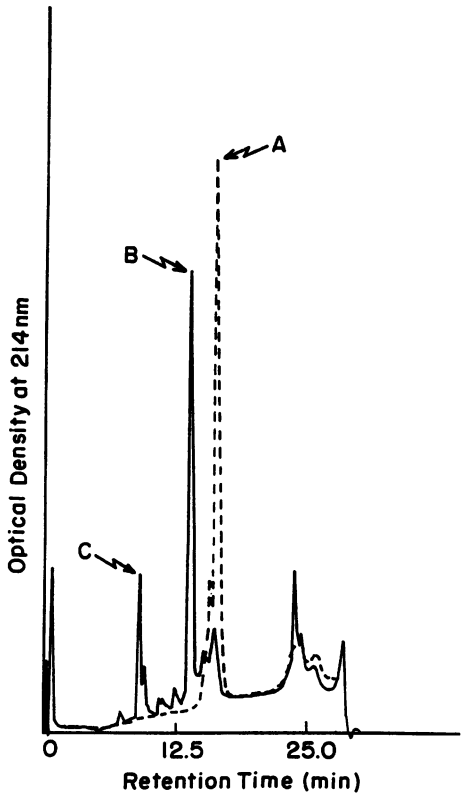

FIG. 2. Reversed-phase retention time of C5apep before and after digestion with SCPA. Solid line, C5apep preincubated for 3.5 h with purified SCPA; dashed line, C5apep incubated for 3.5 h in buffer without SCPA.

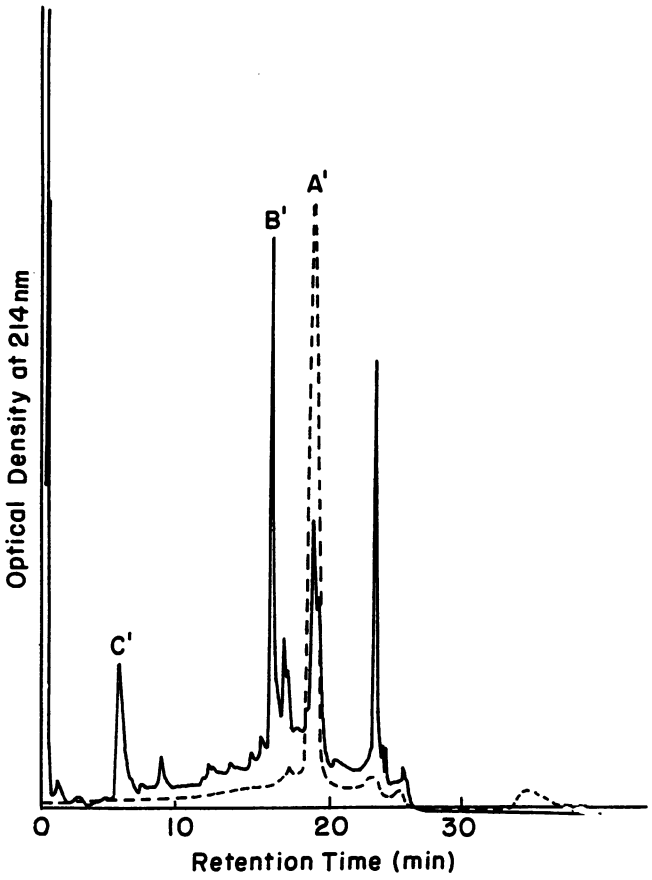

FIG. 3. Reversed-phase retention time of C5apep analog 17C. Solid line, peptide incubated for 3.5 h with SCPA; dashed line, the same peptide incubated for 3.5 h in buffer without SCPA. The reaction mixture and HPLC gradient conditions are described in Materials and Methods.

TABLE 1. Amino acid composition of C5apep fragments

Amino acid	Amt (nmol) of amino acid in:		
	Peak A	Peak B	Peak C
CM-cysteine	0.0 (0)[a]	0.0 (0)	0.0 (0)
Valine	1.6 (2)	1.9 (2)	0.0 (0)
Alanine	2.0 (2)	2.0 (2)	0.0 (0)
Serine	1.6 (2)	1.4 (2)	0.0 (0)
Glutamic acid	2.1 (2)	1.1 (1)	1.1 (1)
Leucine	2.1 (2)	1.0 (1)	1.0 (1)
Arginine	2.0 (2)	1.0 (1)	1.0 (1)
Aspartic acid	2.1 (2)	1.0 (1)	1.0 (1)
Isoleucine	1.0 (1)	1.0 (1)	0.0 (0)
Histidine	1.0 (1)	1.0 (1)	0.0 (0)
Lysine	1.1 (1)	0.0 (0)	1.0 (1)
Methionine	0.9 (1)	0.0 (0)	0.4 (1)
Glycine	1.0 (1)	0.0 (0)	1.1 (1)
Threonine	0.0 (0)	0.0 (0)	0.0 (0)
Proline	0.0 (0)	0.0 (0)	0.0 (0)
Tyrosine	0.0 (0)	0.0 (0)	0.0 (0)
Phenylalanine	0.0 (0)	0.0 (0)	0.0 (0)
Cystine/2 and cysteic acid	0.6 (1)	0.7 (1)	0.0 (0)

[a] Values in parentheses indicate predicted amounts in nanomoles.

TABLE 2. Amino acid composition of C5apep analog 17C

Amino acid	Amt (nmol) of amino acid in:		
	Peak A	Peak B	Peak C
CM-cysteine	0.0 (0)[a]	0.0 (0)	0.0 (0)
Valine	1.7 (2)	1.9 (2)	0.0 (0)
Alanine	2.0 (2)	2.0 (2)	0.0 (0)
Serine	0.8 (1)	0.8 (1)	0.0 (0)
Glutamic acid	3.1 (3)	1.2 (1)	2.0 (2)
Leucine	2.2 (2)	1.1 (1)	1.0 (1)
Arginine	2.0 (2)	1.1 (1)	1.0 (1)
Aspartic acid	2.1 (2)	1.1 (1)	1.0 (1)
Isoleucine	1.0 (1)	0.9 (1)	0.0 (0)
Histidine	1.0 (1)	1.0 (1)	0.0 (0)
Lysine	0.1 (0)	0.1 (0)	0.0 (0)
Methionine	1.0 (1)	0.1 (0)	0.9 (1)
Glycine	1.5 (1)	0.2 (0)	1.0 (1)
Threonine	0.1 (0)	0.0 (0)	0.0 (0)
Proline	0.0 (0)	0.0 (0)	0.0 (0)
Tyrosine	0.0 (0)	0.0 (0)	0.0 (0)
Phenylalanine	0.0 (0)	0.0 (0)	0.0 (0)
Cystine/2 and Cysteic acid	0.0 (1)	0.0 (1)	0.0 (0)

[a] Values in parentheses indicate predicted amounts in nanomoles.

peptide was cleaved by SCPA to yield two fragments (Fig. 3): the N-terminal fragment again contained histidine, whereas the C-terminal fragment contained glutamine (Table 2). This confirmed that His-67 is the P1 amino acid and that Lys-68 did not directly influence specificity. The second analog, peptide 17B, had proline inserted between His-67 and Lys-68. This peptide was resistant to SCPA (data not shown). Perhaps the tendency of proline to exist in the *cis* isomeric form in solutions of small peptides (9) significantly alters the secondary structure of the latter peptide relative to the C5apep, which could account for its resistance to SCPA. An alternative explanation for the resistance of this peptide is that the imide bond between histidine and proline may be more resistant to attack than the amide bond between histidine and lysine.

Native C5 is resistant to SCPA. Substrate conformation is also likely to contribute to the specificity of SCPA. C5a is normally released during complement activation from the alpha chain of C5. This highly restricted proteolytic reaction occurs during the interaction of C5 with the multimolecular C5 convertase complex. If substrate conformation contributes to enzymatic specificity, then one would predict that SCPA would be unable to cleave native C5. Purified C5 was incubated with SCPA and the products were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). No detectable alteration in the mobility of C5 α or β subunits was observed even after 19 h of incubation with SCPA (Fig. 4). This confirmed that C5 was resistant to cleavage by SCPA and indicated that substrate binding residues surrounding the His-Lys bond are buried or inaccessible on the native C5 molecule. Similar experiments using purified C3 protein showed that it was also resistant to proteolytic attack by SCPA (data not shown).

DISCUSSION

The first arm of phagocytic immunological defenses is chemotactic detection of the invading microorganism. A gradient of C5a emanating from the focus of infection, established through activation of the complement pathway, is the primary attractant to phagocytic leukocytes. Our

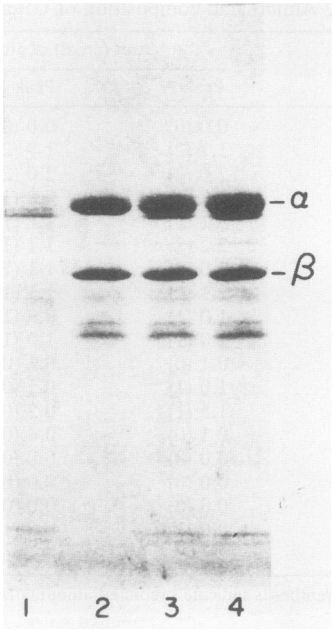

FIG. 4. Susceptibility of native C5 to SCPA. Purified C5 (250 μg/ml) was incubated with 63 μg of SCPA per ml at 37°C in 62.5 mM Tris-HCl (pH 7.4). After incubation, the equivalent of 5 μg of C5 from each sample was boiled in sample buffer containing 2% SDS and 1% 2-mercaptoethanol and analyzed by SDS-PAGE. Lanes: 1, 1.25 μg of SCPA alone; 2, C5 alone; 3 and 4, C5 incubated with SCPA for 1 and 19 h, respectively. α, 110,000-Da subunit of C5; β, 75,000-Da subunit of C5. The gel was stained with Coomassie blue.

discovery that *S. pyogenes* expresses a cell-wall-bound peptidase which rapidly destroys C5a suggested a means by which these human pathogens could avoid immediate detection by phagocytic defenses (20, 22). More recently, group B (11) and G (6) streptococci of human origin were also shown to harbor cell-wall-bound C5a peptidases which are related, possibly identical, to that produced by *S. pyogenes*. These findings suggest that inactivation of chemotactic signals is a universal virulence mechanism of streptococci.

Sequence comparison with those of other proteases predicts that SCPA is a serine protease related to the subtilisin family of enzymes. Siezen et al. (16), in aligning these enzymes by structural and functional domains, placed SCPA in the class I group of enzymes; SCPA is most similar to the lactococcal PrP protease (3, 19). Regions of the protein containing charge-transfer and substrate-binding residues are most conserved.

Our earlier data based on carboxypeptidase sequencing of an N-terminal cleavage product of C5a suggested that SCPA hydrolyzed C5a at the carboxy side of Lys-68. Those experiments, however, did not distinguish between endoproteolytic cleavage and carboxypeptidase activity (20). Experiments presented here show that our previous interpretation of carboxy sequencing data was incorrect. Analysis of cleavage products of a synthetic peptide which corresponds to C5a demonstrates that SCPA is an endopeptidase which severs C5a between His-67 and Lys-68, residues which are within the leukocyte C5a binding site (4). An analog peptide with the P1′ lysine replaced by glutamine also functioned as a substrate for SCPA. This shows that the P1′ side of the scissile bond can be varied and that lysine is not essential for specificity. Although the SCPA used in previous experiments was extracted from cells with trypsin (20, 21), in contrast to that used here which was released from cells with the muralytic enzyme phage C lysin, it is improbable that the method of extraction would change cleavage specificity. Because SCPA extracted by either method, under the conditions employed, would lack the membrane-spanning region, it would seem that this domain of the enzyme has little influence on its specificity. The absence of Ser-66 in the P2 position of two analog peptides, 17B and 17C, had no detectable impact on the cleavage specificity; peptide 17C and C5apep were equally hydrolyzed on the carboxyl side of His-67. Placement of a proline residue between the P1 histidine and lysine blocked SCPA activity. We presume that this insertion alters the secondary structure of the peptide (9) so that the scissile bond is not accessible to the enzyme-active site. These peptides have not been tested for their capacity to inhibit cleavage of C5a.

Recombinant SCPA extracted from *Escherichia coli* carrying the cloned *scpA* gene from serotype M12 streptococci also cleaved C5apep between His and Lys residues (unpublished data). A preference for histidine at the P1 position is unusual. Bohnsack et al., by using plasma desorption mass spectrometry, deduced that a peptidase from group B streptococci preferentially cleaves C5a between His-67 and Lys-68 (2), but we are unaware of other peptidases with this specificity. Other subtilisins have broad substrate specificity, acting adjacent to a variety of residues (16).

The concept that bacterial pathogens make use of specific proteases to counter the action of immunological defenses first emerged with the discovery of enzymes which specifically destroy mucosal immunoglobulin A (14). These proteases are highly specific, with the precise cleavage site in immunoglobulin A being dependent upon the bacterial species. If SCPA evolved to specifically block C5a-mediated chemotaxis, i.e., to function as a virulence factor, then we would expect it to be highly specific, not an enzyme which generally processes protein substrates for metabolic consumption. Previous experiments demonstrated that six proteins, known to have many internal histidine residues, are very resistant to cleavage by SCPA. Only bovine serum albumin, after denaturation with urea and prolonged incubation, showed significant susceptibility to SCPA proteolytic action (20). This narrow specificity of SCPA is in contrast to that of subtilisins and is surprising in light of the marked similarities between their catalytic domains. Residues involved in charge transfer are conserved, as are residues on both sides of the binding pocket. Variability between SCPA and other subtilisins in residues at the entrance and bottom of the pocket (16) could account for differences in specificity. We originally suggested that residues 399 to 418 of SCPA correspond to the S1 specificity crevice (3). Instead, Siezen et al. (16) aligned residues 276 to 295 of SCPA with residues 136 to 156 of subtilisin BPN′ to produce a better match (16). We agree that this region more likely corresponds to the S1 cleft. SCPA and the lactococcal PrP proteases are much larger than most subtilisins. Both have long stretches of sequence between their more-N-terminal catalytic triad and C-terminal cell membrane anchor regions. Vos et al. (18) engineered hybrid proteins to show that amino acids in this intervening region of PrP contribute to substrate specificity (18).

Tertiary and quaternary structures can also alter the susceptibility of a protein to endoproteolytic cleavage. Native C5 is composed of dissimilar α and β subunits. If SCPA functioned specifically to disrupt chemotaxis, then one might expect the P1 scissile bond to be inaccessible to SCPA

before the intact C5 molecule is cleaved by C5 convertase. This, in fact, appears to be true since incubation of C5 or C3 for extended periods of time did not alter the molecular sizes of the subunits of either protein. Although we have not yet tested the susceptibility of C3a, it may also be cleaved by SCPA. The stem domain of this anaphylatoxin, like C5a, contains histidine at position 67. Resistance of these native proteins to SCPA suggests that tertiary or quaternary structure is an important determinant of SCPA substrate specificity.

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